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INHIBITION OF INTERCELLULAR COMMUNICATION IN RAT LEYDIG CELLS IN VITRO BY XENOBIOTIC CHEMICALS

By

Hong Hsu

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ABSTRACT

INHIBITION OF INTERCELLULAR COMMUNICATION IN RAT LEYDIG CELLS IN VITRO BY XENOBIOTIC CHEMICALS

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Hong Hsu

The modulation of gap junctional intercellular communication (GJIC) by xenobiotic chemicals has been investigated in a rat leydig cell line, LC 540. GJIC was measured using the scrape-loading/dye transfer and fluorescence recovery after photobleaching (FRAP) techniques. GJIC was inhibited in these cells following 1 hr treatment with 12-O-tetradecanoyl phorbol-13-acetate (TPA), Dieldrin, Heptachlor (HC), Aroclor-1254 (A-1254) and a number of other xenobiotics. Such dose and time inhibition of GJIC induced by TPA, Dieldrin, and HC was found to be associated with an increase in translocation of protein kinase C (PKC) to the plasma membrane and also an increase in the free intracellular calcium level, $[Ca^{++}]_i$. It was also found that the inhibition of GJIC by A-1254 was preceded by a transient increase in $[Ca^{++}]_i$, but not associated with translocation of PKC. Northern blot analysis of total mRNA demonstrated that LC 540 cells expressed connexin 43, but not connexin 32 mRNA. The level of connexin 43 mRNA was affected by TPA or Dieldrin, increased by 1 hr and decreased by 24 hr treatments. In addition, the 1 hr treatment by either TPA or Dieldrin induced the expression of connexin 26 mRNA.

This results indicates that gap junctional communication can be regulated by multiple mechanisms including functional state and mRNA expression.

To my parents with love

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INTRODUCTION

Gap junctions are clusters of intercellular channels that allow small molecules to pass from one cell to another. It has been demonstrated that gap junctions play an important role in coordinating cellular signals (15). Intercellular communication mediated by gap junctional protein channels was shown to be down-regulated by endogenous agents such as hormones, growth factors as well as exogenous agents such as neurotoxins, drugs, environmental pollutants, etc (26). In addition many xenobiotic chemicals which have been shown to be teratogens, tumor promoters and neurotoxins were linked to their ability to inhibit gap junctional intercellular communication (GJIC) in vitro (21), suggesting that inhibition of GJIC may be a useful biomarker to identify potential environmental toxicants. However, in view of the possibility of differences among structurally diverse xenobiotics on GJIC inhibition, it would be pertinent to compare the modulation of GJIC by different chemicals. Such studies provide meaningful assessment of the potential toxic effects of environmental chemicals.

In the present investigation, a rat testicular leydig cell derived cell culture system was used to examine the effects of several environmentally important xenobiotic chemicals on GJIC. The results indicate that the rat leydig cell line is a useful in vitro model to study cell-cell communication and that different xenobiotics exhibit different effect on blockage of GJIC. One of the biochemical mechanisms in

the regulation of the gap junctions is exemplified by TPA -- a classic tumor promoter, activates the phospholipid/Ca^{++} - dependent enzyme, protein kinase C (41). Furthermore, intracellular free Ca^{++} changes have been observed when the rat leydig cells were exposed to some xenobiotics, suggesting that other biochemical mechanisms underlying GJIC inhibition. In addition, the gap junction expressed in rat leydig cells has been identified as connexin 43. The expression of the mRNA encoding connexin 43 was found to be affected by treatment with some chemicals. Thus, data support the hypothesis that GJIC can be regulated at different levels, i.e. transcriptional, translational or post-translational level. Therefore, it is necessary to study more precisely the biochemical and molecular mechanisms by which xenobiotics affect GJIC.

LITERATURE REVIEW

A. Gap junction: a functional definition

Gap junctions are intercellular structures that link contacting cells and allow ions and small metabolites to pass directly from cell to cell, thereby coupling the cells both electrically and metabolically (1). "Cell-cell communication of this type was first demonstrated physiologically in 1958, but it took more than 10 years to show that this physiological coupling correlated with the presence of gap junction seen in the electron microscope. The initial evidence for cell coupling came from electro-physiological studies of specific pairs of interacting nerve cells in the nerve cord of a crayfish." (1). Later experiments using small fluorescent molecules revealed that the molecules capable of passing through gap junctions are no greater than 1000 to 1500 daltons (1). The pore size of the connecting channels is estimated to be about 1.5 nm. It is also inferred that coupled cells share their small molecules (such as inorganic ions, sugars, amino acids, nucleotides, and vitamins), but not their macromolecules (proteins, nucleic acids, and polysaccharides). In 1966, Potter et al.(2) found that gap-junctional communication was present between all cells in early embryos, in spite of the final developmental fate. The extensive occurrence of gap junctions indicate that they play an important part in coordinating cellular signals, including those involved in growth control and embryogenesis (3). The aim of this

review is to outline current opinion on some properties of gap junctions.

B. Structure of gap junction

Gap junctions are constructed from transmembrane proteins that called connexins encoded by a gene family (1). Each cell contributes with an hemichannel called connexon which consists of 6 subunits. In hepatocytes, each subunit is thought to be constructed of a 27 kD protein (4). In an integrated junction, two such connexons are directed end to end forming a channel of 1.5-2.0 nm diameter. DNA sequencing studies suggest that the polypeptide chain consists of about 280 amino acid residues, and crosses the lipid bilayer as four α helices (1). Two loops of the polypeptide protrude into the intercellular gap, where they interact closely with related loops of the proteins which supplied by the connexon in the plasma membrane of the neighboring cell (4). Gap junction protein has been imaged as a three-domain protein from X-ray diffraction. It is composed of a transmembrane domain, a cytoplasmic domain, and a gating domain (5). A monoclonal antibody to the rat liver 27 kD protein can bind specifically to the junction resulting in obstruction of intercellular communication (6). Recently, cDNA coding for the '27 kD' protein has been cloned. The cDNA sequences derived from rat liver (7) and human liver (8) are almost identical.

C. Importance of gap junction

1. Normal development

In a multicellular organism, the activities of cells in a tissue are coordinated. Such intercellular coordination requires a well developed system for intercellular signaling. Within a target organ, cells can transduce signals metabolites of low molecular weight and share them with neighboring cells through junctional channels. Groups of cells along different developmental pathways may lose the ability to communicate with each other (9). " Gap junctions appear de novo during compaction in the eight-cell stage of mouse development. This is a critical event in the life of the embryo, because gap junctional intercellular communication is an essential requirement for maintaining compaction and , hence , for development of the blastocyst." (10). The variation in the patterns of dye transfer with cell position have been reported in the early Xenopus embryo. Cells located in future dorsal regions of the animal pole communicated with their neighbors both more frequently and more extensively than those in future ventral regions at the 32-cell stage (11). Whether these patterns of dye transfer are linked directly to a developmental role for gap junctions is unknown. However, the evidence for a functional role for gap junctions in development stems from experiments using antibodies to obstruct junctional communication, which produces pattern defects in the region derived from the antibody-injected cell in the early neurula stage of Xenopus (12). The initiation of gap junctions assembly is an accurately timed event when they first present during compaction in the eight-cell stage of mouse development.(Reviewed by Kidder, 13). In 1983, McLauglin et al. found that the timing of gap junction assembly is unaffected by agents that disrupt gene expression, but constant transcription and

protein synthesis were required to the full extent of intercellular coupling (14).

Understanding of the mechanism of gap junction assembly and its temporal regulation is so important that this system would provide valuable insight into early mammalian development (10).

2. Embryogenesis

Cell coupling via gap junctions appear to be important in embryogenesis. In 1979, Loewenstein reported that gap junctions were found between cells of most embryonic and adult tissue. Furthermore, their presence can be detected functionally by monitoring the intercellular passage of electrical currents (ionic coupling) or fluorescent dye (15). Whether the changes in cell coupling are the cause or effect of cell determination become a question when it appears that dye couplings are restricted in groups of embryonic cells. The analysis of the temporal-spatial relation between cell determination and changes in intercellular communication may be the first clue to answer the question. The term "communication compartments" was used to describe those separate domains of cells that are well coupled to each other but uncoupled or poorly coupled to cells beyond a defined boundary (16). The temporal and spatial distribution of these compartments imply that gap junctions may play a role in regulating the ongoing development. In 1984, Warner et al. mentioned more direct experiments about polyclonal antisera directed against purified mammalian gap junction proteins being used to inhibit intercellular communication. The affinity-purified antibodies to the 32-kD rat liver gap junction protein were microinjected

into particular blastomeres in the Xenopus levis embryo , which resulted in both a reduction in junctional conductance and evident defects in the development of dorsoanterio structures, including derivatives of the neural tube (17). Communication through gap junctions was also examined in 8-cell zygotes generated by fertilization of eggs of the DDK inbred strain of mice with spermatozoa of the C3H strain by Mia Buehr et al. (18) in 1987. These authors observed that the zygotes developed from eggs of the DDK strain fertilized by foreign spermatozoa were characterized physiologically by defective gap junctional communication. Improving gap junctional communication is sufficient to allow many zygotes to maintain the compaction, indicating a relationship between compaction and communication through gap junctions. More recently, Gimlich et al. studied Xenopus embryos. They used low-stringency screening of cDNA libraries with cloned mammalian gap junction mRNAs to distinguish mRNAs that potentially encode gap junction proteins in the Xenopus oocyte and early embryo. The levels of these mRNAs show a strikingly different temporal regulation and tissue distribution (19). These results suggest that intercellular communication during oocyte growth and postfertilization development is a complex phenomenon involving the coordinated regulation of several genes.

3. Regulation of cell proliferation.

Under normal conditions, some adult cells never replicate their DNA nor divide (nerve cells), others only rarely do so (liver cells), while others retain a rapid division rate (intestinal cells, bone marrow cells). Usually, cells cooperate with each

other to form integral components of an organ. Based on the results from his and other's laboratories, Loewenstein et al. were the first to hypothesize that communication could be important for regulation of cell growth (15). Within this concept it was considered that growth regulating molecules could penetrate gap junctional channels and thereby maintain the growth of cell and its integrity under tight constraints. It was further postulated that if cells lost junctional communication with neighboring cells, their growth might become unchecked leading to neoplasia (15, 20).

" The process of gap junctional-intercellular communication is operationally dependent on a cell's ability to recognize and dock with another cell, to organize the gap junction subunits into functional channels, to transfer regulatory ions and small molecules and to have appropriate subcellular components to respond to these regulatory signals." (21).

4. Tumorigenesis

Gap junctional intercellular communication is an important biological process which is required to maintain homeostasis within a tissue. The regulatory metabolites exchanged between cells can be modified and result in up or down regulated gap junctional intercellular communication, and thereby provide adaptive means to regulate cell growth, differentiation and the control of differentiated functions in normal tissue (22,23). Thus, blockage of the exchange of important "signal" ions and molecules between normal communicating cells could lead to abnormal cell

proliferation, as well as tumorigenesis (24). The identification of junctional defects has been emphasized by the primary work on the role of junctional communication in tumorigenesis, either by electron microscopy of tumor tissues or the measurement of coupling in tumor cell cultures (25). The gap junctional communication can be blocked either by chemicals (tumor promoters and other xenobiotics), endogenous growth factors, hormones, expressed oncogenes and high intracellular free Ca^{++} levels. The consequence of the inhibition of gap junction at intercellular communication is to promote the expansion of initiated cells, which are unable to terminally differentiate, as a result of escaping the suppressing effect of surrounding and communicating normal cells (26).

5. Cellular toxicity

When chemicals interact with a higher organism, three cellular responses might happen, according to the different circumstances. First of these, the genetic information of cells might change (mutagenic); Second, cell death might occur via the ability of chemicals to disrupt membrane integrity and inhibit vital enzymes, etc (cytotoxic agents); Third , modulation of genetic expression (gene modulation) might be triggered (27). Generally, at the whole organism level, both beneficial/adaptive and harmful/maladaptive responses can be caused when exposure to chemicals occurs. The chemical modulation of gap junctional intercellular communication as a cellular mechanism of chemical toxicity could give rise to a wide variety of harmful results (21). It has also been suggested that intercellular communication may have

a role in tumor promotion. In 1979, Yotti et al. (28) and Murray and Fitzgerald (29) independently reported that metabolic cooperation between cells in culture was blocked by the classical tumor promoter TPA (12-O-tetradecanoyl phorbol-13-acetate). A mechanism of tumor promotion was proposed which hypothesize that the increased proliferation of "initiated" cells and subsequent tumor development might be induced by tumor promoters (e.g., TPA) which block intercellular communication and the transfer of the transfer of growth inhibitory signals via gap junctions. Increased proliferation allows initiated cells to gain a selective growth advantage and increased probability of accumulating further mutational events, resulting in the autonomous, promoter-independent growth recognized as tumor progression (30). In recent years, "genotoxicity" has become a very important concept to study chemical toxicity (31).

It is clear that mutagens can be harmful, but not all harmful chemicals are mutagenic. It has already been showed that genotoxic chemicals at noncytotoxic levels do not inhibit intercellular communication (27). Cytotoxic action of genotoxic and nongenotoxic agents could inhibit intercellular communication indirectly, by causing some contiguous communicating cells to die, thereby physically disrupting intercellular communication (21). Tumor promoters, that can inhibit intercellular communication at noncytotoxic levels, have been considered to act either at the membrane level, or the genetic level, depending on various theories.

D. Mechanisms of gap junction regulation

1. Biochemical mechanism

The permeability of gap junctions is rapidly (within seconds) and reversibly decreased by reducing cytosolic pH or increasing the cytosolic concentration of free Ca^{++}. In some tissues the permeability can be regulated by the voltage gradient across the junction or by extracellular chemical signals. It seems that gap junctions are dynamic structures that can change in response to different stimuli in the cell. Loewenstein (15) reported that regulation of the state of the channels was first attributed to the effects of changes in the intracellular Ca^{++} concentration, with enhanced Ca^{++} producing uncoupling. Spray et al. (33) have indicated that uncoupling may be a subsequent result of increased internal H^{+} concentrations. Based on the experimental observations (34), the physiological potentials of the two ions should be considered. "For Ca^{++}, the gradients are steep and poised to raise $[Ca^{++}]_i$ whenever a cell becomes leaky or its energy metabolism fails. For H^{+}, on the other hand, the electrochemical gradients are precisely in the wrong direction for that" as suggested by Loewenstein, W.R. (35). The reason for the Ca^{++} control seems clear. When a cell dies or is damaged, ions such as Ca^{++}, and Na^{+} move into the cell across the leaky membrane. If the cell were to keep coupled to its healthy neighbors, these two would suffer a dangerous disturbance of their internal chemistry. But the influx of Ca^{++} into the sick cell effectively separates it and prevents damage from spreading by shutting off the gap junction channel (1). Among the cellular ions, Ca^{++} is rather unique in crystal and easily interacts specifically with protein structure. Progress has taken advantage again and again of this special Ca^{++} talent for the signal process (35). The data from Saez et al. (36) propose that halomethanes

decrease gap junction between hepatocytes due to free radical generation via cytochrome P^{450} oxidation metabolism. The action of free radicals may be through direct or indirect oxidation of the gap junction protein of a cytoplasmic regulatory molecule directly or indirectly, or even of the membrane lipids (37).

