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N,N-Dimethylformamide Modulates Acid Extrusion from Murine Hepatoma Cells[1]

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***N,N*-Dimethylformamide (DMF) affects cellular differentiation, causes hepatotoxicity and gastric irritation, and may be carcinogenic. Since these processes involve changes in cellular pH homeostasis, we investigated the effects of DMF on H^+ extrusion and cytosolic pH (pH_i) of mouse hepatoma cells (Hepa 1C1C7). Extracellular pH was monitored using a silicon-based sensor system (Cytosensor microphysiometer) and pH_i was monitored by fluorescence spectrophotometry. Superfusion of cells with DMF (0.25 to 0.5 M) suppressed the extracellular acidification rate (ECAR) below baseline. Following washout of DMF there was a rapid, concentration-dependent, prolonged overshoot of ECAR above baseline rates. Removal of extracellular Na^+ or superfusion with amiloride abolished the overshoot in acidification rate, indicating involvement of Na^+/H^+ exchange. The overshoot was dependent on extracellular glucose, suggesting that it arises from an increase in metabolic acid production. Fluorescence measurements showed that DMF did not change pH_i. Furthermore, DMF did not alter the rate of pH_i recovery of cells acid loaded using nigericin, indicating that DMF does not directly alter Na^+/H^+ exchange activity in these cells. In summary, these data suggest that suppression of acidification rate by DMF is likely due to decreased metabolic acid production. Washout of DMF is then accompanied by increased glucose metabolism and H^+ efflux via Na^+/H^+ exchange. It is possible that alterations in H^+ production and transport contribute to the hepatotoxicity of DMF and its effects on cellular differentiation.**

N,N-Dimethylformamide (DMF) is a solvent used in the manufacture of synthetic fibers, leathers, and films, and in the purification and extraction of other organics, such as vinyl-based polymers, acetylene, and petroleum (Eberling, 1980; IARC, 1989). Worldwide production is estimated as 2.7×10^5 tons per year (Marsella, 1994). DMF is readily absorbed by inhalation, dermal contact, and ingestion (Massmann, 1956; Kimmerle and Eben, 1975; Kennedy, 1986).

The metabolism of DMF is incompletely understood. In humans, DMF is metabolized by liver P-450 2E1 enzymes to produce the urinary metabolites *N*-(hydroxymethyl)-*N*-methylformamide and subsequently *N*-methylformamide (NMF). NMF may be further oxidized to form the reactive intermediate, methylisocyanate, which quickly conjugates with glutathione (Mraz and Turecek, 1987; Mraz *et al.,* 1989). Based upon *in vitro* and *in vivo* studies, DMF was found to inhibit its own P-450 2E1-dependent metabolism (Mraz *et al.*, 1993). Similarly, Yang *et al.* (1991) observed that DMF inhibits P-450 2E1-facilitated metabolism of *N,N*-dimethylnitrosamine.

Chronic exposure to DMF can lead to gastric irritation (Gescher, 1993), pancreatic disorders (Chary, 1974), carcinogenesis (Ducatman *et al.*, 1986; Levin *et al.*, 1987), and hepatotoxicity (Scailteur and Lauwerys, 1987; Van den Bulcke *et al.,* 1994; Chieli *et al.,* 1995). Various investigators have shown that DMF toxicity is characterized by elevation in the serum levels of several hepatic enzymes (Fleming *et al.,* 1990; Van den Bulcke *et al.*, 1994; Chieli *et al.*, 1995), indicating that the liver is a primary target organ. Additionally, rats given 1 week of daily exposure to DMF displayed hepatomegaly with increased cholesterol and glutathione *S*-transferase and decreased hepatic microsomal cytochrome P-450 and protoheme levels (Imazu *et al.,* 1992). Following 12 weeks of repeated exposure there was massive liver fibrosis (Itoh *et al.*, 1987). Even DMF concentrations as low as 1 mM cause elevations in cellular glutathione *in vitro* (Elovaara *et al.*, 1983).

DMF has been shown to stimulate the maturation and differentiation of tumor cells. In some tumor cell lines DMF increases doubling time, reduces saturation density, and induces anchorage dependence (Chakrabarty *et al.,* 1984; Naguib *et al.*, 1987; DeRose and Claycamp, 1989). Naguib and coworkers (1987) have shown alterations in enzymes of pyrimidine metabolism in DMF-treated tumor cells, consistent with decreased anabolic enzyme activity and increased catabolic

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enzyme activity. It is unknown at this time whether the toxic effects of DMF and its ability to induce differentiation are related.

As in other cell types, cytosolic pH regulates the activity of several important enzymes in hepatocytes (Lidofsky *et al.*, 1993). Furthermore, changes in cytosolic pH and transmembrane proton movement have been implicated in the regulation of cellular proliferation and differentiation. Disturbances in cellular pH homeostasis are thought to play a role in anoxia-induced cell damage (Strazzabosco and Boyer, 1996) and may also play a role in hepatotoxicity. Therefore, to gain insights into the effects of DMF on hepatocytes, we employed a sensitive technique (McConnell *et al.*, 1992) to monitor the actions of DMF on real-time acid production and H^+ transport in murine hepatoma cells.

METHODS

Materials and solutions. Dulbecco's modified essential medium (DMEM) (Gibco cat. no. 23800), HCO_3^--free minimum essential medium (MEM) (Gibco cat. no. 41500), MEM (Gibco cat. no. 12370) buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (25 mM), Dulbecco's phosphate-buffered saline, heat-inactivated fetal bovine serum (FBS), and antibiotics (10,000 μg/mL streptomycin; 10,000 U/mL penicillin; 25 μg/mL amphotericin B) were purchased from Gibco BRL, Life Technologies (Burlington, Ontario, Canada). Nigericin and solutions of nonessential amino acids and vitamin supplements were purchased from ICN Pharmaceuticals (Costa Mesa, CA). *N*-methyl-D-glucamine (NMG) and amiloride were purchased from Sigma (St. Louis, MO). 2′,7′-*Bis*(carboxyethyl)-5-or-6-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR) and DMF (99.7%, glass distilled) was purchased from BDH Inc. (Toronto, Ontario, Canada).

