

Activation of Potassium and Chloride Channels by Tumor Necrosis Factor α

ROLE IN LIVER CELL DEATH*

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Hubert H. Nietsch[‡], Michael W. Roe[‡], Jerome F. Fiekers[§], Ann L. Moore[‡], and Steven D. Lidofsky[‡][¶]

From the Departments of [‡]Medicine, [¶]Pharmacology, and [§]Anatomy and Neurobiology, University of Vermont, Burlington, Vermont 05401

Despite abundant evidence for changes in mitochondrial membrane permeability in tumor necrosis factor (TNF)-mediated cell death, the role of plasma membrane ion channels in this process remains unclear. These studies examine the influence of TNF on ion channel opening and death in a model rat liver cell line (HTC). TNF (25 ng/ml) elicited a 2- and 5-fold increase in K^+ and Cl^- currents, respectively, in HTC cells. These increases occurred within 5–10 min after TNF exposure and were inhibited either by K^+ or Cl^- substitution or by K^+ channel blockers (Ba^{2+} , quinine, 0.1 mM each) or Cl^- channel blockers (10 μ M 5-nitro-2-(3-phenylpropylamino)benzoic acid and 0.1 mM *N*-phenylanthranilic acid), respectively. TNF-mediated increases in K^+ and Cl^- currents were each inhibited by intracellular Ca^{2+} chelation (5 mM EGTA), ATP depletion (4 units/ml apyrase), and the protein kinase C (PKC) inhibitors chelerythrine (10 μ M) or PKC 19–36 peptide (1 μ M). In contrast, currents were not attenuated by the calmodulin kinase II 281–309 peptide (10 μ M), an inhibitor of calmodulin kinase II. In the presence of actinomycin D (1 μ M), each of the above ion channel blockers significantly delayed the progression to TNF-mediated cell death. Collectively, these data suggest that activation of K^+ and Cl^- channels is an early response to TNF signaling and that channel opening is Ca^{2+} - and PKC-dependent. Our findings further suggest that K^+ and Cl^- channels participate in pathways leading to TNF-mediated cell death and thus represent potential therapeutic targets to attenuate liver injury from TNF.

Tumor necrosis factor α (TNF)¹ is an inflammatory cytokine that induces programmed cell death in a variety of tissue types (1). In the liver, TNF has been implicated as a mediator of hepatocellular dysfunction and death following toxic injury, viral hepatitis, and sepsis (2–8). It is thought that such pathological conditions lead to the release of TNF by hepatic macro-

phages, with resultant paracrine actions on other liver cells (9). In liver, TNF exhibits pleiotropic effects, ranging from reduction of bile flow to hepatocellular apoptosis (10–12). Experimental evidence supports several mechanisms to account for such effects, including activation of caspases and kinases, generation of free radicals, and down-regulation of membrane organic solute transporters (13–17). Despite this body of evidence, there remain significant gaps in our knowledge regarding the responsible pathways that couple TNF to liver damage.

There is abundant information to suggest that apoptosis is associated with increases in mitochondrial membrane permeability but considerably less is known with respect to the role of the plasma membrane. In a limited number of cell types, increases in plasma membrane permeability to ions represent early responses to apoptotic stimuli. In cultured neurons, serum withdrawal increases voltage-activated K^+ channel activity and cell death, and in lymphocytes, engagement of the cell surface protein Fas leads to K^+ loss, activation of outwardly rectifying Cl^- channels, and apoptosis (18–20). Conversely, pharmacological blockade of K^+ or Cl^- channels blunts apoptosis in these experimental models. Thus, K^+ and Cl^- channels may play important roles in apoptotic processes.

Based on its function in inducing apoptosis, there is reason to believe that TNF could influence cell death through activation of ion channels. In support of this, TNF increases K^+ currents in selected neurons and activates Cl^- currents in neutrophils (21, 22). However, it is unknown whether TNF affects plasma membrane ion channels in other cell types or whether such channels are involved in TNF-mediated cell death. In this study, we have examined the effects of TNF on plasma membrane conductance and death in the model hepatocyte-like cell line HTC. Our results suggest that TNF activates K^+ and Cl^- channels and that channel activation is an early signal in pathways leading to TNF-mediated liver cell death.

EXPERIMENTAL PROCEDURES

Experimental Reagents—Murine TNF was obtained from R & D Systems. Actinomycin D, Ca^{2+} /calmodulin kinase II inhibitor (CaMKII 281–309), chelerythrine, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and PKC inhibitor peptide (PKC 19–36) were purchased from Calbiochem. *N*-phenylanthranilic acid (DPC) was from Alexis. Fura-2 acetoxymethyl ester was obtained from Molecular Probes. 4',6-Diaminido-2-phenylindole (DAPI, in Vectashield mounting medium) was from Vector laboratories. All other reagents came from Sigma.

