Nafenopin Causes Protein Kinase C-Mediated Serine Phosphorylation and Loss of Function of Connexin 32 Protein in Rat Hepatocytes without Aberrant Expression or Localization

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The characteristics and mechanism of the inhibition of connexin-mediated gap junctional communication by the non-genotoxic rodent hepatocarcinogen, nafenopin, has been studied in rat hepatocytes. Nafenopin caused a time- and concentration-dependent inhibition of dye coupling in hepatocytes as assessed by transfer of microinjected lucifer yellow. A half-maximum inhibitory effect of nafenopin occurred at approximately 50 μM, which was not cytotoxic. The inhibitory effect was reversible since a significant recovery of communication was observed 3 h after removal of the chemical. The protein kinase inhibitor G66976 prevented the inhibition of dye coupling, but a tyrosine kinase inhibitor (genistein) did not. Connexin 32 and 26 protein expression, as assessed by immunoblotting, was similar in nafenopin-treated hepatocytes compared to controls, with the exception that in a 10-h culture with nafenopin, the level of connexin 26 was elevated compared to controls. Immunohistochemistry indicated that the localization of plaques containing connexin 32 was not affected in hepatocytes by nafenopin. Immunoprecipitated connexin 32 was, however, detected by an anti-phosphoserine antibody following nafenopin treatment, but not in controls. This serine phosphorylation was prevented in the presence of G66976. The results give further support for a role of protein kinase C in the post-translational inactivation of connexin 32 function in rat hepatocytes by nafenopin.

Key Words: gap junctions; connexin; peroxisome proliferators; nongenotoxic carcinogen; nafenopin.

Peroxisome proliferators are nongenotoxic rodent hepatocarcinogens (see Ashby et al., 1994; Moody et al., 1991) for which elevated cell division and suppression of apoptosis may play a mechanistic role (Bayly et al., 1994; Schulte-Hermann et al., 1990;). A wide range of nongenotoxic carcinogens, including various peroxisome proliferators, has been demonstrated to inhibit connexin-mediated gap junctional intercellular communication (GJIC) (Budunova and Williams, 1994; Klaunig and Ruch, 1990; Mesnil et al., 1995; Trosko, 1987; Trosko et al., 1990). This effect on cell communication mirrors that of various oncogene products and growth factors (for review see Trosko et al., 1990). The inhibition is thought to provide a loss of tumor-suppressor activity normally afforded by the connexin molecules through the regulation of growth of neighboring cells (Trosko and Ruch, 1998; Tsuda et al., 1995). This influence of many nongenotoxic carcinogens may be particularly important in the promotion stage of carcinogenesis, allowing selective mitogenesis of unrestrained, pre-malignant cells.

There are multiple mechanisms of down-regulation of GJ including transcriptional, post-transcriptional, and post-translational events (Bruzzone et al., 1996). Post-translational modification appears to be a major mechanism whereby nongenotoxic carcinogens act on connexins, not surprisingly through a diverse range of interactions. For example, 12-O-tetradecanoylphorbol-13-acetate (phorbol ester; TPA) led to phosphorylation of Cx43 in rat liver epithelial cells and aberrant localization of this connexin leading to inhibition of GJIC (Asamoto et al., 1991; Berthoud et al., 1992). Furthermore, DDT also influences phosphorylated forms of Cx43 in cultured liver cells (Budunova et al., 1993). It has been proposed that the phosphorylation status may affect Cx43 assembly (Lampe, 1994). Furthermore, phenobarbitone, polychlorinated biphenyl, DDT, and the peroxisome proliferator clofibrate decreased GJIC in hepatocytes following treatment of rats in vivo. This was associated with cytoplasmic localization of some Cx32 protein (the major hepatocyte connexin form) and only minimal changes to Cx32 protein levels (Krutzik et al., 1995). Neveu et al. (1994) linked reduced centrilobular Cx32 immunohistochemical staining caused by phenobarbitone treatment of rats to the generation of a NaOH-soluble form of Cx32. Other studies with phenobarbitone have also suggested a transient reduction of Cx32 mRNA in rat liver (Mesnil et al., 1998).

Although several studies have shown that peroxisome proliferators inhibit GJIC in rodent hepatocytes (Ketcham and Klaunig, 1997; Leibold et al., 1994), the mechanism(s) whereby these structurally diverse agents operate are not clear.
Based on protection against inhibition of GJIC by the protein kinase C (PKC) inhibitor, G6976, Leibold et al. (1994) suggested that nafenopin, but not Wy-14,643 and monothiophenethylphthalate (MEHP) may, at least in part, operate through PKC activation. Other studies with Wy-14,643 suggest that G6976 and PKC depletion are in fact also effective in reducing the inhibitory effect of this peroxisome proliferator in rat hepatocytes, though vitamin E was also effective, suggesting a role for both PKC and oxidative stress (Ketcham and Klaunig, 1997).