The latest elements to join the panel of junctional control are protein kinases. Recent studies of the kinase behavior showed phosphorylation of gap-junction proteins and activation of their synthesis (38). The regulation of many cellular functions is modulated by two major signal transducing systems. Some are those receptors linked to the formation of cAMP-dependent protein kinase (PKA). Some studying showed that cAMP is an important regulator of junctional permeability. An elevation in intracellular cAMP level has been shown previously to enhance permeability and to increase the number of gap junction particles (15). On the other hand, C kinase (PKC), an enzyme that is activated by the receptor-mediated hydrolysis of inositol phospholipid, may regulate many Ca^{++} dependent processes depending on a variety of extracellular signal across the membrane. Such a critical role of this protein kinase system seems to give a logical basis for signal transduction, and a new dimension to understand cell-to-cell communication (39). In 1977, PKC was identified as a proteolyticly- activated protein kinase, and is now known to be widespread in various tissues and organs (39). In most tissues, this enzyme is recovered mainly from the soluble fraction as an inactive form, and is obviously translocated to membranes in a Ca^{++} -dependent way when cells are stimulated (39). Kishmoto in 1980 (40) and Kaibuchi in 1981 (41) did kinetic analyses and reported

that a small amount of diacylglycerol dramatically enhanced the affinity of PKC for Ca^{++} , completely activating the enzyme without any change in Ca^{++} levels. Phorbol ester tumor promoters have been shown to specifically bind to and activate PKC by acting as an analog of diacylglycerol (42). Other investigations also confirmed that C-kinase has a role in tumor promotion (43). In 1979, Yotti, et al (28) demonstrated that TPA is a very strong inhibitor of gap junctions. It is also indicated that increased cAMP concentration almost fully protected the cells against the inhibitory effects of TPA. The mechanisms for the effects both in inhibition and promotion need to be investigated, and it is inferred that gap junctional protein may be a direct substrate for PKC and PKA (42) and that PKA and PKC have opposing role in its regulation (44).

2. Molecular mechanism

The regulation of gap junction at the transcription level and probably at the protein level by protein kinases is of major interest (45). The influence of various growth factors on gap junctional intercellular communication was reported in normal human epidermal keratinocytes (NHEK) in 1989 by Madhukar et al.(46). The study demonstrated that growth factor/hormones on normal cell proliferation may be correlated with gap junctional communication . The inhibition of GJIC in the NHEK cells can be caused by the epidermal growth factor (EGF) and transforming growth factor ß (TGF-ß), EGF acted as a mitogen while TGF-ß suppressed DNA synthesis and induced cell differentiation (46). An interesting extension including tyrosine

protein kinases has come from observations in which junctional communication was examined in a temperature-sensitive mutant of cells infected with Rous sarcoma virus at permissive and non-permissive temperatures (47). When the src gene was active, communication through gap junctions was profoundly decreased. The function of tyrosine protein kinases in gating of gap junction channels may involve interaction with PKA and PKC regulatory cascades (45). For example, the PKA inhibitor protein is 80-90% inactivated when tyrosine is phosphorylated by the EGF receptor (48). In some systems, $pp60^{v\text{-}src}$ is known to activate the PKC pathway (49). Hence, PKA and PKC pathways may be invoked by tyrosine kinase. It is possible that the molecular mechanism controlling gap junctional communication is the activation of cAMP-dependent protein kinases by hormone-induced signals passed from receptor-bearing cells to receptorless partners (50). " Of special interest is the hormone-manipulated control of transcription of mRNA for the connexins. So, a great deal remains to be learned" (45).

MATERIALS AND METHODS

A. Cell Culture

The major portion of the work presented in this thesis was carried out using a rat testicular leydig cells line--LC 540, originally obtained from American Type Culture Collection (ATCC), Rockville, MD. While these cells were derived from tumors, they exhibited a significant ability to perform gap junction mediated intercellular communication, and therefore, were considered suitable for studying chemical modulation of intercellular communication. The cells were routinely cultured in the laboratory on a modified DMEM (Dulbeccos' Modified Eagles' Medium), which contained Earle's balanced salt solution with a 50% increase of vitamins and essential amino acids except glutamine, a 100% increase of non-essential amino acids and 1 mM sodium pyruvate, 5.5 mM glucose, 14.3 mM NaCl, 11.9 mM $NaHCO_3$. The medium was supplemented with 5% fetal bovine serum and 50μg/ml gentamicin was used as an antibiotic. The cells were subcultured once every week at a seeding density of 10% confluence. For protein kinase C assays, the cells were seeded in 75 cm^2 Corning tissue culture flasks and were used at confluence. For cell-cell communication assays and for measuring intercellular free calcium, the cells were seeded in 35 mm tissue culture dishes at different densities depending upon the type of assay.

B. Chemicals

TPA (12-O-tetradecanoyl phorbol-13-acetate), Estradiol, and Ethinyl Estradiol were obtained from Sigma Chemicals, St. Louis, MO. HC (Heptachlor), HE (Heptachlor Epoxide) were from Velsicol Chemical Corporation, Chicago, IL. Dieldrin, p,p'-DDT were purchased from Shell Development Corporation, Modesto, CA. Aroclor-1254 and Fire Master BP-6 were a gift from Dr. Matthew Zabik, Pesticide Research Center, Michigan State University. Lindane was obtained from Chemical Services, Westchester, PA. Mirex was a gift from Allied Chemical, New Jersey. All of the above chemicals were dissolved in ethanol, except Mirex which was dissolved in ethanol : Acetone (1:1).

The fluorescent dyes: lucifer yellow, 5,6 carboxyfluorecein diacetate(CFDA) and Fluo-3 were obtained from Molecular Probes Inc., Eugene, OR. Stock solutions of these chemicals were made in phosphate-buffered saline(PBS) (for Lucifer yellow) or ethanol (for 5,6 CFDA and Fluo-3).

[Γ-^{32}P]ATP and [α-^{32}P]dCTP (sp.act.3000 Ci/mmol) were supplied by Amersham International.

All other biochemical used in the investigation were of the highest purity available. Agarose (Low EEO) was from Boehringer Mannheim Biochemical, Indianapolis, IL. Random Primed DNA Labeling Kit was from Boehringer Mannheim, W. Germany. All of the solutions and buffers used for molecular biology were made in DEPC treated water and sterilized either by autoclaving or filtration.

C. Measurement of intercellular communication:

Two different assays were used to measure gap junctional intercellular communication (GJIC) of LC 540 cells under different conditions.

1. Assay for Scrape-Loading / Dye Transfer (SLDT)

a) SLDT (51)

Scrape-loading dye transfer is a rapid and simple technique to study gap junctional intercellular communication. Confluent cultures in 35 mm plates (1.2-1.6 x 10^6 cells) were rinsed several times with PBS and drained after treatment with the test chemicals. 2 ml of 0.05% lucifer yellow in PBS was added to the plates and three scrape lines were made in center of the monolayer with a surgical blade. After 3 minutes to allow dye uptake and transfer at room temperature, the cells were washed several times with PBS (with Ca^{++} & Mg^{++}) and were fixed in 4% phosphate-buffered formalin. The fixed cells were examined under a Nikon epifluorescence phase microscope. Pictures were taken in the regular light and UV light.

b). Quantitation of SLDT assay: (52)

Quantitation of SLDT assay was achieved using the image scan program on the ACAS-570 Laser Spetrocytometer (Meridian instruments, Okemos, MI). Since the dye, Lucifer yellow is fluorescent, an image of the cells loaded with the dye could be quantitated by scanning the formalin fixed cells after scrape loading. A scan of the scrape-loading cell image from a computer program was generated and the integrated value of fluorescence intensity over a boxed area of the scrape line was obtained as

a quantitation of the dye transfer.

2. Laser spectrometric analysis of fluorescence recovery after photobleaching (FRAP) as a measure of intercellular communication: (53)

FRAP (Fluorescence redistribution after photobleaching) is another technique to measure cell-cell communication, which can quantitate rapid dye transfer and detect inhibition of communication by chemical treatment. In this procedure, LC 540 cells growing in 35 mm plates were rinsed with Ca^{++} , Mg^{++} PBS several times and then were stained with 1% 5, 6-carboxyl-fluorescein diacetate dye for 15 minutes in dark at room temperature. After staining, the cells were rinsed several times with PBS, 2 ml of PBS was added and cells were examined on ACAS 570 immediately. 5-6 of labeled contacting cells may then be photobleached by a laser beam, the isolated cell was used as a standard without photobleaching. A computer-generated color image of fluorescence distribution is shown in Fig. 14 that presents the LC 540 cells without treatment (called control sample). The color contour map of each cell presents concentric color rings that join to a white center indicating the nuclear volume (White means the highest fluorescence intensity). At that time just after photobleaching, the labeled cells show green color (low density of dye) inside themselves, however 15 minutes later, the color changes to yellow and red (means higher density of dye). It means that cells were communication competent, the photobleached cells may acquire the dye from the neighboring cells in contact. This recovery of the dye is thus a measure of the gap junction permeability and could be

quantitated using an appropriate software program of the ACAS-570-interfaced computer.

D. Protein Kinase C Assay:

1. Assay for activation of protein kinase C (PKC) -- a calcium and phospholipid dependent protein kinase (54)

a) Preparation of partially purified cytosolic and membrane fractions:

After incubation of the treated cells for the appropriate times, all the operations were processed at 4°C. Cells from two 75 cm^2 flasks were used for each preparation. The cells were rinsed twice with PBS and then rinsed twice with buffer B containing 20 Mm Tris-HCl, pH 7.5, 2 Mm EDTA, 0.5 Mm EGTA, 2 Mm PMSF, 5 Mm 2-mercaptoethanol, and rinsed once with buffer A (Buffer B with 0.33 M sucrose, 100 kIU/ml aprotinin and 25 ug/ml leupeptin). Cells of each preparation or treatment were scraped into 4 ml of buffer A and disrupted in a glass-glass Dounce homogenizer with 30 strokes. The homogenates were sonicated by sonic dismembrator (Fisher, Model 300) at 35% output for 3 x 10 seconds and centrifuged at 25000 rpm (100,000 g) for 1 hour in a Beckman sw 27 rotor. The supernatant was collected as the cytosolic fraction and the pellet was washed twice with buffer B and resuspended with 10 strokes in 4 ml of buffer B in a Dounce homogenizer containing 1% Nonidet P-40 to solubilize PKC tightly associated with the membrane fraction . The solubilized suspension was centrifuged in a Beckman ultracentrifuge at 100,000 g for 1 hour and the supernatant was used as the membrane (particulate) fraction.

PKC from the cytosolic and membrane fractions was partially purified by DE-52 (Sigma, St. Louis, MO) column chromatography as follows: 15 ml of buffer B was used to wash the column and after adding the sample, and the column was washed with 6 ml of buffer B. PKC was eluted with 2 ml buffer B containing 0.1 M NaCl and leupeptin (25 ug/ml). The eluate was used to assay PKC activity.

b) <u>PKC activity assay:</u>

Protein Kinase C activity of the purified fractions was assessed by measuring the incorporation of ^{32}P-Phosphate (from [Γ^{32}P]-ATP) into histone III in the presence of Ca^{++} , phospholipid, and diolein. The reaction was initiated by the addition of 10 μg phosphatidylserine (PS) and 0.5 μg 1,2 diolein (DO) followed by 50 μl of enzyme preparation to reaction mixture. The reaction mixture contained 20 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 0.7 mM $CaCl_2$, 100 μM [Γ^{-32}P]ATP (sp.act.100 cpm/pmol), 250 μg/ml histone III-S, 50 μg/ml leupeptin in a total volume of 250 μl. After incubation at 30°C for 5 minutes, the reaction was terminated by adding 1 ml of cold 25% trichloroacetic acid (TCA) and the solution was kept overnight at 4°C. The precipitates were collected on 0.45 μm cellulose nitrate Millipore filters. The filters were washed 5 times with 3.0 ml of cold 5% TCA, air-dried and counted for radioactivity in a liquid scintillation counter. The kinase activity was expressed as the difference between ^{32}P incorporation into histone in the absence of PS/DO and Ca^{++} from that in the presence of these activators.

c) Lowry Protein Assay (55)

Protein determinations were made according to Lowry's Procedure. Standerization was carried out with BSA. 500 μl of 1:5 or 1:10 diluted protein samples were reacted with 2.5 ml of freshly prepared solution containing 2% Na_2CO_3 in 0.1 N NaOH, 1% $CuSO_4$ and 2% Na^+ K^+ Tartrate. After mixing and standing for 15 minutes at room temperature, 0.25 ml of 1 N feline phenol reagent was added, mixed well and incubated at room temperature for 30 minutes. The absorbance of the samples was read at 660 nm on a Gilford spectro-photometer.

E. Measurement of $[Ca^{++}]_i$ (56)

Intracellular free calcium under different treatment conditions was measured using ACAS-570 laser spectrometer and a new fluorescent dye--Fluo-3 (Molecular Probes). The cells in 35 mm plates were rinsed with Ca^{++} , Mg^{++} PBS several times and stained with Fluo-3 (50μM, dissolved in ethanol) for 1 hour at 37°C. After washing the cells several times with PBS, 1 ml of Ca^{++} , Mg^{++} PBS was left inside the plate. An appropriate computer program (scan image) ACAS 570 was chosen for analyzing the change of Ca^{++} in the cells. After the second scan, 1 ml of PBS containing 2x concentration of the test chemical was quickly added into the plate and changes in calcium distribution were monitored for an additional 8 scans. Any intracellular Ca^{++} increases, in response to the test chemicals, were noted as changes in the intensity of the pseudocolor image of the cells. The transient increase in intracellular free calcium was observed as a transient peak during scanning.

F. Northern blot analysis junction genes

1. Procedure for isolation and purification of plasmid DNA (57)

a) Growth and amplification of plasmid DNA.

Three LB plates (Luria Bertani's Medium of 1.5% agar) containing ampicillin at 50 ug/ml were used to culture HB101, a competent strain of the bacterium, E.coli containing the plasmid vectors carrying cDNA inserts for Connexins (gap junction proteins): Cx26, or Cx32 or Cx43. Those plates were incubated at 37°C over night. A single colony from the culture plate was introduced into 5 ml of LB medium containing 25 μg/ml ampicillin under sterile conditions and incubated over night at 37°C. The LB contained 1% bacto-tryptone, 0.5% bacto-yeast, and 1% NaCl. The pH of the LB medium was adjusted to 7.5 before autoclaving. Four milliliters of the above overnight cultures were added to each 2 liters of LB medium and incubated at 37°C with vigorous shaking. The OD_{600} of the cultures was measured every half hour. When the OD reached 0.5, 1 ml of chloramphenicol (150 mg/ml in EtOH) was added to each liter of culture to inhibit bacterial protein synthesis and amplify plasmids with continuous shaking.

b) Isolation of plasmid DNA

The bacterial cells were centrifuged at 4000 g for 10 minutes. The supernatant was discarded and the bacterial pellet from 2 liters of culture was suspended in 20 ml of solution I (GET buffer: 50 mM Glucose, 10 mM EDTA, 25 mM Tris HCL), 40 ml of solution II (0.2 N NaOH, 1% SDS) and 30 ml of solution III (5 M

potassium acetate, 11.5% glacial acetic acid). The mixture was vortexed well and put on ice for half an hour. The solution was centrifuged at 10,000 rpm for 10 minutes and the supernatant was mixed with 2 volumes of 100% ethanol and kept at -20°C over night. After that, the mixture was centrifuged at 10,000 rpm for 10 minutes. The resulting pellet was dried at room temperature and suspended in 10 ml of TE buffer. 50 μl RNase A (10 ng/ml) was added and incubated at 37°C for 30 minutes. Phenol/chloroform extraction was done after adding 1 ml of 5 M NaCl. The DNA was reprecipitated after adding 1/10 volume of salt (3 M sodium acetate) and 2 volumes of 100% ethanol at -20°C for 1 hour. The pellet was suspended in 200 μl of TE buffer after centrifugation. An appropriate dilution of the plasmid DNA in TE buffer was made, and the OD_{260} was used to calculate the concentration of plasmid DNA (OD_{260}=1=50 μg/ml DNA).

c) Isolation of gap junction cDNA insert:

15-30 ug of DNA was used to react with restriction enzyme at 37°C for 2 hours and the volume of sample was 10 μl including 1 μl of 10 x EcoRI buffer and 1 μl of loading buffer. Lambda Hind III DNA was chosen as size marker. 0.4 g of Low EE0 agarose mixed with 50 ml of 1 x TAE buffer was heated in a microwave oven and cooled to 60°C, and gently dispensed over a clean minigel plate after adding 5 μl of 10 mg/ml ethidium bromide. Comb was put with well former at proper depth, 1 mm above plate surface. Half an hour later, the gel was harden and set in the minigel apparatus with 500 ml of 1 x TAE buffer. Samples and controls

were loaded into wells after removing comb. DNA was electrophoresed from the minus to the plus pole at 80 V for approximately 1 hour or until bromophenol blue dye front is 75% across the gel. After the gel was removed from the tank, it was rinsed with water to remove excess ethidium bromide. The gels were photographed on a UV transillumi-nator to visualize the bands. If result of the minigel was expected, a bigger gel was run as the same way to isolate and purify cDNA insert by electroelution. DNA bands of interest were located with UV and cut out with a sterile razor blade. The piece of agar was carefully transferred to a dialysis bag. One volume of electrophoresis buffer was added so that the piece of agar was completely surrounded without bubbles when the dialysis tube was tight. 150 mA current was applied for 90 minutes when the dialysis tube containing agar piece had been put at the bottom of the gel apparatus filled with buffer. Reverse current was taken for 30 seconds after DNA was eluted. Buffer was transferred from the bag to a Eppendorf tube with filter and was centrifuged to remove the fragment of agar in the buffer. Precipitation was done twice with 0.15 M sodium acetate and 2 volumes of 100% ethanol and then the pellet was resuspended in ddH_2O. This is the insert of gap junction DNA. A minigel was run to estimate the concentration of fragment DNA. Plasmid DNA of known concentration could be used as control.