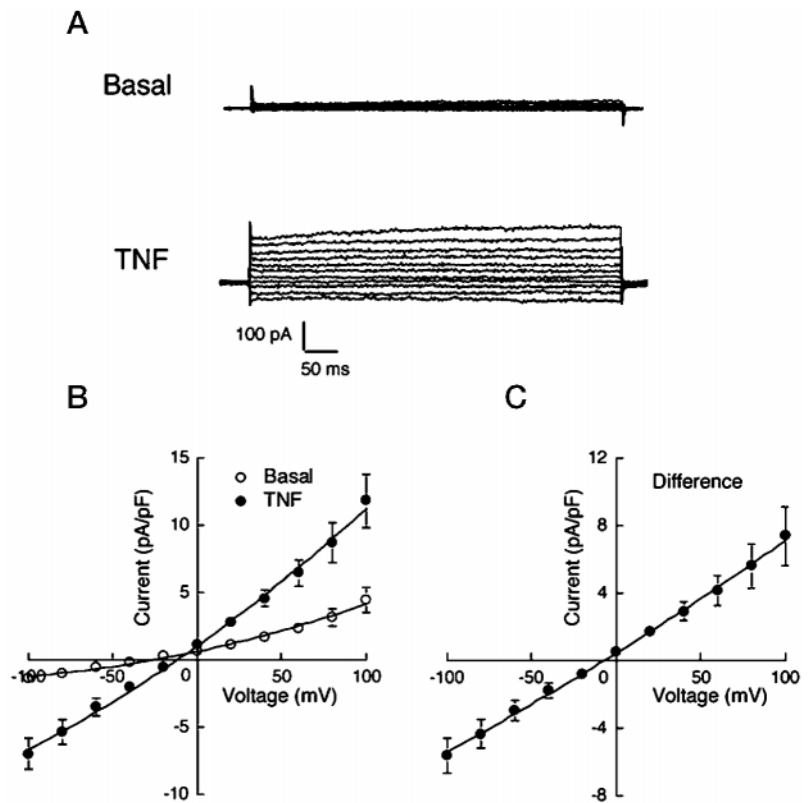
Cell Culture—HTC rat hepatoma cells were grown in minimal essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin and maintained in a humidified 5% CO_2 atmosphere. They were passaged every 3–5 days. Approximately 24 h before study, cells were seeded onto 35-mm diameter tissue culture dishes for electrophysiology experi-

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[¶] To whom correspondence should be addressed: Burgess 414 MFU, University of Vermont, Burlington, VT 05401. Tel.: 802-847-5990; Fax: 802-847-4928; E-mail: steven.lidofsky@uvm.edu.

¹ The abbreviations used are: TNF, tumor necrosis factor; CaMKII, calmodulin kinase II; PKC, protein kinase C; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; DPC, *N*-phenylanthranilic acid; $[Ca^{2+}]_i$, cytosolic calcium concentration; DAPI, 4',6-diaminido-2-phenylindole; I_{-80} , current at -80 mV; I_0 , current at 0 mV; pF, picofarad.

FIG. 1. Actions of TNF on membrane currents in HTC cells. Membrane currents were measured using patch clamp recording techniques in the whole cell configuration as described under "Experimental Procedures." *A*, shown are current transients in a representative cell in response to step changes in membrane potential (-100 mV to $+100$ mV in 20 mV increments) under basal conditions and 10 min following exposure to TNF (25 ng/ml). *B*, current-voltage relation under basal conditions and 10 min following exposure to TNF. Data represent mean \pm S.E. of 20 cells. *C*, relation between membrane voltage and difference current (current 10 min after TNF exposure minus basal current) analyzed from data presented in *B*. In *B* and *C*, currents have been normalized to membrane capacitance.



ments, onto 15 -mm glass coverslips for measurement of cell calcium, or onto 96 -well plates for measurements of cell death.

Electrophysiology—Whole cell currents were measured using patch clamp recording techniques as described previously (23). All experiments were performed at room temperature. The standard extracellular solution contained: 140 mM NaCl, 4 mM KCl, 1 mM KH_2PO_4 , 2 mM MgCl_2 , 1 mM CaCl_2 , 10 mM glucose, and 10 mM HEPES (pH 7.4). The standard intracellular (pipette) solution contained: 10 mM NaCl, 130 mM KCl, 2 mM MgCl_2 , 0.5 mM CaCl_2 , 1 mM EGTA, and 10 mM HEPES (pH 7.3). Under these conditions, K^+ currents reverse near -80 mV, and Cl^- currents reverse near 0 mV (24). In selected experiments, $[\text{Cl}^-]$ was reduced by substitution of sodium gluconate and potassium gluconate for NaCl and KCl, respectively, in both extracellular and intracellular solutions. In other experiments, K^+ was removed from both extracellular and intracellular solutions by isosmotic substitution of NaCl for KCl and Na_2HPO_4 for KH_2PO_4 .

