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Inhibition of intercellular communication in rat hepatocytes by phenobarbital, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and γ-hexachlorocyclohexane (lindane): modification by antioxidants and inhibitors of cyclo-oxygenase

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Several tumour promoting chemicals have been shown to inhibit intercellular communication (IC) through gap junctions in cell cultures. In the present investigation we studied the effect of the hepatic tumour promoters phenobarbital (PB), 1,1,1-trichloro-2,2-(*p*-chlorophenyl)ethane (DDT) and γ-hexachlorocyclohexane (lindane) on IC in rat hepatocyte cultures. IC was evaluated by microinjection of fluorescent Lucifer Yellow CH dye and visualization of dye spread to adjacent hepatocytes. Incubation of hepatocytes with PB (2 mM), DDT (30 μM) and lindane (25 μM) decreased dye-coupling of the cells by about 30%, 42% and 35%, respectively; dye-coupling in untreated cultures was 88.1 ± 0.7%. Inhibition of IC was reversible when the xenobiotics were removed from the medium. The antioxidant vitamin E (100 μM) prevented inhibition of dye-coupling by PB and lindane and partially that by DDT. Superoxide dismutase (100 units/μl) counteracted the effect on dye-coupling by PB, but not that by the insecticides. Similarly, the cyclo-oxygenase inhibitors indomethacin and aspirin only reversed the effect of PB on IC, but not that of DDT or lindane. As indicated by further experiments, prevention by non-steroidal anti-inflammatory agents of PB-induced inhibition of IC is most likely not mediated by inhibition of cyclo-oxygenase. The results indicate significant differences in the action of PB, DDT and lindane on IC in hepatocyte cultures. This is suggested by the differential effects of superoxide dismutase and non-steroidal anti-inflammatory agents on the action of the three tumour promoting chemicals. Whereas superoxide radicals may be involved in the inhibition of dye-coupling by PB, radical intermediates of the insecticides may be responsible for the decrease in dye-coupling by DDT and lindane.

Introduction

Gap junctions are plasma membrane structures formed at the area of contact between two cells. They consist of paired connexons which form cell-to-cell channels and permit the free diffusion of small molecules (<900 Da) between adjacent cells (1–3). Each connexon is built up of six connexins, which are products of a multigene family. To date two gap junction proteins have been better characterized in hepatocytes, connexins 32 and 26 (3). It has been suggested that gap junctional channels are involved in metabolic cooperation, intercellular synchronization of cellular activities, cellular differentiation and growth of a tissue (4). Several lines of evidence suggest that dysfunction of gap junction communication may play an important role in carcinogenesis. Several oncogene products, such as those from *src* and *ras*, have been shown to be associated with a decrease in gap junctional communication (5,6). Many tumour cells express a heritable phenotype of reduced intercellular communication (IC*) competence, i.e. they show a dysfunction of homologous (between tumour cells) or heterologous (between tumour and normal cells) communication (1). Finally, several tumour promoting chemicals inhibit gap junctional IC in cell culture (cf. 2). It has been suggested that the inhibition of intercellular communication could isolate preneoplastic cells, thereby allowing selective growth of initiated cells (1,7).

Phenobarbital (PB), 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT) and γ-hexachlorocyclohexane (lindane) have been shown to promote tumour formation in the liver (8,9) and to inhibit IC between hepatocytes in primary culture (10–12). The mechanisms by which these tumour promoters inhibit IC between cells remain largely unknown. Activation of protein kinase C (PKC), which may underly the action of several skin tumour promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), is unlikely to play a role in DDT-induced inhibition of IC in V79 cells (13,14). Similarly, there is no evidence at present for the involvement of PKC in the hepatic effects of PB (15). Reactive oxygen species and organic peroxides have been shown to be active promoting principles in the skin (16,17), and similarly in the liver free radicals generated during oxygen activation may be involved in tumour promotion (cf. 18, 19). Reactive oxygen species can be generated in the liver, for example, by cytochrome P450, peroxisomes, mitochondria and NADH oxidase of the plasma membrane (18,20). Several findings suggest that induction of cytochrome P450 isoenzymes by compounds such as PB, or 'uncoupling' of the enzymes, causes increased production of superoxide anion radical (21,22).