2.Isolation of total RNA from the cells (58)

Total cellular RNA from cell cultures was isolated by the method of Single-step Method of RNA Isolation by Acid Guanidinium Thiocyanate Phenol-Chloroform

Extraction.

LC 540 cells cultured in 150 cm^2 flask were rinsed 3 times with PBS and lysed by adding 3 ml of solution D (Denaturation solution: 4 M guanidium thiocyanate; 25 mM sodium citrate, pH=7; 0.5% sarcosyl; 0.1 M 2-mercaptoethanol). The lysate was transferred to a 50 ml tube and 0.3 ml sodium acetate (2 M, pH=4), 3 ml water-saturated phenol, and 1.5 ml of chloroform were added, mixed well and shaken vigorously for 10 seconds. After cooling on ice for 15 minutes, the extract was centrifuged at 10,000 g, for 20 minutes at 4°C (9000 rpm in SS34 rotor). The aqueous phase was transferred to a fresh tube and phenol-chloroform extraction was repeated. Three milliliters of isopropanol was added to the aqueous phase and kept at --20°C for at least one hour. The pellet was dissolved in 0.3 ml of solution D and transferred to a 1.5 ml Eppendorf tube and centrifuged at 10,000 g for 20 minutes at 4°C. The same volume of isopropanol was added, mixed and left at -20°C for 1 hour, centrifuged for 10 minutes, and the pellet was suspended in 75% ethanol, centrifuged for 5 minutes and dried in speed vacuum for 10 minutes. The pellet was suspended in 50 μl of TE. ODs were read and the ratio of OD_{260}/OD_{280} was calculated.

3. Analysis of RNA on HCHO-agarose gels and northern blotting (57)

The isolated RNA was electrophoresed on 1% HCHO-agarose gels as follows: For a 1% gel, 1.0 g agarose (Low EE0) was dissolved in 87 ml DEPC-ddH_2O using a microwave oven for 4 minutes. Ten ml of 10 x MOPS (=0.2 M MOPS: 10 mM

sodium acetate, 10 mM EDTA, pH 7.0) buffer was added and allowed to cool to 60°C. In a fume hood, 5.1 ml of formaldehyde (37% solution) was added before the mixture was poured into an 11 x 14 cm tray. The gel was allowed to set one hour. During this time, the samples were prepared for electrophoresis by mixing the following in a sterile Eppendorf tube: 5.5 μl of RNA (10-20 ug/sample), 1.0 ul of 10x MOPS, 3.5 ul of Formaldehyde, 10.0 ul of Formamide , 1 ul of Ethidium Bromide(1 mg/ml), and 2 μl of DEPC-treated 10 x gel loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF). The mixture was centrifugated briefly to mix the contents, heated at 65°C for 15 minutes to denature the RNA and placed on ice immediately. The gel was pre-run for 5 minutes at 5 V/cm (70 V for a 14 cm gel) and RNA samples were loaded into the wells. The gel was run in subermagible mixture of 1 x MOPS and 8% formamide at 3-4 V/cm. When the bromophenol dye migrated to approximately 8 cm, electrophoresis was terminated, and the gel was removed from the electrophoresis unit on to a UV transparent tray. A transparent ruler was aligned along the gel and the gel was photographed by using ultraviolet illumination. Following that, the gel was soaked in 10 x SSC buffer (1.5 M NaCl, 0.1 M NaH_2PO_4-H_2O, pH 7.0) for 20 minutes twice and prepared for northern blotting. The Nylon Membrane (Hybond™ - N, 0.45 Micron. Amersham Corporation, Ailington Heights, IL) was pre-wetted in ddH_2O for 5 minutes and soaked in 10 x SSC buffer for 5 minutes. RNA was transferred in 10 x SSC by capillary action: A glass plate was set inside the tray containing about 150 ml of 10 x SSC buffer, and covered by a wick made by 3M paper. The RNA gel was

up-side down to the wick and the two sides of the wick were inserted into the buffer to keep the RNA transferred in sufficient SSC buffer constantly. The RNA side of the gel was exactly attached by a nylon membrane without any bubbles. Above the membrane two pieces of Whitman (3 mm) and paper towel wads were put on. On the top, there was a glass plate and an half-liter weight. Ten hours later, the membrane was dried by baking at 80°C for 2 hours and exposed to 254 nM UV source for 4 minutes to crosslink the RNA to the membrane.

4. Preparation of α-^{32}P labeled cDNA probes

The cDNA probes of each connexin were made using a Random Primed DNA Labeling Kit (Molecular biology boehringer mannheim) as follow:

The 50 ng (20 μl) of DNA was denatured by heating for 10 minutes at 95°C and subsequently cooling on ice. The following were added to set up reaction: 6 ul of dATP, dGTP, dTTP mixture (prepared by making a 1:1:1 mixture), 4 μl of reaction mixture (hexanucleotide mixture in 10 x concentrated reaction buffer), 10 μl of a-^{32}P dCTP and 2 μl of Klenow enzyme and incubated at 37°C for 1 hour. The reaction was stopped by adding 10 μl of 0.2 M EDTA and the volume was made up to 50 μl. Before adding the mixture to the column, the column (Nu-Clean D50) was prepared by warming to room temperature and inverting several times to resuspend the gel. The top cap was removed first then the bottom cap to avoid creating a vacuum. The excess buffer was allowed to drain through the bottom, and the column was pre-spun for 2 minutes at 1,100-8,500 xg. The column was placed in a fresh collection tube in

an upright position. The entire volume of labeling mixture was applied carefully to the center of the gel bed and the column was recentrifuged for 4 minutes as above. The purified labeled probe in the collection tube was denatured by heating at 95°C for 10 minutes, placed on ice, and used for hybridization immediately.

5. Hybridization of RNA blots with labeled cDNA autoradiography

RNA membrane was pre-hybridized in a sealable bag containing 15 ml pre-hybridization fluid(5 x SSC, 5 x Denhardt's reagent, 0.5% sodium dodecyl sulfate, 250 μg/ml denatured salmon sperm DNA) at 65°C for 1 hour. Then the denatured radiolabeled probe was directly added to 10-15 ml fresh prehybridization fluid and exchanged to the old prehybridization fluid in the sealable bag. The hybridization reaction was done at 65°C over night. The filter (RNA membrane) was then washed in the mixture of 1 x SSC (0.15 M NaCl, 0.01 M NaH_2PO4-H_2O, and 0.001 M EDTA) and 0.1% SDS at room temperature for 20 minutes twice, and in mixture of 0.5% SSC and 0.1% SDS at 65°C for 20 minutes twice. The blots were exposed to a Kodak XAR-2 X-ray film for 24-48 hours at -70°C in a cassette containing intensifying screens. After exposure, the film was processed in an automatic X-ray film processor (Radiology Department, Michigan State University). The intensities of the mRNA bands under different treatments were compared.

RESULTS

A. Effect of xenobiotics on GJIC

The primary purpose of this report was to test the effect of TPA and other xenobiotics on gap junctional intercellular communication in rat leydig cell culture system (LC 540) in vitro. If they can inhibit GJIC in these rat testicular leydig cells, it suggest that inhibition of GJIC by toxic chemicals may be a useful biomarker for identifying potential reproductive/developmental toxicants. Many xenobiotic chemicals which have been shown to be tumor promoters, teratogens and neurotoxins were linked to their ability to inhibit GJIC in vitro (22). In addition, tumor promoting agents, such as TPA, have been reported to block GJIC in various cell types (28,29,59,60). This study shows the effects of ten chemicals on GJIC between LC 540 cells.

1. Effect of TPA and other xenobiotic chemicals on dye transfer (SLDT)

Monolayers of LC 540 cell exposed for 1 hr to TPA (10 ng/ml) and other chemicals (eg. HC, Dieldrin, DDT, etc.,10 μg/ml, see Fig.7 showed different degree of inhibition of GJIC. Slight transfer of dye was observed in the TPA-treated cells (Fig. 4) while control cells (system culture) showed extensive dye transfer on both sides of the scraped line (Fig. 2). Different extent of dye transfer were observed after different chemical treatments. All xenobiotics were added to the cultures at

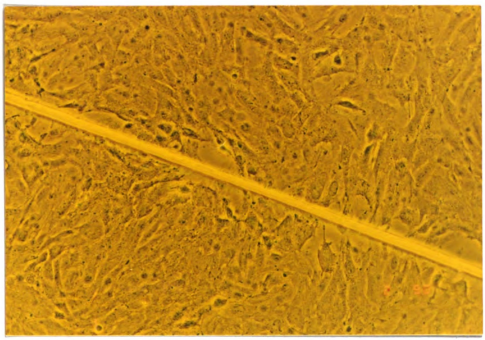


Figure 1. Control in LC 540 Cells (SLDT-Phase)

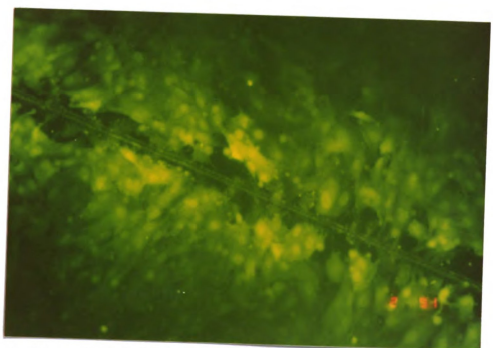


Figure 2. Control in LC 540 Cells (SLDT-Fluorescence)

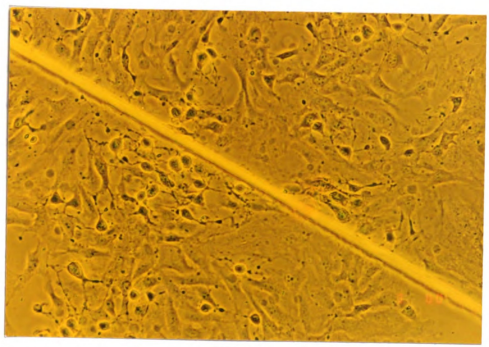


Figure 3. TPA 10 ng/ml, 1 hr. (SLDT-Phase)

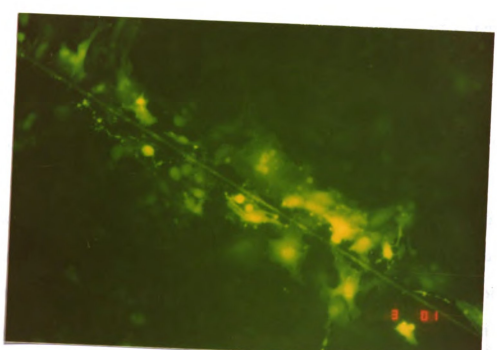


Figure 4. TPA 10 ng/ml, 1 hr. (SLDT-Fluorescence)

noncytotoxic level (Fig. 1, 3). Fig. 1 are the control cells, while Fig. 3 shows the cells treated with 10 ng/ml of TPA treatment at noncytotoxic concentration. With the quantitation procedure of dye transfer taken on ACAS 570, a scan of the scrape-loaded cells image was generated and an integrated value of fluorescence intensity over a boxed area of the scrape line was obtained as a measure of the extent of dye transfer among the different treatments (eg. Fig. 5 & 6). At least 6 areas of each treatment were quantitated and the average fluorescence was calculated. Based on the data, a figure was drawn with Harvard Graphics on the computer to show the different effects of those treatments on GJIC (Fig. 7).

