Kinetics of Phenobarbital Inhibition of Intercellular Communication in Mouse Hepatocytes

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ABSTRACT

Gap junction-mediated intercellular communication in untreated and phenobarbital-treated C57BL/6 × C3H F1 mouse hepatocytes was evaluated by microinjection of fluorescent Lucifer Yellow CH dye. Intercellular communication (dye coupling) was detected in untreated hepatocytes after 0.5 h in culture, reached a maximum level in 24- and 48-h-old cultures (85.2%), and then decreased over the next 72 h. Phenobarbital (20-500 μg/ml) decreased dye coupling in a dose-related manner when added to freshly plated cultures. This inhibitory effect was evident during 0.5-12 h of treatment but was not seen in cultures treated for 24 h. Phenobarbital also decreased dye coupling within 30 min when added to established (24-h-old) hepatocyte cultures. This effect was maximal after 2 h treatment. In these cultures, dye coupling recovered within 15 min after removal of the promoter. Hepatocytes, pretreated with phenobarbital for 24 h, did not show inhibition of dye coupling after reapplication of phenobarbital. Thus, phenobarbital inhibited mouse hepatocyte dye coupling rapidly and reversibly, and the cells became refractory to the inhibitory effect after prolonged treatment.

INTRODUCTION

Cell growth may be regulated by the cell-to-cell exchange of small molecules and ions through gap junctions (i.e., intercellular communication) (1). The loss of gap junctions or a decrease in cell-to-cell communication may predispose cells to enhanced growth (1). Gap junctions are decreased or absent in regenerating liver (2, 3), and intercellular communication can be decreased by growth factors (4) and tumor promoters (5-8). Several types of neoplastic cells have also been shown to have a reduced or a complete loss of intercellular communication (1).

The loss or inhibition of intercellular communication is also thought to be a mechanism involved in tumor promotion (8). Tumor promoters often have mitogenic activity in their target tissue, thus permitting the expansion of the initiated cell population (9, 10). This stimulated cell growth may increase the likelihood of additional genetic events required for complete neoplastic transformation to occur in an initiated cell (10). The mechanism by which tumor promoters exert their mitogenic effect remains unclear. However, it is now known that nearly all tumor promoters inhibit intercellular communication both in vivo (11-13) and in vitro (8). This inhibitory effect appears to be characteristic of tumor promoters, not genotoxic carcinogens or cytotoxic agents (14). Therefore, one possible mechanism by which tumor promoters enhance initiated cell growth may be through their ability to inhibit intercellular communication.

Work in our laboratory has focused on defining the mechanisms by which tumor promoters inhibit hepatocyte intercellular communication (7, 14-16). We have previously assessed hepatocyte intercellular communication by gap-junctional passage of [3H]uridine nucleotides from prelabeled “donor” hepatocytes to nonlabeled “recipient” hepatocytes by autoradiography (7). With this method, we have shown that several liver tumor promoters inhibited hepatocyte intercellular communication, whereas genotoxic carcinogens and hepatocytotoxins did not (7, 14). In addition, the inhibitory effect of liver tumor promoters on rodent hepatocyte intercellular communication correlated with in vivo strain and species sensitivities to the hepatocarcinogenic activity of the promoters (16).

Unfortunately, the [3H]uridine method to detect hepatocyte intercellular communication is limited in that the kinetics of the inhibitory effect of a promoter cannot easily be studied. This is because there is a delay between gap-junctional passage of [3H]uridine nucleotides from donor to recipient cells and incorporation into recipient RNA (7). In hepatocytes, this delay limits the earliest time of detection of intercellular communication to 4 h after establishment of donor-recipient cultures (7). Also, agents that inhibit recipient cell RNA synthesis might artifactually result in observed decreases in intercellular communication. To circumvent these limitations of the nucleotide transfer method, we have in this investigation evaluated hepatocyte intercellular communication by microinjection of fluorescent dye (Lucifer Yellow CH) and observing spread of dye into adjacent cells (dye coupling). Lucifer Yellow CH is plasma membrane impermeable (17) but small enough (Mw 440) to pass through liver cell gap junctions (molecular weight exclusion limit of about 1000; Ref. 18). Thus, detection of hepatocyte intercellular communication using Lucifer Yellow CH dye coupling is nearly instantaneous and dependent only on the existence of permeable gap junctions, not on tracer incorporation. In the present study, we have utilized Lucifer Yellow CH dye coupling to evaluate the time course of the inhibitory effect of phenobarbital, a liver tumor promoter (19), and recovery from the inhibitory action of phenobarbital on male C57BL/6 × C3H F1 (hereafter called B6C3F1) mouse hepatocyte intercellular communication.

