Nonselective Cation Channels as Effectors of Free Radical–Induced Rat Liver Cell Necrosis

LUIS FELIPE BARROS,[1,2] ANDRÉS STUTZIN,[1] ANDREA CALIXTO,[1] MARCELO CATALÁN,[1] JOEL CASTRO,[2] CLAUDIO HETZ,[1] AND TAMARA HERMOSILLA[2]

Necrosis, as opposed to apoptosis, is recognized as a nonspecific cell death that induces tissue inflammation and is preceded by cell edema. In non-neuronal cells, the latter has been explained by defective outward pumping of Na^+ caused by metabolic depletion or by increased Na^+ influx via membrane transporters. Here we describe a novel mechanism of swelling and necrosis; namely the influx of Na^+ through oxidative stress-activated nonselective cation channels. Exposure of liver epithelial Clone 9 cells to the free-radical donors calphostin C or menadione induced the rapid activation of an approximately 16-pS nonselective cation channel (NSCC). Blockage of this conductance with flufenamic acid protected the cells against swelling, calcium overload, and necrosis. Protection was also achieved by Gd^{3+}, an inhibitor of stretch-activated cation channels, or by isosmotic replacement of extracellular Na^+ with *N*-methyl-D-glucamine. It is proposed that NSCCs, which are ubiquitous although largely inactive in healthy cells, become activated under severe oxidative stress. The ensuing influx of Na^+ initiates a positive feedback of metabolic and electrolytic disturbances leading cells to their necrotic demise. (HEPATOLOGY 2001;33:114-122.)

Necrosis is regarded as a nonspecific, accidental type of cell death. Usually observed under pathologic conditions such as hypoxia and oxidative stress, it is characterized by the net gain of Na^+ and water. Eventually, the plasma membrane is broken, the swollen cells release their contents, and tissue inflammation is triggered.[1-3] This is in marked contrast to apoptosis, a nonlytic death preceded by cell shrinking and net K^+ loss through specific membrane channels.[4-6] Necrotic swelling, like apoptotic shrinking, is not just an epiphenomenon but an absolute requirement for cell death.[7-9]

Because Na^+ is the major extracellular osmolyte, necrotic swelling must involve Na^+ overload. In most cell types, this accumulation is generally regarded as passive, *i.e.*, not requiring the activation of specific effectors but due to defective outward Na^+ pumping in low adenosine triphosphate (ATP) conditions.[2,3,10-12] In contrast, neurons can swell and die as a result of an active event called excitotoxicity,[6] which involves the exogenous activation of nonselective cation channels such as the *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) subtypes of glutamate receptors. The resulting Na^+ influx overcomes the extrusion capacity causing net Na^+ gain, swelling, and necrosis.[13] Na^+ influx may also be a major factor for necrosis in non-neuronal cells. For instance, hepatocytes exposed to the free-radical donor menadione swelled up and died much faster than those whose Na^+ pump was inhibited with ouabain.[14] The Na^+/H^+ exchanger and the Na^+/HCO_3^- cotransporter were identified as major pathways of sodium influx in that cell system.[15]

Looking for specific mechanisms for Na^+ overload in non-neuronal cells, we focused on nonselective cation channels (NSCCs). These are ubiquitous ion channels, which are found dormant in healthy cells but can be activated by intracellular signals such as high Ca^{2+}, low ATP, free radicals, and membrane stretching. These channels have been described in liver cells[16-20] and in virtually every other tissue or cell lines studied.[21-26] Our results suggest that on exposure to free radicals, liver cells activate their NSCCs. These channels, which are inhibited by flufenamic acid, cause sodium and calcium overload and eventually lead the cells to their necrotic death.