2. Time course of TPA or Dieldrin effect on dye transfer

It has been shown that inhibition of intercellular communication by TPA in WB cells is reversible and time- and dose-dependent (52). The effect of TPA on GJIC in LC 540 cells was investigated. The maximal blockage of intercellular communication by 10 ng/ml TPA treatment was at 1/2 - 1 hr (Fig. 8) and communication began to return as early as 2 hr. By 24 hr, the cells established normal levels of communication. Similar experiments were done by treating LC 540 cells with 10 μg/ml Dieldrin. For this chemical (Fig. 9), the maximal inhibition was seen at 1 -2 hr (Fig. 11) and the reversal event occurred at later time, about 4 hr (Fig. 12), but it re-established only about 80% normal levels of communication at 18-24 hour (Fig. 13.). Thus both TPA and Dieldrin induce reversible inhibition of cell-cell communication in LC 540 cells, but the start time of reversion and the level of

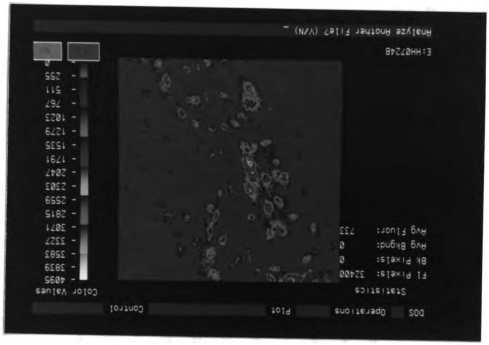


Figure 5. Control. (SLDT Quantitation on ACAS)

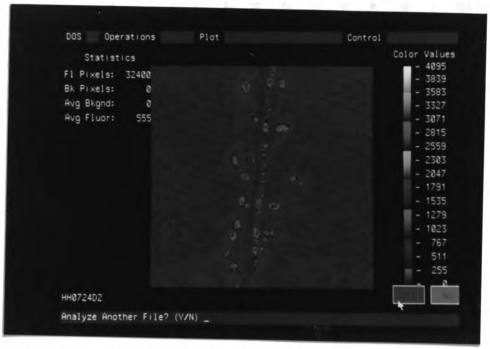
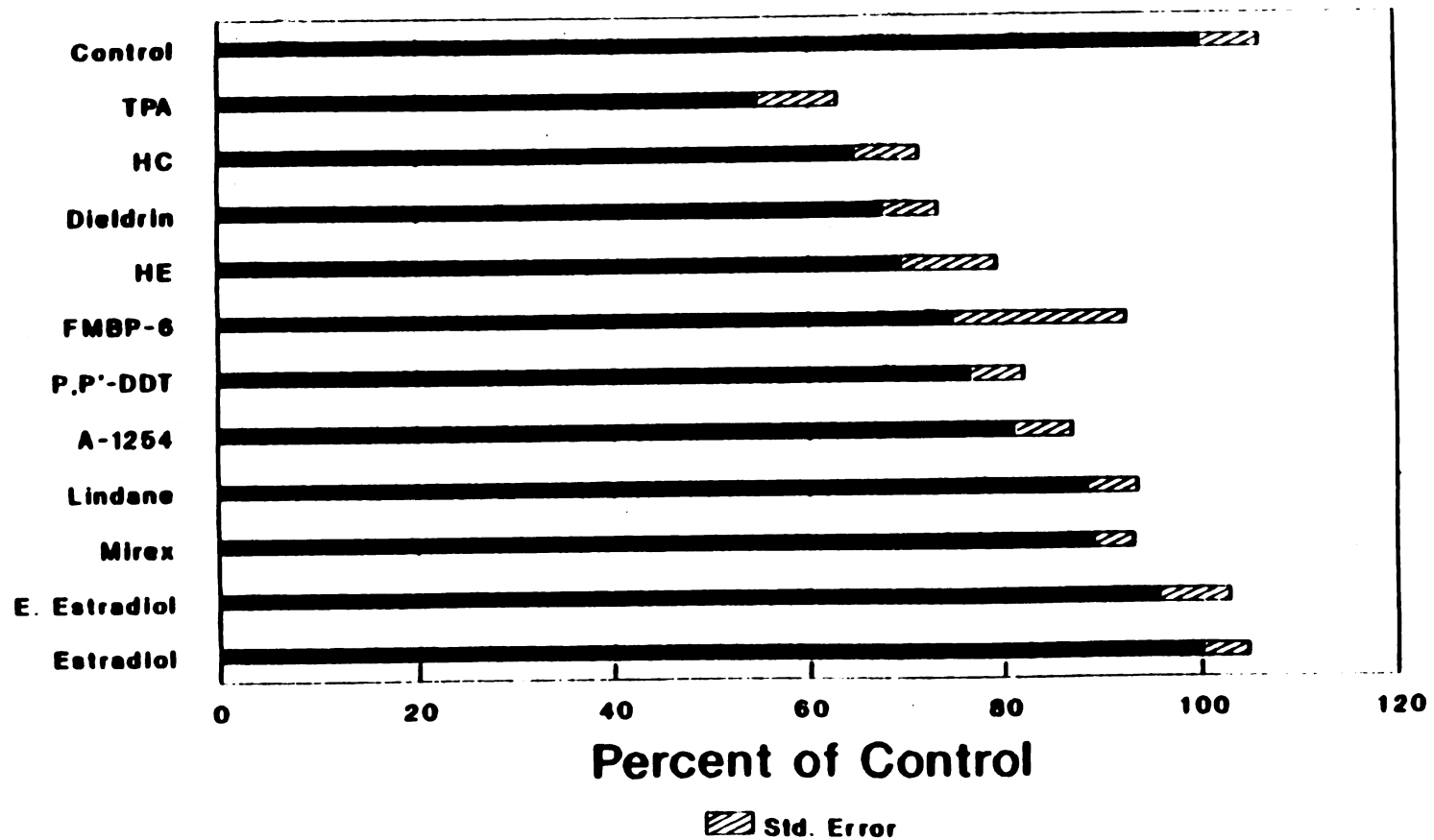


Figure 6. TPA 10 ng/ml. (SLDT Quantitation on ACAS).

FIG.7

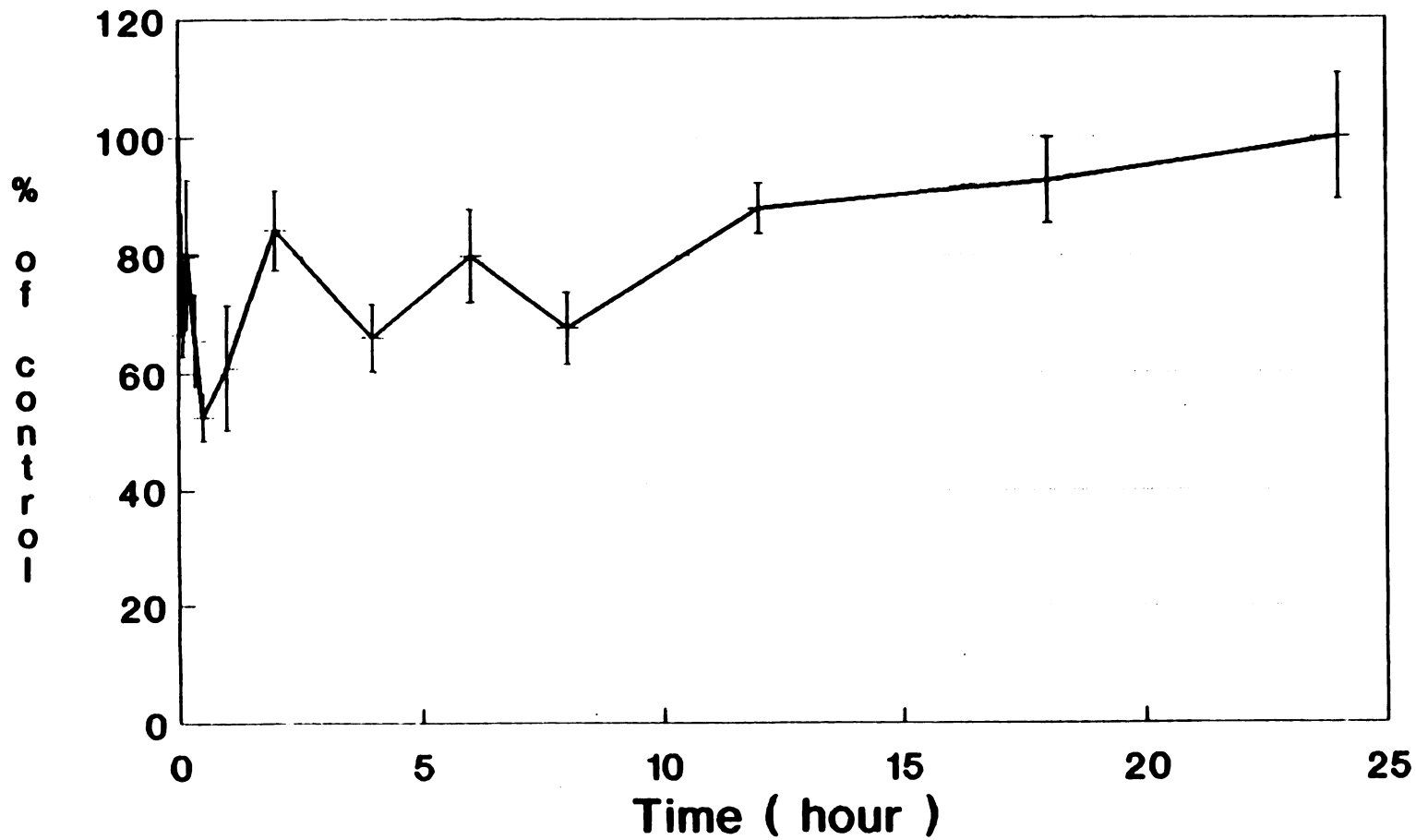
Effect of Xenobiotic Chemicals on GJIC in LC 540 Cells



Quantitation on ACAS 570

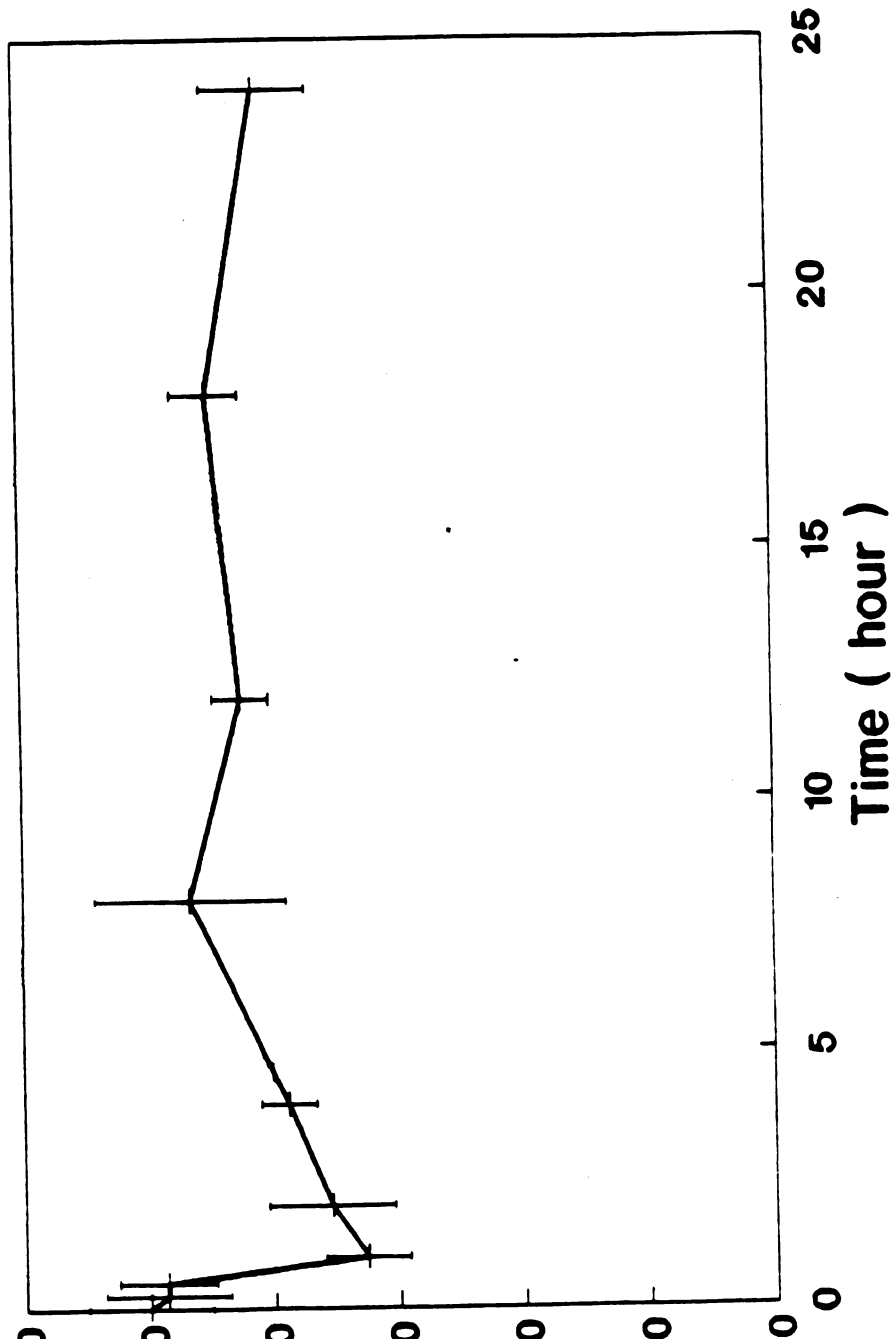
FIG.8

Time Response of TPA Effect on GJIC in LC 540 Cells



Quantitation on ACAS 570

FIG.9 Time Response of Dieldrin Effect on GJIC in LC 540 Cells



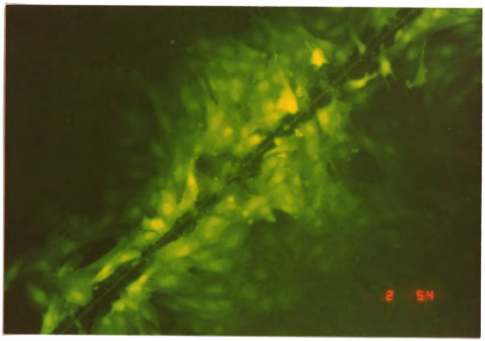


Figure 10. Control. (SLDT)

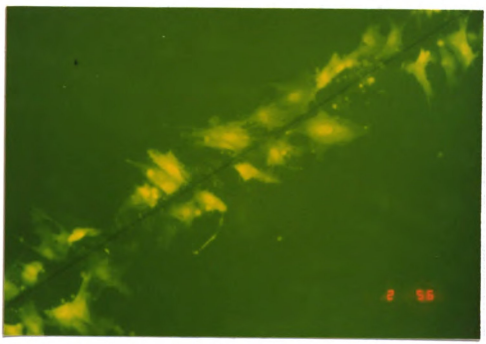


Figure 11. Dieldrin 10 μg/ml, 1 hr. (SLDT)

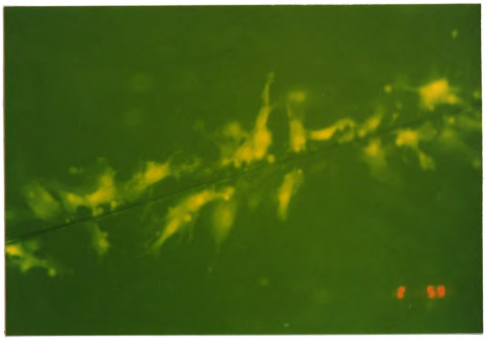


Figure 12. Dieldrin 10 μg/ml, 4 hr. (SLDT)

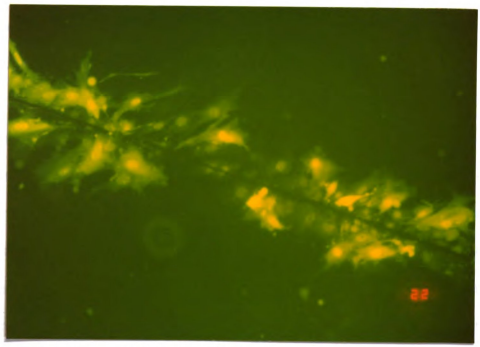


Figure 13. Dieldrin 10 μg/ml, 18 hr. (SLDT)

re-establishment are different (Fig.8 and Fig 9)

3. Dose response of TPA on FRAP

LC 540 cells were treated with different concentrations of TPA (10, 100, 250, 500 ng/ml) for 1 hr to observe the dose effect of TPA. TPA of 10 ng/ml blocked the majority of gap junctional communication, and higher non-cytotoxic concentrations from 100 ng/ml to 500 ng/ml of TPA almost inhibit cell-cell communication completely (Fig. 16, 17, 18 & 19). The data presented in Fig. 15 shows dose response curve of TPA on GJIC between LC 540 cells.

4. Dose effect of HC and Dieldrin on dye transfer

Heptachlor (HC) and Dieldrin were tested for dose-response in LC 540 cells. Cells were treated for 1 hr with different concentrations of HC (HC 0.5, 1.0, 2.0, 5.0, 7.0, & 10.0 μg/ml). The inhibition of GJIC started from the exposure to a very low dose (1.0 μg/ml) of HC (Fig. 21) and increased blockage occurred with the HC treatment of 7.0 μg/ml and higher concentration (Fig. 22 & 23). Fig. 24 shows the dose relationship of HC effect on GJIC. The dose-response of Dieldrin treatment in the different concentrations like HC is also presented (Fig. 29). The blockage of intercellular communication started from 1 μg/ml of Dieldrin (Fig. 26), and increased from 2 μg/ml to higher concentrations of Dieldrin (Fig. 27 & 28). Thus, the inhibition of GJIC by HC or Dieldrin began at similar concentrations and increased in a dose dependent manner (Compare Fig. 24 & 29).

