Estradiol and Chlordecone (Kepone) Decrease Adenosine 3′5′-Cyclic Monophosphate Concentrations in the Ovariectomized Immature Rat Uterus (43921)

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Abstract. **Adenosine 3′5′-cyclic monophosphate (cAMP) has been repeatedly shown to mimic some actions of estrogen in the rat uterus. However, the relationship between estrogens and uterine cAMP remains controversial. The effect of chronic exposure (3 days) to a biologically potent, long-acting estrogen, estradiol benzoate (EB), or the xenoestrogen chlordecone (Kepone), which has a long half-life in the circulation, was examined in ovariectomized immature rats. Both compounds, when administered in doses that provided equal increases in uterine weight, produced equivalent decreases in uterine cAMP content. Although the decrease in cAMP was apparent within 48 hr, it was more pronounced at 72 hr. There was no reduction in cAMP produced in response to direct stimulation of uterine adenylyl cyclase by forskolin, indicating that loss of the enzyme was not a factor in the lowering of cAMP content. The pure anti-estrogen ICI-182,780, in a dose-dependent fashion, prevented the action the estradiol benzoate and chlordecone, suggesting that the lowering of cAMP was dependent on an estrogen receptor. The physiological significance of reduced uterine cAMP with chronic estrogen treatment remains to be determined.** [P.S.E.B.M. 1995, Vol 210]

Steroids are thought to produce their effects in target organs by binding to nuclear receptors that act as transcription-activating factors, which bind to response elements in the promotor region of the specific genes (1). Although introduced several years ago (2) and recently revived (3, 4), the concept of additional steroid receptors, including those on the plasma membrane, that function via traditional second messengers remains controversial (reviewed in Ref. 5). Early studies, extensively reviewed by Singhal (6), indicated that administration of estrogen to ovariectomized rats resulted in a prompt but brief increase in uterine adenylyl cyclase activity and adenosine 3′5′-cyclic monophosphate (cAMP) content. Therefore interest in cAMP as a second messenger for estrogen action was initiated. However, a study that compared changes in cAMP and guanine 3′5′-cyclic monophosphate (cGMP) over a period of several hours in the uteri and vaginas of ovariectomized rats injected with estrogen indicated a reciprocal change in the nucleotides of the two tissues (7). That is, estrogen caused an increase in uterine cGMP but a decrease in cAMP, while the reverse was true for the vagina. These findings were consistent with the view that increased cAMP is associated with cellular differentiation but is a negative regulator of cell proliferation (reviewed in Refs. 8 and 9). In contrast to the latter idea, exogenous cAMP and cholera toxin, a known stimulator of adenylyl cyclase, have been shown to mimic at least some of the actions of estrogen upon the uterus (10–12).

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A recent study (13) found that estradiol and tamoxifen, an antiestrogen with estrogenic activity, when administered for 3 days decreased mouse uterine cAMP content, presumably because of increased phosphodiesterase activity. These findings prompted a reevaluation of cAMP in the rat uterus chronically exposed to estrogen. The present study compares the effects of uterine weight and cAMP content of a biologically potent conjugated and long-acting estrogen (estradiol benzoate) with that of chlordecone (Kepone), an oragnochlorine pesticide that has been shown to have a low affinity (0.04% that of estradiol) for the nuclear estrogen receptor and thus weak uterine estrogenic activity (14, 15). In an attempt to determine whether the effects were mediated via an estrogen receptor, the effect of a pure antiestrogen (ICI-182,780), which has an affinity for the nuclear estrogen receptor only 12% less than that for estradiol (16), on these responses was also examined. Doses of the estrogens that increased uterine wet weight also reduced cAMP content, and these effects could be prevented in a dose-dependent fashion by the anti-estrogen.