We have tested further the hypothesis that Cx32 phosphorylation by PKC mediates the GJIC inhibition by nafenopin in cultured rat hepatocytes and investigated if this inhibition is associated with aberrant localization of plaques in which Cx32 is contained.

MATERIALS AND METHODS

Materials. Nafenopin (2-methyl-2-[p(1,2,3,4-tetrahydro-1-naphthyl)-phenyl]proionic acid) was a gift from Ciba-Geigy, Basel, Switzerland. cDNA for mouse Cx32 was kindly provided by Willette, K. (Institut für Genetik Abt. Molekularargentin, University of Bonn, Germany). Pentobarbitone sodium B.P. (Sagatal, 60mg/ml) was from RMB Animal Health Ltd. (Dagenham, UK). Collagenase type A (Clostridium histolyticum) was from Boehringer Mannheim GmbH (Mannheim, Germany) and fetal calf serum was from Advanced Protein Products, UK. All other tissue culture reagents were purchased from Gibco, UK, or Sigma, UK. Mouse monoclonal anti-Cx26 and anti-Cx32 antibodies were from Zymed Laboratories, Inc. (USA) and anti-mouse IgG (whole molecule) peroxidase conjugate was from Sigma, UK. G6976 was from Calbiochem, UK. Enhanced chemiluminescence (ECL) reagents and ECL hyperfilm were from Amersham, UK. All other chemicals were of analytical grade and were purchased from Sigma Chemical Co., Ltd. (UK) unless otherwise stated. Lucifer yellow CH was from Sigma, UK. All tissue culture plastics were from Nunc, UK unless otherwise stated.

Animals. Male Wistar rats, were from Charles River UK and maintained on standard laboratory chow (41B maintenance diet; Pilbury, Birmingham, UK) and tap water ad libitum.

Preparation of isolated rat hepatocytes. Rat hepatocytes were prepared by collagenase perfusion (Seglen, 1976). Briefly, 180–220 g male (8–10 weeks old) male Wistar rats were terminally anesthetized by intraperitoneal injection of diazabicyclo[2,2,2]octane. Animals were washed once and fresh medium, with or without nafenopin (50 μM) in dimethylformamide (DMF), was added. Control flasks were treated with DMF (vehicle) to the same final concentration of 0.25% (v/v).

Microinjection and assessment of hepatocyte gap junctional intercellular communication (GJIC). Hepatocyte GJIC was determined by the measurement of dye coupling between adjacent cells using microinjection of the fluorescent dye Lucifer yellow CH (5% w/v in 0.1 M LiCl). Micropipettes pulled from 1.0 mm external diameter glass capillaries (with inner filament) and a tip diameter of 0.3–0.5 μm (Intracel Ltd., Herts, UK) with a computer controlled electrode puller (model 763) (Campden Instruments Ltd., UK) were back-filled with Lucifer yellow CH. “Donor” hepatocytes were impaled with the micropipette under phase contrast microscopy. Four min after dye injection, hepatocytes in direct contact with “dye donor” hepatocytes were evaluated for evidence of dye coupling. The percentage of dye-coupled recipients was determined for each experimental condition and statistical analysis (t-test p ≤0.05) performed. Ten cells per culture dish were microinjected in order to assess the incidence of dye transfer to “recipient” hepatocytes, and this was repeated for control and nafenopin-treated cultures. Studies were performed to assess the time required for restoration of GJIC following removal of nafenopin. Nafenopin was removed from cultures after 5 h of nafenopin treatment and washed 3 times with culture medium. At 1, 2, and 3 h thereafter, dye coupling was determined. The effect of nafenopin over a range of concentration (0–200 μM) on GJIC was also determined.