Cells and cell culture. Hepa 1C1C7 mouse hepatoma-derived cells were a gift from Dr. O. Hankinson (University of California, Los Angeles). Cells were grown in DMEM supplemented with sodium bicarbonate (24 mM), glucose (11 mM), HEPES (20 mM), sodium pyruvate (10 mM), lipoic acid (1.0 nM), vitamin B_{12} (1.0 nM), zinc sulfate (0.9 nM), nonessential amino acids (1% v/v), vitamin supplement solution (1% v/v), L-glutamine (2 mM), FBS (10% v/v), and antibiotic solution (1% v/v) in humidified 5% CO_2:95% air at 37°C. Cells were subcultured at a ratio of 1:7 every 2 to 3 days.

Measurement of extracellular acidification rate (ECAR). Cells were seeded at a density of 1.5×10^5 cells/cm^2 on porous polycarbonate membrane capsules (3 μm pore size; Corning Costar Corporation, Cambridge, MA) in supplemented DMEM and incubated for 48 h in humidified 5% CO_2:95% air at 37°C. Cells adhering to polycarbonate membranes were placed in microflow chambers and positioned above silicon-based potentiometric sensors (Cytosensor microphysiometer, Molecular Devices, Sunnyvale CA), which detect changes in extracellular pH of as little as 10^{-3} units (McConnell *et al.*, 1992). Cells were superfused at a rate of 100 μL/min with running medium (HCO_3^--free MEM supplemented with HEPES (1 mM) and bovine albumin (1 mg/mL)) at pH 7.30 $\pm$ 0.02 and 290 $\pm$ 5 mOsm/liter at 37°C. Each chamber was superfused with fluid from one of two reservoirs regulated by a computer-controlled valve.

The Cytosensor uses a light-addressable potentiometric sensor to monitor extracellular pH (pH_o). The relationship of voltage versus pH is linear; at 37°C, a change of 61 mV corresponds to approximately 1 pH unit. Every 90 s, the flow of running medium was halted for 30 s, during which time extracellular acid accumulated. Measurements of the rate of acidification were obtained by linear least squares fit to the slope of the pH_o − time trace during these periods.

For Na^+-dependence experiments, cells were superfused with buffer containing NaCl or NMG chloride (150 mM), KCl (4.4 mM), KH_2PO_4 (1.31 mM), $MgSO_4$ (0.81 mM), HEPES (1 mM), $CaCl_2$ (1.8 mM), glucose (10 mM), and bovine albumin (1 mg/mL), pH adjusted to 7.30 $\pm$ 0.02 and osmolarity adjusted to 290 $\pm$ 5 mOsm/liter. Where indicated, amiloride–HCl (500 μM) was dissolved directly in running medium. For metabolic-dependence experiments, glucose/glutamine-containing medium and glucose/glutamine-free medium were prepared. Each contained DMEM, HEPES (1 mM), bovine albumin (1 mg/mL) with or without glucose (5.6 mM), and L-glutamine (2 mM); pH adjusted to 7.30 $\pm$ 0.02 and osmolarity adjusted to 290 $\pm$ 5 mOsm/liter.

Cytosolic pH measurements. Cells were plated on 60-mm culture dishes in supplemented DMEM at a density of 3.5×10^4 cells/cm^2 and incubated for 48 h in humidified 5% CO_2:95% air at 37°C. Cells were then incubated with BCECF-AM (3 μM) for 20 min and subsequently washed with Na^+ buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES; adjusted to pH 7.30 $\pm$ 0.02 and 290 $\pm$ 5 mOsm/liter). Cells were harvested by incubation for 2 min in nominally Ca^{2+}- and Mg^{2+}-free buffer containing trypsin (0.05%) and EDTA (0.5 mM). Cell suspensions were sedimented at 1200 rpm for 5 min and the pellet was resuspended in HEPES-buffered MEM to a density of approximately 7×10^5 cells/mL. For each determination, a cell sample (1 mL) was sedimented and resuspended in the fluorometric cuvette in 2 mL of the indicated buffer (with constant stirring, at 37°C). Cytosolic pH was monitored using a fluorometer (Model RF-M2004, Photon Technology International, London, Canada) at 495 nm excitation and 525 nm emission. Calibration was performed as described by Dixon and Wilson (1995). Briefly, the cells were lysed with Triton X-100 (0.1% v/v) and the fluorescence intensity was measured. The pH of the medium was measured in the fluorescence cuvette using a pH electrode. Fluorescence intensity and pH of the medium were then recorded following a series of additions of 2-(*N*-morpholino)ethanesulfonic acid (MES) over the range of fluorescence intensities encountered during the experiment. These measurements were used to construct a standard curve of fluorescence intensity vs pH. A correction factor was used to account for quenching of the dye intracellularly. This factor was obtained by suspending BCECF-loaded cells in high K^+ medium supplemented with nigericin (1 μM) and determining the fluorescence ratio before and after lysis with Triton X-100 (0.1% v/v).

Cells were acid-loaded as follows. BCECF-loaded cells were suspended in Na^+- and K^+-free buffer that contained 140 mM NMG chloride, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES; adjusted to pH 7.30 $\pm$ 0.02 and 290 $\pm$ 5 mOsm/liter. Addition of nigericin (10 μM), which causes a rapid exchange of intracellular K^+ for extracellular H^+ (Dixon and Wilson, 1995), produced a decline in pH_i of approximately 1 pH unit within 30 s. Two hundred seconds after the addition of nigericin, DMF (0.25 M final) or amiloride (500 μM final) was added to some samples. NaCl (200 mM) was added 100 s later to initiate recovery of pH_i through Na^+/H^+ exchange.

Statistical analysis.

All extracellular acidification rates were normalized as a percentage of the baseline acidification rate prior to introduction of test substance. Normalization compensated for differences in cell numbers amongst chambers of the Cytosensor and permitted comparisons between experiments. One- or two-way analysis of variance with repeated measures, followed by Tukey–Kramer multiple comparison test, was used for comparisons amongst means. Values are expressed as means $\pm$ SE with $p < 0.05$ being considered significant.