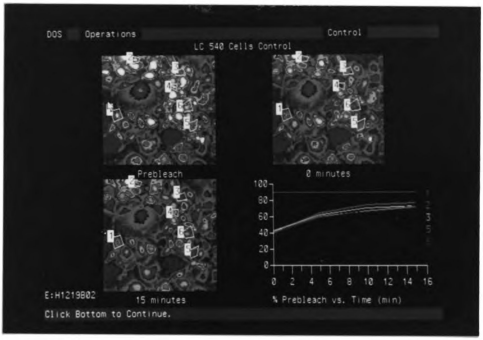
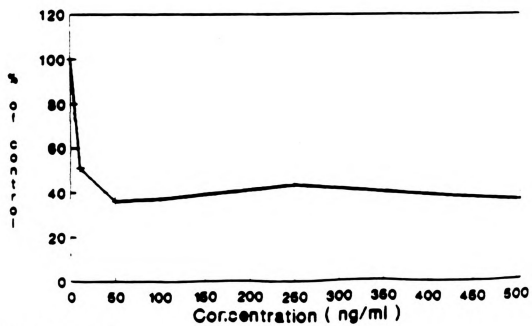


Figure 14. Control (FRAP)

FIG.15. **Dose Response of TPA Effect on GJIC in LC 540 Cells**



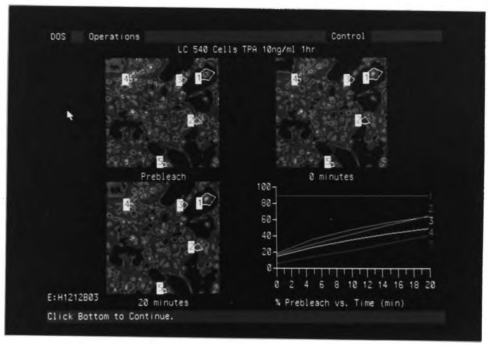


Figure 16. TPA 10 ng/ml, 1 hr. (FRAP)

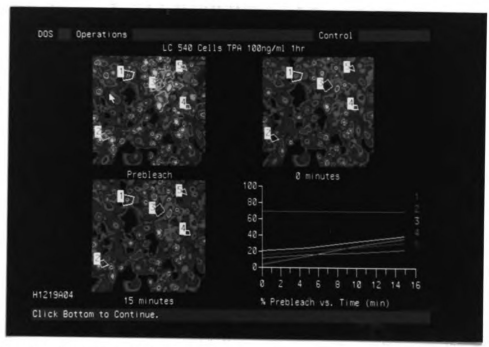


Figure 17. TPA 100 ng/ml, 1 hr. (FRAP)

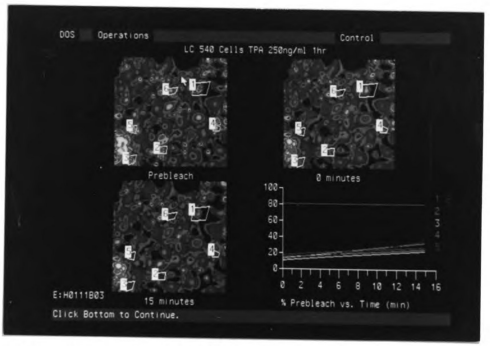


Figure 18. TPA 250 ng/ml, 1 hr. (FRAP)

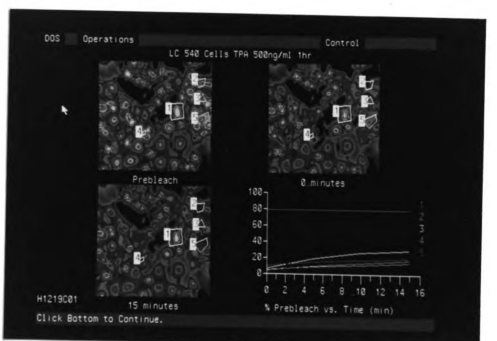


Figure 19. TPA 500 ng/ml, 1 hr. (FRAP)

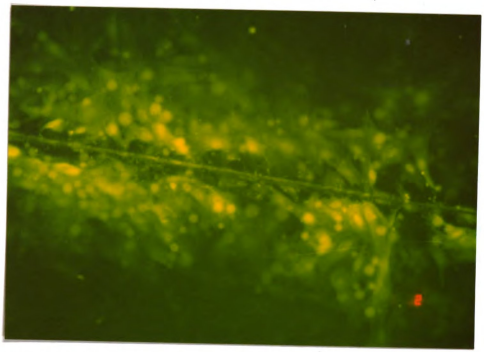


Figure 20. Control. (SLDT)

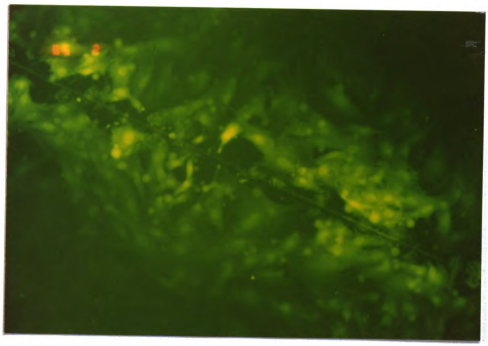


Figure 21. HC 1.0 μg/ml, 1 hr. (SLDT)

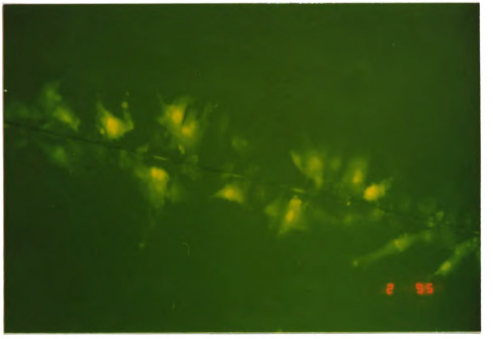


Figure 22. HC 7.0 μg/ml, 1 hr.

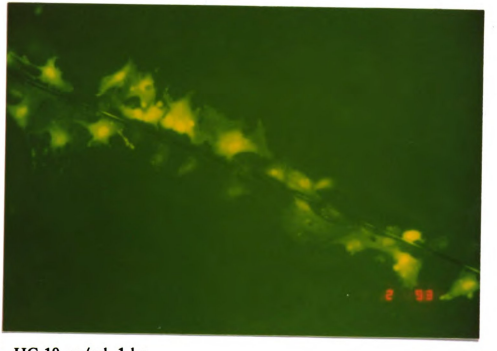
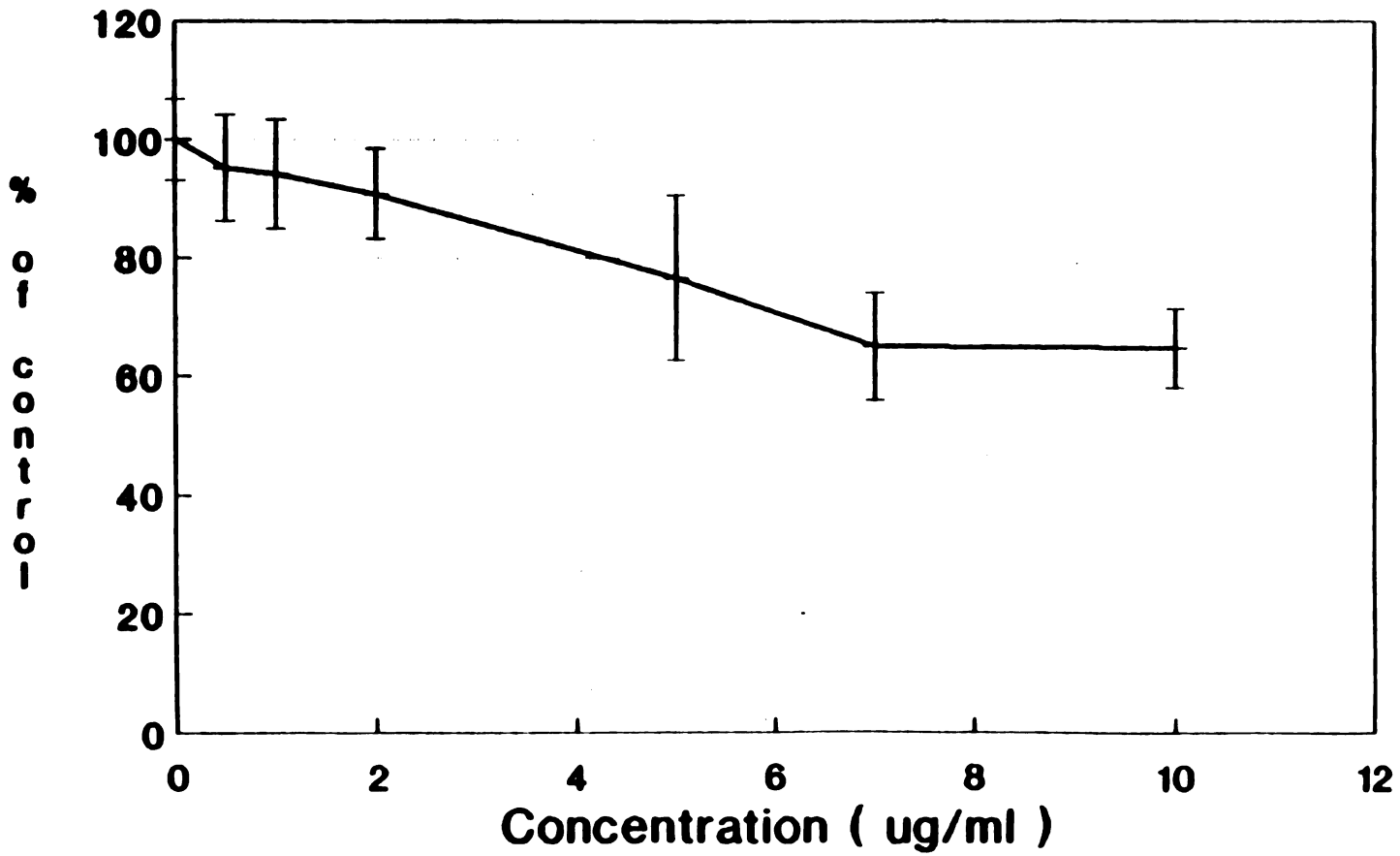


Figure 23. HC 10 μg/ml, 1 hr.

FIG.24 **Dose Response of HC Effect On GJIC in LC 540 Cells**



Quantitation on ACAS 570

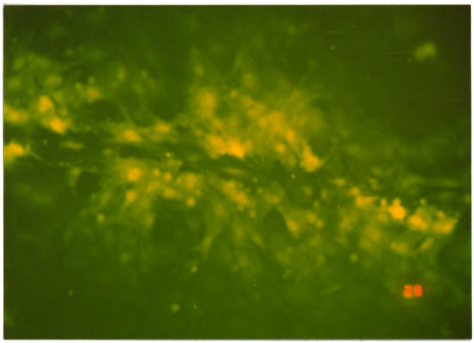


Figure 25. Control. (SLDT)

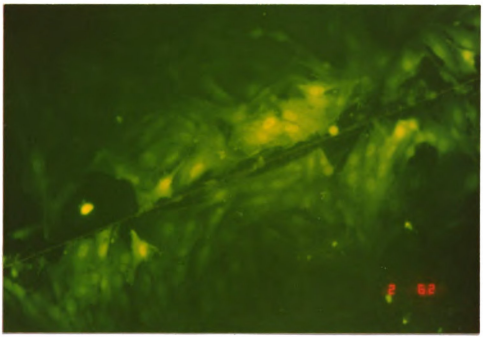


Figure 26. Dieldrin 1.0 μg/ml, 1 hr. (SLDT)

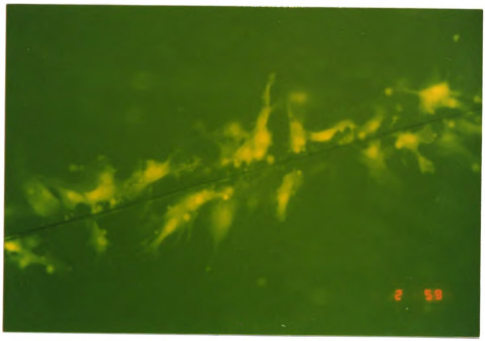


Figure 27. Dieldrin 5.0 μg/ml, 1 hr. (SLDT)

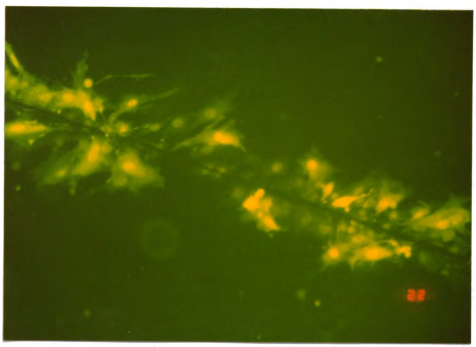
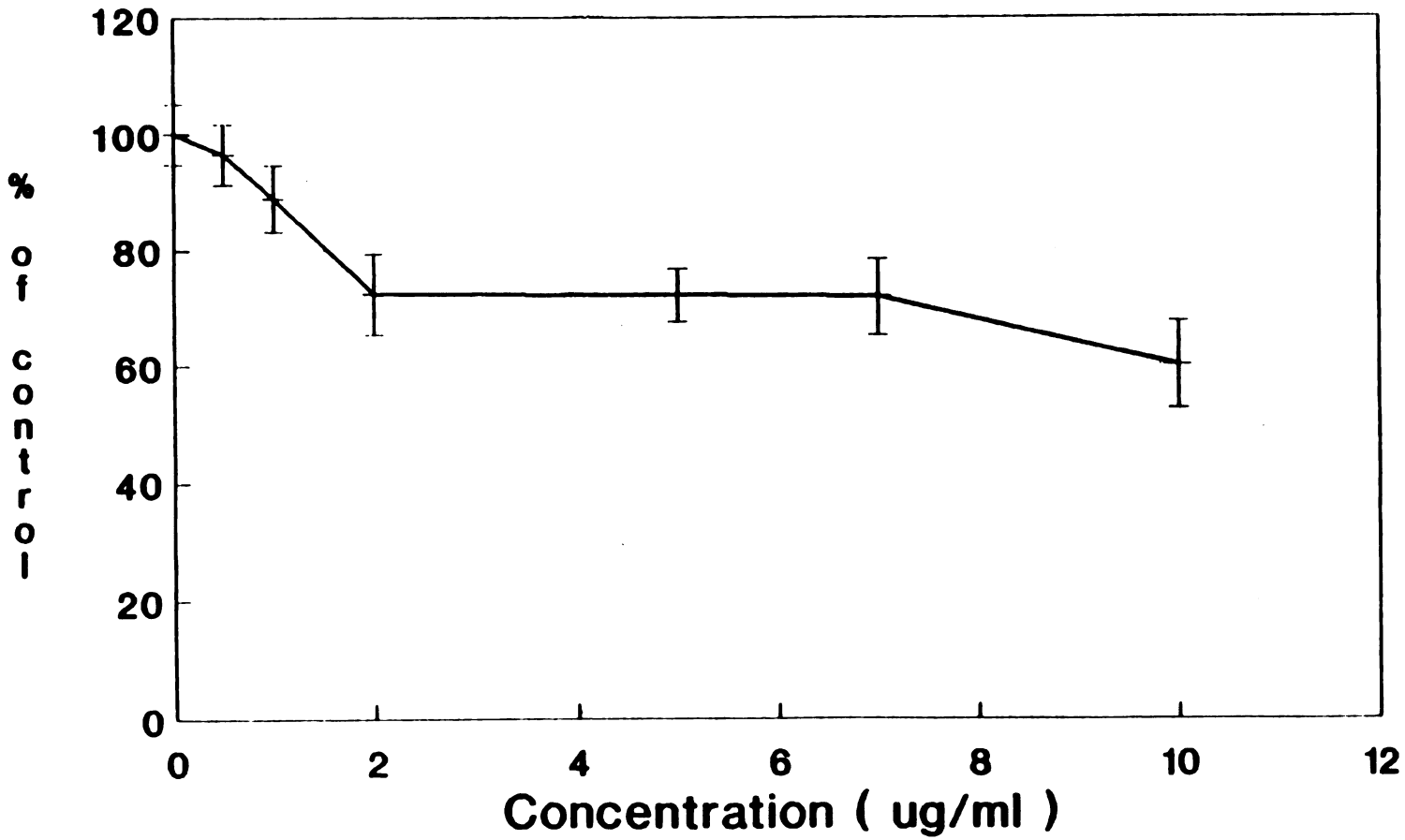