Immunoblot analysis. The technique was based on that of Neveu, et al. (1994). Cells in tissue culture flasks (25 cm²) were washed with warm phosphate-buffered saline (PBS) and scraped in 0.02% EDTA, centrifuged (2000 × g) to pellet the cells, which were resuspended in 0.5 ml of 0.1 M NaHCO₃ (pH 7.0) containing 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium molybdate, and 2 μg/ml leupeptin. An equal volume of 20 mM NaOH (freshly prepared) was added, followed by 30 s of sonication at 50% power with a Branson sonifer 250 microtip. Samples were centrifuged at 12,000 × g for 15 min and NaOH-insoluble pellets recovered, resuspended in the 20 mM NaOH/NaHCO₃, buffer described above and sonicated for 30 s. Samples were re-centrifuged at 12,000 × g for 15 min and the pellet suspended in 0.5 ml NaHCO₃, buffer described above. Protein concentrations were determined according to Bradford (1976). Proteins were stabilized in 2% SDS, 62.5 mM Tris–HCl, 1% glycerol, and 50 mM sodium dithiothreitol (pH 6.8) for 30 min at room temperature (RT), and then loaded on 3% stacking and 12.5% separating SDS–polyacrylamide gel cast in minigel apparatus (Bio-Rad, Richmond, CA). After electrophoretic transfer to nitrocellulose membrane (Bio-Rad, modified Towbin transfer buffer (100 mM methanol) at 300 mA for 90 min at 4°C, the membrane was blocked overnight at 4°C with a quenching solution (2 g of bovine serum albumin (BSA) in 20 ml of 40 mM Tris–HCl, pH 7.4, 0.1% Tween-20). The SDS–polyacrylamide gels were stained with Coomassie blue R-250 (Bio-Rad) to evaluate equal loading and transfer of proteins. Blots were incubated with primary antibodies for 2 h at RT, washed several times in TBS (50 mM Tris–HCl, pH 7.4, 0.9% NaCl), followed by incubation with a peroxidase-linked secondary antibody for 1 h and subsequent detection with enhanced chemiluminescent ECL reagents.

Localization of connexin protein. The fixed hepatocytes were cultured in 24-well plates at a density of 1 × 10⁶ cells per well, each well containing a 9 mm round glass coverslip. Following 5 h nafenopin treatment, the hepatocytes were washed 3 times with phosphate buffered saline (PBS) at 37°C and fixed with acetone (100%) for 4 min. Following a further 3 washes in PBS (37°C), the cells were incubated with bovine serum albumin (1% w/v) in PBS for 1 h at room temperature. The cells were subjected to a final 3 washes in PBS at 37°C before being transferred to a 24-well plate partially filled with a hydrophobic resin.

Each coverslip was overlaid with 50 μl of mouse monoclonal Cx32 antibody (diluted 1/1000 in PBS) and incubated in a humidified atmosphere for 2 h at room temperature. The excess antibody was removed by washing 3 times with PBS at 37°C before incubating with a goat anti-mouse IgG fluorescein isothiocyanate (FITC) conjugated secondary antibody, (diluted 1/75 in PBS) for 1 h in a humid atmosphere. The coverslips were subjected to a final 3 washes in PBS before mounting onto slides, using 90% glycerol/10% PBS containing 2.5% (weight/volume) diazabicyclo[2,2,2]octane.

The fixed hepatocytes were observed using Biovision software (Improvis, Coventry, UK) driving an Axiovert 135TV inverted fluorescent micro-
scope (Carl Zeiss Welwyn, Garden City, UK) fitted with a Hamamatsu CCD camera (C3077). Primary hepatocytes were located using light microscopy to avoid quenching of fluorescence. Each coverslip was systematically scanned across the center region of the coverslip to check for the presence of fluorescent-labeled plaques containing Cx32.

Serine phosphorylation of Cx protein. Three flasks were set up for control, TPA (1 μM) and nafenopin (50 μM) treated cultures, and immunoprecipitate samples generated after treatment. The flasks were rinsed in cold PBS and then scraped into lysis buffer (RIPA, Loo et al., 1995; 300 μl). Following a 60-min incubation on ice, the samples were vortexed and centrifuged at 250 × g for 10 min. The lysate (supernatant) was transferred to clean tubes and centrifuged at 10,000 × g for 1 h at 4°C. Mouse monoclonal Cx32 antibody (5 μl) was added to each eppendorf and incubated for 1 h at 4°C. Protein G-covered beads (100 μl) were added to each eppendorf and incubated for 1 h at 4°C with continuous rocking. Following a subsequent centrifugation at 10,000 × g, for 15 s at 4°C, the supernatant was discarded and the pellet was resuspended in lysis buffer (500 μl). The pellet was centrifuged at 12,000 × g for 15 s at RT and the supernatant removed using a bent 23-gauge sterile needle. The pellet was then washed a further 3 times in lysis buffer (500 μl), before pooling the contents of the 3 eppendorfs into one and resuspending the pellet in 2X Lammeli buffer (25 μl). The antibody-antigen immunoprecipitated samples were stored at −70°C.