Equally, it has been suggested that some of the biological effects of PB may be mediated by arachidonate cyclo-oxygenase products (23,24). Thus pretreatment of rats with indomethacin and 5,8,11,14-eicosatetraynoic acid inhibited induction of ornithine decarboxylase (ODC) by PB. ODC, the rate limiting enzyme in polyamine biosynthesis, plays an essential role in normal as well as in neoplastic growth (25). Recently Pereira *et al.* (26) compared the ability of different barbiturates to induce ODC activity and to enhance the formation of putative preneoplastic hepatocyte foci. Only the more potent ODC inducers exhibited tumour promoting activity. Furthermore, Denda *et al.* (24) showed that feeding rats inhibitors of arachidonic metabolism, including inhibitors of phospholipase A_2 and cyclo-oxygenase, significantly decreased hepatic tumour promotion by PB.

The present study addresses the question of whether reactive oxygen species and products of the arachidonate cyclo-oxygenase pathway are involved in the inhibition of IC of hepatocytes in primary culture by PB, DDT and lindane.

*Abbreviations: AA, arachidonic acid; Asp, aspirin; BSA, bovine serum albumin; Cat, catalase; DDT, 1,1,1,-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; EGTA; ethyleneglycol bis(β-aminoethylether)-*N*-*N*'-tetraacetic acid; IC, intercellular communication; ODC, ornithine decarboxylase; PB, phenobarbital; PBS, phosphate-buffered solution; SOD, superoxide dismutase.

Materials and methods

Chemicals

Calf thymus DNA, collagenase A and fatty acid-free bovine serum albumin (BSA) were from Boehringer Mannheim (Mannheim, Germany); bisbenzimide from Calbiochem (Frankfurt, Germany); Dulbecco's minimal essential medium (DMEM), penicillin/streptomycin (10 000 U/10 000 mg/ml) from Biochrom KG (Berlin, Germany); EGTA and LiCl from Merck (Darmstadt, Germanny); Lucifer Yellow CH and dimethylsulphoxide (DMSO) from Fluka Feinchemikalien (Neu-Ulm, Germany); dexamethasone from Merck, Sharp & Dohme (München, Germany); [1-^{14}C]arachidonic acid from Amersham Buchler (Braunschweig, Germany) and Perkoll™ from Pharmacia (Freiburg, Germany). All other chemicals and enzymes were obtained from Sigma Chemie GmbH (Deisenhofen, Germany) in the highest quality commercially available.

Animals

Male Wistar rats (7–10 weeks old, 160–240 g), maintained with free access to food and water, were obtained from the GSF breeding colony.

Isolation of hepatocytes

After anaesthetization with sodium pentobarbital (100 mg/kg body wt, 4 ml/kg) the abdominal cavity was opened by a midline incision and the liver was perfused via the portal vein for about 5 min with Ca^{2+}-free modified Hanks' solution containing 100 μM EGTA at a flow rate of 40 ml/min. Subsequently the perfusion was continued with the same Ca^{2+}-free solution without EGTA for 5 min. Finally, the liver was perfused for 15 min with DMEM containing 1.8 mM $CaCl_2$ and 0.13 U/ml of collagenase before Glisson's capsule was opened and the cells gently dispersed in DMEM. After filtration through an 80 μm and subsequently a 40 μm nylon mesh gauze the cells were washed three times in DMEM. Viability was greater than 85%, as determined by exclusion of 0.4% trypan blue.

In some experiments hepatocytes were purified by Percoll iso-density centrifugation to remove non-parenchymal cells, as described by Kreamer *et al.* (27). In brief, nine volumes of Percoll solution were added to one volume of 10-times concentrated Hanks' balanced salt solution (80.0 g/l NaCl, 4.0 g/l KCl, 2.0 g/l $MgSO_47H_2O$, 0.6 g/l KH_2PO_4, 10.0 g/l glucose, 0.6 g/l $Na_2HPO_42H_2O$ and 17.8 g/l HEPES, pH 7.4). Four millilitres of this solution were mixed with 6 ml DMEM containing 50×10^6 cells maximum and centrifuged for 10 min at 50 *g*. After aspiration of the supernatant the pellet containing purified hepatocytes was washed twice with DMEM.

Cell culture

For the investigation of IC, 1×10^6 viable hepatocytes were plated on 60 mm plastic culture dishes coated with rat tail collagen. The cells were suspended in 2 ml Leibovitz L-15 medium supplemented with 1.8 g/l $NaHCO_3$, 20 mM HEPES, 0.1 μM dexamethasone, 10 mU/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin. After 1.5 h of attachment cultures were washed once with HEPES-buffered PBS and refed with 2 ml fresh medium. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

The conditions of cell culture for determining arachidonic acid (AA) metabolism were similar to those described above, except that 2×10^6 Percoll-purified viable hepatocytes were plated. The medium was changed after 2 h and contained 0.5% fatty acid-free BSA.