Materials and Methods

Chemicals. Estradiol benzoate (EB), benzyl benzoate, sesame seed oil, TRIS, EDTA, ATP, 3-isobutyl-1-methylxanthine (IB-MIX), and forskolin (7β acetoxy,1α,6β,9α-trihydroxy-8,13-epoxy-labd-14 en-11 one) were purchased from Sigma Chemical Co. (St. Louis, MO). Chlordecone, 99% pure (Kepone; decachlorooctahydro-1,3,4-metheno-2H-cyclobuta-[cd]pentalen-2-one) was purchased from Radian Corporation (Austin, TX). ICI 182,780 was a gift from ICI Pharmaceutical Co. (Macclesfields, United Kingdom). Chlordecone, EB, and ICI-182,780 were dissolved in benzyl benzoate before dilution with sesame seed oil to produce a 40:60 (v/v) ratio. Trichloracetic acid was purchased from Fisher Scientific (St. Louis, MO). The kit for CAMP [^{125}I] radioimmunoassay was purchased from Biomedical Technologies Inc. (Stoughton, MA) and used according to the directions provided.

Animals. All procedures using animals were reviewed and approved by the University of Kansas Medical Center committee for the care and use of animals using the guidelines set forth by the U.S. Public Health Service.

Anesthetized immature female rats, 22–24 days old (Holtzman strain; Harlan Laboratory Animals, Madison, WI), were ovariectomized through a mid dorsal skin incision and dorso-lateral incisions in the body wall. The animals were maintained in temperature-controlled (22 ± 1°C) quarters on a daily cycle of 14 hr of light and 10 hr of darkness. Laboratory Rodent Chow #5001 (PMI Feeds Inc., St. Louis, MO) and tap water were constantly available.

Four days after surgery, the animals were divided into groups of at least five rats each and injected (subcutaneous) with estradiol benzoate (EB), chlordecone, or one of these compounds plus ICI-182,780, each dissolved in 0.1 ml of oil; combination treatments were given at different sites at least 5 cm apart. Control rats were injected at the same time with oil vehicle. The animals were injected daily for 3 days with autopsy 24 hr after the last dose. In one study, animals were sacrificed 24, 48, or 72 hr after the first dose. The uteri were quickly removed, trimmed of connective tissue and fat, weighed, frozen on dry ice, and stored at −70°C until assayed for cAMP.

cAMP Assay. Two homogenization methods were used depending upon whether adenylyl cyclase activity was also to be measured. In the first method, uteri were homogenized in 1 ml of 6% trichloracetic acid in a glass-teflon homogenizer. The homogenate was centrifuged at 2000*g* for 30 min (4°C). Tritiated cAMP was added to the supernatant fluid to monitor recovery and then extracted four times with 2 ml diethyl ether that was saturated with acidified water. The aqueous phase was lyophilized and dissolved in 0.15 *M* sodium acetate buffer (pH 6.2) for determination of cAMP by radioimmunoassay. All assays were performed in duplicate and expressed as pmole/mg of uterus. In the second method, the uteri were cut, after weighing, into 5-mm pieces and placed in 50 m*M* Tris (pH 7.4) containing 4 m*M* EDTA and 0.5 m*M* IB-MIX before freezing. The tissue was thawed and homogenized in a glass-teflon homogenizer before being centrifuged at 2000*g* for 30 min. The supernatant fluid was collected, assayed for cAMP content, and also tested for adenylyl cyclase activity. For the latter, 25 μl of the homogenate was incubated for 15 min at 25°C in a total volume of 1 ml of Tris buffer containing 1 m*M* ATP, 3 m*M* $MgSO_4$, 10 m*M* theophylline, and 10 μ*M* forskolin. The reaction was stopped by placing the tubes in boiling water for 5 min. After centrifugation the cAMP content of the supernatant fluid was measured by RIA. The intra- and interassay coefficients of variation did not exceed 10%.

Statistics. Results were compared by one-way analysis of variance followed by Newman-Keuls multiple comparison test; *P* values less than 0.05 were considered significant.

Results

The mean body weight at autopsy for all animals was 117.1 ± 1.7 g, and none of the treatments had any effect on this weight. Figure 1 summarizes uterine weights obtained in various assays using daily administration of EB or chlordecone. The smallest dose of EB to produce a significant weight increase was 0.25 μg/kg, while 15 mg/kg chlordecone was required. However, a linear increase in weight was seen with doses of chlordecone between 7.5 and 30 mg/kg.