Samples were equilibrated at RT, and then centrifuged at 7000 × g for 2 min at RT. Twelve μl of each IP sample was loaded on a 12.5% SDS gel. Conditions for gel electrophoresis are previously described in immunoblot analysis, with the only addition being that the transfer buffer contained sodium orthovanadate (100 μM). Membrane filters were blocked in membrane blocking solution (Zymed) overnight at 4°C with constant rocking. The filters were washed 3 times in an excess of 40 mM TBS (40 mM Tris–HCl, pH 7.4, 0.9% NaCl), before being gently rocked in a container with rabbit polyclonal antibody (Zymed) to phosphorylated serine (1:1000) in blocking solution for 2 h at RT. The membrane was then washed for 1 × 15 min and 3 × 10 min in 40 mM TBS, before rocking with a peroxidase conjugated secondary antibody (1:2000) in membrane blocking solution for 60 min. Again, blots were washed several times in 40 mM TBS, before detection with enhanced ECL.

Analysis of Cx mRNA. Total RNA from the cell was isolated, using the Gibco BRL TRIZOL reagent according to the manufacturers’ recommended protocol, based on the method of Chomczynski and Sacchi (1987). Four 25 cm² flasks were set up for control and nafenopin treated cultures at each time point and total RNA was isolated after 2 h and 5 h nafenopin treatment, respectively.

An optical density (OD) reading at the wavelengths of 260 nm and 280 nm was taken using the GeneQuant II DNA/RNA calculator (Pharmacia-Biotech). This was performed in triplicate. The ratio of OD₂₆₀ to OD₂₈₀ was used as a measure of RNA purity; RNA with a ratio within the range 1.8–2.0 was indicative of purity. The yield of RNA was determined using an OD₂₆₀ of 1.0 absorbance unit to be equivalent to 40 μg/ml of single-stranded RNA (Sambrook et al., 1989).

The integrity of the isolated RNA was confirmed by 1% denaturing agarose gel electrophoresis (AGE) according to the method of Lehrach, et al., (1977). RNA samples were denatured at 65°C for 10 min and cooled to RT. Samples were mixed with 5X RNA loading buffer (containing EtBr 50 μg/ml) in a ratio of 1:2 and 10 μg of RNA was loaded for each sample. Samples were electrophoresed at 5V/cm and the integrity of the RNA was confirmed, based on the visualization of the 28S and 18S ribosomal RNA bands by UV transillumination. RNA (20 μg) was loaded per well and subsequently electrophoresed at 5V/cm. The gel was set up with several steps in DEPC-treated H₂O before being transferred to pre-soaked (20X sodium citrate buffer), nylon membrane (Hybond N⁺), and UV irradiated at 120 mJoules, using the auto-cross link setting on a Stratalinker UV Crosslinker (Stratagene).

Filters were prehybridized in Ambion prehybridization solution (1 h at 65°C) prior to hybridization (10 h at 42°C) with cDNA probe, non-isotopically labeled using Ambion Psoralen-Biotin reagent. Relative quantification of

![FIG. 1. Time-dependent effect of nafenopin on dye transfer in primary rat hepatocytes. Nafenopin was added after 2 h of culture and dye transfer was recorded from 20 cells injected with Lucifer yellow per time point. To monitor recovery from nafenopin treatment, cells were washed with fresh medium without nafenopin at 4 h. Results are presented as means ± SEM of data from 3 independent experiments. Dye transfer values between control and nafenopin treatments were considered by Students t-test; *p ≤ 0.05, **p ≤ 0.01.

mRNA transcripts from Northern analysis was accomplished by densitometric scanning of the film after development. This was performed using a Biorad imaging densitometer (GS-670) and Molecular Analyst™/PC Windows software version 1.4 (Biorad).

RESULTS

Time Course of Maintenance of GJIC in Rat Hepatocytes

Intercellular communication (dye coupling) was observed during the first 14 h of primary rat hepatocyte culture (Fig. 1). In control cultures, dye coupling increased from 28 ± 3% at 2 h culture duration to a maximum value of 88 ± 3.0% after 7 h. Subsequently, a gradual decrease was observed, until no dye coupling could be detected after 14 h of culture. However, the culture period for retention of gap junction function was sufficient for the short-term inhibitory effects of nafenopin to be studied.