Tumour promotors and other test substances, dissolved either in water or DMSO, were added after plating the cells. DMSO concentration never exceeded 0.3% (v/v). Before addition to the cultures AA was preincubated in the BSA-containing medium for 20 min at 37°C. Fatty acid free-BSA did not modulate the effects of test compounds on IC.

Assessment of hepatocyte gap junctional intercellular communication

IC between hepatocytes was detected by microinjection of fluorescent Lucifer Yellow. Injection needles with a guaranteed diameter of 0.3–0.5 μm were filled with 5% (w/v) Lucifer Yellow CH in 0.1 M LiCl (Femtotips™; Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Hepatocyte cultures 5–8 h old were observed under a Zeiss Axiovert 10 epifluorescence microscope (Oberkochen, Germany) and 20–30 cells/dish were injected with the fluorescent dye solution using an automatic pneumatic micromanipulator–injector system (Micromanipulator model 5170, Microinjector model 5242; Eppendorf). Dye solution was injected into cells with a pressure of 30–50 hPa for 0.7 s. Five minutes after dye injection hepatocytes in direct contact with 'dye-donor' hepatocytes were evaluated for evidence of dye-coupling. The percentage of dye-coupled neighbouring cells and of non-coupling dye-donors was determined for each experimental condition.

Labelling of hepatocytes with [1-^{14}C]AA

Cells were plated in the presence of 25 μM [1-^{14}C]-arachidonic acid (1 μCi/25 nmol). After 2 h of labelling, 61.5 ± 6.4% (n=4) of the added AA was cell-associated. Cultures were then washed twice with HEPES-buffered PBS and refed with 2.6 ml of fresh medium (without AA) containing the test compounds. Cultures were maintained up to 24 h and aliquots of the medium were taken at various time points. These aliquots were centrifuged for 5 min at 10 000 *g* to remove dead cells and debris. Radioactivity in the supernatant was determined by liquid scintillation counting.

Qualitative analysis of radioactivity released into the medium

After centrifugation supernatants were acidified with 25 μl 0.1 N HCl per 0.5 ml supernatant, to get a final pH value of 2.9 and extracted twice with 0.5 ml ethylacetate. Extracted lipids were dried, redissolved in 100 μl ethylacetate and subjected to thin layer chromatography according to Fischer *et al.* (28). AA, PGE_1, PGE_2 and $PGF_{2\alpha}$ were taken as reference substances.

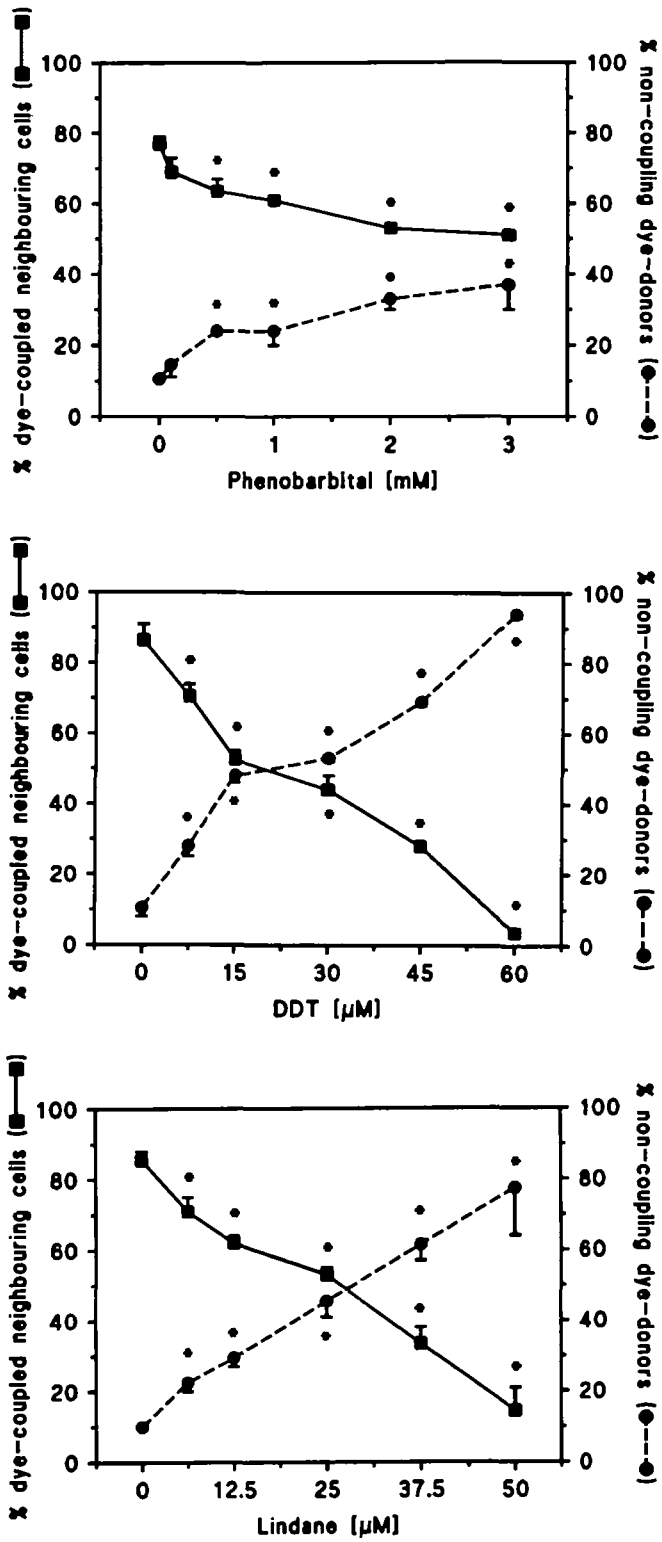
TLC sheets (Merck, Darmstadt, Germany) were scanned for radioactivity with a Rita Radio-TLC-Analyser 3200 (Straubenhardt, Germany).