MATERIALS AND METHODS

Animals. Male B6C3F1/Cnibr mice, 4-6 months old, were purchased from Charles River Laboratories, Inc. (Wilmington, MA), and used exclusively in this study. Mice were housed in polycarbonate cages and fed Purina Lab Chow Blox (Ralston Purina Co., St. Louis, MO) and water ad libitum.

Chemicals. Phenobarbital and Lucifer Yellow CH were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and tissue culture supplies were purchased from sources previously indicated.

Hepatocyte Isolation and Culture. Hepatocytes were isolated by two-stage collagenase perfusion through the portal vein (20) and plated out at 1 × 10⁷ viable cells per 60-mm plastic dish in 3 ml medium. Initial viability of the isolated cells, determined by trypan blue dye exclusion, was always above 90%. The cells were cultured in Leibovitz's L-15 medium supplemented with glucose (1 mg/ml), dexamethasone (1 μg/ml), gentamicin sulfate (50 μg/ml), and fetal bovine serum (10%, v/v). Hyclone Laboratories, Logan, UT) at 37°C (21). The cultures were refed with 3 ml medium/dish after a 2-h attachment period.

Detection of Hepatocyte Intercellular Communication by Lucifer Yellow Dye Injection. Microelectrodes were pulled from 1.5-mm-diameter single barrel glass Kwik-Fil capillaries (World Precision Instruments, 

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2519
Inc., New Haven, CT) using a Narishige model PE-2 vertical microelectrode puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan). Microelectrode tip diameters measured approximately 1 μm. Microelectrode tips were backfilled with 5% (w/v) Lucifer Yellow CH in 0.1 M LiCl, and microelectrode barrels were backfilled with 0.1 M LiCl. Hepatocyte cultures (2-120 h old) were observed under a Nikon Optiphot UFX-II epifluorescence microscope (Nikon, Inc., Garden City, NJ) at ×100 at room temperature. “Donor” hepatocytes were impaled with the microelectrode under phase contrast microscopy and dye was iontophoretically ejected using continuous 3 nA current for 1 min. Five min after cessation of dye injection, hepatocytes in direct contact with donor hepatocytes (i.e., recipient hepatocytes) were evaluated under epifluorescence for evidence of dye accumulation (dye coupling). All recipients in contact with injected donors were evaluated for evidence of dye coupling. The percentage of dye-coupled recipients was determined for each treatment and sampling time. Differences in the number of dye-coupled and non-dye-coupled recipients between treatment groups were statistically evaluated by 2 x 2 x2 analysis (22). Dye-coupled cells were photographed by epifluorescence-phase contrast microscopy with Tri-Pan film (ASA 400) (Eastman Kodak Co., Rochester, NY).

Occurrence of Dye Coupling in Nontreated Mouse Hepatocytes during the First 120 h of Culture. To determine the extent of intercellular communication (dye coupling) in nontreated hepatocytes over a prolonged culture period (120 h), nontreated cultures were sampled at 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, and 120 h after initial plating and evaluated for dye coupling. The cultures were refed just prior to the 2-, 24-, 48-, 72-, 96-, and 120-h sampling times.

Effects of Phenobarbital on Dye Coupling in Mouse Hepatocyte Cultures. Initial experiments were performed to determine if phenobarbital could inhibit intercellular communication (dye coupling) between newly cultured cells and if the effect was sustained during the first 48 h of culture. Immediately after plating, cultured hepatocytes were treated with phenobarbital (20, 100, or 500 μg/ml) in DMSO or with DMSO (0.2%). After 2 h attachment, the cultures were refed and retreated with phenobarbital or DMSO only. Dye coupling in phenobarbital- and DMSO-treated cultures was determined at 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h after plating. The cultures were refed and retreated with phenobarbital prior to the 2-h sampling time but not at 24 and 48 h.

To determine if phenobarbital could inhibit intercellular communication in established cultures (with preexisting gap junctions) and to assess the minimum duration of treatment necessary for an inhibitory effect to be seen, 24-h-old cultures were treated with phenobarbital (500 μg/ml) or DMSO (0.2%) only and evaluated for dye coupling 0.25, 0.5, 1, 2, 3, and 4 h after treatment.