RESULTS

Effects of DMF on ECAR of Hepatocytes

ECAR was measured at 1.5-min intervals in cells superfused intermittently with running medium or running medium containing DMF. When superfused only with running medium, ECAR remained stable for several hours. ECAR was altered

during exposure to DMF and following washout of DMF (Fig. 1). In some cases, low concentrations of DMF (0.063 M) induced a slight elevation in ECAR, whereas higher concentrations consistently suppressed ECAR in a concentration-dependent manner. Figure 1A shows responses of individual cell samples to DMF (0.063 to 0.5 M), whereas Fig. 1B shows mean responses from at least four separate cell preparations. Responses to higher concentrations of DMF were not examined in detail because the suppression of ECAR was irreversible at concentrations of 1.0 M or greater.

Following washout of DMF, there was a concentration-dependent overshoot of ECAR and then a gradual return toward baseline acidification rates (Fig. 1). The data in Fig. 1C further illustrate the concentration-dependent nature of the suppression and overshoot of ECAR. The maximal suppressions shown are those obtained immediately prior to washout (Fig. 1C, DMF treatment). The acidification rates at 10.5 min after DMF washout, a time that coincided in most instances with the peak overshoot, are also shown (Fig. 1C, post-DMF treatment). It is worthy of note that a low concentration of DMF (0.125 M), which did not significantly suppress ECAR, afforded a significant overshoot upon washout.

Effect of Amiloride on DMF-Induced Changes in ECAR

A major pathway for transmembrane proton transport in hepatocytes is Na^+/H^+ exchange (Anwer and Nolan, 1988; Strazzabosco and Boyer, 1996). To assess the possible participation of Na^+/H^+ exchange in cellular responses to DMF, we investigated the effects of amiloride, a reversible inhibitor of this transporter (Wakabayashi *et al.,* 1997).

On its own, amiloride (0.5 mM) rapidly suppressed the acidification rate to approximately 80% of baseline ECAR (Fig. 2A). Upon washout, ECAR returned rapidly to baseline with little overshoot. DMF (0.5 M) suppressed ECAR to the same extent as amiloride (Fig. 2B). However, the suppression induced by DMF developed more slowly. As noted above, washout of DMF yielded a large, significant overshoot. When DMF and amiloride were given together, there was suppression of ECAR similar to that produced by DMF alone (cf. Fig. 2C with Figs. 2B or 2D). However, the continuing presence of amiloride during washout of DMF completely inhibited recovery and overshoot of ECAR.

To determine whether amiloride acts during the suppression phase or recovery phase to block the overshoot, cells were exposed to DMF alone and then amiloride was applied for the first 15 min following removal of DMF (Fig. 2D). Under these conditions, amiloride completely prevented recovery and overshoot (cf. Figs. 2B and 2D). In cells previously exposed to DMF, removal of amiloride caused rapid overshoot of ECAR (Figs. 2C and 2D). Taken together, these data suggest that the overshoot response involves proton extrusion mediated by the Na^+/H^+ exchanger.

Dependence of DMF-Induced Suppression and Recovery of ECAR on Extracellular Na^+

To confirm the involvement of the Na^+/H^+ exchanger, we compared responses to DMF in the presence and absence of extracellular Na^+. To this end, Na^+-containing and nominally Na^+-free buffers were prepared using Na^+ and NMG^+ as the principal cations, respectively. All cell samples were initially superfused with running medium and then parallel samples were superfused with Na^+ buffer, running medium, or Na^+-free buffer (Fig. 3). Superfusion with Na^+ buffer increased baseline ECAR to approximately 115% (this may be due to the slightly greater glucose concentration in this buffer than in the regular running medium). In contrast, superfusion with Na^+-free buffer decreased baseline ECAR to approximately 40%, indicating an important role for Na^+-dependent processes in acid extrusion by these cells.

DMF (0.25 M) suppressed ECAR under each of these three conditions. Comparable declines were observed for cells superfused with Na^+ buffer and running medium, whereas a smaller decline was observed for cells superfused with Na^+-free buffer. Washout of DMF in Na^+ buffer and running medium afforded large overshoots. In contrast, there was recovery but no overshoot of ECAR with washout of DMF in Na^+-free buffer.

Replacement of buffers with running medium led to a small decline in ECAR in cells previously exposed to Na^+ buffer, and a transient overshoot in cells previously exposed to Na^+-free buffer. These data indicate that the DMF-induced suppression of ECAR is, at least in part, independent of extracellular Na^+. In contrast, the overshoot is completely dependent on Na^+, consistent with the involvement of Na^+/H^+ exchange.

Effects of DMF on Cytosolic pH and Recovery from Acid Load

DMF could suppress ECAR, either by inhibiting H^+ efflux or by reducing metabolic acid production. When measured fluorometrically, using BCECF, resting pH_i was approximately 7.3. If DMF inhibited H^+ efflux, then a decline in pH_i would be expected. However, there was no significant difference in pH_i between cells treated with DMF (0.25 M) and parallel samples of control cells (data not shown).

We also considered the possibility that DMF inhibits Na^+/H^+ exchange, but Na^+/H^+ exchange may not have been active at resting values of pH_i. Accordingly, we activated Na^+/H^+ exchange by acid-loading cells to a pH_i of approximately 6.0 using nigericin in a Na^+- and K^+-free buffer. The rate of recovery of pH_i following addition of Na^+ was then monitored in the presence and absence of DMF (0.25 M) or amiloride (0.5 mM) (Fig. 4).