Inhibition of Dye Coupling by Nafenopin in Rat Hepatocytes

Nafenopin caused a time-dependent (Fig. 1) and dose-dependent (Fig. 2) decrease in the incidence of dye coupling. Although nafenopin (50 μM)-treated cultures showed similar dye coupling to that of control for the first 3 h (Fig. 1), by 4 h, nafenopin had had a significant effect (p ≤ 0.05), showing a 20 ± 5% decrease in dye coupling compared to the respective control. From 4 h of nafenopin treatment onwards, a lower level of dye coupling was observed compared to controls, until
the hepatocytes became noncommunicative after 11 h of culture. Figure 2 shows that dye coupling was reduced after a 5-h treatment with nafenopin in a concentration-dependent manner. A half-maximal inhibitory effect of nafenopin on dye coupling (IC$_{50}$) was observed at approximately 50 $\mu$M. There was no observable difference in hepatocyte morphology with nafenopin (50 $\mu$M) treatment, compared to control cells, and viability based on LDH leakage was maintained above 95%, in contrast to evidence of cytotoxicity at 200 $\mu$M.

Recovery of GJIC Subsequent to Treatment with Nafenopin

The inhibitory effect of nafenopin on dye coupling levels was reversible. Figure 1 shows that removal of media, washing, and replacement of medium containing no nafenopin allowed cells to recover from the inhibitory effect of nafenopin. At 3 h following removal of nafenopin, a significant increase ($p \leq 0.05$) of 25 $\pm$ 3% in dye coupling was observed, but no evidence of recovery was seen up to 2 h after removal. By a 10-h culture duration, 43 $\pm$ 2% dye coupling was observed in hepatocytes treated for only 4 h with nafenopin, whereas there was no observable communication in hepatocytes subjected to continued nafenopin treatment.

Effect of Selective Signal Transduction Inhibitors on GJIC in Rat Hepatocytes

Dye coupling levels were observed during the first 12 h of primary rat hepatocyte culture in the presence of nafenopin (50 $\mu$M), nafenopin (50 $\mu$M) + Gö6976 (10 $\mu$M) and nafenopin (50 $\mu$M) + genistein (10 $\mu$M) (Fig. 3). Gö6976 specifically inhibits the classical PKCs, but not cAMP- or cGMP-dependent protein kinases (Martiny-Baron et al., 1993) and genistein is a potent inhibitor of tyrosine protein kinases at the concentration used (Akiyama, 1987).

In this experiment, dye coupling in the presence of nafenopin was similar to that seen in Figure 1. An identical trend in dye coupling levels was observed for rat hepatocytes treated with a combination of nafenopin and genistein. However, in cells treated with a combination of nafenopin and the PKC inhibitor Gö6976, although the same general trend of time dependency for dye coupling was observed, the maximal level of dye coupling was 73 $\pm$ 3%, greater than that seen in the absence of Gö6976. It has previously been shown (Leibold et al., 1994) that Gö6976 does not affect dye coupling in control (untreated) rat hepatocytes. This maximal level was maintained for 3 h and the subsequent decrease in dye coupling was similar to that observed in control cultures (Fig. 1).

Effect of Nafenopin on Cx32 Protein Expression in Primary Rat Hepatocyte Cultures

Figure 4 shows the expression of Cx32 protein in membrane protein-enriched fractions (NaOH insoluble fraction) of primary rat hepatocytes. The photographs are representative of 6 independent experiments. Two bands were seen, one at 27 kDa and one at 54 kDa, representing the monomer and dimer forms, respectively (Musil and Goodenough, 1990). In control cul-

![FIG. 2. Concentration-dependent effect of nafenopin on dye transfer in primary rat hepatocytes. Nafenopin was added after 2 h of culture and dye transfer was recorded from 20 cells injected with Lucifer yellow per point. Results are presented as means $\pm$ SEM of data derived from 3 independent experiments.](image-url)
tures, comparable expression was noted at 2 and 5 h, but by 10 h of culture, Cx32 protein was barely detectable (note that these time points are following the initial 2 h plating period). In nafenopin-treated cultures, the same trend in protein expression was observed with comparable expression at 2 and 5 h, but again, by 10 h of culture Cx32 was greatly reduced. There was no change in Cx32 protein expression when hepatocytes were subjected to 5 h nafenopin treatment in the presence of the PKC inhibitor Go6976. Coomassie blue-stained polyacrylamide gels confirmed that protein separation and equal loading of samples were achieved.

The cytoplasmic fraction (NaOH soluble fraction) of the cultured hepatocytes was also analyzed for evidence of Cx32 protein (data not shown). There was a small amount of Cx32 protein detected in control cultures at 4 h, in both monomer and dimer form, which both declined with time. In nafenopin-treated samples, the slightest evidence of Cx32 protein was observed after 2 h (but not at 5 or 10 h) nafenopin treatment in both monomer and dimer form. This indicated that no movement of Cx32 protein from the membrane-enriched fraction to the cytoplasmic fraction had occurred.