Studies were also performed to assess how rapidly hepatocyte dye coupling recovered following removal of phenobarbital. Twenty-four-h-old cultures were treated with phenobarbital (500 μg/ml) or DMSO (0.2%) for 2 h and then evaluated for dye coupling. Similar cultures were treated with phenobarbital (500 μg/ml) or DMSO (0.2%) for 2 h and then washed 3 times with culture medium and refed with 3 ml of culture medium. At 0, 0.25, 0.5, and 1 h after refeeding, dye coupling was determined in the phenobarbital- and DMSO-pretreated cultures.

Experiments were also performed to determine if the hepatocytes became refractory to the inhibitory effect of phenobarbital on dye coupling after prolonged exposure (0-24 h in culture). Hepatocyte cultures were treated with phenobarbital (500 μg/ml) or DMSO (0.2%) at plating (0 h) and after attachment and refeeding (2 h) and then assayed for dye coupling after 8 and 24 h. Additional phenobarbital- and DMSO-treated cultures were refed after 24 h treatment and secondarily treated with either phenobarbital (500 μg/ml) or DMSO (0.2%). Dye coupling was determined in these cultures 2 h later.

RESULTs

Fig. 1 depicts phase contrast-fluorescence photomicrographs of dye-coupled mouse hepatocytes after 2 h (Fig. 1a) or 24 h (Fig. 1b) in culture. Intercellular communication (dye coupling) in nontreated mouse hepatocyte cultures over the first 0.5–120 h of culture is shown in Fig. 2. Dye coupling increased rapidly during the first hours of culture to a value of 56.4% dye-coupled...
recipient cells at 2 h. This level of dye coupling displayed a more gradual increase during the next 22 h in culture to a maximal level at the 24-h sampling time of 89.2%. Dye coupling remained stable in 48-h cultures but then decreased markedly over the next 72 h in culture to 39.2% dye-coupled recipients. Refeeding of cultures with fresh medium had no effect on dye coupling when compared to nonrefed cultures (data not shown). This suggests that factors in the medium did not contribute to the decline in dye coupling over the 120-h culture period.

When phenobarbital (20–500 μg/ml) was added to the newly plated cultures and dye coupling was assessed over the next 0.5–48 h, a dose-responsive inhibition of dye coupling was observed in the cultures sampled up to 12 h (Fig. 3). However, no statistically significant difference in dye coupling was observed in the cultures sampled at 24 and 48 h, indicating that the hepatocytes had recovered from the phenobarbital inhibitory effect.

Phenobarbital also inhibited dye coupling when administered to established (24-h-old) hepatocyte cultures (Fig. 4). The initial dye coupling level in the 24-h-old cultures was 84.0%, and this level remained similar over the next 240 min in DMSO-treated control cultures. When phenobarbital (500 μg/ml) was added to the cultures, a statistically significant inhibition of dye coupling was evident after 30 min exposure. Dye coupling levels then decreased further with increasing duration of phenobarbital exposure to a minimum of 31.9% after 2 h exposure. This level of inhibition remained similar in the 3- and 4-h-exposed cultures.

The recovery from phenobarbital-mediated inhibition of dye coupling in the 24- and 48-h-treated cultures (Fig. 3) suggested either that the hepatocytes had become unresponsive to the inhibitory effect of phenobarbital after prolonged exposure or that phenobarbital had been altered or removed from the media. To test these two hypotheses, two experiments were performed. First, the hepatocytes were preexposed to phenobarbital (500 μg/ml) for 24 h and then evaluated for their dye coupling response to a second administration of phenobarbital (500 μg/ml) (Table 1). In cultures that were treated with phenobarbital at plating (0 h) and refeeding (2 h), dye coupling was significantly decreased after 8 h (Table 1). However, after 24 h treatment with phenobarbital, dye coupling returned to the control level (Table 1). These cultures were then refed at 24 h and treated with a second application of phenobarbital (500 μg/ml) or DMSO (0.2%) for 2 h. In DMSO-preexposed cultures, secondary application of phenobarbital resulted in a significant inhibition of dye coupling, while in hepatocyte cultures preexposed to phenobarbital, secondary application of phenobarbital had no effect on dye coupling (Table 1). In a second experiment, hepatocyte cultures were treated with phenobarbital (20, 100, or 500 μg/ml) or DMSO (0.2%) for 24 h. After the 24-h treatment period, “conditioned” media from the treated cultures were transferred to nontreated, 24-h-old hepatocyte cultures. After 2 h incubation in the “conditioned” media, dye coupling was assayed. There was a dose-related inhibition of dye coupling by phenobarbital in these cultures (Table 2) indicating that phenobarbital was still present in an active form in “conditioned” media. Thus, these two experiments indicate that the recovery from the inhibitory effect of phenobarbital on hepatocyte dye coupling in 24-h-treated cultures was not due to loss or alteration of phenobarbital, but instead a change in the hepatocyte response to the promoter.