Results

The dye-coupling level in rat hepatocyte cultures was 88.1 ± 0.7% (n=34) 5 h after plating. It remained at this high level during the following 4 h of experimentation in both untreated and DMSO-treated control cultures. The tumour promoting chemicals PB, DDT and lindane significantly inhibited dye-coupling (Figures 1–3). Inhibition of IC by the three chemicals was dose related. Phenobarbital inhibited dye-coupling by about 30% at a concentration of 2–3 mM. Lindane and DDT were considerably more effective than the barbiturate. The concentrations of lindane and DDT required to inhibit dye-coupling by about 30% were at least 80–100-fold lower than the concentration required to achieve a similar inhibition by PB. The course of the concentration–effect curves became steeper above 25 μM lindane and 30 μM DDT. The increasing effectiveness of the insecticides at higher concentrations may be due to secondary effects, such as cytotoxicity. Light microscopically, cells treated with more than 25 μM lindane or 30 μM DDT showed the first signs of morphological alterations (stress fibres). In order to exclude cytotoxicity effects, DDT and lindane were only used at concentrations of 30 μM and 25 μM, respectively, in the following experiments. At these concentrations inhibition of IC was reversible when the test compounds were removed from the medium (Figure 4). As shown in Figures 1–3, the decrease in the percentage of dye-coupled neighbouring cells resulted from the increased number of dye donors which had lost the ability to communicate with any of their neighbours.

To examine whether reactive oxygen species may be involved in the action of PB, DDT and lindane we studied the effect of antioxidants on inhibition of dye-coupling in hepatocyte cultures. Vitamin E effectively counteracted the inhibition of IC by PB and lindane and partially reversed the inhibition by DDT (Figure 5). The antioxidant vitamin E (100 μM) is not specific in its radical scavenging ability, reacting with several organic radicals. To address more specifically the question of whether superoxide radicals are involved, similar experiments were performed using superoxide dismutase. Addition of superoxide dismutase (100 units/ml) almost completely prevented the inhibition of IC caused by PB, but not that produced by DDT and lindane (Figure 6). Catalase did not modify inhibition of IC by the xenobiotics (Figure 6). Similar results were also obtained when the effects of SOD and vitamin E were examined at lower concentrations of the tumour promoters (1 mM PB, 15 μM DDT and 12.5 μM lindane) (data not shown).

Recent results suggested involvement of cyclo-oxygenase in the tumour promoting activity of PB. We therefore studied whether inhibitors of cyclo-oxygenase, aspirin and indomethacin, affect the action of PB, DDT and lindane on dye-coupling in hepatocyte cultures. Both inhibitors significantly reversed the effect of 1 and 2 mM PB on IC, however they did not alter the response of the hepatocytes to 30 and 15 μM DDT or 25 and 12.5 μM lindane (Figure 7; data for indomethacin and the lower



Figs 1–3. Dose–response effects of PB, DDT and lindane on rat hepatocyte intercellular communication after 5 h of treatment with the tumour promotors. Dye spreading was assayed 5 min after injection. 20–35 separate injections into single hepatocytes (dye-donors) were performed for each point in each experiment. Results represent the mean and range of two separate experiments. Asterisks denote that the values of each of the two experiments are significantly different from their respective untreated controls ($P<0.05$, paired t-test).

concentrations of the tumour promoters not shown). Even at a concentration of 1 mM aspirin did not affect the inhibition by the insecticides (data not shown). Further experiments were