Effect of Nafenopin on Cx26 Protein Expression in Primary Rat Hepatocyte Cultures

The protein expression pattern observed for Cx26 in primary rat hepatocytes (Fig. 5) showed a single band (monomer form), of approximately 21 kDa. In control cultures, comparable expression was noted at 2 and 5 h after the start of the treatment period, but by 10 h, Cx26 protein expression was barely detectable. For nafenopin (50 μM)-treated cultures, following 2-h and 5-h nafenopin treatments (4-h and 7-h culture duration), there was no difference in protein expression compared to controls. However, by 10-h nafenopin treatment (12-h culture duration), in contrast to control cultures, there was evidence of Cx26 protein being present. Equal protein loading was confirmed by Coomassie blue staining.

Phosphorylation of Cx32 in Rat Hepatocytes Associated with Nafenopin Treatment

Cx32 was immunoprecipitated from rat hepatocytes using Cx32 antibody (Fig. 6a). A distinct band was observed in each lane, representing Cx32 monomer (27kDa). Since an equal volume of sample was loaded in each lane, a higher level of Cx32 protein appeared to have been immunoprecipitated in control and TPA-treated hepatocytes as compared with nafenopin-treated hepatocytes.

In Figure 6b, the immunoprecipitated samples have been probed with an antiphosphoserine antibody. The result indicated a single band for both nafenopin- and TPA-treated hepatocytes and the position of the band was at approximately 27 kDa, the same Mr observed for Cx32 antibody. The band for TPA-treated hepatocytes was stronger than that observed for nafenopin treatment. No evidence of a band was seen in control cultures.

Therefore, despite there being more protein immunoprecipitated from control cultures, there was less phosphorylation

FIG. 4. Immunoblot detection of Cx32 in the membrane enriched fraction (NaOH-insoluble) from rat hepatocytes with (N) or without (C) pretreatment with nafenopin (50 μM). Values 2, 5, and 10 refer to the pretreatment time periods. N5/G indicates the equivalent of N5 in the presence of the PKC inhibitor Go6976. Twenty μg of protein was loaded per lane. A sample of gap junction enriched, rat liver membrane lysate protein (6 μg) (not shown) was used as a control. Migration of monomer (~27 kDa) and dimer (~54 kDa) forms of Cx32 are shown. The photograph is representative of 6 independent experiments.

FIG. 5. Immunoblot detection of Cx26 in rat hepatocytes (NaOH insoluble fraction) with (N) or without (C) pretreatment with nafenopin (50 μM). Values 2, 5, and 10 refer to the pretreatment time periods. Twenty μg of protein were loaded per lane. Migration of monomer (~21 kDa) is shown. The photograph is representative of 3 independent experiments.

FIG. 6. Immunoblot detection of Cx32, (a) following immunoprecipitation from rat hepatocytes with or without (C) pretreatment with nafenopin (50 μM; N) or TPA (1 μM) for 5 h, and immunoblot detection of phosphoserine (b) associated with the immunoprecipitated Cx32. The Mr of ~27 kDa, is indicated from the ECL protein markers used (M). The photograph represents the result of 2 separate experiments.
observed in comparison to both nafenopin- and TPA-treated hepatocytes.

Localization of Cx32 in Rat Hepatocytes following Nafenopin Treatment

The effect of nafenopin treatment on the localization of Cx32 within GJ plaques (detected by immunocytochemistry and fluorescence confocal microscopy) is shown in Figure 7. No change in the localization of Cx32 could be detected after nafenopin treatment, as Cx32 protein could be observed clearly in the membrane of both control and nafenopin-treated (50 μM, 5 h) hepatocytes. There was no visible evidence of movement of Cx32 from the cell membrane to the cytoplasm of the hepatocytes.

DISCUSSION

The mechanisms by which the nongenotoxic peroxisome proliferator, nafenopin, is a hepatocarcinogen in rodents are not well understood. However, it has been proposed that both suppression of apoptosis and induction of mitosis may be key to the carcinogenic process (reviewed in Chevalier and Roberts, 1998). The finding that nafenopin can inhibit GJIC in rat hepatocytes (Elcock et al., 1998; Leibold et al., 1994) but not in hepatocytes of the guinea pig (Elcock et al., 1998) (a nonresponsive species regarding mitogenesis and peroxisome proliferation) suggests an important role for this effect. This is in accord with the known cell growth regulatory role of GJIC (Yamasaki and Naus, 1996) and the finding that many nongenotoxic carcinogens tested, including a range of peroxisome proliferators, inhibit this function (Budunova and Williams, 1994; Klaunig and Ruch, 1990; Mesnil et al., 1995; Trosko and Ruch, 1998).

