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Control of Hepatocyte DNA Synthesis by Intracellular pH and Its Role in the Action of Tumor Promoters

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The mechanisms of tumor promotion in liver by various xenobiotics of diverse structure are not well understood. However, these tumor promoters share the ability to exert growth-stimulatory effects on hepatocytes. Our laboratory has been utilizing normal rat hepatocytes under defined conditions of primary cultures, to investigate growth-stimulatory actions of liver tumor promoters. We have shown that most, if not all, of the liver tumor promoters tested stimulate hepatocyte DNA synthesis when added in combination with epidermal growth factor (EGF), insulin, and glucocorticoids. In the present study, we sought evidence for the role of the Na^+/H^+ antiporter and cytoplasmic alkalinization in the direct growth-stimulatory actions of tumor promoters on hepatocytes. Hepatocytes cultured under conditions (bicarbonate-buffered medium) where intracellular pH (pH_i) was independent of extracellular pH (pH_e), EGF- and insulin-stimulated rates of DNA synthesis were unaffected by modest changes in pH_e. However, under conditions (HEPES-buffered medium) where pH_i varied in a linear fashion with pH_e, rates of EGF- and insulin-stimulated DNA synthesis were highly dependent on pH_e. Similarly, 12-O-tetradecanoylphorbol-13-acetate (TPA) and α-hexachlorocyclohexane (HCH)-stimulated DNA synthesis were pH_e-dependent but were stimulatory over different pH_e ranges, suggesting that these promoters may act by distinct mechanisms. Chemicals that are capable of inducing rapid cytoplasmic alkalinization, ammonium chloride (1 and 15 mM) and monensin (0.5 μM), were found to stimulate hepatocyte DNA synthesis. The role of the Na^+/H^+ antiport in controlling pH_i of hepatocytes was demonstrated by artificially acidifying 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl (BCECF)-loaded isolated hepatocytes with 20 mM sodium acetate and the use of specific inhibitors. Amiloride and its analogues inhibited pH_i recovery from the acid load in a dose dependent manner and the relative potency of these inhibitors paralleled their K_i values for the Na^+/H^+ antiport. At concentrations that stimulate hepatocyte DNA synthesis, some liver tumor promoters phenobarbital (PB) and HCH, were found to cause a rapid rise pH_i in isolated hepatocytes which was sensitive to amiloride and its analogues. Taken together, our data suggest that activation of Na^+/H^+ antiport activity may be one mechanism whereby some liver tumor promoters stimulate hepatocytes DNA synthesis. This study has implications for the mechanisms of tumor promotion in liver carcinogenesis. J. Cell. Physiol. 195: 61–69, 2003.

The Na^+/H^+ antiporter is the most widely studied intracellular pH (pH_i) regulator in vertebrate cells. It is now known to be a ubiquitous feature of the plasma membrane of mammalian cells (reviewed by Madshus, 1988; Grinstein et al., 1989), including rat hepatocytes (Arias and Forgac, 1984; Moseley et al., 1986a; Anwer and Nolan, 1988; Renner et al., 1989a). In response to a fall in pH_i, the Na^+/H^+ antiporter functions to expel H^+ from the cytoplasm, using the inwardly directed Na^+-gradient created by Na^+/K^+-ATPase.

The Na^+/H^+ antiporter is believed to play a role in regulating various metabolic events that are dependent on pH_i, including DNA synthesis. The first evidence to

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associate activation of Na^+/H^+ antiporter and initiation of growth came from studies on the activation of the sea urchin eggs (Johnson et al., 1976). A rapid increase in pH_i after fertilization was seen as a result of the activation of amiloride-sensitive exchange of extracellular Na^+ for intracellular H^+. Substantial evidence now suggests that activation of the antiport and/or subsequent cytoplasmic alkalinization may be required for cell proliferation. Many mitogens and co-mitogens, including growth factors, serum, and phorbol esters, have been shown to rapidly (within 1 min) activate the antiporter in many cell types, thus producing an intracellular alkalinization of about 0.15-0.3 pH units (Frelin et al., 1988; Quinn et al., 1996; Di Sario et al., 2001). The observation that many growth factors increase pH_i has led to the notion that increased pH_i may be a necessary early signaling event in growth factor action. Interestingly, Na^+/H^+ antiporter-dependent intracellular alkalinization is also considered to be an early event in malignant transformation, and appears to play an essential role in the development of transfomation-associated phenotypes (Reshkin et al., 2000). Further evidence to link Na^+/H^+ antiport with growth comes from studies using specific inhibitors of the antiporter. Amiloride and its analogues have been shown to inhibit cell division in sea urchin (Johnson et al., 1976) and growth factor-induced DNA synthesis in several mammalian cell types (reviewed by Soltoff and Cantley, 1988; Grinstein et al., 1989). Koch and Leffert (1980) have further shown that DNA synthesis can be re-initiated upon removal of amiloride, consistent with the notion that Na^+/H^+ antiporter plays a role in events preceding DNA synthesis.