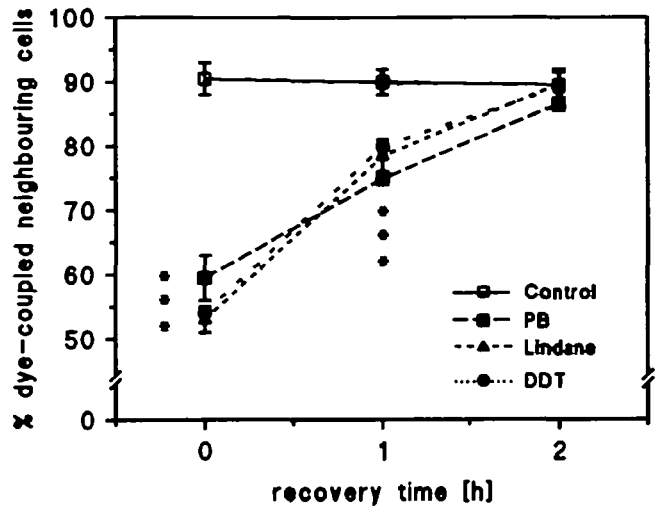


Fig. 4. Recovery of rat hepatocyte intercellular communication after removal of PB (2 mM), DDT (30 μM) and lindane (25 μM) from the culture medium. Hepatocyte cultures were treated with the tumour promoters for 5 h. Subsequently dye-coupling was evaluated (0 h). The cultures were washed twice and refed with fresh medium and dye-coupling was evaluated over the next 2 h. Results represent the mean and range of two separate experiments. Asterisks denote that the values of each of the two experiments are significantly different from their respective untreated controls ($P<0.05$, paired t-test).

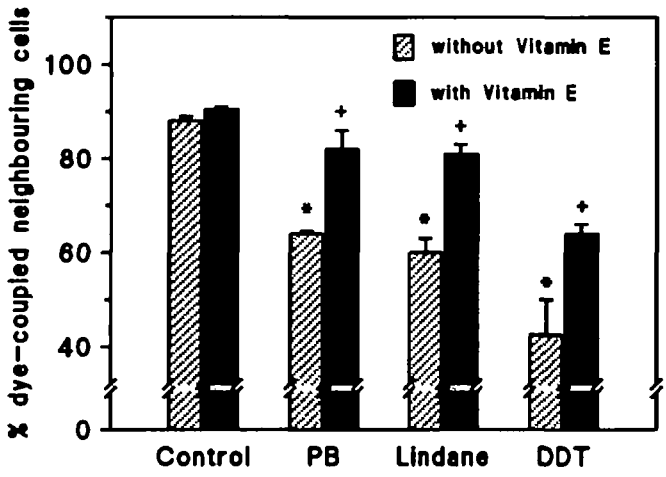


Fig. 5. Effect of vitamin E on PB-, DDT- and lindane-induced inhibition of IC. Hepatocyte cultures were treated with PB (2 mM), DDT (30 μM) or lindane (25 μM) with or without vitamin E (100 μM). Dye-coupling was evaluated after 5 h. Results represent the mean and range of two separate experiments. Asterisks denote that the values of each of the two experiments are significantly different from their respective untreated controls, + denotes that the values in the presence of the tumour promoters and vitamin E are significantly different from values in the presence of the tumour promoters only ($P<0.05$, paired t-test).

carried out to clarify whether inhibition of cyclo-oxygenase is responsible for the removal of the effect of PB on IC in rat hepatocyte cultures. It has been suggested that in contrast to non-parenchymal liver cells (e.g. Kupffer and endothelial cells), hepatocytes have only a very low capacity to synthesize prostaglandins (29). Consequently the effect of the non-steroidal anti-inflammatory agents could possibly be mediated by non-parenchymal liver cells contaminating hepatocyte cultures. To exclude this possibility we purified the liver cell suspension by centrifugation in a Percoll gradient to remove contaminating non-parenchymal cells. As shown in Figure 8, PB inhibition of IC,