Figure 28. Dieldrin 10.0 μg/ml, 1 hr. (SLDT)

FIG.29 **Dose Response of Dieldrin Effect on GJIC in LC 540 Cells**



Quantitation on ACAS 570

B. Translocation of PKC

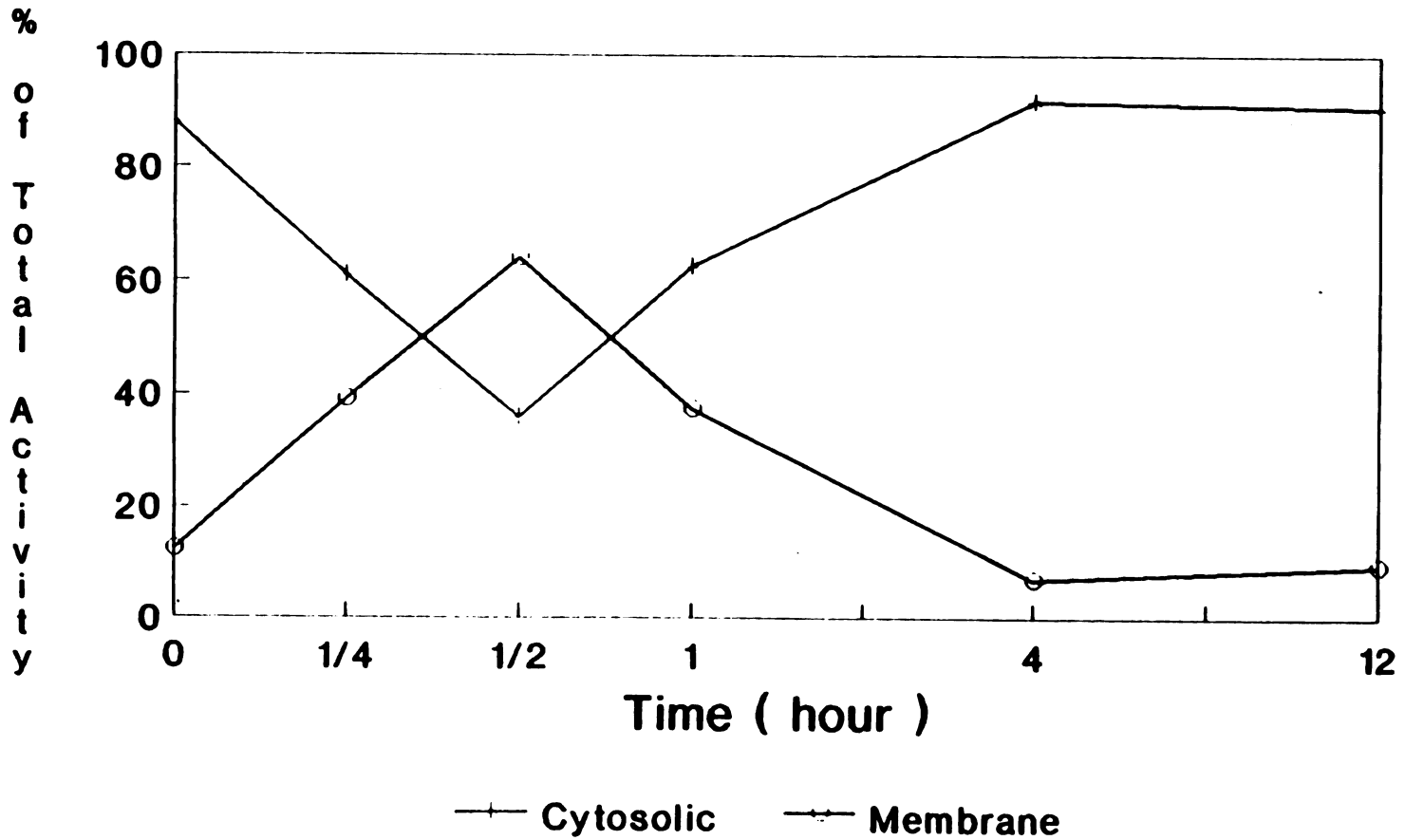
Protein Kinase C is an phospholipid/Ca^{++} - dependent enzyme, which is an important in signal transduction. Several investigations have correlated activation of PKC by the TPA with down regulation of gap junctional communication (38). The results of the present studies have examined the effect of TPA and other xenobiotics on GJIC in LC 540 cells to determined whether PKC is involved in the inhibition of gap junctional communication.

1. Time response relationship of TPA effect on PKC translocation and GJIC inhibition

The inhibition of GJIC and PKC translocation after treatment with the same concentration of TPA (10 ng/ml) in LC 540 cells at different time intervals was studied. Data presented in Fig. 30 indicate that the majority of PKC activity in control cells (untreated cells) was recovered from the cytosol. However, in TPA-treated cells, there was a translocation of PKC from cytosol to the membrane as soon as 15 minutes post-treatment. The maximal activity of PKC in the membrane fraction was about 5 times that of control at 1/2 hr and about 2 times that of control at 1 hr. It dropped below that of control at 4 hr and maintained a similar level by 12 hr, which indicated that the translocation of PKC activity caused by TPA was time dependent. Although TPA affect the distribution of PKC, the total activity of the enzyme was not affected over time. Comparing Fig. 30 and Fig. 8, it is clear that translocation of PKC to the membrane was correlated to the inhibition of GJIC in LC 540 cells at 1/2-1 hr. By 4 hr, a partial recovery was detected and by 12 hr, to

FIG. 30

Time Response of TPA on PKC in LC 540 Cells



nearly normal concentration level, corresponding to the returned of PKC translocation into the membrane. Hence, it appears to be a direct relationship of TPA time-response effect on PKC translocation to the plasma membrane and inhibition of GJIC.

2. Dose response relationship of TPA effect on PKC translocation and GJIC inhibition.

The blockage of GJIC and PKC translocation were determined following treatment of LC 540 cells at different doses of TPA treatment (from 10 ng/ml-500 ng/ml) for 1 hr. Fig. 31 shows that PKC activity in the membrane reached about 2 times that of control when LC 540 cells were treated with 10 ng/ml of TPA. Furthermore, it reached a maximum of the level equal or higher than the cytosolic level in control cells when treated with 100 or 500 ng/ml of TPA. Comparing to Fig.4, translocation of PKC to the membrane appears to correspond well with the blockage of GJIC in LC 540 cells at the concentration of 10 ng/ml of TPA. With TPA treatment at 100 ng/ml, the maximal translocation of PKC correlated closely to the extent of GJIC inhibition. This finding suggests that there is an intimate association between dose-dependent changes in PKC translocation and inhibition of GJIC.

3. The effect of Dieldrin, HE and A-1254 on PKC translocation and GJIC inhibition

LC 540 cells treated by 10 μg/ml of Dieldrin, HE (Heptachlor Epoxide) and

FIG.31

Dose Response of TPA on PKC in LC 540 Cells

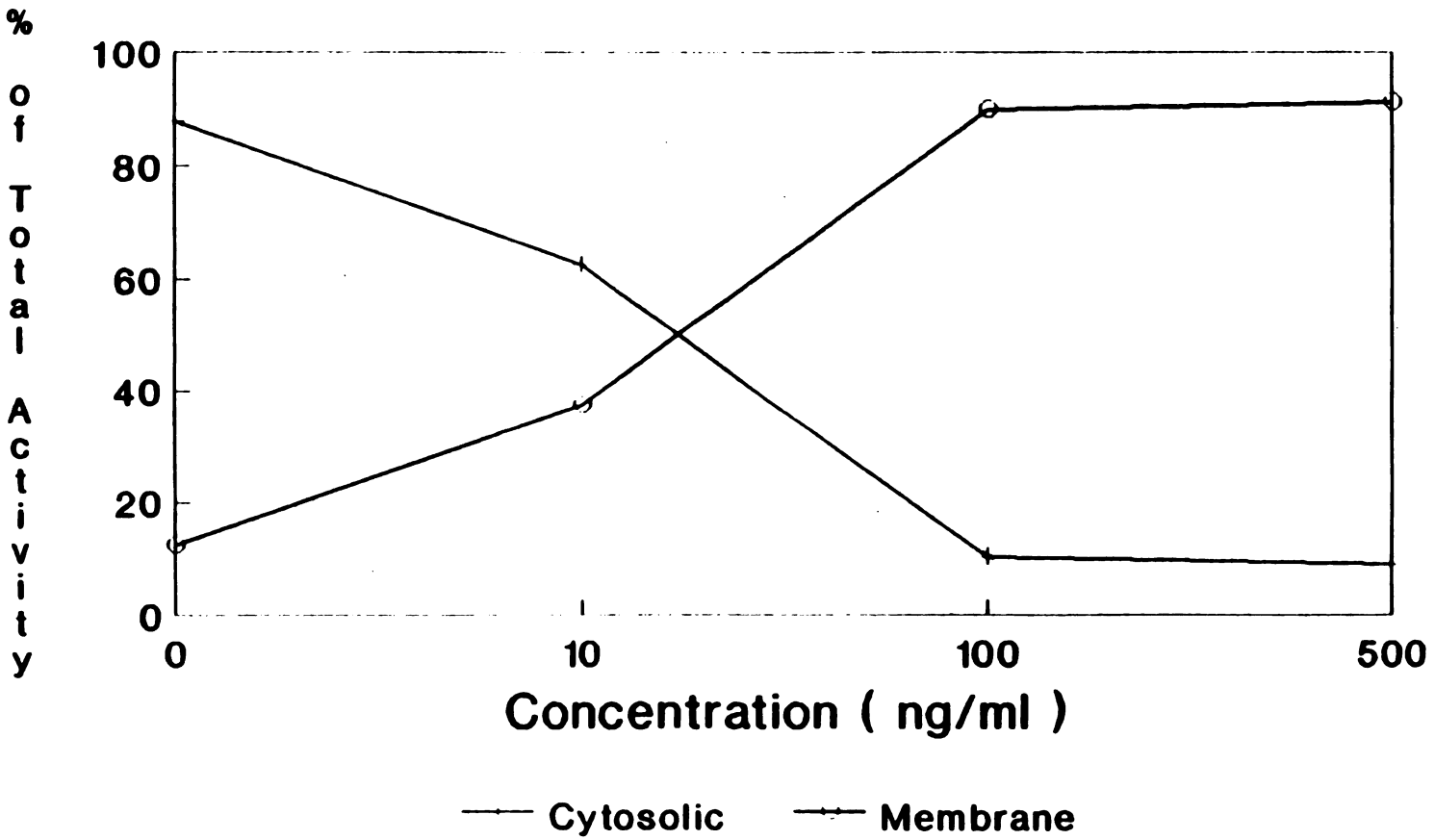
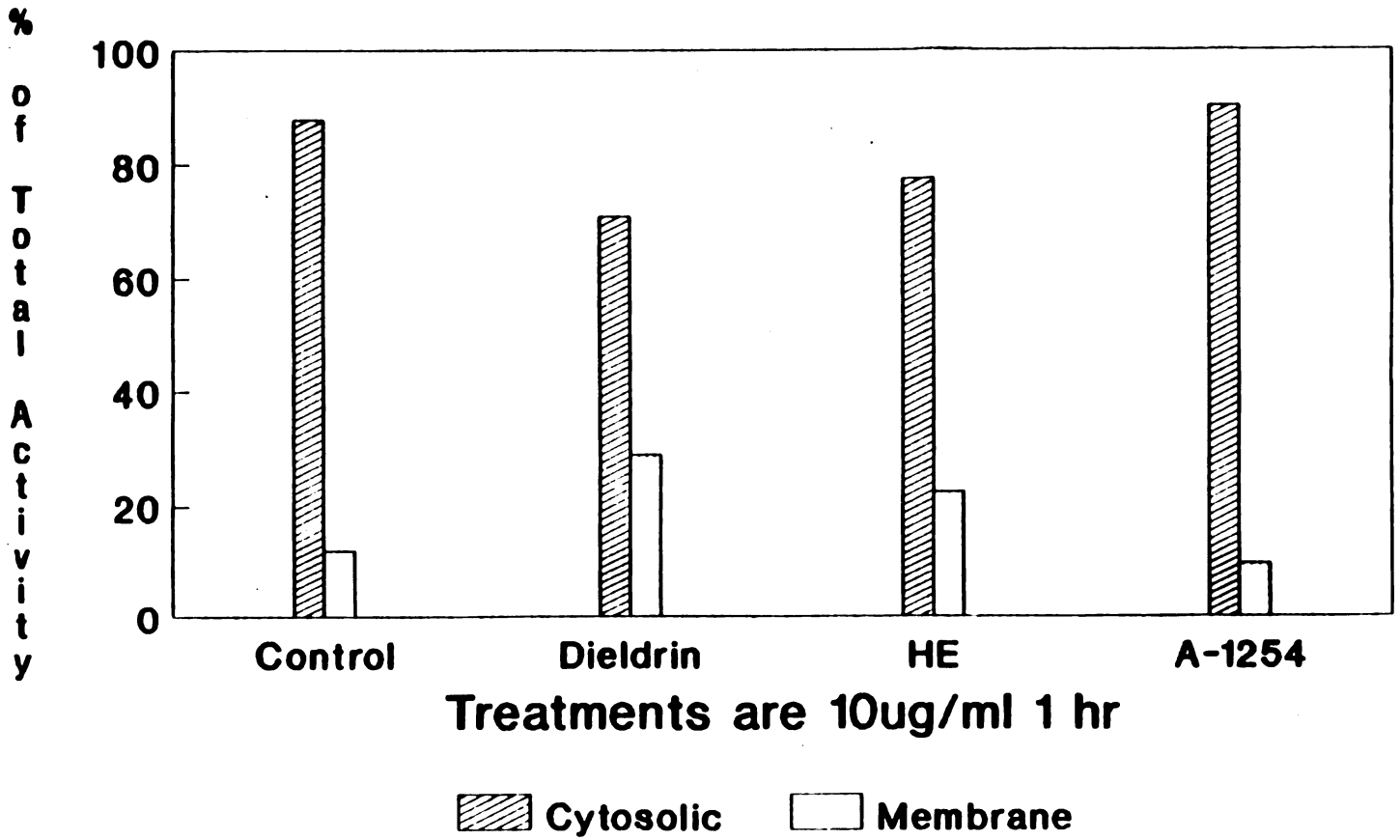


FIG.32

Efffects of Dieldrin ,HE, A-1254 on PKC in LC 540 Cells



Aroclor-1254 for 1 hr were studied to determine the relationship between PKC translocation to the plasma membrane and inhibition of GJIC in LC 540 cells. The PKC activity in membrane was about 2 times that of control in Dieldrin-treated cells and about 1.5 times that of control in HE-treated cells. However, there was no PKC translocation in A-1254-treated cells (Fig. 32). A comparison with Fig. 7 shows that translocation of PKC from cytosol to membrane by treatment of dieldrin or HE corresponds to the inhibition of GJIC. The lack of PKC translocation in A-1254-treated cells, implies that some other mechanism was involved in regulation of GJIC besides activation of PKC.