Both control and DMF-treated cells recovered rapidly, whereas little recovery of pH_i occurred in amiloride-treated cells (initial rate of recovery of pH_i in amiloride-treated cells was 0.05 pH units/min, Fig. 4). In contrast, the initial rate of

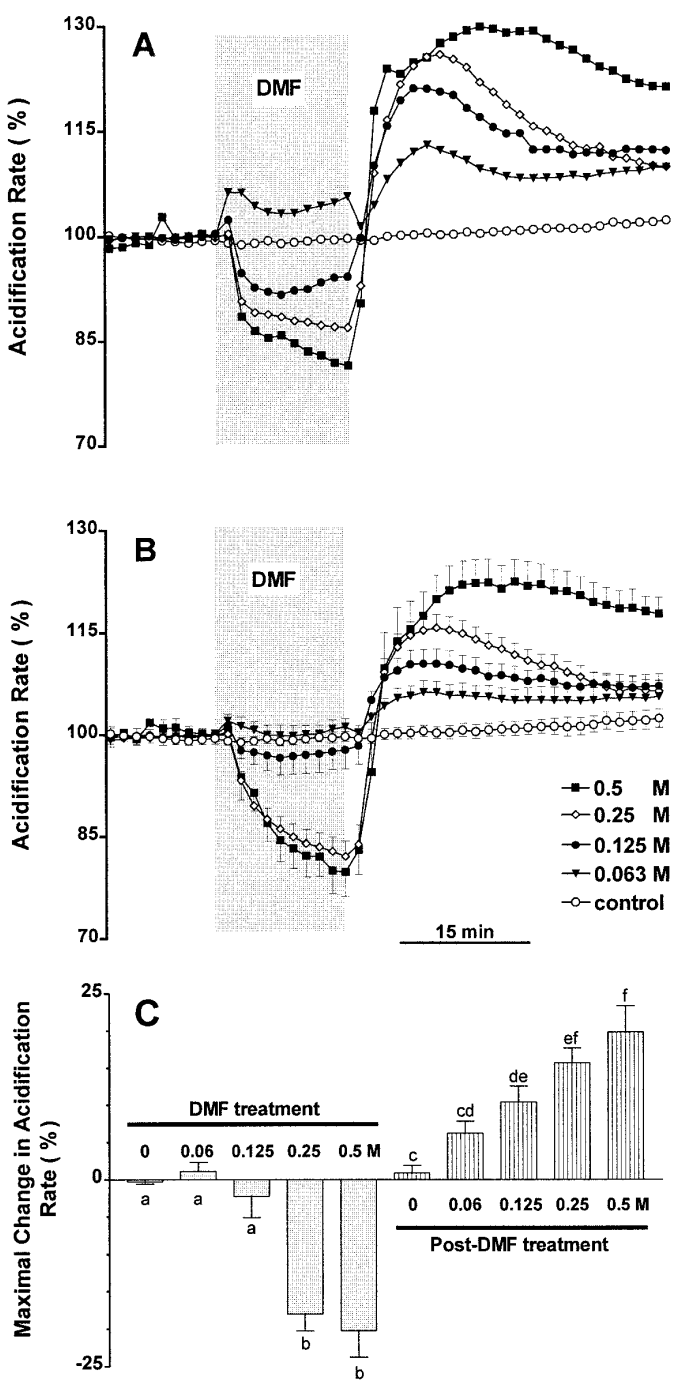

FIG. 1. Effects of DMF on ECAR of hepatocytes. Hepa 1C1C7 cells, cultured on porous polycarbonate membranes, were installed in microflow chambers of a microphysiometer. Cells were superfused with running medium (100 μL/min) and ECAR was monitored at 1.5-min intervals. (A) Representative responses from five individual chambers, each of which contained hepatocytes. Baseline acidification rates were normalized to 100% to compensate for small differences between chambers. During the period indicated by the shaded area, each cell sample was exposed to DMF (in running medium) at the concentration indicated. Control samples were exposed to running medium alone. After 15 min of exposure to DMF, cells were superfused by running medium alone (washout). The suppression of ECAR at high concentrations of DMF and the subsequent overshoot of ECAR can be noted. (B) Mean ECAR responses to the indicated concentrations of DMF. Data points are means ± SE, n = 5 to 15 determinations. Initial baseline rates of acidification were as follows (in $-\mu$V/s): control, 134 ± 35 (n = 5); DMF 0.063 M, 118 ± 16 (n = 7); 0.125 M, 132 ± 26 (n = 8); 0.25 M, 111 ± 16 (n = 15); and 0.5 M, 91 ± 8 (n = 5). ECAR returned toward baseline values within 30 min of washout for all concentrations except 0.5 M DMF, where ECAR declined over a period of 1 to 2 h. (C) Maximal suppression and overshoot of ECAR induced by various concentrations of DMF. Suppression of ECAR (during DMF treatment) was quantified immediately prior to washout of DMF. Overshoot of ECAR (post-DMF treatment) was quantified after 10.5 min of washout, a time when overshoot was generally maximal. Data are changes in ECAR expressed as percentage of baseline and are means ± SE (n = 5 to 15 determinations from at least four separate experiments). Independent statistical analyses of the suppression (DMF treatment) and overshoot (post-DMF treatment) data were performed. Bars labeled with the same lowercase letter are not significantly different from each other.

recovery of pH_i of approximately 0.4 pH units/min, did not differ between DMF-treated and control cells. These data indicate that DMF does not influence pH_i, nor does it directly alter the activity of Na^+/H^+ exchangers.

Dependence of DMF-Induced Suppression and Recovery of ECAR on Extracellular Glucose

Since DMF does not directly affect pH_i or Na^+/H^+ exchange, it is likely that DMF modulates metabolic activity in hepatocytes. Therefore, we investigated the effect of removing the metabolic substrates, glucose and glutamine, from the extracellular medium.

Cell samples were initially superfused with running medium and then parallel samples were superfused for 15 min with running medium, DMEM (with glucose and glutamine), or DMEM (glucose- and glutamine-free) (Fig. 5). Superfusion with glucose-free medium rapidly decreased ECAR to approximately 60% of baseline, indicating that acid production by these cells is highly sensitive to changes in the availability of metabolic substrates.

Cells were exposed to DMF (0.25 M) for 6 min under each of these three conditions. It should be noted that a shorter time of exposure to DMF was used in these experiments as full recovery of ECAR did not occur in cells superfused with glucose-free medium for prolonged periods. Under each condition, DMF suppressed ECAR to the same extent (Fig. 5). Washout of DMF in running medium and glucose-containing medium led to significant overshoots of ECAR. In contrast, there was recovery but no overshoot of ECAR with washout of DMF in glucose-free medium.

Restoration of the original running medium had little influence on ECAR in cells previously exposed to the running medium or glucose-containing medium. However, the ECAR of control cells previously exposed to glucose-free medium returned promptly to baseline. Interestingly, the cells exposed additionally to DMF demonstrated significant overshoot upon restoration of the original running medium.