MATERIALS AND METHODS

Cell Culture and Viability Assays. Clone 9 cells, epithelial cells originally derived from normal rat liver, were obtained from the American Tissue Culture Collection (ATCC; Rockville, MD). Briefly, cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphothericin B at 37°C in an atmosphere of 5% CO_2-95% air. Cells were passaged once a week and used between passage number 12 and 25. These cells were chosen as a model to ensure reproducibility by avoiding the rapid changes in biological properties that are inherent to hepatocytes in primary cultures (discussed by Schlenker et al.[27]). Also, as Clone 9 cells are not transformed, they were deemed more likely to maintain properties of the original tissue as compared with transformed cell lines. Cells were exposed to experimental conditions in Krebs-Ringer-HEPES buffer (136 mmol/L NaCl, 20 mmol/L HEPES, 4.7 mmol/L KCl, 1.25 mmol/L $MgSO_4$, 1.25 mmol/L $CaCl_2$, pH 7.4) supplemented with 25 mmol/L glucose (KRH-glc). In selected experiments, Na^+ was re-

Abbreviations: ATP, adenosine triphosphate; NSCC, nonselective cation channel; KRH-glc, Krebs-Ringer-HEPES-glucose; EGTA, ethyleneglycoltetraacetic acid; LDH, lactate dehydrogenase; TUNEL, TdT-mediated dUTP nick-end labeling; FITC, fluorescein isothiocyanate; PKC, protein kinase C; PI, propidium iodide.

From the [1]Instituto de Ciencias Biomédicas Facultad de Medicina, Universidad de Chile, Santiago, Chile; and [2]Centro de Estudios Científicos (CECS), Casilla, Valdivia, Chile.

Received February 2, 2000; accepted October 2, 2000.

Supported by Fondecyts 1990782 and 1980718. Institutional support to the Centro de Estudios Científicos (CECS) was from Fuerza Aérea de Chile, I. Municipalidad de Las Condes, and a group of Chilean private companies (AFP Provida, CODELCO, Empresas CMPC, Telefónica del Sur y Masisa S.A.) is also acknowledged. Support was also obtained through the International Program of the Howard Hughes Medical Institute and Cátedra Presidencial en Ciencias (to Francisco V. Sepúlveda). CECS is a Millennium Science Institute.

Address reprint requests to: Luis Felipe Barros, M.D., Ph.D., CECS, Casilla 1469, Valdivia, Chile. E-mail: fbarros@cecs.cl; fax: (56) 63 234515.

0270-9139/01/3301-0016$3.00/0
doi:10.1053/jhep.2001.20530

placed equimolarly by *N*-methyl-D-glucamine, whereas in others Ca^{2+} was chelated with ethyleneglycoltetraacetic acid (EGTA). To assess viability, cells in 35-mm dishes were exposed to 0.2 % trypan blue in KRH-glc for 2 minutes at room temperature. Microscope fields showing approximately 1,000 cells were recorded using a video camera and quantified for blue-stained cells. Lactate dehydrogenase (LDH) activity in cell supernatants was determined by a colorimetric endpoint kit according to the manufacturer's instructions (Sigma, St. Louis, MO). A calibration curve was produced to ensure linearity in the range was studied. Results are expressed as the percentage of maximum release, measured in the presence of 100 μmol/L digitonin. DNA fragmentation was detected by TdT-mediated dUTP nick-end labeling (TUNEL) assay as indicated by the manufacturer (Promega, Madison, WI). Externalization of phosphatidylserine was assessed by annexin V-fluorescein isothiocyanate (FITC) binding using both epifluorescence microscopy and flow cytometry, as suggested by the kit's manufacturer (Roche, Mannheim, Germany). For flow cytometry, cells were subjected to experimental conditions and then harvested by trypsinization (0.025 mg/mL). A total of 10,000 cells per sample were analyzed using a FACScan (Becton Dickinson, Mountain View, CA) with the Cell Quest software.