Cells were exposed to TNF by the addition of a concentrated aliquot (5 μl) to the culture dish (1 -ml volume). In experiments involving ion channel blockade, the test compound was added to the extracellular solution. In experiments involving kinase inhibition, the test compound was included in the pipette solution. Membrane currents were acquired and analyzed using pClamp software (Axon Instruments). Currents were normalized to membrane capacitance to account for differences in cell size. All data have been presented as mean \pm S.E. Statistical analysis was performed using unpaired or paired *t* tests, as appropriate, with a *p* value less than 0.05 required to achieve significance.

Cell Calcium—Cytosolic calcium concentration ($[\text{Ca}^{2+}]_i$) was measured in HTC cells using the calcium-sensitive dye fura-2, using a modification of methods previously described (25). Coverslips containing fura-2-loaded HTC cells were placed in a perfusion chamber mounted on a Nikon Diaphot microscope, and ratiometric microfluorimetry was performed with a PTI Deltascan system. All measurements were performed at room temperature.

Cell Death—Cell death was assessed by uptake of the impermeant dye trypan blue. On the day of study, medium from wells in a 96 -well plate was exchanged with culture medium alone or medium containing actinomycin D (1 μM) and/or TNF (25 ng/ml), in the absence or presence of selected ion channel blockers. K^+ channel blockers included BaCl_2 (0.1 mM) and quinine (0.1 mM), and Cl^- channel blockers included DPC (0.1 mM) and NPPB (10 μM). At defined time intervals following medium exchange, wells (2–3/condition) were briefly incubated with 0.4% trypan blue (in 0.9% NaCl), their contents were exchanged with 0.9% NaCl, and they were then viewed under a microscope. Fields of view contain-

ing at least 100 cells were evaluated for stained and unstained cells. A cell stained with trypan blue was counted as a dead cell. Cell death was expressed as the percentage of trypan blue-stained cells. All experiments were performed three to four times.

Apoptosis was assessed by the presence of fragmentation and condensation in cell nuclei stained with DAPI. Cells plated on 15 -mm coverslips were incubated in 96 -well plates and treated with TNF and actinomycin D, in the presence or absence of ion channel blockers, as described above. At defined time points, coverslips were removed and incubated with DAPI as per the manufacturer's directions and mounted on glass slides. Fields of view containing at least 100 cells were evaluated by using a fluorescence microscope. Apoptosis was expressed as the percentage cells containing fragmented or condensed nuclei (26). All experiments were performed three times.

RESULTS

TNF Increases Membrane Currents—Under basal conditions, membrane currents in HTC cells were small in magnitude (less than 100 pA at 100 mV), similar to previously published values (23, 24). Exposure to TNF (25 ng/ml) led to a significant increase in membrane currents that were outwardly rectifying (Fig. 1). The increase in membrane currents was detectable between 3 and 5 min after TNF exposure and reached maximal levels by 10 min; currents remained stable for up to 15 min (data not shown). At a holding potential of -80 mV (close to the reversal potential for K^+), inward current (I_{-80}) increased from a basal value of -0.99 ± 0.35 pA/pF to a maximum value of -5.38 ± 0.92 pA/pF ($n = 20$). Similarly, at a holding potential of 0 mV (close to the reversal potential for Cl^-), outward current (I_0) increased from 0.64 ± 0.08 pA/pF to a maximum value of 1.12 ± 0.15 pA/pF ($n = 20$). The increases for each of these currents were statistically significant ($p < 0.04$ by paired *t* test). The reversal potential for the difference current (current after TNF exposure minus basal current) was approximately -9 mV, between the reversal potentials for K^+ and Cl^- under the experimental conditions employed (Fig. 1).

Origin of TNF-evoked Membrane Currents—To resolve the ionic basis of TNF-activated membrane currents, we performed experiments involving ion substitution and exposure to se-