Koch and Leffert (1979, 1980) provided the initial evidence for a possible role of Na^+/H^+ antiport in hepatocyte growth. Addition to long-term hepatocyte cultures of a mixture of insulin, glucagon and epidermal growth factor (EGF), was found to stimulate rapid influx of $^{22}Na^+$, followed 24–28 h later by incorporation of thymidine into DNA. Such stimulation of DNA synthesis was blocked by removal of Na^+ or administration of amiloride (Koch and Leffert, 1979, 1980). Na^+/H^+ antiport activity has been shown to be increased in liver plasma membranes from hepatectomized and neonatal rats, further suggesting a link between the antiporter and hepatocyte growth (Moseley et al., 1986a; Goodrich and Suchy, 1990). Growth-stimulatory agents such as EGF, hepatocyte growth factor, and TPA, which are known to increase DNA synthesis in hepatocytes, have also been shown to activate the Na^+/H^+ antiport (Smith and Boyer, 1986; Moule and McGivan, 1990; Tanaka et al., 1994; Kaneko et al., 1995). Other related findings in liver include inhibition by amiloride of nafenopin-stimulated DNA synthesis (Ochsner et al., 1990) and mitogen-induced protooncogenes expression in primary rat hepatocytes (Kruijer et al., 1986; Leffert et al., 1990). Similarly, inhibition by amiloride and its analogues of intracellular alkalinization and DNA synthesis have been observed in hepatoma cells (Strazzabosco et al., 1995; Garcia-Canero et al., 1999).

Our laboratory has been utilizing normal rat hepatocytes under defined conditions of primary cultures, to investigate growth-stimulatory actions of tumor promoters, an important element in the promotion stage of carcinogenesis (Yusof and Edwards, 1990; Lee and Edwards, 2001a,b). The aims of the present study are: (1) to clarify the significance of pH_i changes in control of hepatocyte DNA synthesis; and (2) to investigate whether liver tumor promoters act like growth factors to rapidly activate the Na^+/H^+ antiport, thereby inducing intracellular alkalinization.

MATERIALS AND METHODS

Animals and materials

Male wistar rats (Porton strain), weighing about 200 g, given free access to laboratory chow and water, were used for hepatocyte preparation in all experiments. Williams' Medium E (WME) was obtained from Flow Laboratories (Irvine, Scotland), Swim's S-77 medium and antibiotics were from GIBCO (Grand Island). LHSA medium was prepared as described by Oliver et al. (1978). Collagenase was from Boehringer Mannheim GmbH (Penzberg, Germany); α-hexachlorocyclohexane (HCH) and 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane (DDT) were from Serva Feinbiochemica (Heidelberg, Germany) or from Aldrich (Milwaukee, WI); phenobarbital (PB) or its sodium salt were from Prosanna Laboratories (Carole Park, Queensland, Australia). Pregnenolone-16α-carbonitrile (PCN), 12-*o*-tetradecanoylphorbol-13-acetate (TPA) and all other chemicals were from Sigma (St. Louis, MO). Amiloride analogues, 5-(N-ethyl-N-isopropyl)-amiloride (EIA), 5-(N,N-hexamethylene)-amiloride (HMA), 5-(N-methyl-N-(guanidino-carbonylmethyl)-amiloride (MGA), and 5-(N-methyl-N-tert-butyl)-amiloride (MBA), were provided by Dr. Edward J. Cragoe, Jr., as part of a collaborative study. The fluorescent dye, 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was from Molecular Probes (Eugene, OR). Type I collagen was extracted from rat tails, essentially as described by Michalopoulos and Pitot (1975). Culture dishes were collagen-coated using a solution of 0.3 mg collagen/ml 0.1% acetic acid. Stock solutions of test agents were prepared weekly. The final concentration of dimethylsulfoxide (DMSO) when added to cultures was 0.2% or less and had no effect on DNA synthesis.

Hepatocyte isolation and culture conditions

Hepatocytes were isolated using a two-step procedure as previously described (Whiting and Edwards, 1979). Low centrifugal speeds (50 ×*g*) were used during washing of cells with LHSA medium to minimize the contamination of hepatocytes with smaller non-parenchymal cells. The washed hepatocyte pellet was resuspended in warm WME containing antibiotics (penicillin, 100 U/ml, streptomycin sulphate 100 μg/ml), 300 nM insulin, and 3% fetal bovine serum. For DNA synthesis experiments, cells were plated on 50 mm collagen-coated dishes at a density of about $3.5–4 \times 10^4$ cells/cm^2. Higher cell density, as indicated below, were used for other types of experiments. Cells were allowed to attach for 3–4 h at 37°C in a humidified atmosphere of 5% CO_2 in air. After the attachment period, cells were maintained in fresh WME (serum-free) containing antibiotics as above. This is termed 'standard culture medium'. Insulin (300 nM), dexamethasone (30 nM), and EGF (10 ng/ml) were added

depending on the nature of the experiments as indicated in each figure legend. Other test compounds were added either after attachment, or with subsequent daily medium changes, or both, as indicated in the figure legends.

Measurements of the rate of hepatocyte DNA synthesis

After various times in culture, medium was replaced with fresh standard medium containing 0.08 μCi/ml [methyl-^{3}H]thymidine (20 Ci/mmol, New England Nuclear, Boston, MA), and cells incubated for 3 h. Following extensive washing, DNA was extracted (Yusof and Edwards, 1990), with one aliquot taken for scintillation counting and another to determine DNA concentration (Burton, 1956). The values shown in each figure are means ± SD from groups of four dishes per treatment obtained with a single hepatocyte preparation. Results were confirmed using independent cell preparations.