Electrophysiology. Currents were measured from isolated Clone 9 cells at room temperature by patch-clamp techniques with an EPC-7 amplifier (List Medical, Darmstadt, Germany) as described elsewhere.[21,28] The pipette solution contained (in mmol/L): Na^+ (or K^+) gluconate 140, $MgCl_2$ 1.3, $CaCl_2$ 2.6, KCl 5, HEPES 10, pH 7.4 adjusted with Tris. The bath solution contained (in mmol/L): Na^+ (or K^+) gluconate 40, $MgCl_2$ 1.3, $CaCl_2$ 2.6, KCl 5, HEPES 10, sucrose 100, glucose 5.6, pH 7.4 adjusted with Tris (E_{rev} cations = 29 mV). The tonicity was adjusted at 300 mOsm per kg H_2O, measured by freezing-point depression (Advanced Instruments, Norwood, MA). The signal was low-pass filtered at 0.2 kHz (−3 dB) and digitized at 2 kHz. Acquisition, analysis, and fitting were done with the pClamp 6.0 software suite (Axon Instruments, Inc., Foster City, CA). For analysis of P_0, patches were held at the desired potential for at least 3 minutes. For records containing more than one amplitude level, the P_0 was calculated as described previously.[21]

Calcium and Cell Volume Estimations. To estimate $[Ca^{2+}]_i$, cells grown on glass coverslips (N°1) were loaded for 20 minutes (room temperature) with 5 μmol/L Fluo-3 in its acetoxymethyl ester form (Fluo-3 AM) in KRH-glc containing 0.02% pluronic acid. Fluorescence was imaged with an LSM 410 Zeiss confocal microscope as described previously.[29] Background noise was measured in segments of the field devoid of cells and found to be not significantly different from the signal recorded in dye-depleted cells (100 μmol/L digitonin). This value was subtracted from cell measurements. To compare values from different cells, data were standardized by assigning baseline fluorescence (F_0) the value of 1. This method to estimate $[Ca^{2+}]_i$ in Clone 9 cells was validated using a Mn^{2+} quenching protocol.[30,31] Cell height at room temperature was estimated from 3D images of calcein-loaded cells using confocal microscopy.[29]

RESULTS

To explore the possible role of NSCCs in cell death, Clone 9 rat liver epithelial cells were first subjected to oxidative cell death. When exposed to calphostin C, a free-radical donor, the cells rapidly swelled, increasing their average height (μm) from 6.4 ± 0.1[72 cells] to 15 ± 0.6[72 cells]. Most cells swelled up as domes, with no apparent variation in cross-sectional areas immediately above the substrate, which would suggest a 2-3 fold increase in average cell volume. As depicted in Fig. 1A, swelling was heterogeneous, with some cells reaching a height of over 20 μm. These cells became quasispherical and started to detach from the substrate as evidenced by the formation of gaps in the monolayer (small arrow in Fig. 1A). Cell swelling was associated with a large increase in cytosolic calcium $[Ca^{2+}]_i$ (Fig. 1B) that reached a plateau of 12.4 ± 3[8]-fold over basal levels for calphostin C and 11.1 ± 3[7]-fold for menadione, a different free-radical donor. Because the K_d of Fluo-3 for Ca^{2+} lies in the region of 300 nmol/L, it can be concluded that the exposure to the oxidants increased $[Ca^{2+}]_i$ to levels at least into the micromolar range.

Volume and calcium increases were followed by cell lysis, which was quantified using trypan blue uptake and LDH release. Fig. 1C shows that there was a good correlation between the two methods, with the better sensitivity of trypan blue uptake that is to be expected from the much lower molecular weight of the dye as compared with LDH. The effect of oxidative damage on cell viability was time and dose dependent (Fig. 1D). As a reference, from the pooled data of two experiments the concentration of calphostin C capable of inducing a 50% decrease in cell viability after 4 hours incubation (LD_{50}) was estimated at 48 ± 10 nmol/L (Fig. 1D). Both calphostin C and menadione have been shown to induce dose-dependent necrosis and/or apoptosis in a number of cell lines and organs, including the liver.[14,32-34] Even though calphostin C is used routinely to inhibit protein kinase C (PKC), an enzyme that has been shown to modulate NSCCs in liver cells,[35] it seems unlikely that the effects of calphostin C under our experimental conditions were significantly related to PKC inhibition. First menadione, a free-radical donor that is not known to affect PKC, was toxic for Clone 9 (Fig. 1). Second, 2-hour exposure of Clone 9 cells to the protein kinase C inhibitor chelerythrine (1 μmol/L) did not affect cell viability (1 ± 2[3]% and −0.87 ± 1.0[3]% of cell death as measured by trypan blue uptake and LDH release, respectively). Third, LDH release by 2-hour exposure to menadione (25 ± 3[3]%) was not significantly affected in the presence of chelerythrine (29 ± 6[3]%; $P = .27$ in Student's t test). In addition, dissociation between PKC inhibition and toxicity by calphostin C has been reported previously.[34] The character, degree, and time course of Clone 9 cell parameters during free radical–induced cell death were similar to those of hepatocytes exposed to chemical hypoxia.[36]