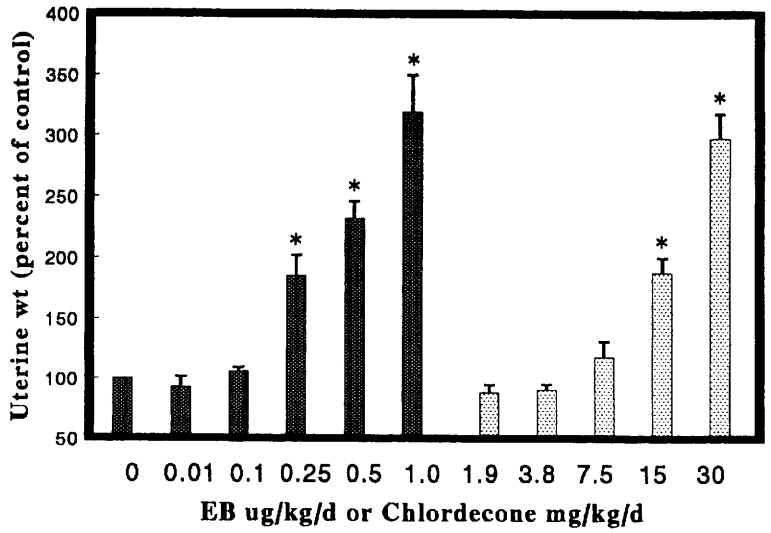

Figure 1. Uterine weight of ovariectomized immature rats injected (sc) daily for 3 days with various doses of estradiol benzoate (EB) (dark bars) or chlordecone: animals were autopsied 24 hr after the last injection. The results of several assays are combined and expressed as a percentage of uterine weight of control animals in each assay that were treated with oil vehicle. SEM for groups of 6–15 animals is shown by vertical line. The asterisk indicates significant ($P < 0.05$) difference from control calculated on nontransformed data.

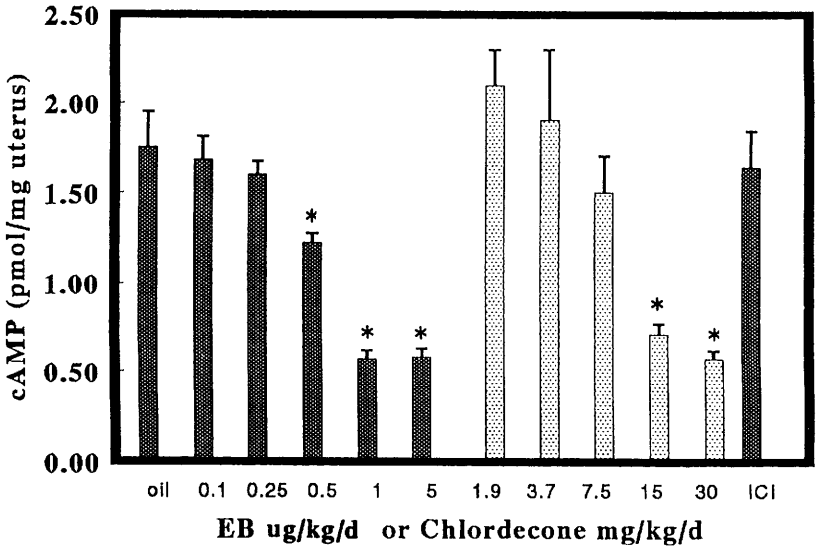

Figure 2. The uterine cAMP content at 72 hr after the first of three doses of estradiol benzoate (dark bars) or chlordecone. The cAMP content of uteri of animals exposed to 0.5 mg/kg of the anti-estrogen ICI-182,780 (ICI) is shown on the right. SEM for groups of at least five animals is shown by vertical lines. Values with an asterisk are significantly ($P < 0.05$) different from those of control animals treated with oil vehicle.