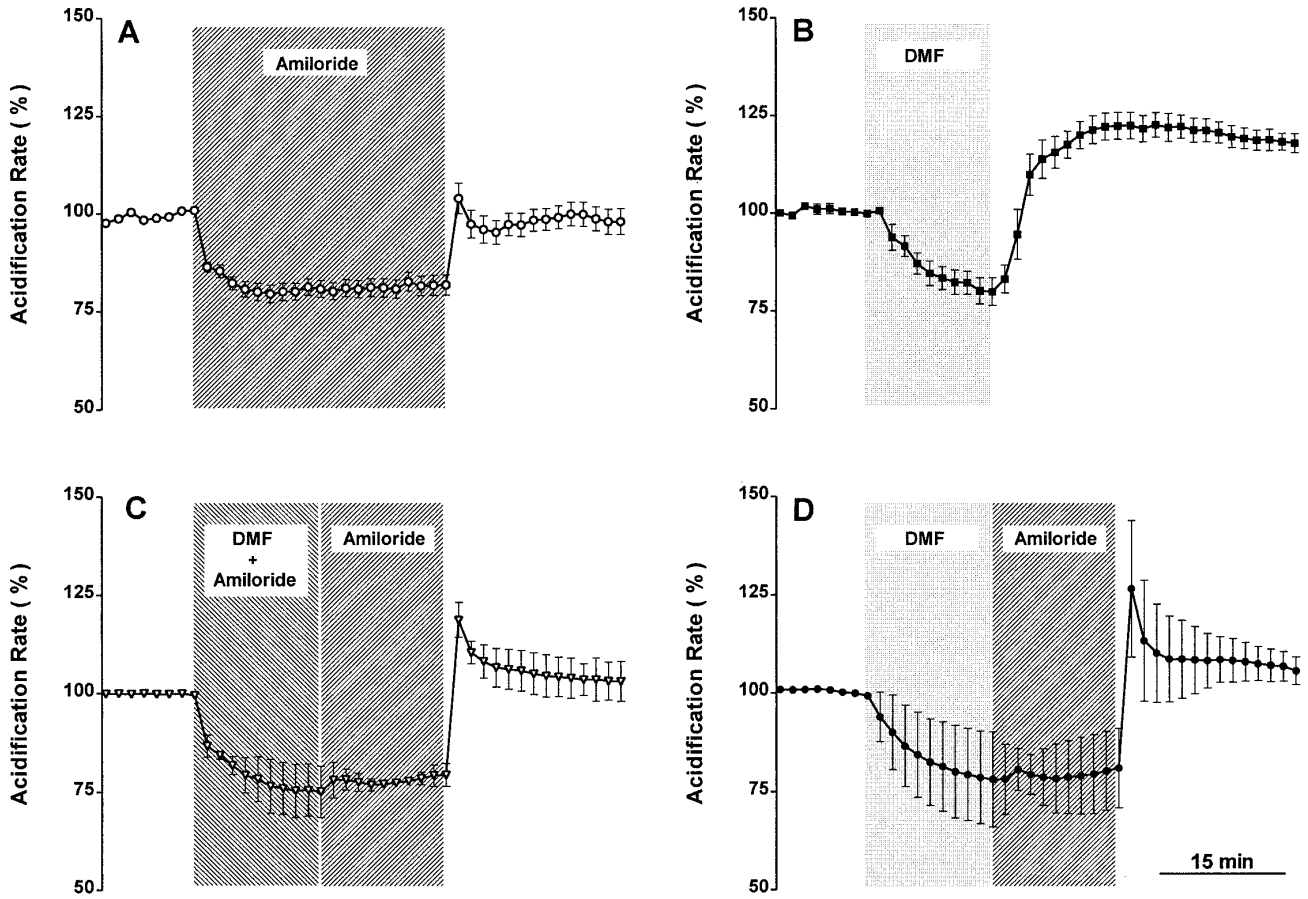

FIG. 2. Effect of amiloride on DMF-induced changes in ECAR. Illustrated are mean ECAR responses of Hepa 1C1C7 cells measured using microphysiometry. Cell samples were superfused initially with running medium. Subsequently, cells were exposed to the indicated substances (shaded areas), followed by washout with running medium. Data are ECAR, expressed as percentage of baseline, and are means ± SE from at least three separate experiments. (A) Response to the Na^+/H^+ exchange inhibitor, amiloride (0.5 mM in running medium for 30 min). The initial baseline rate of acidification was −123 ± 21 μV/s (n = 5 determinations). (B) Response to DMF (0.5 M in running medium for 15 min). The initial baseline rate of acidification was −91 ± 8 μV/s (n = 5 determinations). (C) Response of cells, exposed initially to a combination of DMF (0.5 M) and amiloride (0.5 mM) in running medium for 15 min, followed by amiloride (0.5 mM) alone in running medium for a further 15 min. The initial baseline rate of acidification was −131 ± 32 μV/s (n = 3 determinations). (D) Response of cells, exposed initially to DMF (0.5 M) in running medium for 15 min, followed by amiloride (0.5 mM) in running medium for a further 15 min. The initial baseline rate of acidification was −142 ± 17 μV/s (n = 5 determinations).

These data indicate that the DMF-induced suppression of ECAR is not dependent on the presence of extracellular glucose. In contrast, development of the overshoot is glucose dependent.

DISCUSSION

Baseline ECAR in Hepa 1C1C7 Cells

Baseline acidification rates were in the range of 0.05 to 0.15 pH units/min. Variation probably arose from differences in cell density from sample to sample. In hepatocytes, the major transporters responsible for efflux of H^+ or H^+ equivalents include Na^+/H^+ exchangers and Na^+-HCO_3^- cotransporters (Henderson *et al.*, 1987; Lidofsky *et al.*, 1993; Strazzabosco and Boyer, 1996). However, it is unlikely that Na^+–HCO_3^- cotransport contributed to acid extrusion in these experiments as the superfusion media were nominally HCO_3^--free.