To address the role of NSCCs in oxidative cell necrosis, we first confirmed their presence in Clone 9 cells. In high Ca^{2+} and in the absence of permeant anions, excised inside-out patches revealed multiple level single-channel currents with a slope conductance of approximately 16 pS and linear current-voltage relationship between −60 and 60 mV, similar to that previously described.[21,37] The channel was found to discriminate poorly between monovalent cations with a selectivity sequence $K^+ \approx Rb \geq Na^+ > Cs^+ > Ca^{2+}$, and it was fully and reversibly blocked by 100 μmol/L flufenamic acid, an inhibitor of Ca^{2+}-activated NSCCs[38,39] (Fig. 2A-C) but not by 20 μmol/L Gd^{3+}, an inhibitor of the stretch-activated NSCC[40,41] (not shown). We next explored the effect of free radicals on the activity of the channel *in situ*, by recording its activity in the cell-attached configuration. As shown in Fig. 2D to E, exposure to calphostin C rapidly (<3 minutes) increased the open probability of the channel. Exposure to menadione 100 μmol/L also increased the open probability of a cationic channel of similar amplitude to that observed in the presence of calphostin C (Fig. 2G-H). The channel, as activated either by calphostin C or menadione, was inhibited by flufenamic acid (Fig. 2) but not by 20 μmol/L Gd^{3+} (not shown). Chelerythrine 1 μmol/L was without effect on silent patches wherein the NSCC could later be activated by calphostin C (Fig. 2J-L),