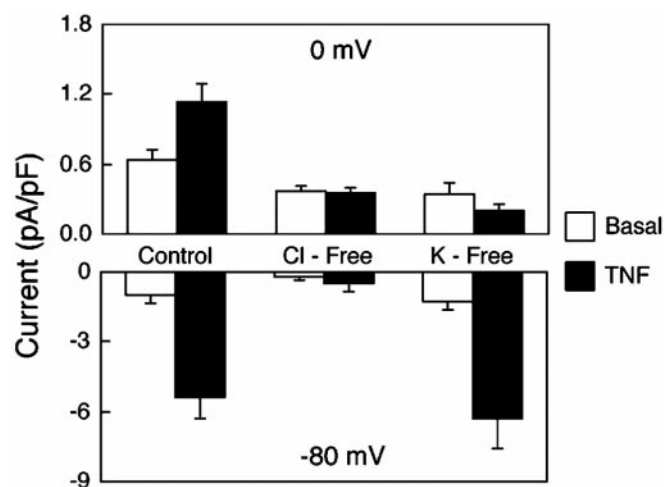


FIG. 2. Effect of ion substitution on TNF-evoked membrane currents. Currents were measured at 0 mV (reversal potential for Cl^- , upper panel) and at -80 mV (reversal potential for K^+ , lower panel) under basal conditions (open bars) and 10 min following TNF (25 ng/ml) exposure (filled bars). For Cl^- -free conditions, gluconate was isosmotically substituted for Cl^- as described under "Experimental Procedures." For K^+ -free conditions, Na^+ was substituted for K^+ as described under "Experimental Procedures." Data represent mean \pm S.E. of nine or more cells for each condition, and currents have been normalized to membrane capacitance.

lected channel blockers. Removal of K^+ eliminated the TNF-elicited increase in I_0 but did not significantly affect the increase in I_{-80} (Fig. 2). These data suggested that I_0 was attributable to K^+ currents under these experimental conditions. Furthermore, replacement of Cl^- with the impermeant anion gluconate abolished the TNF-elicited increase in I_{-80} , consistent with the concept that I_{-80} reflected Cl^- currents. Interestingly, gluconate substitution also inhibited the TNF-elicited increase in I_0 , raising the possibility that the K^+ -dependent increase in I_0 evoked by TNF was Cl^- -dependent. Collectively, these data suggested that TNF increased both K^+ and Cl^- currents.

Experiments with ion channel blockers supported this hypothesis. In the presence of the K^+ channel blocker Ba^{2+} (0.1 mM), TNF-elicited increases in I_0 were significantly inhibited (Fig. 3). A similar effect was seen with exposure to quinine (0.1 mM), which also blocks K^+ channels. By contrast, neither Ba^{2+} nor quinine prevented TNF-mediated increases in I_{-80} , indicating that these agents did not affect Cl^- currents (Fig. 3). As shown in Fig. 3, the Cl^- channel blockers NPPB (10 μM) and DPC (0.1 mM), each attenuated TNF-elicited increases in I_{-80} , but they did not affect TNF-mediated increases in I_0 . Taken together, these findings were consistent with activation of K^+ and Cl^- channels by TNF.

Mechanisms of TNF-mediated Increases in Membrane Currents—The delay in activation of membrane currents following exposure to TNF suggested the participation of intracellular signaling cascades. Previous work has supported a potential role for intracellular Ca^{2+} and protein kinases (22), each of which has been shown to affect K^+ and Cl^- permeability in liver cells (27, 28). We therefore sought to examine the effects of (a) lowering $[\text{Ca}^{2+}]_i$ or (b) protein kinase inhibition on the increases in membrane currents produced by TNF.

To determine the role of intracellular Ca^{2+} in channel activation, we lowered intracellular $[\text{Ca}^{2+}]_i$ by increasing the concentration of EGTA in the pipette solution to 5 mM in the absence of Ca^{2+} and observed the effects on membrane currents. As shown in Fig. 4, reduction of $[\text{Ca}^{2+}]_i$ significantly inhibited TNF-mediated increases in both I_0 and I_{-80} . This suggested that TNF-mediated activation of K^+ and Cl^- chan-

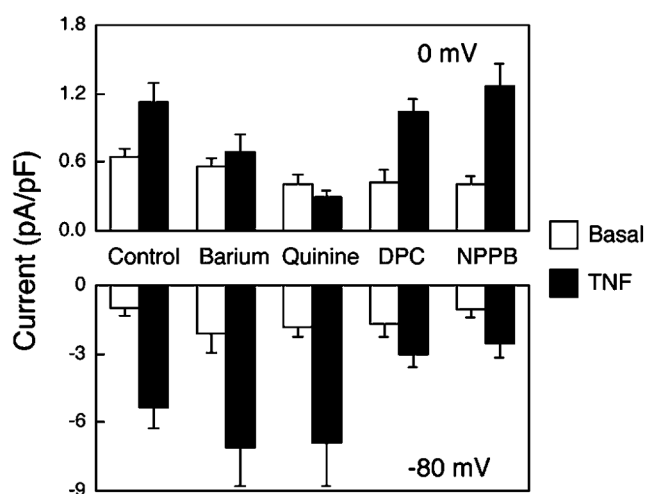


FIG. 3. Effect of ion channel blockade on TNF-evoked membrane currents. Currents were measured at 0 mV (upper panel) and at -80 mV (lower panel), in the absence (control) or presence of individual ion channel blockers, under basal conditions (open bars) and 10 min following TNF (25 ng/ml) exposure (filled bars). The concentrations of blockers were as follows 0.1 mM Ba^{2+} , 0.1 mM quinine, 0.1 mM DPC, and 10 μM NPPB. Data represent mean \pm S.E. of 10 or more cells for each condition, and currents have been normalized to membrane capacitance.