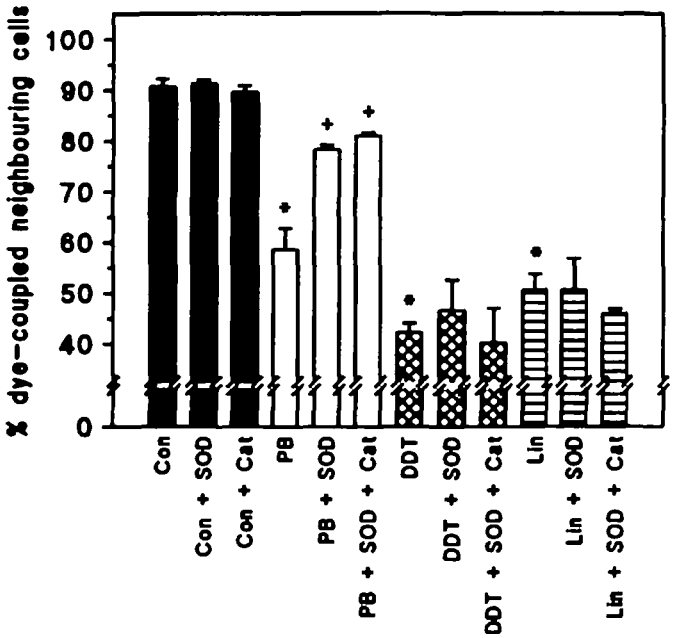


Fig. 6. Effect of SOD and catalase on inhibition of hepatocyte intercellular communication by PB, DDT and lindane. Hepatocyte cultures were treated with PB (2 mM), DDT (30 μM) or lindane (25 μM) with or without SOD (100 U/ml) and catalase (Cat, 100 U/ml), respectively. Dye-coupling was assayed after 5 h. Results represent the mean ± SEM of three separate experiments. Asterisks denote that the values in the presence of the tumour promoters are significantly different from untreated controls, + denotes that the values in the presence of the tumour promoters and antioxidants are significantly different from values in the presence of the tumour promoters only ($P<0.05$, paired t-test).

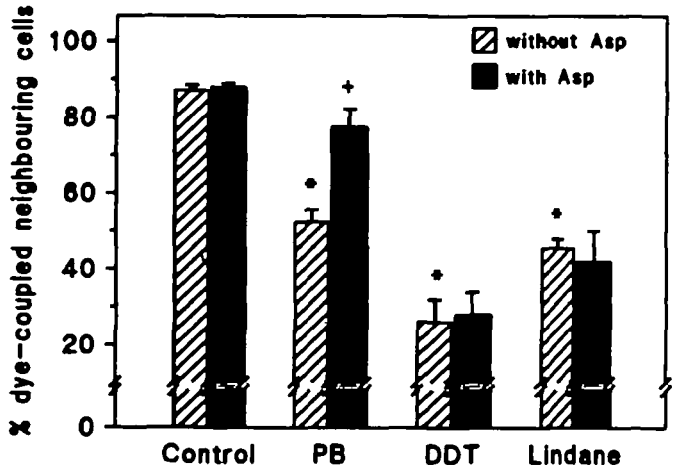


Fig.7. Effect of aspirin on inhibition of hepatocyte intercellular communication by PB, DDT and lindane. Hepatocyte cultures were treated with PB (2 mM), DDT (30 μM) or lindane (25 μM) in the presence or absence of aspirin (Asp, 100 μM). Dye-coupling was evaluated after 5 h. Results represent the mean ± SEM of three separate experiments. Asterisks denote that the values in the presence of the tumour promoters are significantly different from untreated controls, + denotes that the values in the presence of the tumour promoters and aspirin are significantly different from values in the presence of the tumour promoters only ($P<0.05$, paired t-test).

and aspirin counteraction of the effect, were similar in parent and Percoll centrifuged hepatocytes. If PB activates cyclo-oxygenase it would be expected to induce an increase in arachidonic acid metabolism in liver cell cultures. Thus hepatocytes were prelabelled with [^{14}C]arachidonate and the