C. $[Ca^{++}]_i$ changes by xenobiotics

Some chemicals (eg. TPA, Dieldrin and HE) inhibited GJIC by a mechanism associated to translocation of PKC to the plasma membrane, but others (eg. Aroclor-1254) showed no effect on the distribution of PKC. Therefore, other mechanisms such as changes in free intracellular Ca^{++} in response to chemical treatment of LC 540 cells were studied. Changes in free intercellular Ca^{++} concentration are shown by the Figures from the experimental results done with a program on the ACAS 570. LC 540 cells treated by TPA 10 ng/ml, Dieldrin 10 μg/ml or HC 10 μg/ml for 1 hr showed an increase of cytosolic free Ca^{++}. But some differences between treatments were observed. A marked Ca^{++} increase occurred at 4 minute post-treatment when LC 540 cells were exposed to TPA (Fig. 33); For Dieldrin and HC the increase in intracellular free Ca^{++} occurred at about 2 minute after treatment (Fig. 34 & 35).

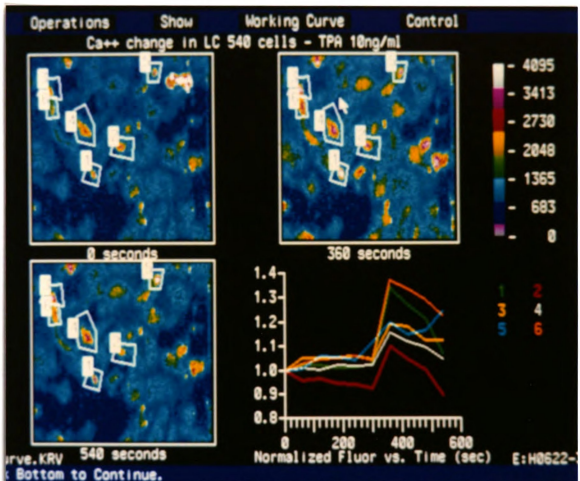


Figure 33. $[Ca^{++}]_i$ -- TPA 10 ng/ml

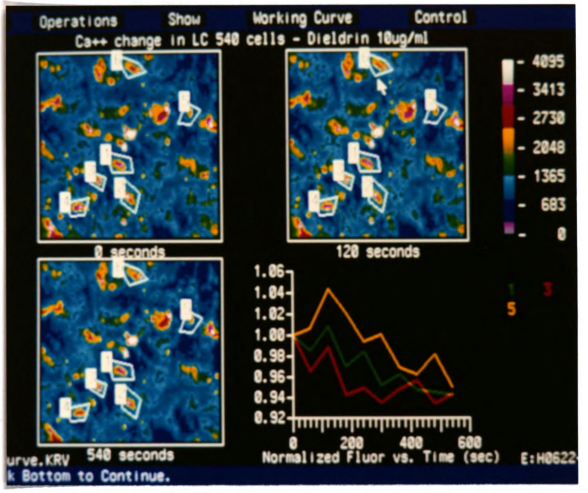


Figure 34. [Ca^{++}]$_i$ -- Dieldrin 10 ug/ml

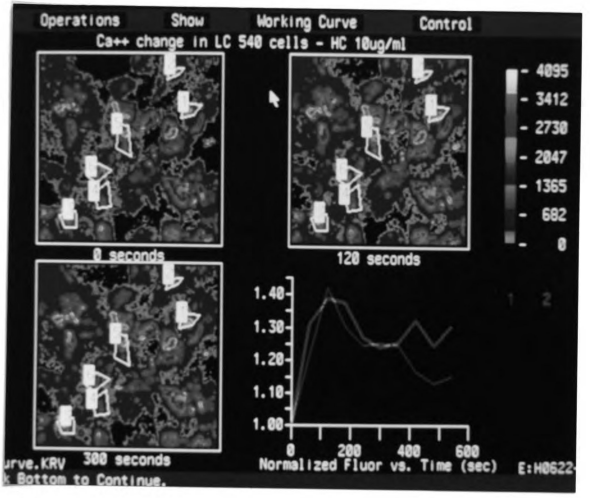


Figure 35. [Ca^{++}] -- Heptachlor 10 ug/ml

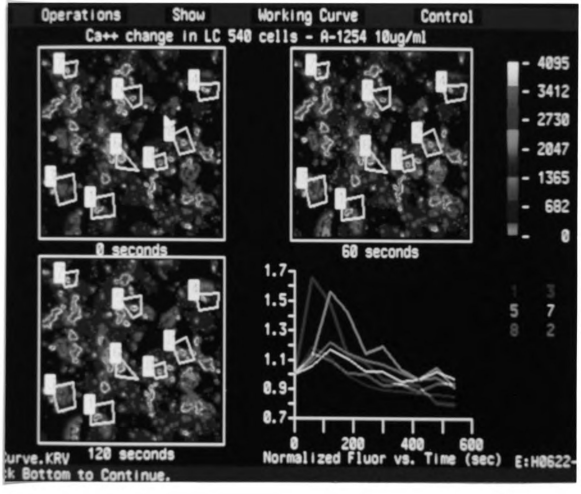


Figure 36. $[Ca^{++}]_i$ -- Arachlor-1254 10 ug/ml

Cells treated with Aroclor-1254 showed rapid increase in $[Ca^{++}]_i$ (Fig. 36). Despite failing to affect translocation of PKC, Aroclor-1254 enhanced the level of calcium inside the cells. Thus, all four chemicals tested here induce transient increase in intracellular free Ca^{++} followed by inhibition of GJIC in LC 540 cells.

D. Expression of connexins

1. plasmid DNA and fragment DNA of connexins

The plasmid DNA carrying Cx26, or Cx32 or Cx43 were isolated from E. coli containing those vectors and digested with restriction enzyme (EcoRI). The three kinds of plasmid DNA were cut completely by the enzyme as shown on Fig. 37. Purification of DNA fragments was done by electroelution (Fig. 38). Compared to DNA marker (EcoR I Hind III), all of the three DNA inserts appear (shown the right size). cDNA insert of Cx43 is 1.4 kb, Cx32 is 1.5 kb and Cx26 is 1.1 kb.

2. Northern blot analysis

Total RNA was isolated from LC 540 cells and separated by electrophoresis using gels containing formaldehyde. Fig. 39 shows the RNA isolated from LC 540 cells. The tRNA and the 18 S and 28 S rRNA were clearly visible. the size of 18 S rRNA is 1.9 kb and 28 S rRNA is 4.7 kb. RNA of LC 540 cells without any treatment and RNA of the cells treated with either TPA 10 ng/ml or Dieldrin 10μg/ml for 1 hr were electrophoresed and transferred to a same membrane (Fig. 40). After Northern blot, hybridization of specific probes was viewed by autoradiography. Fig.40

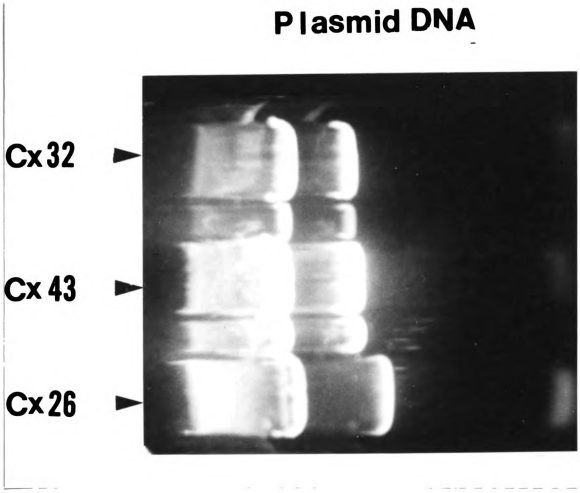


Figure 37. Plasmid DNAs of connexins

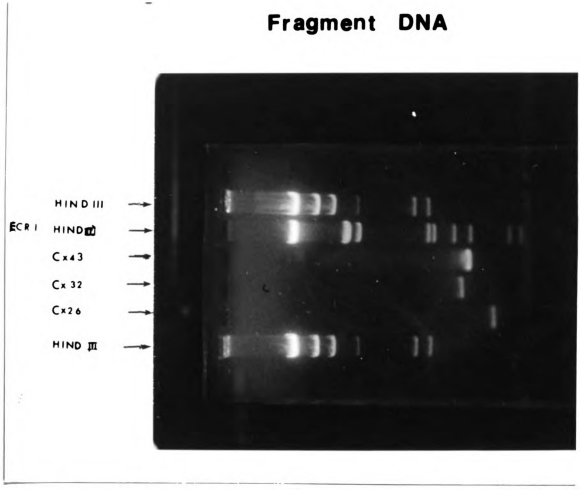


Figure 38. Fragment DNAs of connexins.

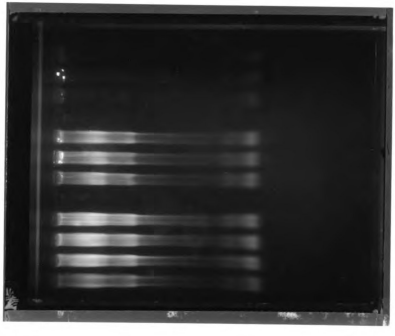


Figure 39. Total RNAs from LC 540 cells.

shows mRNA of Cx43 (3 kb) specifically hybridized to the probe (cDNA insert of Cx43). The intensity of the bands increased dramatically following treatment with either TPA or Dieldrin in 1 hr, indicating that LC 540 cells expressed the gene for Cx43 gap junction protein and that treatments of 10 ng/ml TPA and Dieldrin 10 μg/ml for 1 hr enhanced the expression of that gene.

A similar experiment was done by treating LC 540 cells with TPA (10 ng/ml) and Dieldrin (10 μg/ml) for 24 hr. The opposite effect was observed. The intensity of the bands following treatment with TPA and Dieldrin decreased (Fig. 41). Another interesting result was obtained when a different probe (Cx26) was hybridized with the same RNA membrane used for the Cx43 probe. It did not show the specific bands to Cx26 mRNA on the RNA of LC 540 cells without treatment, but it did show the specific bands on the RNA (2.5 kb) from cells treated with TPA and Dieldrin (Fig. 43). This suggests that the treatments of 10 ng/ml TPA and 10 μg/ml Dieldrin for 1 hr induced the expression of the gene for Cx26 gap junction protein. Finally, the cDNA of β Actin was used as a control probe to the hybridize with RNA from LC 540 cells (Fig. 43), the results confirmed that the RNA of LC 540 cells could be translated to protein.

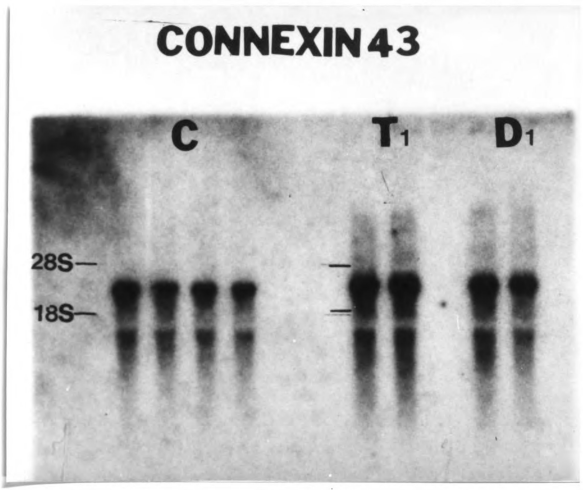


Figure 40. Northern blot detection of mRNA encoding for Cx43 in LC 540 cells treated with TPA and Dieldrin for 1 hr.

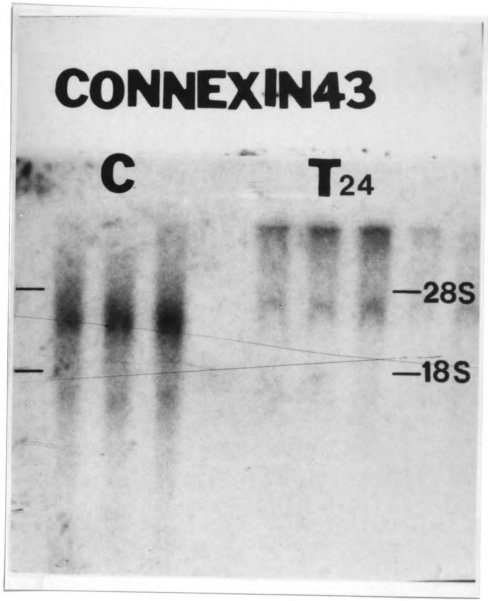


Figure 41. Northern blot detection of mRNA encoding for Cx43 in LC 540 cells treated with TPA for 24 hr.

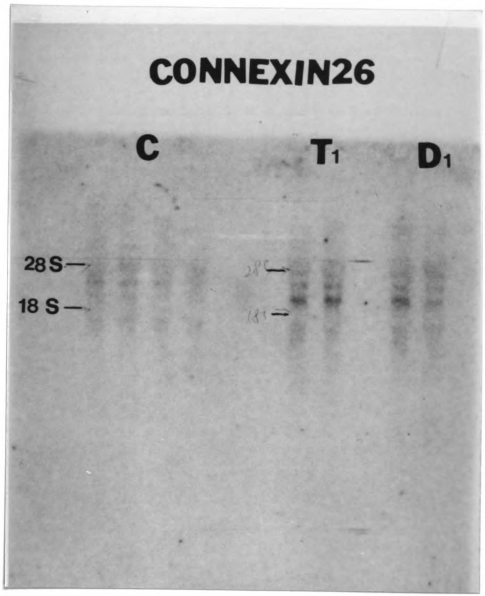


Figure 42. Northern blot detection of mRNA encoding for Cx26 in LC 540 cells treated wit TPA and Dieldrin for 1 hr.