There are a number of potential mechanisms whereby nafenopin may inhibit GJIC. We found that the inhibition was rapid (within 4 h of nafenopin addition, and therefore not subsequent to peroxisome proliferation). The inhibition was at least partially reversible within 3 h after cessation of treatment. No alteration in the levels of Cx32 or Cx26 proteins was found to be associated with nafenopin-mediated inhibition of GJIC. Moreover, mRNA levels for the major hepatocyte connexin form (Cx32) were unaffected by nafenopin during the inhibitory time period (analyzed by Northern blot, data not shown). These results indicate that an alteration of transcriptional control of connexins is unlikely to provide an explanation for the observed inhibition. Sequence information on the promoter region for cx32 has been published by Miller et al. (1988) and we have also determined this sequence (unpublished) for the Wistar rat. We found no consensus sequence for binding of the peroxisome proliferator activated receptor α (PPARα; Tugwood et al., 1996) in the regulatory region of the cx32 gene based on a “Transfac” database search. Alternatively, connexin function is altered by perturbation, at least in part, of rat hepatocyte second messenger systems by nafenopin and posttranslational modification; cAMP-dependent protein kinase, protein kinase C (PKC), and Ca²⁺/calmodulin-dependent protein kinase II, as well as tyrosine kinase, and mitogen-activated protein kinase have all been demonstrated to participate in connexin modulation (see Bruzzone et al., 1996). However, different Cx isoforms can have different functional properties under similar phosphorylating conditions; possibly different patterns of phosphorylation of target sites on the Cx proteins could account for the opposite functional effects that have been observed (Bruzzone et al., 1996).

We were able to confirm the finding of Leibold et al. (1994)
that inhibition of PKC protects against the inhibition of GJIC by nafenopin. No such protection was afforded by the tyrosine kinase inhibitor, genistein. Since Gö6976 was found to inhibit only the classical Ca\(^2+\)-dependent PKC isoenzymes at the concentration used in this study (Martiny-Baron et al., 1993) the α isoform (expressed in the liver, Wetsel et al., 1992) is a likely mediator. Previous studies have shown PKC to phosphorylate more than one site on Cx32 (see Saez et al., 1998). Moreover, activation of PKC by peroxisome proliferators was found to correlate with their relative ability to induce tumors in rodents (see Bojes et al., 1997a,b). Activation has been proposed to be through inhibition of ketogenesis and to involve tumor necrosis factor α (TNFα) (Bojes et al., 1997b). Indeed TNFα signaling through TNFα receptor 1 (TNFR1) has been proposed to be necessary for the suppression of apoptosis and elevation of hepatocyte proliferation induced by peroxisome proliferators (Rolfe et al., 1997; West et al., 1999). Experiments to investigate whether peroxisome proliferators affect intracellular signaling showed a transient increase in cytosolic Ca\(^2+\) levels as one of the earliest events in response of rat hepatocytes to nafenopin (Bieri, 1993; Ochsner et al., 1990), and the peroxisome proliferator, cipirofibrate (Bennet and Williams, 1993). It has been well established that increases in intracellular Ca\(^2+\) are known to close GJs (see Horz-Wagenblatt and Shalloway, 1993; Loewenstein, 1981). It has been hypothesized that Ca\(^2+\)-activated calmodulin may alter GJ function via Ca\(^2+\)/cell adhesion molecule (CAM) protein kinases (Jansen et al., 1996). Although calcium may mediate phosphorylation events in the action of nafenopin, it has also been shown that proteolytic degradation of Cx32-containing GJs can be mediated by Ca\(^2+\)-activated calpains (Elvira et al., 1993). Moreover, coenzyme A (CoA) esters of several hypolipidemic drugs, including the peroxisome proliferator ciprofibrate, potentiated diacylglycerol-activated PKC by decreasing the requirement of the enzyme for phosphatidylserine (Bronfman et al., 1989; Orellana et al., 1990). In addition, activation of other signal transduction pathways, specifically mitogen-activated protein kinase (Rokos and Ledwith, 1997) and elevation of several phosphoproteins. (Passilly et al., 1995) has also been associated with peroxisome proliferators. Thus, activation of complex cell survival signaling pathways appears to be permissive for a response that nonetheless remains entirely dependent upon the presence of sufficient functional PPARα (Gonzalez, 1997).