In the present study, removal of extracellular Na^+ caused a 60% decline in baseline ECAR. This is consistent with the findings of Anwer and Nolan (1988), who demonstrated that, in isolated rat hepatocytes, 35 to 50% of H^+ efflux is Na^+ dependent. These authors found that the majority of Na^+-independent H^+ extrusion in rat hepatocytes is due to efflux of CO_2 and lactic acid. It is likely that a substantial portion of H^+ extrusion in murine hepatoma cells is also due to efflux of lactic acid, as removal of glucose led to a 40% decline in baseline ECAR. It is possible that the remaining acid production is due to glycogen metabolism leading to lactic acid and CO_2 efflux.

Possible Mechanisms Underlying DMF-Induced Suppression of ECAR

In some cases slight elevations in ECAR were observed with 0.063 M DMF, indicating enhanced metabolic activity.

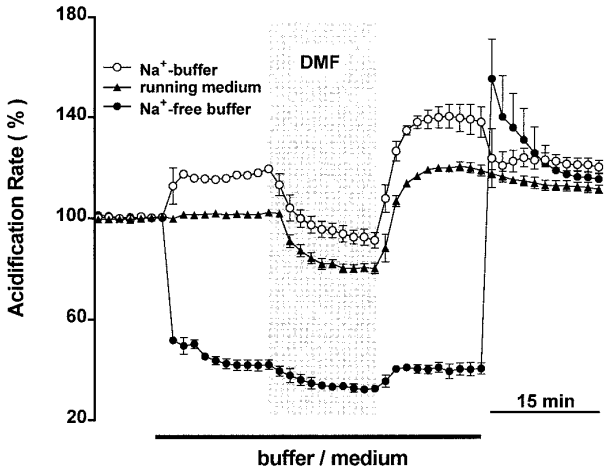

FIG. 3. Dependence of DMF-induced changes in ECAR on extracellular Na^+. Illustrated are mean ECAR of cell samples exposed to DMF in regular running medium, Na^+-containing, or Na^+-free buffers. In each case, cells were initially superfused with running medium and then with the buffer/medium indicated for 45 min. Following equilibration (0 to 15 min), cells were exposed to DMF (0.25 M) in the appropriate buffer/medium (15 to 30 min). From 30 to 45 min, cells were superfused with the appropriate buffer/medium (washout). At 45 min, cells were superfused with regular running medium. The initial baseline rates of acidification were as follows (in $-\mu$V/s): 109 ± 15 (Na^+ buffer), 126 ± 21 (running medium), and 132 ± 12 (Na^+-free buffer) ($n = 3$ for each). Data are ECAR expressed as percentage of baseline and are means ± SE from three separate experiments.

Whether this reflects the catabolism of DMF itself or stimulation of hepatocyte metabolism is not known. Overall, low concentrations of DMF (0.063 to 0.125 M) did not modify ECAR significantly. In contrast, higher concentrations of DMF (0.25 to 0.5 M) consistently suppressed ECAR. Thus, it appears that a threshold concentration between 0.125 and 0.25 M DMF exists for suppression of ECAR. At concentrations up to 0.5 M, the effects of DMF on ECAR were reversible, indicating a lack of acute cytotoxicity.

DMF-induced suppression of ECAR could be due to inhibition of proton efflux and/or a depression of proton production. We considered the possibility that DMF directly suppressed Na^+/H^+ exchange. However, DMF induced slight suppression of ECAR in the absence of extracellular Na^+, suggesting that inhibition of Na^+/H^+ exchange could not fully account for this effect of DMF. The influence of DMF on Na^+/H^+ exchange was then examined directly by determining the effects of DMF on the Na^+-dependent recovery of pH_i following cytosolic acidification. The absence of any significant effect of DMF indicated that the solvent does not inhibit Na^+/H^+ exchange, implying that suppression of ECAR arises from a decline in metabolic acid production.

Metabolic effects of DMF have been reported in other systems. For example, DMF exerts an inhibitory effect on muscle contraction (Escalona de Motta *et al.,* 1982), which may arise through decreased energy metabolism (Le Tallec *et al.,* 1996). It is of interest that DMF has a self-inhibitory effect on its own metabolism (Lundberg *et al.,* 1981; Mraz *et al.,* 1993). In our study, the removal of extracellular glucose depressed baseline ECAR in hepatocytes. However, removal of glucose did not influence DMF-induced suppression of ECAR, suggesting that this action of DMF is mediated by inhibition of a glucose-independent metabolic pathway.

Possible Mechanisms Underlying the Overshoot of ECAR

Removal of DMF yielded rapid, concentration-dependent increases in ECAR above baseline. It is unlikely that the increase in ECAR simply reflects recovery from prior suppression because a significant overshoot occurred at 0.125 M DMF, a concentration that did not suppress ECAR. The overshoot of ECAR is likely due to an increase in H^+ transport and/or H^+ production.

The overshoot was abolished by amiloride, consistent with the involvement of Na^+/H^+ exchange. Hepatocytes express the NHE-1 isoform of the Na^+/H^+ exchanger (Orlowski *et al.,* 1992), which is sensitive to inhibition by amiloride (Wakabayashi *et al.,* 1997). The absence of an overshoot in medium that did not contain Na^+ further supports the involvement of Na^+/H^+ exchange. Alternatively, the effects of amiloride and Na^+ removal may have been due to inhibition of acid production. However, upon washout of amiloride, or reintroduction of Na^+, a rapid, transient overshoot of ECAR still occurred. This delayed overshoot is consistent with an accumulation of H^+ from stimulated metabolism occurring upon removal of DMF (when Na^+/H^+ exchange was blocked). Upon reactivation of Na^+/H^+ exchange, rapid efflux of H^+ occurs resulting in the overshoot. In the absence of DMF, temporary suppression of Na^+/H^+ exchange by amiloride did not give rise to an overshoot of ECAR.

While DMF suppressed ECAR in glucose-free medium, no overshoot occurred upon washout. Reintroduction of glucose produced a sustained overshoot similar to that observed upon washout of DMF in the continuing presence of glucose. Thus, it appears that glucose metabolism is required for the burst of acid production occurring upon washout of DMF.