Larger doses of this compound were not tested to avoid acute toxic effects. Although larger doses of EB produced larger uteri (i.e., 5 μg/kg EB produced a 60% increase over those found with 1 μg [196.1 ± 0.4 vs 120.6 ± 0.3 mg/100 g]), the smaller dose was used for all other studies.

Uterine cAMP content measured at 72 hr is shown in Figure 2. A dose of 0.5 μg/kg, EB reduced cAMP content significantly, but 15 mg/kg of chlordecone was needed to obtain a significant reduction. The reductions obtained with 1 μg/kg EB and 30 mg/kg chlordecone were similar, as were the uterine weights produced, and this formed the basis for the rationale of using these two doses for other studies. Changes in uterine cAMP content with time after initial exposure to the compounds are shown in Figure 3. Although chlordecone significantly reduced cAMP content within 48 hr, 72 hr was required to obtain the same reduction using EB. In these groups, uterine weight was significantly increased at 24 hr in animals given

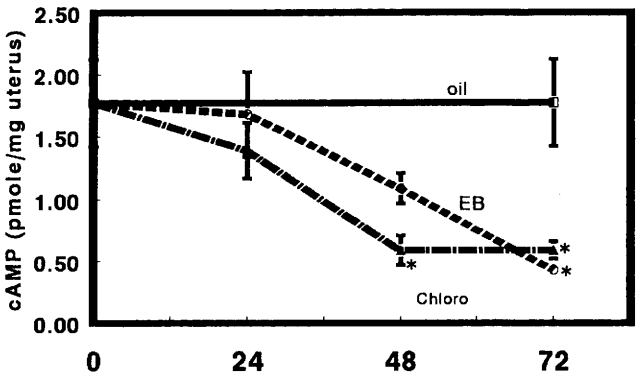

Figure 3. Uterine cAMP content, determined after the first (24 hr), second (48 hr), or third (72 hr) dose of 1 μg/kg estradiol benzoate or 30 mg/kg chlordecone, compared with that of animals injected with oil vehicle. Vertical lines indicate SEM for groups of at least five animals. Asterisks indicate values significantly different from those of control animals.

chlordecone, but not until 48 hr in those receiving EB (Fig. 4). However, there were no significant differences in uterine weights obtained with EB compared with those with chlordecone at any of the times examined.

The pure anti-estrogen ICI-182,780 inhibited the uterotropic effect of both EB and chlordecone (Fig. 5). A dose of 0.1 mg/kg/day reduced the effect of 1 μg EB/kg by 83% and with 0.5 mg/kg/day uterine weight was slightly, but not significantly, below that of control animals injected with oil vehicle. The anti-estrogen was somewhat less effective against chlordecone than against EB, and even with a dose of 0.4 mg/kg/day uterine weight was significantly higher than that of control animals treated with vehicle. However, a dose of 0.5 mg/kg of ICI-182,780 reduced uterine weight to that of control animals.

Similar to its effect on uterine weight increases, ICI-182,780 prevented, in a dose-dependent fashion, the decrease in uterine cAMP seen with 1 μg/kg EB or 30 mg/kg chlordecone (Fig. 6). As was the case with uterine weight reduction, ICI-182,780 was more effective at counteracting the effect of EB on uterine cAMP than that of chlordecone. With 0.2 mg/kg of ICI-182,780 the effect of 1 μg/kg EB was completely prevented but even with 0.4 mg/kg of the anti-estrogen cAMP was lower ($P < 0.01$) in animals treated with chlordecone than in control animals treated with oil. However, the same cAMP levels found in controls were obtained with 0.5 mg/kg/day of ICI-182,780. The basal level of uterine cAMP was not significantly depressed by treatment of the animals with oil plus 0.5 mg/kg ICI-182,780 (Fig. 2).