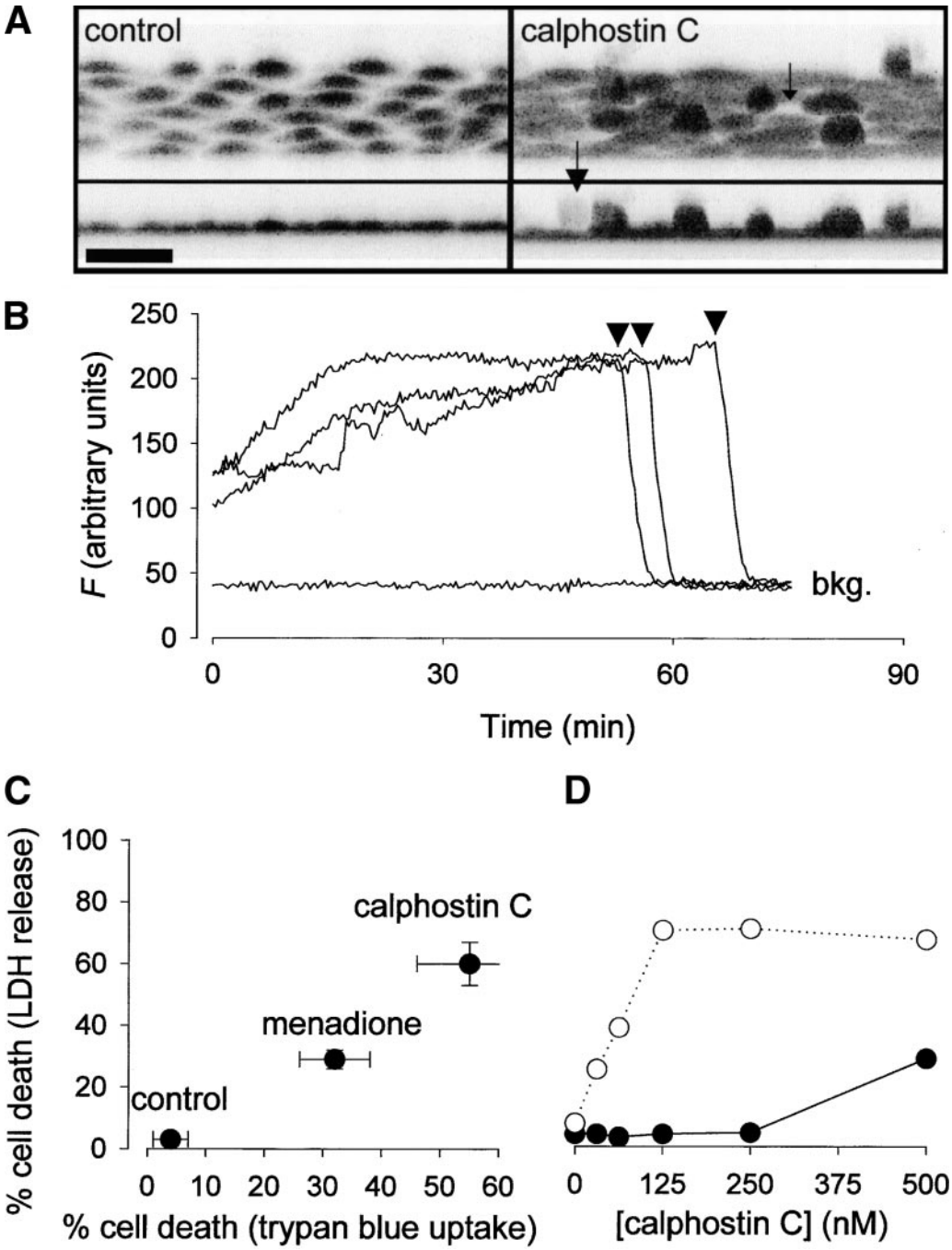

FIG. 1. Swelling, calcium overload, and necrosis of Clone 9 cells exposed to oxidative stress. (A) Cells in confluent monolayers were exposed to 500 nmol/L calphostin C or vehicle for 60 minutes and then loaded with calcein. *Upper panels*, 10° angle; *lower panels*, 0° angle. *Bar* = 50 μm. (B) Fluo-3–loaded cells were exposed to 500 nmol/L calphostin C for 60 minutes prior to fluorescence measurements. Data are from 3 single cells and are representative of 14 separate experiments. (C) Cell death was estimated after exposure to 500 nmol/L calphostin C or 100 μmol/L menadione for 2 hours (trypan blue uptake) or 4 hours (LDH release). Data are mean ± SEM.[4-5] (D) Cell death was estimated by LDH release after exposure to increasing concentrations of calphostin C for 4 hours (*open symbols*) or 2 hours (*closed symbols*). Means of 2 experiments performed in duplicate. *Large arrows* in A and B indicate cell lysis events. The *small arrow* in A points to a gap in the monolayer.

strongly suggesting that PKC inhibition does not mediate the activation of the channel.