Measurements of changes in intracellular pH

Buffers used were based on those described by Moolenaar et al. (1983) for studies with fibroblasts. Freshly isolated hepatocytes (50 mg wet weight/ml) were resuspended in cold Buffer A (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.1), and incubated with 5 μM BCECF-acetoxymethyl ester (pKa 6.98) for 30 min at 37°C. Cells were continuously gassed with oxygen and shaken constantly (100 rpm) during this period. The non-fluorescent BCECF-AM readily entered the cells where cytosolic esterases cleaved the ester bonds, releasing the anionic BCECF which exhibits pH-sensitive fluorescence. Cells were pelleted (30 sec at 50 ×*g*) and resuspended at a density of 30 mg wet weight/ml in Buffer B (same as Buffer A except at pH 7.4). A further 15–20 min incubation at 37°C was necessary to allow complete intracellular de-esterification of BCECF-AM. Hepatocytes were then pelleted (30 sec at 50 ×*g*) and resuspended (30 mg wet weight/ml) in 2.5 ml of warm Buffer B at pH 7.4 and put into a pre-warmed magnetically-stirred cuvette, maintained at 30–32°C, unless otherwise indicated. The fluorescence emission was monitored using the an Aminco Bowman Spectroflourimeter (in earlier experiments) or a Perkin Elmer LS 50 Spectrometer (in later experiments) with excitation at 500 nm and emission at 530 nm. Calibration of cytoplasmic dye flourescence as a function of pH_i was done by the H^+ equilibration method (Thomas et al., 1979) using the K^+/H^+ ionophore nigericin (10 μM) and Buffer C, which has high K^+ concentration (150 mM KCl, 10 mM HEPES, pH 7.0) approximating intracellular K^+ concentration. Nigericin was added to cells after determining the position of the baseline. Under this condition, $[H^+]_i/[H^+]_o = [K^+]_i/[K^+]_o$. Changes in fluorescence were recorded with successive additions of 1 μl aliquots of 1 M KOH, until the pH of the solution was 7.6. External pH_i was measured using a Corning pH meter. For measurements of changes in pH_i upon treatment with test agents, a baseline pH_i was typically achieved first. Results presented in Figures 5–7 are representatives of at least three experiments from the same and different batches of isolated hepatocytes.

RESULTS

Effects of varying pH of HEPES- and bicarbonate-buffered medium on the ability of hepatocyte to synthesize DNA

Studies by Pollock (1984) on primary cultured rat hepatocytes have shown an almost linear relationship between pH_i and external pH of HEPES-buffered medium in the pH range 6.8–8.0. In contrast, pH_i of cultured hepatocytes in bicarbonate: CO_2 medium was relatively constant over the pH_i range tested. Such findings provided an opportunity to monitor possible changes in the rate of DNA synthesis upon modulation of pH_i of cultured hepatocytes.

Previous studies conducted in this laboratory used bicarbonate: CO_2 medium, and it is not known whether HEPES itself has any effect on hepatocyte DNA synthesis. Therefore, the first step taken was to test directly, effects of HEPES on the ability of cultured hepatocytes (in bicarbonate-buffered medium) to synthesize DNA. A concentration-dependent inhibitory effect of HEPES on [^{3}H]thymidine incorporation into DNA is shown in Figure 1. At concentrations up to 10 mM, HEPES had no effect on DNA synthesis. Higher concentrations were apparently inhibitory although statistically significant inhibition was seen only at 25 mM HEPES. For the studies below with HEPES-buffered media, 10 mM HEPES was employed.

Bicarbonate buffer (WME) was equilibrated with 5% CO_2 at 37°C and adjusted to desired pH. Similarly, HEPES-buffered (bicarbonate-free) WME was equili-

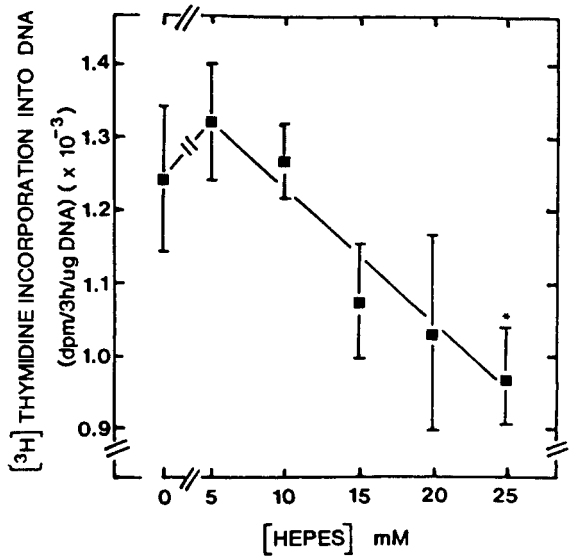

Fig. 1. Concentration-dependent effect of HEPES on hepatocyte DNA synthesis in bicarbonate-buffered medium. Freshly isolated hepatocytes were plated onto collagen-coated dishes (35×10^3 cells/cm^2), and after 4 h for cell attachment in the presence of insulin (300 nM) and 3% fetal calf serum, medium was replaced with 'fresh standard culture medium'. Cultures were maintained with daily medium changes. After 40 h in culture, [^{3}H]thymidine was added to determine rates of DNA synthesis as described in Materials and Methods. Each value is mean ± SD (n = 8). Where treatment with HEPES resulted in a mean value significantly different ($P < 0.001$) from control (without HEPES), this is shown by an asterisk.