Fig. 5 illustrates that the 24-h-old hepatocytes recovered from the inhibitory effect of phenobarbital on intercellular communication in a rapid fashion when fresh medium was added to the cultures. When 24-h hepatocyte cultures were treated with phenobarbital (500 μg/ml) for 2 h, a significant decrease in dye coupling was seen compared to DMSO control cultures (39.4%

### Table 1 Development of refractoriness to phenobarbital inhibition of mouse hepatocyte dye coupling

<table>
<thead>
<tr>
<th>Treatmentsa</th>
<th>Dye coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h and 2 h</td>
</tr>
<tr>
<td>DMSO</td>
<td>8</td>
</tr>
<tr>
<td>PB</td>
<td>8</td>
</tr>
<tr>
<td>DMSO</td>
<td>24</td>
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<td>PB</td>
<td>26</td>
</tr>
<tr>
<td>PB</td>
<td>26</td>
</tr>
</tbody>
</table>

* Hepatocyte cultures were treated for 24 h (or 8 h) with either PB (500 μg/ml) or DMSO (0.2%). After 8 h, 2 groups of cultures (DMSO and PB treated) were assessed for dye coupling. After 24 h, an additional 2 groups of cultures (PB and DMSO treated) were evaluated for dye coupling. Medium was removed from the remaining cultures and replaced with medium containing either PB or DMSO and evaluated for dye coupling after 2 h of treatment.

* PB, phenobarbital.

* * P < 0.05; x2 test.
MOUSE HEPATOCYTE INTERCELLULAR COMMUNICATION

Table 2  Effects of "conditioned" phenobarbital-containing media on mouse hepatocyte dye coupling in 24-h-old cultures

<table>
<thead>
<tr>
<th>Conditioned mediaa</th>
<th>Coupled/noncoupled recipients</th>
<th>% coupled</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>40/3</td>
<td>93.0</td>
</tr>
<tr>
<td>DMSO 0.2%</td>
<td>42/4</td>
<td>91.3</td>
</tr>
<tr>
<td>PB, 20 µg/ml</td>
<td>32/10</td>
<td>76.2</td>
</tr>
<tr>
<td>PB, 100 µg/ml</td>
<td>28/19a</td>
<td>59.6</td>
</tr>
<tr>
<td>PB, 500 µg/ml</td>
<td>19/23a</td>
<td>45.2</td>
</tr>
</tbody>
</table>

a  Hepatocyte cultures were treated for 2 h with "conditioned" media that had been transferred from other hepatocyte cultures that had been treated for 24 h with phenobarbital (PB; 20, 100, or 500 µg/ml) or DMSO or nontreated (NT).

PB, 100 µg/ml Coupled/noncoupled

Fig. 5. Recovery of dye coupling in established (24-h-old) mouse hepatocyte cultures following removal of phenobarbital (PB) after 2 h pretreatment (a P < 0.05 versus DMSO-pretreated control group).

versus 87.1%, respectively). This level of inhibition was similar to that seen in cultures treated with phenobarbital (500 µg/ml) for 2 h (Fig. 4). When phenobarbital was removed from the cultures, the levels of dye coupling in phenobarbital-pretreated cultures rapidly approached control levels (Fig. 5). Immediately after washing and refeeding (0 min recovery time) dye coupling had increased to 58.1% but was still significantly less than the control level (83.9%). However, after 15 min recovery, dye coupling in the phenobarbital-pretreated cultures was equivalent to the control level and remained so over the remaining sampling times.

DISCUSSION

Intercellular communication through gap junctions may be one mechanism by which cell growth is regulated (1). Tumor promoters might enhance tumor formation in vivo by inhibiting intercellular communication (8). In the present study, we evaluated the kinetics of the development of intercellular communication in untreated and phenobarbital-treated cultured B6C3F1 mouse hepatocytes. Previously we have examined intercellular communication between rodent hepatocytes by following the gap junctional passage of [3H]uridine nucleotides from prelabeled donor cells to nonlabeled recipient cells (7, 14–16). The [3H]uridine method cannot be used to evaluate the short-term effects of tumor promoters on hepatocyte intercellular communication or the kinetics of the inhibitory effect of the promoter. Therefore, in the present study, we have utilized fluorescent dye coupling techniques to study the kinetics of the development of intercellular communication in nontreated and phenobarbital-treated hepatocyte cultures.