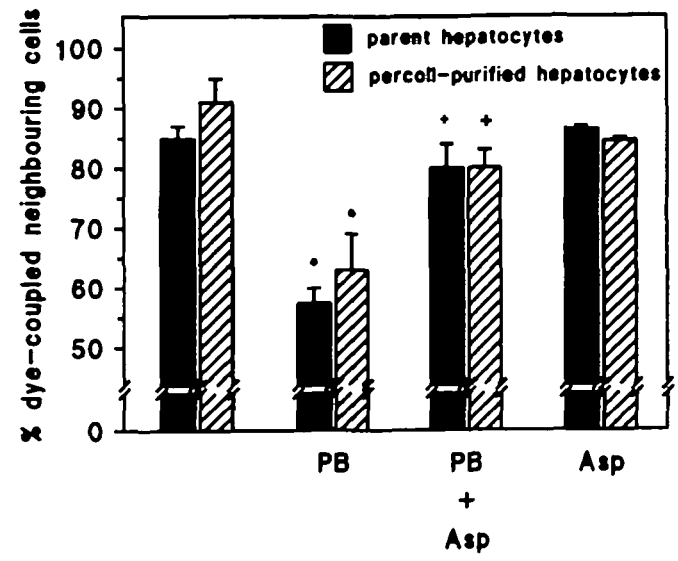


Fig. 8. Effect of aspirin on PB-induced inhibition of intercellular communication in cultures of parent and Percoll-purified hepatocytes. Percoll centrifugation of hepatocytes was performed as described in Materials and methods. Hepatocyte cultures were treated with PB (2 mM) in the presence or absence of aspirin (Asp, 100 μM). Dye-coupling was evaluated after 5 h. Results represent the mean and range of two separate experiments. Asterisks denote that in each of the two experiments the values in the presence of PB are significantly different from their respective untreated controls, + denotes that the values in the presence of PB and aspirin are significantly different from values in the presence of PB only ($P<0.05$, paired t-test).

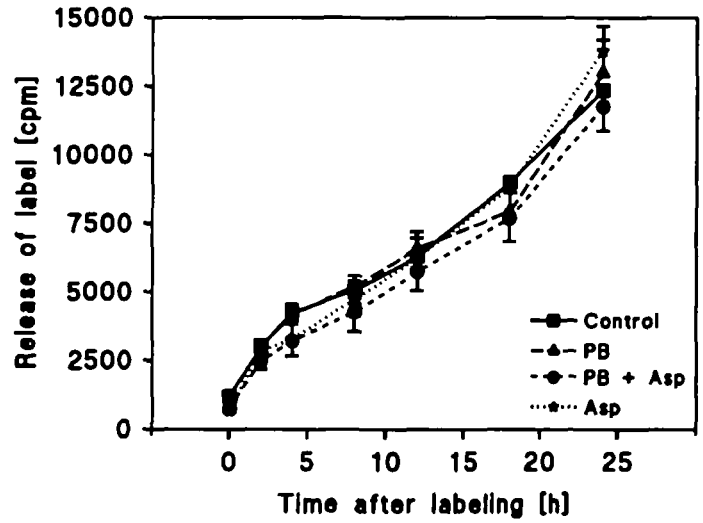


Fig. 9. Effect of PB and aspirin on the release of radioactivity from hepatocytes prelabelled with [^{14}C]arachidonic acid. Isolated hepatocytes were plated in the presence of 25 μM [^{14}C]arachidonic acid (1 μCi/25 nmol). After 2 h cultures were washed twice and refed with fresh medium containing the test compounds (2 mM PB, 100 μM Asp).

release of radioactivity into the medium was determined. Incorporation of radioactivity in the hepatocytes was greater than 61% after a 2 h incubation in the presence of 25 μM [^{14}C]arachidonate. Within the observation period of 24 h there was a constant release of radioactivity into the medium (Figure 9). Addition of PB (2 mM) and aspirin (100 μM) did not affect this process. Ethylacetate extracts of the medium were further analysed by thin layer chromatography (TLC). Arachidonic acid gave a strong signal, however, no cyclo-oxygenase products could be detected in samples of untreated or PB- or aspirin-treated cultures (data not shown). Moreover, supplementation of the

medium with 20 μM arachidonic acid did not enhance inhibition of IC by PB (data not shown). Finally, we studied whether the main prostaglandins determined in the liver, PGE_2 and $PGF_{2\alpha}$, affect IC in hepatocyte cultures. Neither of the prostaglandins affected dye-coupling of the cells, even when tested at the high concentration of 8 μM. Dye-coupling of untreated, PGE_2- and $PGF_{2\alpha}$-treated cells was 85, 83 and 88% respectively.

Discussion

A common feature of many tumour promoting chemicals is their ability to inhibit IC (2). In the present study we investigated the effect of three hepatic tumour promoters, PB, DDT and lindane, on IC in primary cultures of rat hepatocytes. IC was evaluated by microinjection of fluorescent dye and observing inhibition of spread of dye into adjacent cells. The barbiturate and insecticides decreased dye-coupling between hepatocytes. This finding is in good agreement with the observations of Klaunig and colleagues who studied dye-coupling between mouse hepatocytes (10,30). Inhibition of dye-coupling was completely reversible within 2 h (Figure 4), indicating that the effects observed were not due to cytotoxicity. At a cellular level inhibition of IC by the tumour promoters constitutes an all or none reaction: either all gap junctions of a cell are functionally active or all gap junctions are closed, i.e. either a dye donor communicates with all or with none of the neighbouring hepatocytes. This interpretation is suggested by the steady increase of dye donors which lost the ability to communicate with any of their neighbours (Figures 1–3).