In an attempt to determine whether estrogens reduced cAMP by lowering adenylyl cyclase, we used forskolin to stimulate directly the enzyme in uterine

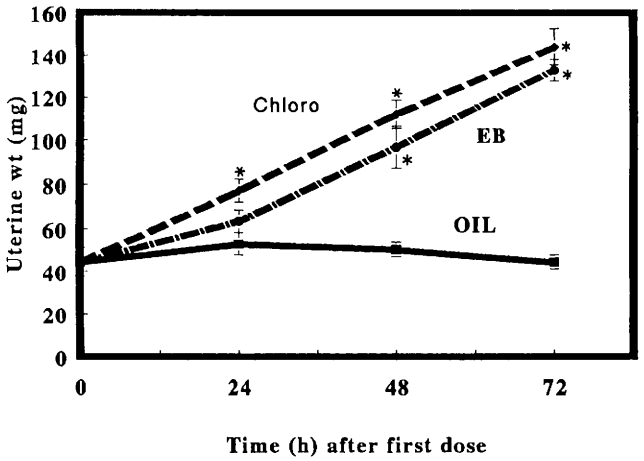

Figure 4. Uterine weight changes after the first (24 hr), second (48 hr), or third (72 hr) injection of EB or chlordecone. Vertical lines indicate SEM for groups of five animals, and asterisks indicate significant increases over values obtained in control animals.

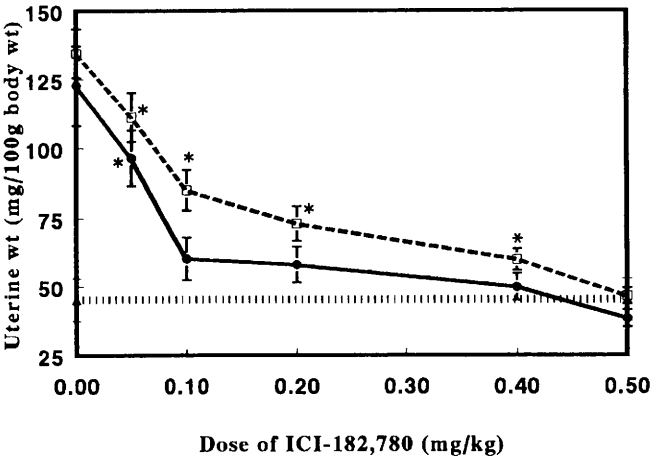

Figure 5. Inhibition of uterine weight increased by 1 μg/kg estradiol benzoate (solid line) or 30 mg/kg chlordecone (dashed line) injected (sc) daily for 3 days at a site different from that for the injection of anti-estrogen (ICI-182,780). The bottom dotted line represents uterine weight of animals given the anti-estrogen plus 0.1 ml of oil vehicle. Vertical lines indicate SEM for groups of at least five animals. Significant differences from control values are indicated by an asterisk.

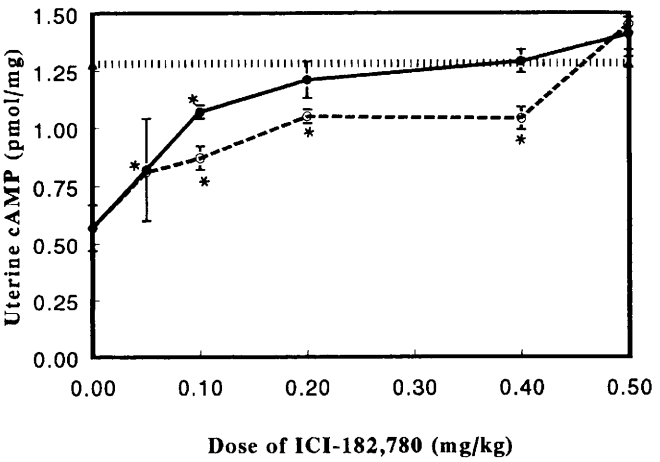

Figure 6. Immunoassayable cAMP extracted from uteri of ovariectomized immature rats injected daily with 1 μg/kg estradiol benzoate (solid line) or 30 mg/kg chlordecone (dashed line) plus the indicated dose of anti-estrogen (ICI-182,780). The cAMP content of control animals injected with 0.1 ml oil is indicated by the top horizontal line. Vertical lines indicate SEM for groups of at least five animals. Significant differences from control values are indicated by an asterisk.

homogenates. Forskolin has been shown to activate all six mammalian forms of adenylyl cyclase (17). As shown in Figure 7, the adenylyl cyclase activity in uterine membranes was retained in animals treated with EB or chlordecone. The simultaneous exposure to antiestrogen EB or chlordecone restored the cAMP content but had little effect upon the inherent cyclase enzyme activity.