It is unlikely that removal of glucose would lead to acute inhibition of Na^+/H^+ exchange. Furthermore, had glucose withdrawal simply inhibited H^+ efflux, a rapid, transient overshoot, as seen upon reintroduction of Na^+, would have been expected. Instead, the onset of the overshoot was more gradual, similar to that occurring with washout of DMF under standard conditions (e.g., Fig. 1). The overshoot is not simply due to a temporary suppression of metabolic activity, since a significant overshoot did not occur upon recovery from glucose depletion in the absence of DMF. In short, it appears that exposure to DMF suppresses glucose-independent metabolic acid production, whereas washout of DMF is accompanied by increased glucose metabolism and H^+ efflux via the Na^+/H^+ exchanger. It is possible that DMF inhibits a stage in metabolism resulting in an accumulation of an intermediate. Washout of DMF

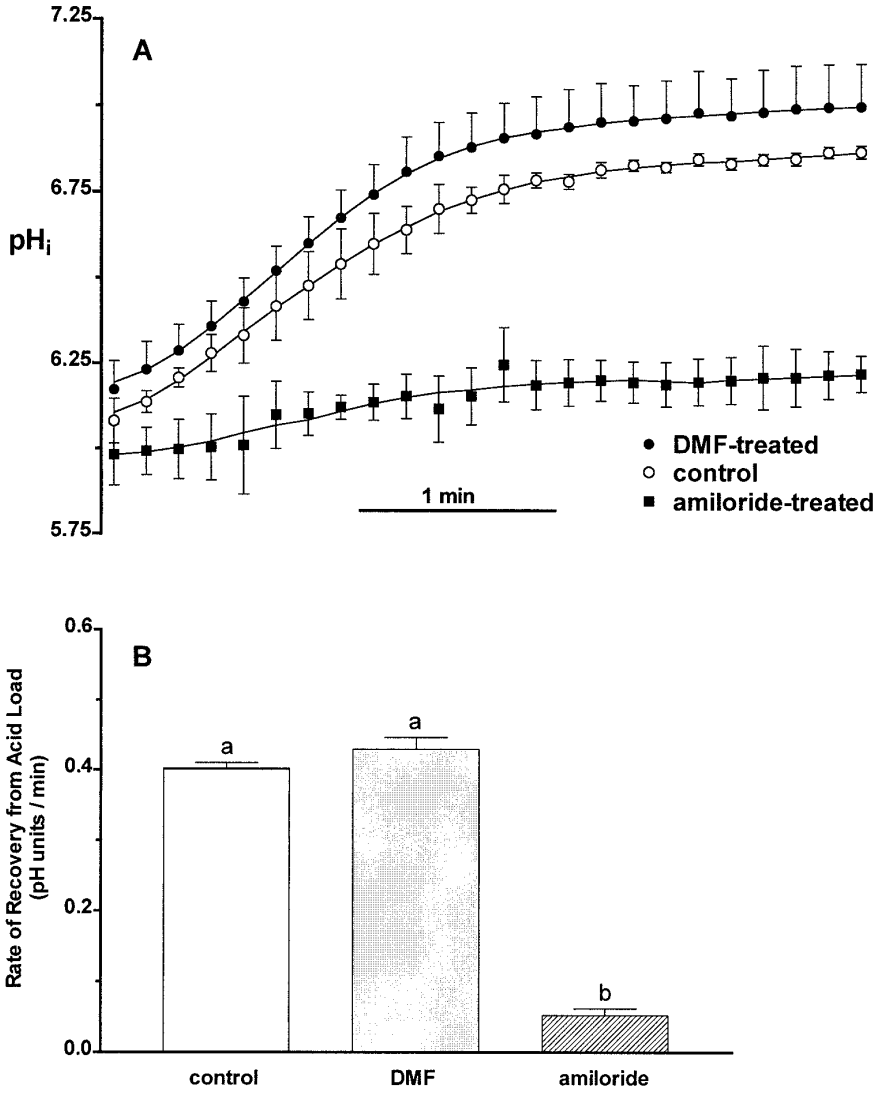

FIG. 4. Effects of DMF on recovery of cytosolic pH (pH_i) from an acid load. Fluorescence spectrophotometry was used to investigate the possible effect of DMF on Na^+/H^+ exchange. Hepa 1C1C7 cells were loaded with the pH-sensitive fluorescent probe, BCECF, and then suspended in Na^+- and K^+-free buffer in a fluorometric cuvette. Cytosolic pH was continuously monitored. Cells were acid-loaded by exposure to nigericin (10 μM) for 200 s. Equal volumes of Na^+- and K^+-free buffer (control, ○), DMF (0.25 M final, ●), or amiloride solution (0.5 mM final, ■) were then added. NaCl (200 mM final) was added 100 s later to induce recovery of pH_i. (A) Time course of changes in pH_i following addition of NaCl. Data are means ± SE (n = 3 cell samples from two separate experiments). Data were corrected for quenching of BCECF fluorescence by amiloride (~15%). (B) Initial rate of recovery, expressed as pH units/min, for each treatment. These rates were determined by linear regression analysis of the changes in pH_i during the first 60 s after NaCl addition. Bars labeled with the same letter are not significantly different from each other. Values of cytosolic pH before and immediately following acid-loading were as follows: control, 7.25 ± 0.03, then 5.95 ± 0.16; DMF-treated, 7.31 ± 0.08, then 6.16 ± 0.10; and amiloride-treated, 7.27 ± 0.07, then 6.01 ± 0.10.

restores the metabolic pathways for the intermediate, whose metabolism results in production of H^+.

It has been suggested that DMF influences the composition and fluidity of membranes and modifies ion channel activity (Gustafson and Tagesson, 1985; Larsen *et al.,* 1996). Other investigators have shown that DMF alters estrogen receptor binding (Sasson and Notides, 1988) and ADP and adrenergic receptors in platelets (Imbriani *et al.,* 1986). It is unlikely that the effects of DMF arise simply from changes in osmolarity, because the related compound, formamide (0.9 M), did not change the water content of muscle even after prolonged exposure (Escalona de Motta *et al.,* 1982). Moreover, Larsen *et al.* (1996) examined the influence of DMF on the lysis of secretory vesicles, whose rates of lysis are directly correlated to osmotic changes. There was no influence on vesicular lysis at concentrations of DMF up to 1.25 M, higher than the concentrations used in the present experiments.