Using the metabolic cooperation assay, Ruch and Klaunig (31) showed that PB, DDT and lindane inhibit gap-junctional passage of [^{3}H]uridine nucleotides from prelabelled 'donor' mouse hepatocytes to non-labelled 'recipient' mouse hepatocytes, and that both the antioxidant vitamin E (100 μM) and superoxide dismutase (SOD) (100 units/ml) prevented inhibition of IC by all three chemicals. These results suggested that activated oxygen species are produced during treatment with the hepatic tumour promotors and are responsible for the inhibition of IC in cultured hepatocytes by these chemicals. In the present study, using rat hepatocytes, we also found that vitamin E (100 μM) efficiently counteracted inhibition of dye-coupling by PB and lindane and partially counteracted inhibition by DDT. SOD (100 units/ml), however, was only effective in PB-treated cultures (Figure 6). At present we have no explanation for the discrepancy between our results obtained in rat hepatocytes and those of Ruch and Klaunig in mouse hepatocytes (31). Our findings suggest that PB, on the one hand, and DDT and lindane, on the other, act via different radical mechanisms. In the case of PB superoxide radicals are most likely involved, since SOD reversed the effect of the barbiturate. The source of the oxygen radicals, however, is not known. In principle chemicals may cause oxidative stress by acting on cellular enzymes or structures which are capable of producing reactive oxygen species (such as cytochrome P450, mitochondria, peroxisomes and plasma membrane oxidase) (18,20). Since SOD has been shown to be efficiently taken up by rat liver cells (32) the generation of reactive oxygen species may take place both in the cytoplasm and at the plasma membrane. Cytochrome P450 may represent an intracellular site of the production of reactive oxygen (18, 33); increased generation of superoxide radicals and H_2O_2 has been shown in microsomes or cell homogenates after treatment of rats with enzyme inducers (19,21,22) and as a result of 'uncoupling' of cytochrome P450 by certain substrates such as hexobarbital and hexachlorobiphenyl (33). Induction of cytochrome P450 is highly unlikely to take place in the present experiments, since the tumour promoting chemicals were only present in the cultures for 5 h. On the other hand it is not known to what extent PB may induce the production of reactive oxygen species by 'uncoupling' in the intact cell. In contrast to the barbiturate, superoxide radicals are unlikely to play an essential role in the inhibition by DDT and lindane. The insecticides may affect IC primarily via the formation of radical intermediates and following reactions such as lipid peroxidation. Formation of a pentachlorocyclohexane radical from lindane and of a reactive free-radical intermediate from DDT have been proposed (34,35) and could represent a source of oxidative stress (36). Recently it has been suggested that the biological effects of PB in the liver may be mediated by products of arachidonic acid metabolism, since inhibitors of cyclo-oxygenase, such as aspirin and indomethacin, inhibit ODC and tumour promotion in the liver (23,24). Similarly, the present results showed that aspirin and indomethacin significantly prevented inhibition of dye-coupling by PB. However, this finding does not necessarily imply that cyclo-oxygenase products are involved in the action of PB on IC. Firstly, parenchymal liver cells have been reported to synthesize almost no prostaglandins, suggesting a very low cyclo-oxygenase activity in hepatocytes (29). The possibility that the effect of PB on IC was mediated by prostaglandin synthesis of contaminating non-parenchymal cells was excluded by subjecting hepatocytes to Percoll density centrifugation prior to cultivation. Inhibition of IC by PB was not altered in the purified hepatocytes. Secondly, PB did not affect the release of radioactivity from hepatocytes prelabelled with [^{14}C]arachidonate. Thirdly, analysis of ethylacetate extracts of the culture medium by thin layer chromatography revealed no changes in the radioactivity distribution in samples of untreated, PB- or aspirin-treated cultures. Fourthly, the main prostaglandins in the liver, PGE_2 and $PGF_{2\alpha}$, did not inhibit IC in cultured hepatocytes.

Besides inhibition of cyclo-oxygenase, several other mechanisms of action have been discussed to explain the biological effects of non-steroidal anti-inflammatory agents, such as inhibition of Ca^{2+}-uptake in stimulated neutrophils (37), inhibition of aromatic amino acid decarboxylase (38) and folate-dependent enzymes (39). High intracellular concentrations of Ca^{2+} have been shown to decrease gap junction permeability (40). However, we are not aware of any report showing a severe disturbance of Ca^{2+} homeostasis by PB in hepatocytes and it is not known whether aspirin and indomethacin modulate Ca^{2+} concentrations in liver cells.

In summary, the present results suggest significant differences in the mechanism of inhibition of IC by the three tumour promoting chemicals studied. SOD and aspirin/indomethacin prevented inhibition of dye-coupling by PB but not by DDT and lindane. The present data, however, do not suggest involvement of cyclo-oxygenase in the action of PB on IC.

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