NSCC blockers significantly inhibited oxidative necrosis in Clone 9 cells (Fig. 3A). Indomethacin failed to protect, which suggests that the effect of flufenamic acid was unrelated to its ability to inhibit cyclooxygenase. Indomethacin showed no effect on the fenamate-sensitive NSCC found in Clone 9 cells (not shown), a result that is consistent with previous observations.[38] The potency of the fenamate to inhibit cell death (IC_{50} = 42 ± 5 μmol/L; Fig. 3B) was similar to that of its inhibition of NSCCs in fibroblasts (IC_{50} = 38 μmol/L) and rat exocrine pancreas (IC_{50}= 50 μmol/L).[38,39] Gd^{3+} has been shown to have no short-term effect (<3 hours) on free-radical production by liver cells exposed to oxidants.[42,43] As predicted from the selectivity of this channel, removal of Na^+ from the bathing medium mimicked the protective effect of the NSCC blockers (Fig. 3C). This result helps rule out a putative role of other known targets of these inhibitors such as chloride channels and voltage-gated calcium channels. Necrosis induced by 100 μmol/L menadione was also sensitive to NSCC inhibition. As assessed by LDH release, menadione-induced cell necrosis was inhibited by 59 ± 2[3]% in the presence of 100 μmol/L flufenamic acid, by 53 ± 1[3]% in the presence of 20 μmol/L Gd^{3+} and by 90 ± 8[2]% in the absence of Na^+. Indomethacin (100 μmol/L) not only failed to protect but actually increased menadione-induced cell death by 68 ± 28%[3]. In a previous study, menadione-induced death of hepatocytes had also been prevented by removal of extracellular Na^+.[15]