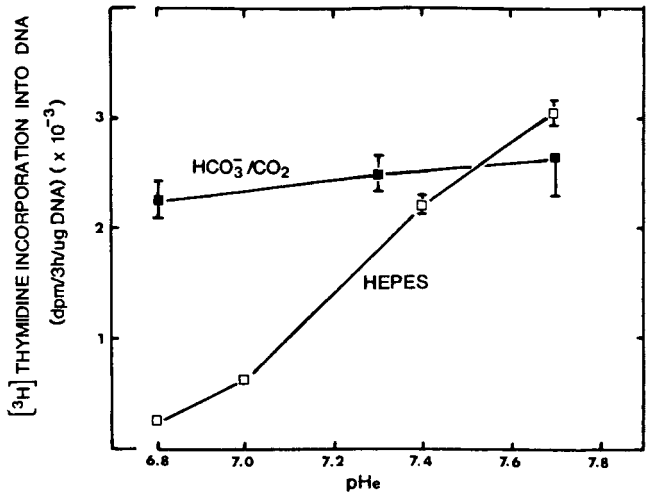

Fig. 2. The effects of varying pH of HEPES- and bicarbonate-buffered media on the ability of hepatocytes to synthesize DNA. Hepatocytes were plated as described in Materials and Methods, and maintained after a 4-h attachment period with either 'standard culture medium' (bicarbonate-buffered) incubated under an atmosphere of 5% CO_2 in air (■) or 10 mM HEPES-buffered bicarbonate-free Williams' medium E (WME) and incubated with an air atmosphere (□). For each type of medium, initial pHs were adjusted to the values shown. EGF (10 ng/ml), dexamethasone (30 nM), and insulin (300 nM) were present in all cultures and daily medium changes were conducted. Rates of DNA synthesis in all cultures were measured after 40 h using the 'standard culture medium' (pH 7.4) containing titrated thymidine, as described for all other DNA synthesis assays, according to the protocol in Materials and Methods.

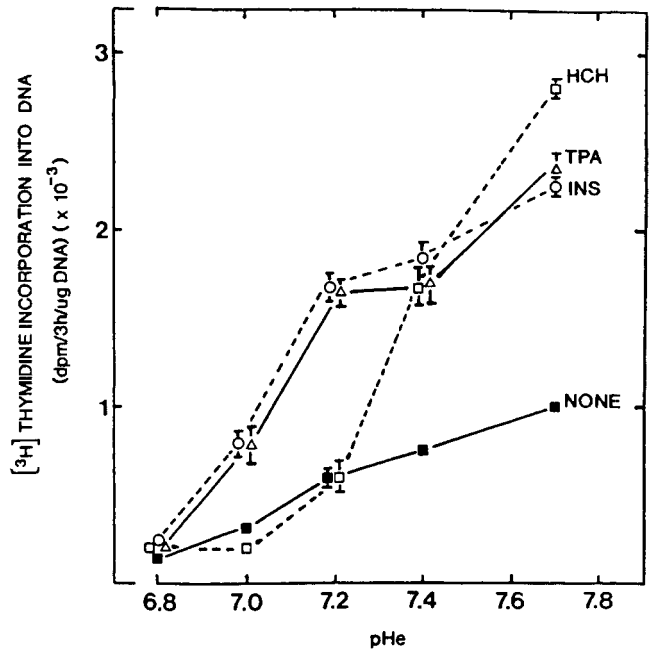

Fig. 3. Effects of test agents on hepatocyte DNA synthesis in HEPES-buffered media of varying pH. Hepatocytes were isolated and plated as described in Materials and Methods. After 4 h for cell attachment, medium was replaced with 10 mM HEPES-buffered (bicarbonate-free) WME at various pHs as shown. Also present in the medium were antibiotics, EGF (10 ng/ml) and dexamethasone (30 nM). Some cultures had no addition (■), insulin (300 nM) (○), TPA (300 nM) (△), or HCH (30 μM) (□). Daily medium changes were conducted and rates of DNA synthesis were measured after 40 h in culture as described in Materials and Methods. Each value is mean ± SD (n = 4).

brated at 37°C and adjusted to the desired pH. Figure 2 shows the effects of varying pH of HEPES- and bicarbonate-buffered medium on hepatocyte DNA synthesis measured after 40 h in culture. There were no significant changes in the rate of DNA synthesis over the external pH range of 6.8–7.7 when bicarbonate: CO_2 medium was used. In contrast, [^{3}H]thymidine incorporation into DNA was seen to increase with increasing pH of HEPES-buffered medium.

Effects of insulin or tumor promoters on hepatocyte DNA synthesis in HEPES-buffered media or varying pH

Since the rate of DNA synthesis was found to increase with increasing pH of HEPES-buffered medium, it was deemed useful to test the effects of promoters on hepatocyte DNA synthesis at these various pHs. Results of this study are presented in Figure 3. None of the compounds tested had an effect on DNA synthesis at external pH of 6.8. As in the previous study, the EGF-stimulated rate of DNA synthesis increased with increasing pH and the ability of insulin, TPA, or HCH to stimulate was also pH-dependent. However, the pH dependence of HCH action differed from that of TPA and insulin. Low pH (6.8–7.2) blocked the stimulatory effect of HCH whereas near-maximal stimulatory effects of insulin or TPA over the effect for EGF alone were seen at pH 7.2. There was little additional stimulation by insulin or TPA as the pH increased from 7.2 to 7.8 whereas stimulation by HCH increased markedly over this range.