Discussion

In the adult rat, only the uterine liminal epithelial cells show mitogenic activity in response to estrogen,

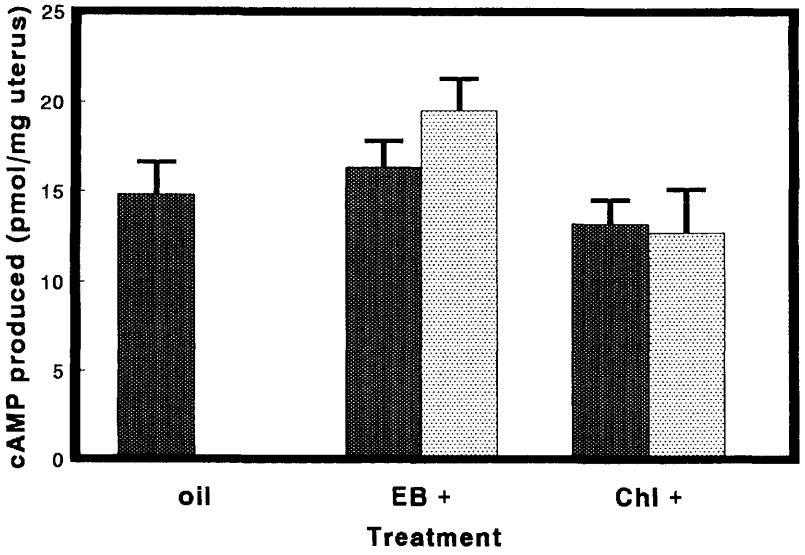

Figure 7. Cyclic AMP produced by uterine homogenates incubated with 10 μ*M* forskolin for 15 min at 25°C. The darker bars indicate the cAMP produced by homogenates obtained 72 hr after exposure to either oil, 1 μg/kg estradiol benzoate (EB), or 30 mg/kg chlordecone (CHL). Values for animals treated with ICI-182,780 plus the estrogen (+) are indicated by the lighter bars. SEM for groups of five animals is shown by vertical line.

whereas in immature rats all uterine cells respond (18). Furthermore, a cardinal estrogenic response, upregulation of the progesterone receptor, is not produced by chlordecone in adult, but only immature rat uteri (19). Therefore, in the present study we chose to use uteri of ovariectomized immature animals 4 days after ovary removal. We believe this a sufficient time for full recovery from the stress of the surgery and for the complete disappearance of the already low levels of endogenous estrogens in these animals.

Clearly, exposure to estradiol or chlordecone for at least 48 hr produced a significant reduction in uterine content of cAMP. Furthermore, there was a rough correlation with doses of these estrogens that increased uterine weight and those that lowered cAMP content (Fig. 1 and 2). Lowering of cAMP by estrogen has been reported by Flandroy and Galand (7), who found that 1 μg of 17β-estradiol slightly, but significantly, reduced immature rat uterine cAMP when measured 24 hr later; the acute effect was an increase in cAMP within 8 min. We did not find a decrease in cAMP within 24 hr, even though uterine weight was significantly elevated by chlordecone, but not estradiol, at this time. Exposure to the estrogens for 48 hr, and especially 72 hr, however, resulted in more than a 50% decrease in cAMP. Fanidi *et al.* (13) reported that 1 μg/kg of estradiol reduced cAMP content of the mouse uterus by 74%, while increasing uterine phosphodiesterase (PDE) activity by 50%. They also found that an increase in dose to 5 μg/kg doubled uterine weight over that seen with 1 μg/kg but increased PDE only another 13%, and was without further effect upon uterine cAMP content. These authors concluded that estrogen lowered cAMP by upregulating PDE activity but did not provide evidence that PDE was quantitatively increased (i.e., only activating factors may be involved). Although we did not measure PDE activity, the lowered uterine cAMP found in the present study, coupled with the fact that adenylyl cyclase stimulated directly by forskolin was not altered, is also consistent with such a conclusion. The fact that a potent antiestrogen, which binds the nuclear estrogen receptor with an affinity only 12% less than that of estradiol (16), prevented the loss of cAMP, also without altering adenylyl cyclase activity, indicates that the mechanism(s) responsible for cAMP reduction involve an estrogen receptor-mediated response.