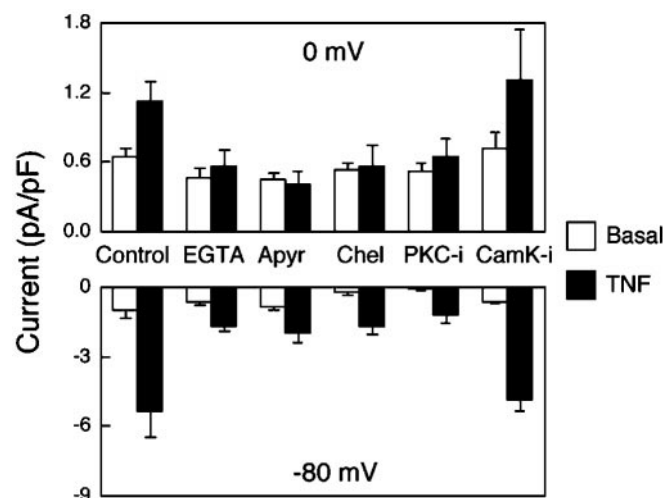


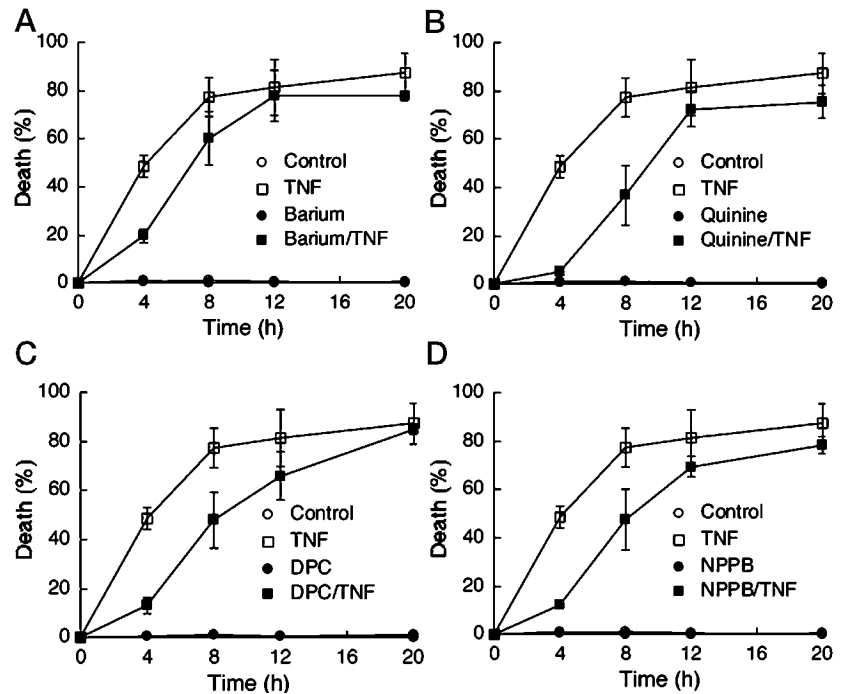
FIG. 4. TNF-evoked membrane currents are dependent on Ca^{2+} and PKC. Currents were measured at 0 mV (upper panel) and at -80 mV (lower panel), in the absence (control) or presence of selected inhibitors in the patch pipette, under basal conditions (open bars) and 10 min following TNF (25 ng/ml) exposure (filled bars). The inhibitors employed were EGTA (5 mM), apyrase (Apyr, 4 units/ml), chelerythrine (Chel, 10 μM), PKC 19-36 (PKC-i, 1 μM), and CaMKII 281-309 (CaMK-i, 10 μM). Data represent mean \pm S.E. of eight or more cells for each condition, and currents have been normalized to membrane capacitance.

nels was Ca^{2+} -dependent.

Based upon the Ca^{2+} dependence of TNF-evoked K^+ and Cl^- currents, we sought to determine if TNF increased $[\text{Ca}^{2+}]_i$. Interestingly, when HTC cells were exposed to TNF (25 ng/ml), $[\text{Ca}^{2+}]_i$ did not change ($n = 4$, data not shown). These data implied that activation of K^+ and Cl^- channels by TNF involved a Ca^{2+} -dependent process but did not require global increases in $[\text{Ca}^{2+}]_i$.