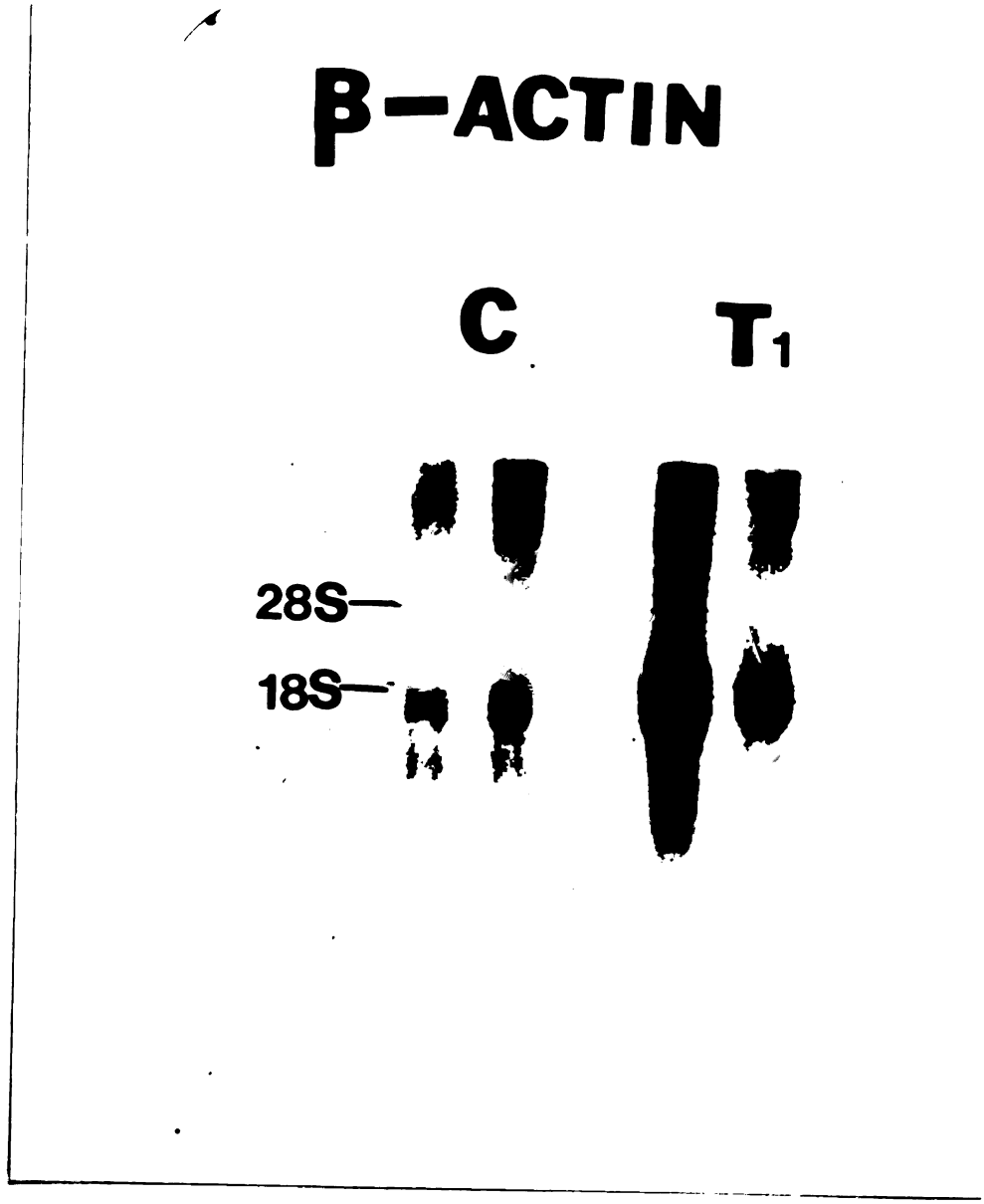


Figure 43. Northern blot detection of mRNA encoding for β - Actin in LC 540 cells treated with TPA for 1 hr.

DISCUSSION

In this study, LC 540 tumor-derived rat Leydig cells were shown to form gap junction-mediated intercellular communication. Furthermore, GJIC in these cells is inhibited by some xenobiotic chemicals.

Ten xenobiotic chemicals including TPA, Dieldrin, HC, HE and Aroclor-1254 were compared for their effects on inhibition of GJIC in LC 540 cells. The results indicated that there were differences in the degree of inhibition of GJIC among these chemical treatments using the scrap loading / dye transfer technique. While some of the chemicals (eg TPA, HC, Dieldrin) inhibited GJIC, some did not inhibit (eg. Estradiol and Epoxide Estradiol) at all. Others (eg. p,p'-DDT, Aroclor-1254) showed the inhibition of GJIC but to a lesser degree than TPA, HC and Dieldrin.

TPA, Dieldrin and HC were chosen for a detailed kinetic analysis of inhibition of gap junctional communication because of their marked inhibitory effect on GJIC in this cell culture model. The inhibition of GJIC in LC 540 cells by TPA or Dieldrin was spontaneously reversible. The duration of maximum inhibition was between 1/2 - 2 hr. Cells treated with TPA reversed to 100% of control, but cells treated with Dieldrin reversed to 80% of control at 24 hr after treatment. Dose response differences could also be seen among TPA, Dieldrin and HC treatment. All three chemicals began inhibiting GJIC at very low concentrations, then significant blockage

of GJIC happened at different concentrations: TPA at 7.0 ng/ml; Dieldrin at 2.0 μg/ml; and HC at 5.0 μg/ml. For 1 hr treatments, the inhibition of GJIC by 10 ng/ml-TPA was more effective than by 10 μg/ml-Dieldrin or HC.

Inhibition of GJIC by chemicals seems to be cell specific. TPA has been shown to block gap junctional communication in various cells types (28,29,59,60). Oh et al.(52) examined the modulation of GJIC by TPA on a normal rat liver epithelial cell line (WB-F344 cells). The inhibition of gap junctional communication in both WB and LC 540 cells showed a similar time- and dose-response by treatment with the same concentrations of TPA. However, the reversal of GJIC inhibition in LC 540 cells reached 100% of control, whereas in WB cells, it reached only 93% of control. Yotti et al.(28) investigated TPA effect on metabolic cooperation in Chinese hamster V-79 cells and the positive results indicate that the TPA effect was irreversible.

Klauning et al.(61) compared the effect of phenobarbital, DDT, and Lindane on mouse hepatocyte gap junctional intercellular communication, their results provide some insight into the mechanisms of inhibition of GJIC. Based on the finding that 2-4 hr were necessary to see maximum inhibition of GJIC by the three promoters tested , and that the reversal to normal intercellular communication following their removal was relatively rapid, it is suggested that direct damage or degradation of the gap junction protein was not the mechanism of inhibition. Direct interaction of chemicals with the plasma membrane or gap junction protein would be expected to occur rapidly.

The idea that the cell membrane may be an important target site for the

phorbol ester class of tumor promoters, such as TPA, has been supported by recent observations. TPA is a highly lipid-soluble molecule (62) and has been shown to associate rapidly with a cell membrane-rich fraction isolated from 3T3 cells previously exposed to the drug (63). In addition, the time-dependent nature of the inhibitory response suggests that uptake and /or metabolism of the tumor promoter may be necessary for the inhibition of intercellular communication. Furthermore, it was demonstrated that TPA is capable of stimulating certain membrane bound enzymes (Na+, K+-ATPase and 5'-nucleotidase), while, modulating membrane protein conformation (64). Protein kinase C has been considered to be a receptor for TPA (65) and its activation has been shown to correlate with the inhibition of GJIC in WB cells (52). The present study with LC 540 cells provides an additional model where TPA induce translocation of the PKC to the plasma membrane. The TPA-induced inhibition of GJIC in LC 540 cells was also transient and correlated to the initial translocation of PKC from the cytosol to the membrane followed by the subsequent loss of PKC from the plasma membrane. This was presumably due to the enhanced phosphorylation of gap junction protein by PKC (66), resulting in the decreased channel permeability. It has also been reported that cAMP-dependent protein kinase has the opposite effect in the regulation of GJIC in different cell types. Saez et al.(44) reported that enhanced phosphorylation of hepatocyte gap junction protein by cAMP-dependent protein kinase increased channel permeability.

The present results confirmed that the level of TPA translocated PKC corresponded with the degree of inhibition of GJIC by TPA, showing dose- and time-

related responses. Dieldrin and HE induced some translocation of PKC, but less than TPA. However, Aroclor-1254 showed no correlation between PKC activity and inhibition of GJIC.

Intracellular free calcium change was also studied as possible mechanism. TPA, Dieldrin, and HC were found to block GJIC and to increase the intracellular free calcium. Aroclor-1254 was also found to increase intracellular free calcium in LC 540 cells. There were time differences for the increase of intracellular communication among the four chemicals tested. The transient increase of $[Ca^{++}]_i$ in LC 540 cells caused by 10 ng/ml-TPA was at 4 minutes post-treatment. After treatment with dieldrin and HE (Heptachlor Epoxide) at about 2 minutes, and Aroclor-1254 at 1-2 minutes post-treatment.

It has been observed that there was a good corresponding effects between inhibition of GJIC and PKC activation in WB cells (52), and as a result of this study, in LC 540 cells treated with TPA. The correlation may exist for inhibition of GJIC, and the increase in intracellular Ca^{++} in LC 540 cells treated with Aroclor-1254. But the results from Madhukar et al.(46) indicate that neither PKC nor an increase in intracellular Ca^{++} were involved in the modulation of GJIC by epidermal growth factor or transforming growth factor-β in human keratinocytes. These finding suggests that other mechanisms may be involved in the inhibition of GJIC in these cells.

It was reported that the upregulation of intercellular communication by cAMP could occur through the enhancement of gap junction gene expression (67). The mRNA encoding the gap junction protein, connexin (Cx), was identified in LC 540

cells by northern blot analysis. LC 540 cells expressed the mRNA for Cx43 gap junction protein. The mRNA level of Cx43 was affected by treatment with TPA and Dieldrin. Treatment for 1 hr with either TPA (10 ng/ml) or Dieldrin (10 ug/ml) increased the Cx43 mRNA level and simultaneously inhibited the GJIC. After 24 hr of treatment with either TPA or Dieldrin, levels of Cx43 decreased and was associated to the reversion of inhibition seen after 24 hr treatment. The cells treated with either TPA or Dieldrin for 1 hr also increased the expression of Cx26 which was not detected in control cells.

Fritz and Tung (68) reported that the development and regulation of normal physiological functions of male reproductive cells were dependent on intercellular communication in testes. It was also indicated that gap junctional communication correlated with the regulation of tissue homeostasis, cell growth and differentiation, as well as synchronization of tissue reactions and tissue regeneration (69). Gap junctions have been shown to be present in various cell types in testes (69). LC 540 cells used in my study are rat leydig cells from the testes. Based on the observation, it was confirmed that LC 540 cell line is a good model to study GJIC, and that inhibition of GJIC by toxic chemicals may be a useful biomarker for identifying potential reproductive / developmental toxicants.

The differences of GJIC inhibition induced by various xenobiotic chemicals could be explained by the several possible mechanisms. TPA has been reported to block gap GJIC in various cell types, including WB cells (52), V 79 cells (28), and human cells (70) as well as LC 540 cells tested here. The mechanism by which TPA

blocks GJIC was most likely mediated by the phosphorylation of the gap junction protein by PKC. Phorbol ester tumor promoters such as TPA have been confirmed to bind specifically to and activate the phospholipid/ Ca^{++} -dependent protein kinase (PKC) by acting as an analog of diacylglycerol (DG) (71). Both DG which activates PKC and inositol 1,3,4 triphosphate (IP^3) which releases Ca^{++} ions from internal stores (endoplasmic reticulum), are hydrolysis products of polyphosphoinositide (PIP^2) mediated enzymatically (39). Thus PKC has been considered as a receptor for TPA. The regulation of many cellular functions including GJIC is mediated by this signal transduction system. It was noted that a small amount of diacylglycerol enhanced the affinity of PKC for Ca^{++} , completely activating the enzyme without any change in Ca^{++} levels (71).

This is the first time that TPA was shown to increase the intracellular Ca^{++} in rat leydig cells using the Fluo-3 as a probe on the ACAS-570. Because the enhancement of intracellular Ca^{++} occurred within few minutes and quickly returned to the original level, it could not explain the effect of TPA on GJIC at 2 hr post-treatment. Although, the calcium change was found to precede the GJIC blockage, it may not be major mechanism like PKC activation. It was demonstrated that the 27-kD major component protein for rat liver gap junctions was phosphorylated by protein kinase C in vitro (66) and in rat hepatocytes (72). It was also reported that the gap junction protein from the rat heart (Connexin 43) was homologous to gap junction protein from the liver (73). Cx43 has been demonstrated to be a phosphoprotein (74) and it could be phosphorylated by protein kinase C (75). In this

study, It was found that the gap junction protein of LC 540 cells is connexin 43 and so the Cx43 here is likely to be phosphorylated by PKC. Since the inhibition of GJIC by TPA happened very soon after treatment (within 15-30 minutes), it can not be explained by new protein synthesis, Although it can be explained by a gating mechanism through phosphorylation of the gap junction.

During maximal inhibition of GJIC the mRNA of Cx43 increased along with the new expression of Cx26 (when the rat leydig cells were treated with 10 ng/ml of TPA for 1 hr). The lack of GJIC in the presence of higher levels of connexin mRNA suggested that TPA induced inhibition of protein translocation. Thus, mRNAs were accumulated but not translated into connexins, leading to a reduction in GJIC due to a reduction in the amount of connexin in the membrane. This interpretation is consistent with the early finding that TPA induce a reduction in the size and number of gap junction plaques (76). Moreover, the mRNA levels of Cx43 and Cx26 decreased after 24 hr TPA treatment and GJIC was recovered to almost control condition, suggesting that the mRNA has been translated to connexin that formed new functional GJIC and then the mRNA was degraded.

Based on these results, it is proposed that TPA affect a feedback system that control the synthesis of connexin. Reviewing the results of Dieldrin and HC treatment on inhibition of GJIC, PKC activation and intracellular calcium changes, suggests that these two chemicals have a similar mechanism as TPA in blocking GJIC in LC 540 cells.

Aroclor-1254 increased in intracellular calcium, but did not translocate PKC.

Although the mechanism might involve the changes in $[Ca^{++}]_i$, it does not occur through PKC activation. The inhibition of GJIC as a result of an increase in $[Ca^{++}]_i$ may be mediated indirectly through phosphorylation of tyrosine residues of gap junction protein by calcium dependent protein kinases. Further investigation of the various processes of gap junctional phosphorylation is necessary to delineate the exact mechanism of GJIC blockage by various xenobiotic chemicals.

CONCLUSIONS

The rat leydig cell line LC 540 is proficient in gap junctional intercellular communication. The cell system provides a good model to study the inhibition of GJIC by various xenobiotics and to identify potential reproductive toxicants. Among the various structurally-diverse chemicals studied for their ability to inhibit GJIC in this cell culture system, some were found to inhibit GJIC. There were differences among the xenobiotics that inhibit GJIC. The inhibition of GJIC by TPA, a classical tumor promoter, was time and dose dependent. The inhibitory action was transient and could be partially reversed in about 2 hrs and completely reversed at 24 hr. Among the chlorinated hydrocarbon insecticides tested, Dieldrin and Heptachlor were found to exhibit a dose-related effect on GJIC inhibition. The inhibitory action by Dieldrin was reversed to 80% of control since GJIC between LC 540 cells was very sensitive. It is suggested that inhibition of GJIC may be a useful biomarker to identify potential environmental toxicants.

The present study also demonstrates that TPA could induce the translocation of the calcium and phospholipid-dependent protein kinase C (PKC) in a dose and time dependent manner. The translocation was transient and followed by the transient inhibition of GJIC by this agent, suggesting that membrane association of PKC might be involved in the inhibition of GJIC induced by TPA. The inhibitory

action of other chemicals such as Aroclor-1254 on GJIC was not associated to translocation of PKC, since they failed to translocate PKC to the membrane but were capable of inhibiting GJIC under the condition. All of the chemicals including TPA, Dieldrin, HC and Aroclor-1254 induced a early and transient increase in intracellular calcium. Since high intracellular calcium levels have been associated to inhibition of GJIC (77), it is proposed that intracellular calcium change induced by these xeobiotics might be involved in the inhibition of GJIC.

Studies of gap junction expression demonstrated that the rat leydig cells expressed the mRNA for connexin 43. The mRNA level of Cx43 was increased by 1 hr treatment of the cells with TPA or Dieldrin and decreased at 24 hr post-treatment. In addition, 1 hr treatment by TPA or Dieldrin also increase the expression for connexin 26.

Based on these data, it is proposed that the increase in mRNAs level could be a feedback response to the decrease in the number of gap junctions on cell membranes. Alternatively, the transient increase in mRNA levels could be due to a transient inhibition in protein translocation and therefore accumulation of mRNAs.

These results indicate that gap junctional communication can be regulated by multiple mechanisms including gating and mRNA expression.

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