Effects of monensin and ammonium chloride on hepatocyte DNA synthesis

Monensin, a Na^+-ionophore (Pressman, 1976), capable of collapsing Na^+ and H^+ gradients has been shown to raise pH_i of many cells (Mills et al., 1985; Church et al., 1989) including hepatocytes in this study (data not shown). Ammonium chloride (NH_4Cl) is also known to raise pH_i of cells immediately upon addition (Moolenaar et al., 1983; Madshus, 1988). This (result not shown) and previous studies from this laboratory (results not shown) showed similar effects of NH_4Cl on pH_i of hepatocytes. It was thus of interest to test the effects of both monensin and NH_4Cl on hepatocyte DNA synthesis. Bicarbonate: CO_2 medium was used, and EGF and insulin were present throughout the experiments. Rates of DNA synthesis were measured after 40 h in culture. High concentrations of NH_4Cl (15 mM) and monensin (0.5–5 μM), when present throughout the experiments had non-specific effects causing cells to detach after 1 day in culture (data not shown). Results in Figure 4 show that maintenance of hepatocytes with media containing a lower initial concentration of NH_4Cl (1 mM) or brief exposure (5 h) to a high concentration of NH_4Cl (15 mM), resulted in significant stimulation of DNA synthesis. Low concentrations of monensin (0.05–0.1 μM) had no significant effect while modest stimulation was seen in cultures exposed for 5 h to 0.5 μM monensin.

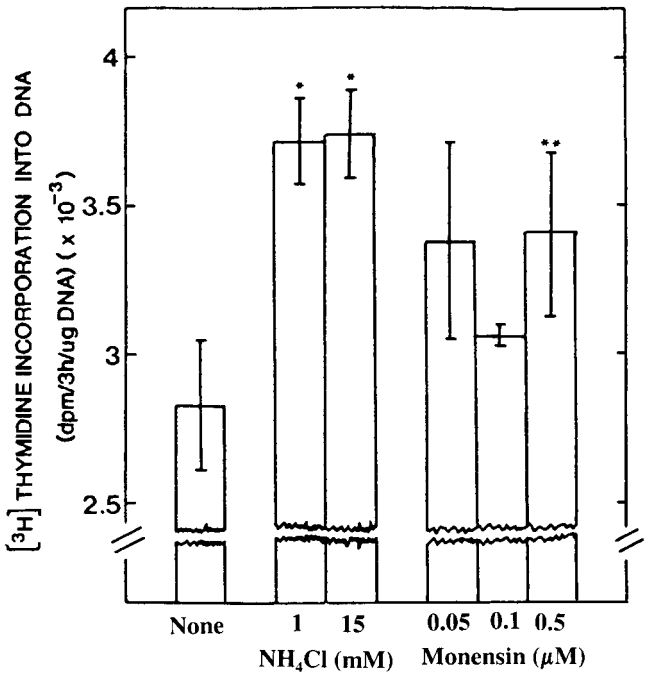

Fig. 4. Effects of ammonium chloride and monensin on hepatocyte DNA synthesis. Hepatocytes were isolated and plated as described in Materials and Methods. After 4 h for cell attachment, medium was changed to 'standard culture medium' containing EGF (10 ng/ml), insulin (300 nM), and additions of NH_4Cl and monensin as indicated. All cultures had NH_4Cl and monensin throughout the experiment except for cultures treated with 15 mM NH_4Cl and 0.5 μM monensin, where treatment was only for 5 h after which medium was replaced with 'standard culture medium' containing EGF and insulin. All cultures were subjected to daily medium changes and rates of DNA synthesis were measured after 40 h in culture as described in Materials and Methods. Each bar is mean ± SD (n = 4) where treatment resulted in a mean value significantly different from control (no addition), this is shown by superscript: $^{*}P < 0.001$, $^{**}P < 0.02$.

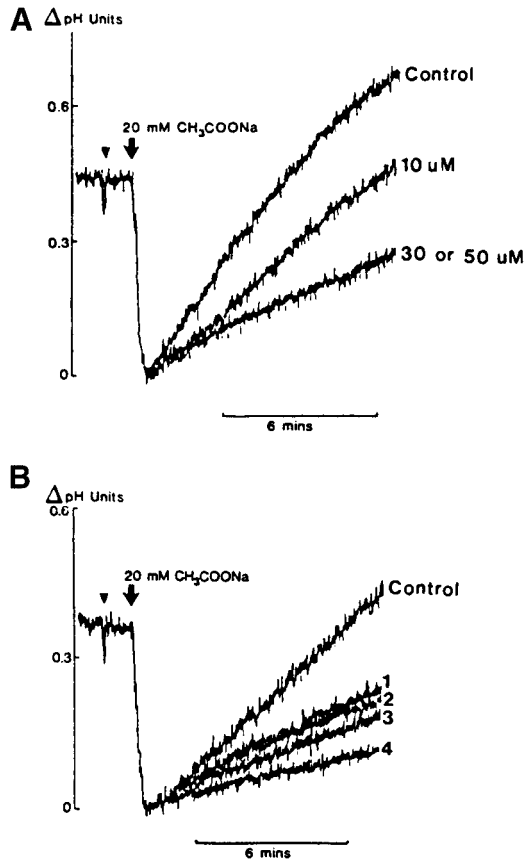

Fig. 5. Effects of amiloride analogues on pH_i recovery from an acid load in isolated hepatocytes. Hepatocytes were loaded with BCECF, washed and resuspended with warm Buffer B, pH 7.4 as described in Materials and Methods. Inhibitors as shown by the arrow (↓), were added, 1 min before the addition of 20 mM sodium acetate (CH_3COO Na). At this time, DMSO (solvent for the inhibitors) was added to control cells (final concentration = 0.1%). The tracings of different treatments were superimposed to allow comparison of pH_i recoveries. **A**: Dose dependent inhibition by EIA of pH_i recovery. **B**: Relative effects of various amiloride analogues as inhibitors of pH_i recovery. The inhibitors were: 1, 30 μM MGA; 2, 30 μM MBA; 3, 30 μM EIA; 4, 10 μM HMA.