The role of cAMP in uterine physiology is unresolved. Administration of exogenous cAMP or cholera toxin, which increases endogenous cAMP, can produce estrogenic effects in the uterus (10–12). A recent study has shown that cAMP, but not cGMP, increased progesterone receptor content of cultured rat uterine cells to the same extent as estradiol or insulin-like growth factor-I (12). Furthermore, this study found that the antiestrogen ICI-164,384, which has a similar structure but a lower affinity for the estrogen receptor than ICI-182,780, inhibited this action of cAMP. But if cAMP can mimic the actions of estrogen, what could be the physiological significance of lowering uterine cAMP with longer exposure to estradiol? We believe that logic requires an examination of the functions that involve both genomic estrogen responses and cAMP actions (i.e., phase I and phase II responses) (20). A possible example of this is provided by the cystic fibrosis transmembrane regulator (CFTR) gene which codes for synthesis of a chloride channel and whose expression in the uterus has been shown to be controlled by estrogen (21). CFTR is functionally activated by cAMP, and thus increased levels of the nucleotide would be expected to be an important factor in uterine water imbibition seen after initial exposure to estrogen (20). The latter is only a transient response, and continued elevated levels of the CFTR activator (cAMP) that would provide fluid transfer into the uterine lumen would not appear appropriate for

delayed responses to estrogen, particularly those associated with pregnancy.

The estrogenicity of chlordecone, a polychlorinated hydrocarbon, has been established by several experimental studies (14, 15, 22). The compound has been shown to induce uterine growth, increase synthesis of progesterone receptors in immature uteri and initiate embryo implantation and maintain pregnancy in hypophysectomized rats treated with progesterone (14, 15, 22). Although the compound has only a low affinity for the estrogen receptor (10^{-5} *M*) its estrogenic activity *in vivo* may be related to its long biologic half-life (initially 8.5 days) (23), which allows long-term interaction with the estrogen receptor. The results of the present study using ICI-182,780 support the conclusion that chlordecone estrogenicity probably involves an estrogen receptor. However, the amount of anti-estrogen needed to inhibit completely the activity of such a weak estrogen was somewhat surprising. With the low affinity of chlordecone for the estrogen receptor, one might expect a higher efficiency of the anti-estrogen on the action of chlordecone. However, until the pharmokinetics of chlordecone and ICI-182,780 have been resolved predictions regarding competitive receptor inhibition are unreliable.

In conclusion, exposure to a potent estrogen, estradiol, or a weak xenoestrogen, chlordecone, has a reciprocal effect upon uterine weight and cAMP content. Response of the uterine membranes to direct stimulation forskolin indicates that the loss of cAMP is not due to a loss adenylyl cyclase. Because the decrease in cAMP and the increase in uterine weight were prevented by simultaneous exposure to a potent pure antiestrogen suggests that the mechanism(s) involved in both responses is a result of estrogen receptor function. The consequences, or the significance of the reduction in cAMP requires further study into interactions between nuclear and membrane receptor functions.