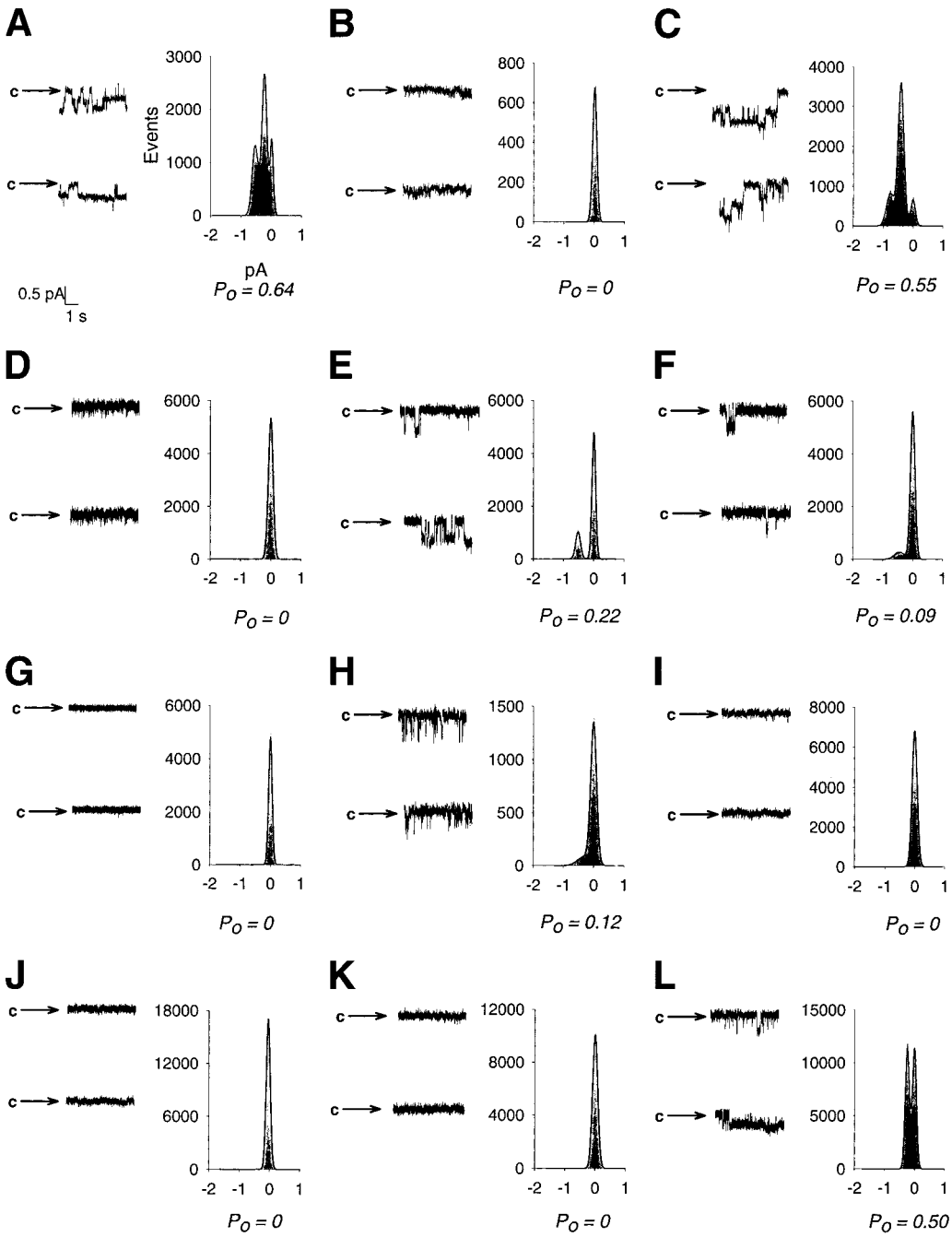

FIG. 2. NSCC activated by calphostin C and menadione and blocked by flufenamic acid. Representative current traces, the *arrows* indicate the zero-current (closed) level. The corresponding all-point amplitude histograms and gaussian fits are shown, indicating the number of active channels in the patch and the unitary current amplitude. (A) Control, excised inside-out patch, Vm −20 mV; (B) 3 minutes after 100 μmol/L flufenamic acid application to the bath; (C) 3 minutes after washout of flufenamic acid. Similar results were obtained in 3 separate experiments. (D) Cell-attached patch (pipette potential +20 mV relative to membrane potential) before the addition of calphostin C, (E) 3 minutes after addition of 500 nmol/L calphostin C to the bath solution, and (F) 3 minutes after addition of 100 μmol/L flufenamic acid to the bath solution. Similar results were obtained in 5 separate experiments. (G) Cell-attached patch (pipette potential +20 mV relative to membrane potential) before the addition of menadione, (H) 3 minutes after the addition of 100 μmol/L menadione to the bath solution, and (I) 3 minutes after the addition of 100 μmol/L flufenamic acid to the bath solution. Similar results were obtained in 3 separate experiments. (J) Control cell-attached patch (pipette potential +20 mV relative to membrane potential), (K) 5 minutes after addition of 1 μmol/L chelerythrine to the bath solution, and (L) 4 minutes after the addition of 500 nmol/L calphostin C to the bath solution. Similar results were obtained in 2 separate experiments.

Because high cytosolic calcium has been proposed as a final common condition for cell death, we investigated the role of NSCCs on the calcium rise elicited by free radical donors. Figure 4 shows that NSCC blockers were good inhibitors of the $[Ca^{2+}]_i$ rise elicited by calphostin C. Preincubation of cells with 100 μmol/L flufenamate or 20 μmol/L Gd^{3+} inhibited the rate of $[Ca^{2+}]_i$ increase elicited by calphostin C by 90 ± 8%[4] and 95 ± 6%[4], respectively (Fig. 4A). Both blockers were also effective at reducing $[Ca^{2+}]_i$ once it has reached fluorophore saturation levels (Fig. 4B), which indicates the participation of their targets during perpetuation of the Ca^{2+} overload. Previous reports, both *in vitro* and *in vivo*, have discarded possible artifactual interference of Gd^{3+} with Fluo-3 $[Ca^{2+}]_i$ measurements.[44] Chelation of extracellular calcium with EGTA failed to prevent the initial $[Ca^{2+}]_i$ rise induced by calphostin C (Fig. 5A), indicating that most of the calcium comes from intracellular stores. A similar observation was made in leukemia cell lines exposed to calphostin C[34] and also in an insulin-secreting cell line, where the early rise in Ca^{2+} induced by hydrogen peroxide was insensitive to extracellular Ca^{2+} removal.[22] This result in Clone 9 cells was somewhat unexpected as Ca^{2+} chelation effectively protected against cytotoxicity (Fig. 3C). However, $[Ca^{2+}]_i$ displayed a long-term tendency towards control values in EGTA-treated cells (Fig. 5B). This effect was not caused by dye bleaching, because a $[Ca^{2+}]_i$ rise could be re-elicited by exposing the cells to millimolar Ca^{2+} concentrations (Fig. 5B). Swelling, evidenced by an increase in cell height, was inhibited by pretreatment of cells with Gd^{3+} (94 ± 1[72]% inhibition), by flufenamic acid (91 ± 2[72]% inhibition), or by isosmotic