Inhibition by amiloride analogues of hepatocyte pH_i recovery from an acid load: evidence for a role of Na^+/H^+ antiporter

The role of Na^+/H^+ antiporter in experimental cells can be shown by artificially acidifying cells, either by removal of cells from NH_4Cl-containing media or by addition of sodium acetate or isobutyrate (Moolenaar et al., 1984, 1986; Isom et al., 1987). Figure 5 shows the effect of adding 20 mM sodium acetate to BCECF-loaded hepatocytes. In aqueous solution, acetic acid exists in its dissociated and undissociated forms. The undissociated form readily diffuses across the plasma membrane into the cells. Once inside the cells, dissociation of acetic acid occurs, resulting in the accumulation of H^+, thereby causing a sharp drop in pH_i. In the absence of inhibitors, this is followed by a slow recovery of pH_i, due to the role of Na^+/H^+ antiport, extruding excess H^+ out of the cells (control cells in Fig. 5). Figure 5 shows that EIA, a specific inhibitor of the Na^+/H^+ antiport inhibited pH_i recovery in a dose-dependent manner, with maximal inhibition at 30 μM. Similar dose-dependent inhibition by other amiloride analogues (HMA, MBA, and MGA) was observed (data not shown). The relative potency of these analogues as inhibitors of pH_i recovery and their maximal-effective concentrations (Fig. 5) can be summarized as follows: 10 μM HMA > 30 μM EIA > 30 μM MBA > 30 μM MGA. This parallels the K_i values of the inhibitors for Na^+/H^+ antiport (Simchowitz and Cragoe, 1986). Such data shows that pH_i recovery observed from an acid load was mediated predominantly by the Na^+/H^+ antiport. The failure of recovery curves to reach a plateau probably reflects the leakage of dye as described below.

Use of probenecid to prevent dye leakage

Various investigators had reported that hepatocytes can secrete BCECF (Petzinger et al., 1988; Renner et al., 1989; Ochsner et al., 1990). Similar problems have also been encountered in this lab. This is problematic in experiments where a reproduceable stable pH_i baseline is required. Probenecid, a blocker of organic anion transport (Di Virgilio et al., 1988), has been shown to block efflux of the fluorescent calcium indicators fura-2

and indo-1 in pancreatic and astrocytoma cells (Arkhammar et al., 1989, 1990; McDonough and Button, 1989). Probenecid was, therefore, tested for effects on hepatocytes pre-loaded with BCECF. Resuts showed that addition of probenecid blocked the progressive increase in baseline fluorescence in hepatocyte suspensions and had no effect on recovery from an acid load produce by treatment with sodium acetate (data not shown). Similarly, probenecid did not prevent pH_i recovery from an acid load produced by treating cells with 15 mM NH_4Cl then transferring cells to NH_4Cl-free medium (data not shown). Figure 6 shows measurement of pH_i changes in response to TPA in the presence of probenecid with BCECF secretion blocked by probenecid. TPA caused a clear elevation of pH_i and this effect was inhibited by EIA, suggesting that the effect of TPA was mediated by the Na^+/H^+ antiport. In experiments where stable pH_i baseline was achieved in the absence of probenecid treatment, TPA also reproducibly elevate pH_i (Fig. 7).

Effects of test agents on pH_i of isolated hepatocytes

Various agents, including liver tumor promoters, were tested for effects on hepatocytes pH_i. Figure 7 shows results from an experiment, typical of many with other separate cell preparations. The phorbol ester, TPA, was found to increase pH_i of about 0.1 pH units compared to the control over a 6.5-mins period. Both PB (0.08 pH units) and HCH (0.05 pH units) were also found to further increase pH_i compared to the control (Fig. 7) over similar time period. In some cases, control cells (0.1% saline, 0.1% DMSO, or no addition) showed a slight increase in fluorescence over a 15 min period, reflecting dye leakage (see above). Other compounds tested for effects on hepatocytes pH_i include monensin and prolactin. 0.5 μM monensin and 20 mU/ml prolactin were found to raise the pH_i approximately 0.05 pH units

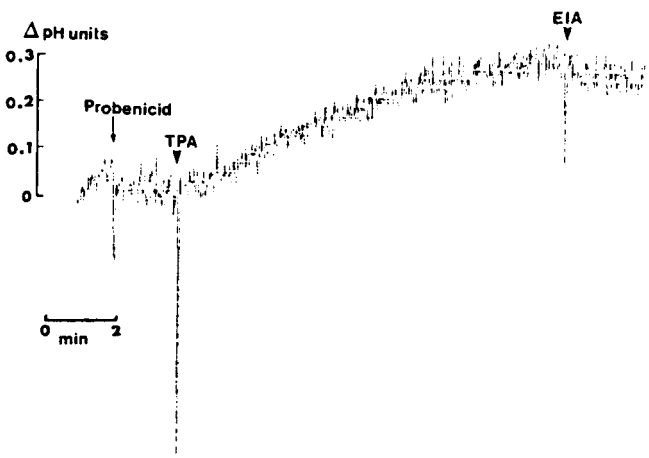

Fig. 6. Effect of TPA on pH_i of hepatocyes, previously treated with probenecid. Freshly isolated heaptocytes were loaded with BCECF (5 μM), washed and resuspended in warm Buffer B, pH 7.4 as described in Materials and Methods. One millimolar probenecid was added 1 min after an initial fluorescence was obtained and followed by an addition of 600 nM TPA 2 min later, as shown. Fluorescence was monitored over a period of 15 min. Thirty micrometer EIA was added 2 min prior to termination of fluorescence measurement.