In nontreated, freshly plated mouse hepatocyte cultures, dye coupling increased during the first 48 h in culture and then decreased over the next 3 days in culture (Fig. 2). The nonlinear development of intercellular communication suggests that there is a rate-limiting component or process of hepatocyte gap junction formation. This may be the availability of gap junction subunits (connexons (2)) in the cultured cells, the number of hepatocyte-to-hepatocyte contacts, and/or de novo synthesis of connexon protein subunits. The peak dye coupling level seen in the nontreated cultures (85.2%) was similar to the level of intercellular communication detected by the [3H]uridine method (83.0%) (7). However, intercellular communication was not detected with the [3H]uridine assay until 4 h in culture (7), whereas dye coupling was seen after 0.5 h in culture in the present study. This suggests that the sensitivities of the two methods for detecting peak levels of hepatocyte intercellular communication are similar but that the dye injection method is better suited to analyzing the rates of development of intercellular communication.

Phenobarbital inhibited dye coupling in freshly plated hepatocyte cultures after 0.5–12 h of treatment (Fig. 3). Intercellular communication was decreased at the first sampling time of 0.5 h and remained below control levels throughout 12 h of treatment. In addition, phenobarbital inhibited dye coupling between hepatocytes of 24-h-old cultures that had established gap junctions (Fig. 4). The inhibitory effect occurred rapidly, being evident 30 min after phenobarbital addition, and reaching full effect after 2 h treatment. Dye coupling in established hepatocyte cultures also recovered rapidly (within 15 min) following phenobarbital removal (Fig. 5). Thus, in both freshly plated and established hepatocyte cultures, phenobarbital inhibited intercellular communication within 30 min after treatment and rapid recovery from the inhibition occurred following removal of the phenobarbital.

The mechanism by which phenobarbital inhibited hepatocyte intercellular communication is not likely to be due to gap junction degradation or decreased synthesis of gap junction protein. Rapid recovery from the inhibition as seen in Fig. 5 would not be expected if de novo gap junction synthesis had to occur following phenobarbital-induced gap junction degradation. Instead it is conceivable that phenobarbital inhibited hepatocyte intercellular communication by either affecting an endogenous intracellular system that controls gap junction permeability and/or by altering the structure of the gap junction itself. Control of hepatocyte gap junction permeability may be up-regulated by cyclic AMP-dependent protein kinases (23) and down-regulated by protein kinase C activation (24–26) and by increased intracellular levels of H+ or Ca2+ ions (27). Inhibition of mouse hepatocyte intercellular communication by phenobarbital may be due to phenobarbital effects on hepatocyte cyclic AMP levels (28), oxygen free radical production (15), activation of protein kinase C (29) or effects on hepatocyte Ca2+ levels (30). Recently, Chauhan and Brockerhoff (29) have provided evidence that phenobarbital competes with diacylglycerol for the protein kinase C receptor site. Activation of protein kinase C by 12-O-tetradecanoylphorbol-13-acetate or synthetic diacylglycerols results in the rapid inhibition of intercellular communication in many types of cells (24–26). In addition, barbiturates are capable of disordering membrane lipids (31) and binding to membrane proteins (32). These effects, alone or in combination, might also contribute to the mechanism of inhibition of hepatocyte intercellular communication by phenobarbital.

Mouse hepatocytes became refractory to the inhibitory effect of phenobarbital on intercellular communication (Tables 1 and...
2) suggesting that in vivo the inhibition of hepatocyte intercellular communication may be only an initial, transient effect of phenobarbital treatment during the prolonged tumor promotion process. Sugie et al. (13), in contrast, have shown that phenobarbital administration to rats for 2–8 weeks resulted in a decrease in the size of hepatocyte gap junctions and in the area of the plasma membrane occupied by the gap junctions. It is also possible that preneoplastic “initiated” hepatocytes are more responsive to the inhibitory effects of phenobarbital on intercellular communication and/or are less capable of developing refractoriness to the inhibitory effect. If intercellular communication is a regulatory component of cellular replication (1), then the latter hypothesis may be substantiated by the results of Schulte-Hermann et al. (33). They demonstrated that normal hepatocytes in rat liver cease responding to the hyperplastic effect of phenobarbital after prolonged treatment whereas putatively preneoplastic focal cells continue to proliferate during chronic phenobarbital administration.

REFERENCES

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