We next assessed the potential role of protein kinases in channel activation. If activation of K^+ and Cl^- channels by TNF involved protein kinases, depletion of intracellular ATP would be predicted to prevent channel activation. To test this prediction, we included apyrase (grade VI, 4 units/ml), which

FIG. 5. Effect of ion channel blockade on TNF-induced cell death. Cell death was measured by the percentage of cells stained with trypan blue (see "Experimental Procedures") under conditions in which TNF (25 ng/ml) and/or a selected channel blocker was present or absent (control). For all conditions, actinomycin D (1 μ M) was present. The blockers used were (A) 0.1 mM Ba²⁺, (B) 0.1 mM quinine, (C) 0.1 mM DPC, and (D) 10 μ M NPPB. Data represent mean \pm S.E. of three to four experiments for each condition, with each experiment performed in duplicate or triplicate.



hydrolyzes ATP (24), in the pipette solution. Apyrase prevented TNF-elicited increases in membrane currents (Fig. 4). These data were consistent with the potential involvement of protein kinases in channel activation. Intracellular dialysis with the PKC inhibitor chelerythrine (10 μ M) or the PKC 19–36 inhibitor peptide (1 μ M) each inhibited TNF-mediated increases in both I_0 and I_{-80} . By contrast, intracellular dialysis with CaMKII 281–309 (10 μ M), a peptide inhibitor of CaMKII, did not significantly block TNF-mediated increases in I_0 and I_{-80} . These data suggested that activation of K⁺ and Cl⁻ channels by TNF was dependent on PKC but not CaMKII.

Ion Channel Blockade and TNF-mediated Cell Death—Liver cell death is a process that takes hours to occur (11, 12). Because our data suggested that openings of K⁺ and Cl⁻ channels occurred within minutes after TNF exposure, we asked whether blockade of such channels influenced TNF-mediated cell death. Consistent with observations in liver cells by others (11, 12), we found that exposure to TNF alone (at concentrations up to 100 ng/ml) for periods of up to 24 h did not affect the proportion of trypan blue-stained cells compared with controls (data not shown). However, in the presence of the transcriptional inhibitor actinomycin D (1 μ M), TNF (25 ng/ml) induced death in nearly 90% of cells within 20 h of exposure (Fig. 5). Actinomycin D alone did not influence cell viability over this time period nor did it influence activation of K⁺ and Cl⁻ channels by TNF. Exposure of HTC cells to actinomycin D (1 μ M) in combination with TNF (25 ng/ml) increased I_{-80} from -0.81 ± 0.17 pA/pF to -5.38 ± 0.92 pA/pF and increased I_0 from 0.64 ± 0.08 pA/pF to 1.12 ± 0.15 pA/pF ($n = 13$). Currents reached maximal values by 10 min after TNF exposure, but by 1 h, currents had fallen to basal levels (data not shown).

When K⁺ or Cl⁻ channel blockers were present, the progression to cell death by TNF was attenuated (Fig. 5). At 4 h following TNF exposure, blockers of K⁺ channels (Ba²⁺ and quinine) and Cl⁻ channels (DPC and NPPB) each reduced by at least 50% the proportion of cells stained with trypan blue (compared with the absence of channel blockers). The relative reduction of cell death produced by channel blockade remained statistically significant for up to 8 h after exposure to TNF ($p < 0.05$). Ion channel blockade did not reduce cell death occurring after more prolonged periods of TNF exposure, and it did not

affect cell viability in the absence of TNF (Fig. 5). Taken together, these data suggest that blockade of K⁺ or Cl⁻ channels delayed but did not prevent TNF-mediated cell death.

To further examine the mechanisms by which TNF-mediated cell death occurred, we asked whether TNF induced apoptosis and if so, whether ion channel blockade affected this process. As shown in Fig. 6, in the presence of actinomycin D (1 μ M), TNF (25 ng/ml) produced nuclear morphological changes characteristic of apoptosis in ~70% of cells within 20 h of exposure. However, at earlier time points, the proportion of cells exhibiting fragmented nuclei was considerably less than the proportion of cells stained with trypan blue. These findings suggested that apoptosis was not the sole contributor to cell death in this experimental model and that necrosis was likely occurring in parallel. Nonetheless, at time points up to 8 h after exposure to TNF, blockers of K⁺ channels (Ba²⁺ and quinine) and Cl⁻ channels (DPC and NPPB) each significantly reduced the proportion of cells with apoptotic nuclear morphology ($p < 0.05$). In aggregate, these observations imply that K⁺ or Cl⁻ channel blockade delayed TNF-induced apoptosis.