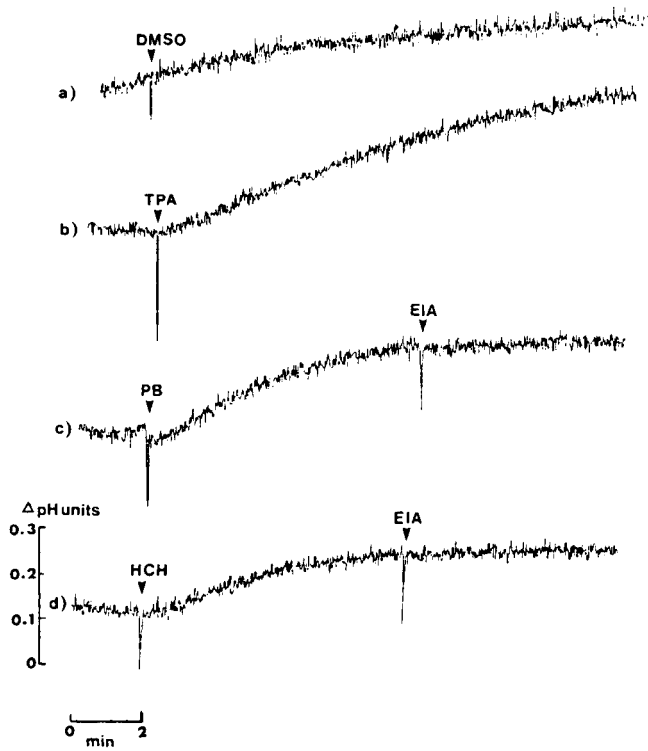

Fig. 7. Effects of test agents on pH_i of isolated hepatocytes. Freshly isolated hepatocytes were loaded with BCECF (5 μM), washed and resuspended in warm Buffer B, pH 7.4 as described in Materials and Methods. Test agents were added after 2 min of initial fluorescence, and were monitored for a period of 15 min. The tracings were from the same batch of isolated hepatocytes and are representative from at least 3 experiments. The additions were: (a) 0.1% DMSO; (b) 600 nM TPA; (c) 2 mM PB; (d) 30 μM HCH.

(data not shown). Figure 7 also shows the ability of EIA to decrease the continuous rise in pH_i following treatment with PB and HCH, suggesting that the Na^+/H^+ antiport is involved in the elevation of pH_i by these liver tumor promoters. EIA alone had no effect on the baseline pH_i (data not shown).

DISCUSSION

This laboratory is interested in determining the mechanisms by which liver tumor promoters stimulate hepatocyte DNA synthesis (Yusof and Edwards, 1990; Petronijevic and Edwards, 1993; Lee and Edwards, 2001a,b). Based on previous studies that the skin tumor promoter, TPA, and growth factors caused rapid elevation of pH_i in hepatocytes (Smith and Boyer, 1986; Tanaka et al., 1994; Kaneko et al., 1995), and that early elevation of pH_i is frequently associated with subsequent stimulation of DNA synthesis in many cell types (see Introduction), we hypothesized that liver tumor promoters may elevate hepatocyte pH_i and this may be relevant to their stimulatory effects on DNA synthesis. There are two major parts in this study. The first section focuses on evidence that hepatocyte DNA synthesis is pH-dependent. The second part focuses on determining the role of the Na^+/H^+ antiport in controlling hepatocyte pH_i. The latter section includes measuring the effects liver tumor promoters on pH_i of isolated hepatocytes.

Pollock (1984) showed that when hepatocytes were incubated without HCO_3^- (so that HCO_3^--dependent mechanisms are inoperative) and in HEPES-buffered media, the ability of HEPES to move through the hepatocyte membrane results in conditions where pH_i varies in linear fashion with pH_e. These observations provided a method for experimental manipulation of pH_i for studies on its relevance to control of DNA synthesis. By contrast, it is known that in HCO_3^--containing media, hepatocytes can maintain relatively constant pH_i in the presence of a range of pH_e. As shown in Figure 2, under conditions where pH_i is largely unaffected by pH_e (HCO_3^--buffered medium), pH_e had little effect on DNA synthesis. In contrast, in HEPES-buffered medium where pH_i varies with pH_e, rates of EGF- and insulin-stimulated DNA synthesis became strongly dependent on pH_e. In this study, the pH_e (and hence pH_i) were controlled throughout a 36-h period from after cell attachment until beginning the DNA synthesis measurements, so it is possible that pH_i is influencing one or more events at any points in the sequence from initial actions of EGF or insulin through to S-phase entry. Results are consistent with findings by Moolenaar et al. (1986) that a pH_i above 7.2 is a permissive requirement for human fibroblasts to initiate DNA synthesis.