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1. Carson-Jurica MA, Schrader WT, O'Malley BW. Steroid receptor family: Structure and functions. Endocr Rev **11**:202–211, 1990.
2. Szego CM. Mechanisms of hormone action: Paralels in receptor-mediated signal propagation for steroid and peptide effectors. Life Sci **35**:2383–2396, 1984.
3. Mobbs CV, Kaplitt M, Kow L-M, Pfaff DW. PLCα: A common mediator of the action of estrogen and other hormones. Mol Cell Endocrinol **80**:C187–C191, 1991.
4. Anuradha P, Khan SM, Karthikeyan N, Thampan RV. The non-activated estrogen receptor (naER) of the goat uterus is a tyrosine kinase. Arch Biochem Biophys **309**:195–204, 1994.
5. Wehling M. Nongenomic actions of steroid hormones. Trends Endocrinol Metab **5**:347–353, 1994.
6. Singhal RL. Cyclic adenosine 3′5′-monophosphate and estrogen stimulation of uterine metabolism. Adv Pharmacol Chemoth **11**:99–150, 1973.
7. Flandroy L, Galand P. Changes in cGMP and cAMP content in the estrogen-stimulated rat uterus: Temporal relationship with other parameters of hormonal stimulation. J Cyclic Nucl Res **4**:145–158, 1978.
8. Boynton AL, Whitfield JF. The role of cyclic AMP in cell proliferation: A critical assessment of the evidence. In: Greengard P, Roison GA, Eds. Advances in Cyclic Nucleotide Research. New York: Raven Press, Vol **15**:pp193–294, 1983.
9. Burgering BMT, Bos JL. Regulation of Ras-mediated signalling: More than one way to skin a cat. TIBS **20**:18–22, 1995.
10. Rath NC, Prasad RN. Action of 3′5′-adenosine monophosphate on the uterus of the ovariectomized rat. Fertil Steril **25**:724–730, 1974.
11. Stewart PJ, Webster RA. Intrauterine injection of cholera toxin induces estrogen-like uterine growth. Biol Reprod **29**:571–679, 1983.
12. Aronica SM, Katzenellenbogen BS. Progesterone receptor regulation in uterine cells: Stimulation by estrogen cyclic adenosine 3′5′-monophosphate and insulin-like growth factor I and suppression by antiestrogens and protein kinase inhibitors. Endocrinology **128**:2045–2052, 1991.
13. Fanidi A, Ahnadi C, Fayard JM, Pageaux FJ, Laugier C. Opposite regulation of cAMP concentration in the quail oviduct and the mouse uterus by tamoxifen. Correlation with estrogen-antagonist and estrogen-agonist activity. J Steroid Biochem Mol Biol **41**:571–577, 1992.
14. Hammond B, Katzenellenbogen BS, Krauthammer N, McConnell J. Estrogenic activity of the insecticide chlordecone (Kepone) and interaction with uterine estrogen receptors. Proc Natl Acad Sci USA **76**:6641–6645, 1979.
15. Eroschenko VP. Estrogenic activity of the insecticide chlordecone in the reproductive tract of birds and mammals. J Toxicol Environ Health **8**:731–742, 1981.
16. Wakeling AE, Dukes M, Bowler J. A potent specific pure antiestrogen with clinical potential. Cancer Res **51**:3867–3873, 1991.
17. Iyengar R. Molecular and functional diversity of mammalian Gs-stimulated adenylyl cyclases. FASEB J **7**:768–775, 1993.
18. Tachi C, Tachi S, Linder HR. Modification by progesterone of oestradiol-induced cell proliferation, RNA synthesis and oestradiol distribution in the rat uterus. J Reprod Fertil **31**:59–76, 1972.
19. Eckols K. Williams J, Uphouse L. Effects of chlordecone on progesterone receptors in immature and adult rats. Toxicol Appl Pharmacol **100**:506–516, 1989.
20. Huett YM, Dey SK. Role of early and late oestrogenic effects on implantation in the mouse. J Reprod Fertil **81**:453–458, 1987.
21. Rochwerger L, Buchwald M. Stimulation of the cystic fibrosis transmembrane regulator expression by estrogen in vivo. Endocrinology **133**:921–930, 1993.
22. Johnson DC, Sen M, Kogo H, Dey SK. Initiation of embryo implantation and maintenance of early pregnancy in the rat by chlordecone (Kepone). Proc Soc Exper Biol Med **195**:44–50, 1990.
23. Guzelian PS. Comparative toxicology of chlordecone (Kepone) in humans and experimental animals. Annu Rev Pharmacol toxicol **22**:89–113, 1982.