DISCUSSION

In this study, we have examined the role of plasma membrane ion channels in TNF signaling. Our observations suggest that TNF increases membrane permeability to K⁺ and Cl⁻ in HTC cells through activation of ion channels and that TNF-mediated cell death is delayed by blockade of these channels. Our data are thus consistent with the hypothesis that channel opening is an early event in a TNF-mediated pathway that leads to liver cell death.

Two principal findings support the concept that TNF activates K⁺ and Cl⁻ channels. First, TNF increased membrane currents (I_0 and I_{-80}) at potentials corresponding to the opening of K⁺ and Cl⁻ channels. Second, the TNF-evoked increases in these currents were selectively prevented by either K⁺ or Cl⁻ removal or by exposure to K⁺ or Cl⁻ channel blockers, respectively. Our data thus extend observations in neurons, in which TNF activates K⁺ channels (21), and neutrophils, in which TNF activates Cl⁻ channels (22), and demonstrate that TNF can activate K⁺ and Cl⁻ channels in the same cell type.

Unexpectedly, the TNF-elicited increase in K⁺ current (I_0)

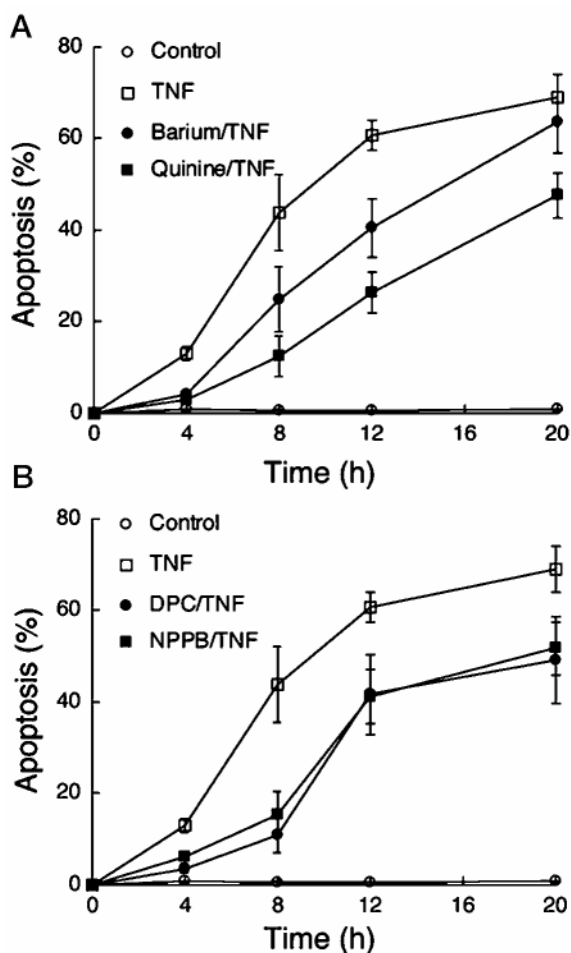


FIG. 6. Effect of ion channel blockade on TNF-induced apoptosis. Apoptosis was measured by the percentage of cells with DAPI-stained nuclei that exhibited fragmentation and/or condensation (see "Experimental Procedures"). Cells were incubated with TNF (25 ng/ml) in the absence or presence of selected channel blockers or in the absence of TNF (*control*). Actinomycin D (1 μ M) was present for all conditions. The blockers used in *A* were Ba²⁺ (0.1 mM) and quinine (0.1 mM), and the blockers used in *B* were DPC (0.1 mM) and NPPB (10 μ M). Data represent mean \pm S.E. of three experiments for each condition, with each experiment performed in duplicate or triplicate.

was inhibited by Cl⁻ removal. However, this current was not attenuated by Cl⁻ channel blockers. This suggests that K⁺ channel opening does not depend on Cl⁻ movement *per se*. Rather, it raises the possibility that Cl⁻ binding itself affects K⁺ channel kinetics. Precedent for such an effect exists in corneal epithelia, where extracellular Cl⁻ is necessary for K⁺ channel opening (29). The significance of this Cl⁻ dependence in HTC cells is uncertain, but such a mechanism could coordinate the concerted opening of K⁺ and Cl⁻ channels.

The onset of K⁺ and Cl⁻ current activation occurred within 3–5 min after TNF exposure. These observations implied that channel opening was mediated by an intracellular signaling cascade. Although the precise details of this cascade have not been defined in this study, our data suggest that PKC activation is involved. Consistent with this interpretation, the increase in both K⁺ and Cl⁻ currents was blocked by (*a*) chelation of intracellular Ca²⁺ with EGTA, (*b*) hydrolysis of intracellular ATP with apyrase, and (*c*) two structurally unrelated PKC inhibitors with distinct mechanisms of action, chelerythrine and PKC inhibitory peptide. In contrast to findings in neutrophils (22), our observations in liver cells indicated that TNF did not increase [Ca²⁺], and that TNF-activated currents were not attenuated by inhibition of CaMKII. Thus, mechanisms that

couple TNF to channel activation appear to exhibit tissue specificity.