It has been reported previously that formamide causes de-

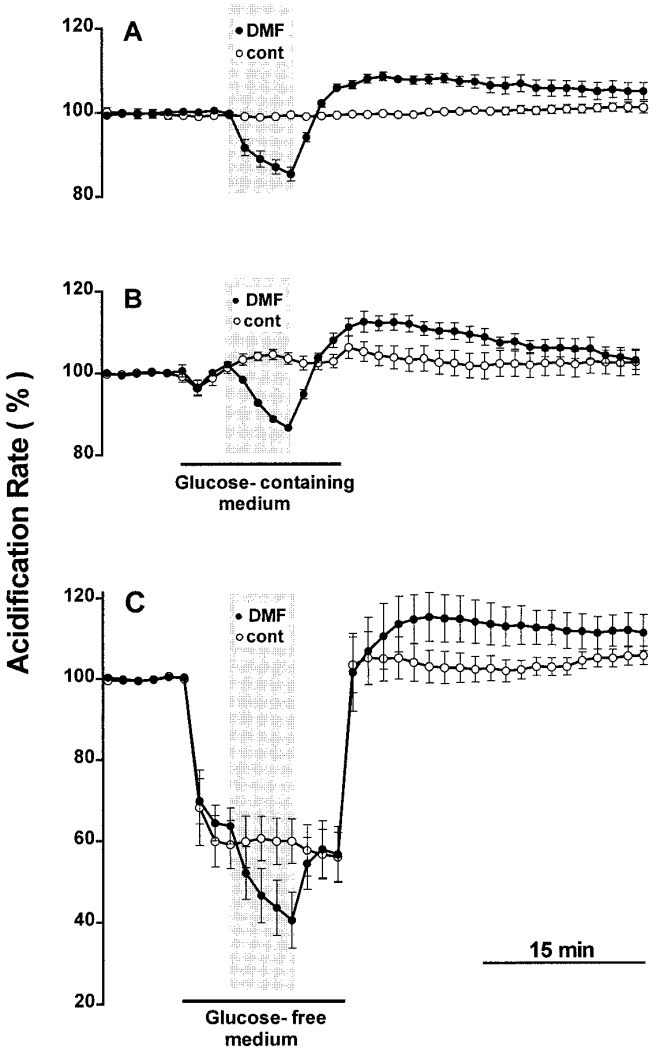

FIG. 5. Dependence of DMF-induced changes in ECAR on extracellular glucose. Illustrated are the effects of DMF on ECAR of cell samples superfused with regular running medium (A), glucose/glutamine-containing medium (B), or glucose/glutamine-free medium (C). In each case, cells were initially superfused with running medium and then the medium indicated for 15 min. Following equilibration (0 to 4.5 min), cells were exposed to DMF (0.25 M, ●) in the appropriate medium or medium alone (control, ○) (4.5 to 10.5 min). From 10.5 to 15 min, cells were superfused with the appropriate medium (washout). At 15 min, cells were superfused with regular running medium. The initial baseline rates of acidification were as follows (in $-\mu$V/s): 130 ± 22 (running medium control), 68 ± 15 (DMF in running medium), 52 ± 13 (glucose-containing control), 65 ± 21 (DMF in glucose medium), 76 ± 36 (glucose-free control), and 73 ± 11 (DMF in glucose-free medium) ($n = 3$ for each). Data are ECAR expressed as percentage of baseline and are means ± SE from three separate experiments.

polarization of frog skeletal muscle (Escalona de Motta *et al.*, 1982). It is of interest that this response was not observed during exposure to formamide, but appeared upon washout and dissipated over 1 to 2 h. It is possible that formamide may similarly enhance the accumulation of an active intermediate, the removal of formamide then leading to a biological response.

Implications for DMF-Induced Toxicity and Differentiation

DMF is hepatotoxic. As reviewed by Gescher (1993), several authors have proposed that the toxic effects of DMF are associated with generation of the reactive metabolite, methylisocyanate. While an *in vivo* time course of damage caused by methylisocyanate has not been determined, whole animal studies point to a progressive toxic response, which appears to be maximal 48 h following DMF administration (Van den Bulcke *et al.*, 1994). In contrast, the present study has shown that DMF causes rapid changes in cellular metabolism, which are reversible within 1 to 2 h. At the present time it is not known whether the observed effects of DMF are produced by the parent compound or an early metabolite. It is possible that the acute changes in cellular metabolism produced by DMF make the cell more susceptible to damage.

During exposure to DMF, there is inhibition of cellular metabolism followed by a prolonged overshoot of ECAR upon washout. As mentioned above, the suppression may arise from inhibition of a metabolic process by DMF or a metabolite of DMF. When DMF is removed it is possible that an accumulated intermediate undergoes rapid metabolism, generating H^+ that are removed via Na^+/H^+ exchange. Activation of Na^+/H^+ exchange would lead to Na^+ loading, placing further metabolic demands on the cell.

The effects of DMF on cellular differentiation may involve activation of Na^+/H^+ exchange. Enhanced Na^+/H^+ exchange is associated with mitogenesis in many cell types, including hepatocytes (Strazzabosco and Boyer, 1996). In this regard, hepatocellular Na^+/H^+ exchange has been shown to be activated during liver regeneration stimulated by partial hepatectomy or experimentally induced cirrhosis in rats (Dallenbach *et al.*, 1994; Elsing *et al.*, 1994). DMF could cause an initial insult to hepatocytes. Removal of DMF then leads to increased Na^+/H^+ exchange activity, which may promote cellular regeneration and differentiation. It is of particular interest that, in our study, Na^+/H^+ exchange was activated without evidence of acute cellular toxicity. Moreover, Na^+/H^+ exchange was activated following the removal of low concentrations of DMF that did not suppress cellular metabolism.

DMF is employed as a solvent of lipophilic compounds in various biological investigations. The present study indicates that it has effects that must be considered. Use of the microphysiometer has uncovered early cellular responses to DMF, which may contribute to our understanding of the mechanisms of the toxic and differentiating effects of this compound. This approach may be useful in evaluating mechanisms of action of other toxic agents in a variety of cellular systems.

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