We have demonstrated that a phosphorylation event is involved in the action of nafenopin on Cx32, by the use of an antiphosphoserine antibody. The specific site of the serine residue and the possibility of other sites being phosphorylated are yet to be established. Takeda et al. (1989) demonstrated that phosphorylation of rat liver Cx32 was entirely dependent on Ca\(^2+\) and was highly specific for serine residues. Serine at position 233 has been identified as a primary site for phosphorylation both by protein kinase (PKA) and PKC, the latter also phosphorylating other sites (Saez et al., 1990). PKC phosphorylation of Cx32 may have different outcomes depending on the balance of other signals, the sites of phosphorylation, and the cell type. Despite evidence that PKC may contribute to inactivation of GJIC mediated by Cx32 (Leibold and Schwartz, 1994; Ren et al., 1998) serine 233 phosphorylation alone (by cAMP-dPK) enhanced Cx32 channel permeability (see Saez et al., 1998 for review). However, PKC phosphorylation can also protect against Cx32 degradation by calpains (Elvira et al., 1993). It was of interest that the positive control TPA (known to cause phosphorylation of Cx43; Berthoud et al. (1992) also phosphorylated serine in Cx32, which is in agreement with the finding of Cx32 phosphorylation by Takeda et al. (1989). However, TPA has been reported not to inhibit GJIC mediated by Cx32 channels in transfected cells (Kwak et al., 1995) and we have found it ineffective in the inhibition of GJIC in rat hepatocytes (unpublished). The inhibition of GJIC by TPA has subsequently been shown to be dependent on the cell type rather than the Cx isoform (Ren and Ruch, 1997). The fact that TPA induces phosphorylation of Cx32 but does not inhibit communication between rat hepatocytes suggests that PKC activation is possibly not the only factor in the inactivation of gap junctions by nafenopin. With respect to post-translational modifications, no phosphorylation analyses were performed on the minor rat hepatocyte connexin Cx26, since this appears not to contain phosphorylation sites for PKC (see Saez et al., 1990, 1998).

Evidence suggests that phosphorylation of connexins may influence both connexin assembly and channel gating (see Bruzzone et al., 1996). Our findings indicate that there was no relocation of Cx32 from its membrane location in association with serine phosphorylation. Evidence for this comes from both investigation of NaOH soluble and insoluble fractions and immunocytochemistry for identification of Cx32-containing plaques, which remained associated with the cell membrane of cultured hepatocytes, following treatment with nafenopin. The effect of nafenopin therefore appears not to be on the transport and assembly of Cx32 to the membrane. This contrasts with at least one of the effects of another peroxisome proliferator, clofibrate, which resulted in an aberrant localization of Cx32-containing plaques in the liver following in vivo treatment of rats (Krutovskikh et al., 1995). The fact that more than one mechanism exists for inhibition of GJ by peroxisome proliferators is supported by the finding of Leibold et al. (1994) that PKC inhibition overcame the inhibitory effect of nafenopin but not of Wy-14,643 or MEHP.

Further work on the necessity of inhibition of GJIC and altered secondary messenger signal in the hepatocarcinogenicity of nafenopin and other peroxisome proliferators is now required to assess their relative importance, in addition to the recognized requirement for PPARα in many responses to peroxisome proliferators (Gonzalez, 1997). It has been postulated that both PPARα-dependent and -independent signal transduction pathways coordinately contribute to cell proliferation and carcinogenesis by peroxisome proliferators (Lapinskas and
Cort, 1999). However, since the growth and tumorigenic response to PPs is absent in the PPARα null mouse (Gonzalez et al., 1997), it seems more likely that such PPARα-independent pathways are common to many biological responses and are permissive for a response to peroxisome proliferators that is entirely dependent upon and specific to PPARα (Gonzalez et al., 1997). Since the species-specificity of inhibition of GJIC correlates with the species specificity of effects on cell proliferation (Elcock et al., 1998), a potential link between this effect and the expression of cyclin-dependent kinases (CDKs) and cyclins should be investigated in the light of the recent established link between connexin (Cx43) expression and their regulation (Chen et al., 1995). A discordant expression of CDKs and cyclins has been observed in response to the peroxisome proliferator Wy-14,643 (Rininger et al., 1996). However, we find that the peroxisome proliferator nafenopin causes alterations in CDK and cyclin expression that are similar to those seen with classic mitogens such as EGF (Chevalier and Roberts, 1999).

In summary, we have shown that the peroxisome proliferator nafenopin inhibits GJIC through a mechanism involving PKC-mediated phosphorylation of Cx32. Previous data link inhibition of GJIC by nafenopin to subsequent proliferation of hepatocytes from rat but not from guinea pig.

REFERENCES