PKC has been identified as a mediator for several actions of TNF (30–34), but the PKC isoforms responsible for the K⁺ and Cl⁻ channel opening in HTC cells remain to be determined. Clues to the types of PKC isoforms involved come from a distinct experimental model of liver cell death, in which the bile acid glycochenodeoxycholic acid produces apoptosis in a PKC-dependent fashion. In this model, glycochenodeoxycholic acid elicits membrane translocation of PKC- α , PKC- δ , and PKC- ϵ (26). This raises the possibility that one (or more) of these PKC isoforms may be relevant to the actions of TNF described in the present study. In HTC cells, PKC- α appears to mediate the opening of K⁺ channels in response to oxidants and Cl⁻ channels in response to swelling (27, 28). It is thus conceivable that PKC- α may also couple TNF to K⁺ and Cl⁻ channel activation. This issue is worthy of experimental pursuit.

Our findings regarding TNF and its effects on cell death mirror observations made by others in model liver cell systems (11, 12). In particular, TNF-mediated cell death required the addition of the transcriptional inhibitor actinomycin D. This implies that TNF activates parallel transcription-dependent pathways (which appear to involve the transcription factor NF- κ B, *cf.* Ref. 11), which serve to prevent cell death. In our hands, liver cell death appeared to occur through both apoptosis and necrosis, given that TNF increased trypan blue uptake (characteristic of necrotic cell death) and the extent of nuclear condensation and/or fragmentation (characteristic of apoptosis) with similar kinetics. Of note, TNF-mediated K⁺ and Cl⁻ channel activation (within minutes) and cessation of channel activation (by 1 h) occurred much earlier than the onset of cell death (within 4 h). This suggests that these channels occupy early positions in TNF-mediated signaling pathways. Furthermore, our findings raise the possibility that one of these pathways could lead to cell death. Consistent with this interpretation, we have shown that K⁺ and Cl⁻ channel blockers delay the progression to TNF-mediated liver cell death. With this in mind, K⁺ and Cl⁻ channels may represent attractive therapeutic targets to attenuate liver injury from TNF.

It should be emphasized that TNF alone was sufficient to evoke K⁺ and Cl⁻ channel opening in HTC cells, but cell death required the addition of actinomycin D. This implies that signaling cascades enabled by K⁺ and Cl⁻ channel opening would not overcome cytoprotective pathways disabled by actinomycin D (see above). In the presence of actinomycin D, K⁺ and Cl⁻ channel blockade reduced cell death for up to 8 h after TNF exposure, a time in which near maximal cell death had occurred in the absence of channel blockade. However, K⁺ and Cl⁻ channel blockade did not ultimately prevent TNF-mediated cell death. A possible interpretation is that K⁺ and Cl⁻ channels participate in the early phases of TNF-mediated signaling pathways that lead to cell death but that later onset, channel-independent pathways are also involved in the death response to TNF. Precedent for this concept exists in biphasic activation of Jun and p38 kinases by TNF, the early phase of which is anti-apoptotic and the later phase of which appears to be linked to apoptosis (35). Similarly, in selected instances, the anti-apoptotic protein Bcl-2 may only delay cell death (36, 37), suggesting the existence of parallel pathways to cell death that exhibit distinct temporal characteristics.

Although our data support a role for K⁺ and Cl⁻ channels in TNF-mediated liver cell death, two additional caveats apply. First, Ba²⁺, quinine, NPPB, and DPC, each of which delayed cell death induced by TNF, may have had effects other than blockade of K⁺ or Cl⁻ channels. The use of structurally dissimilar agents renders this less likely, but the possibility of non-

specific effects cannot be discounted. Second, the observations reported here may not apply to all mammalian tissues. In particular, in astrocytes, TNF has been shown to inhibit rather than activate K^+ currents (34). Thus, the results reported in the present study must be extrapolated with care.

An important question is how activation of K^+ and Cl^- channels contributes to downstream effects of TNF. One intriguing possibility is that K^+ and Cl^- efflux through conductive pathways leads to liver cell shrinkage. Although speculative, this could have at least two consequences. First, cell shrinkage itself can lead to apoptosis (38–41). A second consequence could be reduction of bile formation, achieved through volume-sensitive inhibition of insertion of organic anion transporters into the apical membrane (42, 43). This too is consistent with effects of TNF, which reduces both bile flow as well as the abundance of plasma membrane organic anion transporters in hepatocytes (10, 16). These areas are worthy of further study and could lead to new insights with respect to TNF action.

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