NH_4Cl exposure was found to significantly stimulate hepatocyte DNA synthesis even under conditions where HCO_3^- pH_i-regulating mechanisms are operative (Fig. 4). Although a rapid alkalinization effect of NH_4Cl has not been directly demonstrated in hepatocytes in HCO_3^--buffered medium, it is likely that NH_4Cl has a similar effect to that seen in mesangial cells (Ganz et al., 1989). Brief exposure (5 h) to NH_4Cl or monensin may be approximating signaling events and provide some indication that relatively short periods of pH_i increase may be sufficient to set cell cycle events in train and that elevated pH_i may not be required for full pre-S-phase period. In fact, this notion is supported by our observation that both NH_4Cl and monensin induced hepatic c-*myc* mRNA expression measured after 2 h of exposure (Lee and Edwards, 2003). Furthermore, effects of a single addition of NH_4Cl may of limited duration since NH_4Cl is rapidly metabolized by liver (Williamson et al., 1974). Taken together, our data provided good evidence that hepatocyte DNA synthesis is dependent on pH_i and early cytoplasmic alkalinization plays a role in regulating the growth of hepatocytes.

Under conditions where HCO_3^- pH_i-regulating mechanisms are inoperative, insulin and TPA exhibited parallel pH dependence with maximal effect near pH 7.2 (Fig. 3). In contrast, HCH had no effect at low pH range (6.8–7.2) but strongly enhanced EGF-stimulated DNA synthesis as the pH increased from 7.2 to 7.8. These observations suggest that growth-stimulatory effects of HCH are more pH-dependent and differ in mechanisms from that of TPA and insulin.

Some difficulties were encountered in our hands when the fluorescent dye, BCECF, was used as an indicator of pH_i. This is consistent with findings from several other laboratories (Gleeson et al., 1989; Renner et al., 1989; Ochsner et al., 1990). Probenecid has been used to block efflux of fluorescent calcium indicators from other cell types (Arkhammar et al., 1989, 1990; McDonough and Button, 1989). We, therefore, opted to pre-treat hepatocytes with probenecid prior to testing agents for effects on pH_i. Results showed probenecid could block the gradual rise in fluorescence in control incubations of hepatocytes (Fig. 6) and did not prevent pH_i recovery from an acid load (data not shown), suggesting that it does not interfere with Na^+/H^+ antiport activity. The rise in pH_i stimulated by TPA in hepatocytes previously treated with probenecid, was sensitive to amiloride analogue EIA, suggesting that the effect seen was due to the activation of Na^+/H^+ antiporter and that probenecid does not block the response to one-well known activation of the antiporter. Having overcome the difficulty of obtaining reproduceable baseline fluorescence and established that, in our cultures, the Na^+/H^+ antiport indeed plays an important role in regulating pH_i in hepatocytes (Fig. 5), the direct effect of liver tumor promoters on hepatocytes pH_i was explored. Our results consistently showed that PB and HCH caused an elevation of pH_i (0.05–0.08 pH units) over that in control cells, and that the elevation of pH_i was amiloride analogue-sensitive (Fig. 7). Our preliminary data showed that other liver tumor promoters (eg., DDT and PCN) which stimulated DNA synthesis, also induced cytoplasmic alkalinization in hepatocytes (data not shown). In contrast, the estrogen groups of tumor promoters (Lee and Edwards, 2001a) had no effect on pH_i of hepatocytes (data not shown). This is consistent with the view that estrogenic liver tumor promoters act via distinct mechanisms in stimulating hepatocytes DNA synthesis (Lee and Edwards, 2001a).

The exact molecular events underlying the activation of Na^+/H^+ antiporter by the various liver tumor promoters (PB, HCH, DDT, PCN) are currently not known. These xenobiotics of diverse structures could directly or indirectly affect activity of the antiporter. Other signaling mechanisms, involving mediators such as protein kinase C (Gillies et al., 1991; Ho et al., 1989; Lowe et al., 1990; Tanaka et al., 1994), cyclic AMP (Moule and McGivan, 1990), intracellular Ca^{2+} (Little et al., 1988; Alvarez et al., 1989; Hendey et al., 1989) and tyrosine kinase (Moule and McGivan, 1990; Tanaka et al., 1994) have been shown to increase Na^+/H^+ antiport activity in different cell types. While the effects of TPA in liver cells presumably involve activation of protein kinase C, liver tumor promoters had no effect on protein kinase C activity and do not elevate cyclic AMP and Ca^{2+} levels in hepatocytes (Yusof and Edwards, results not shown), thereby, ruling out the possible role on these signaling mechanisms as mediators. Further studies will be required to determine whether tyrosine kinase activation or any other mechanisms mediate the action of liver tumor promoters in activating the Na^+/H^+ antiporter in hepatocytes. Future studies will also include examination on the effect of liver tumor promoters on individual hepatocytes rather than the entire population of isolated hepatocytes as described in this study.

In conclusion, this study has provided for the first time evidence that hepatocyte DNA synthesis is dependent on pH_i and that elevation in pH_i is at least permissive in the stimulation of DNA synthesis. Some liver tumor promoters were found to elevate pH_i of isolated hepatocytes within minutes via activation of Na^+/H^+

antiport. Taken together, results from this study suggest that activation of Na^+/H^+ antiport may be one mechanism by which some liver tumor promoters stimulate growth in hepatocytes.

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