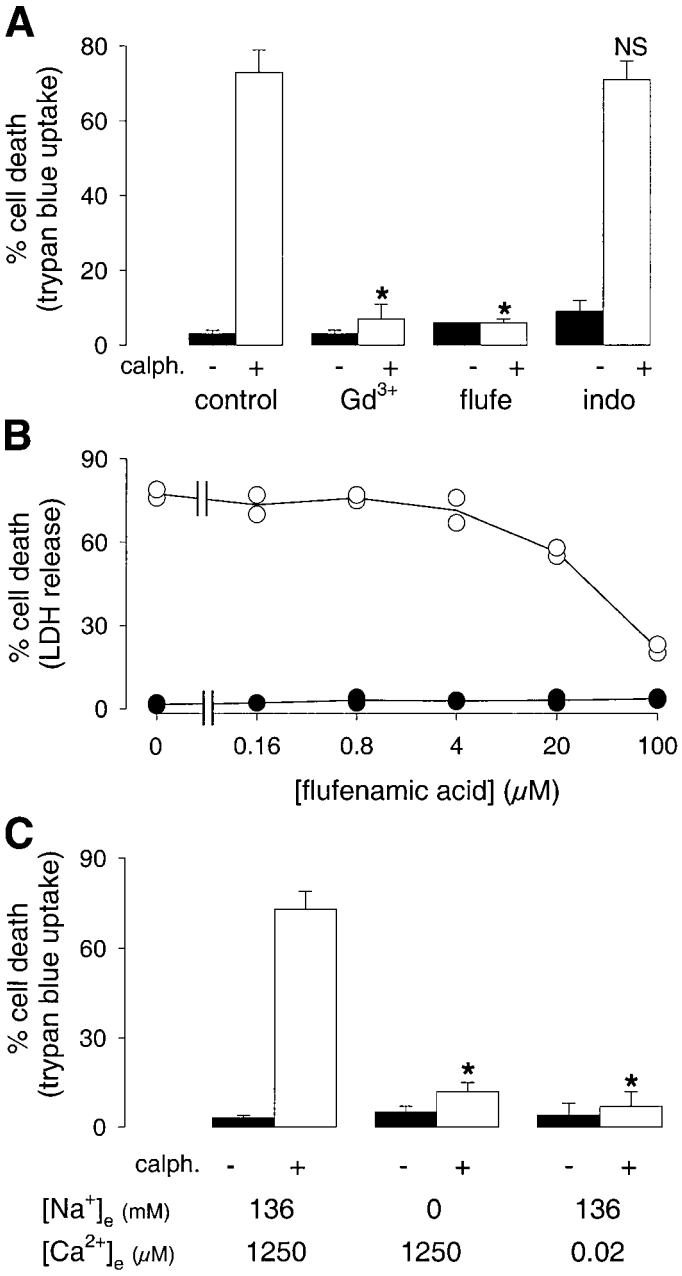

FIG. 3. Inhibition of cell death by NSCC blockers and extracellular sodium removal. (A) Cells were exposed for 2 hours to 20 μmol/L Gd^{3+}, 100 μmol/L flufenamate, or 100 μmol/L indomethacin in the presence (*open bars*) or absence (*solid bars*) of 500 nmol/L calphostin C. (B) Cells were incubated for 4 hours with flufenamic acid in the absence (*closed circles*) or presence (*open circles*) of 500 nmol/L calphostin C. (C) Cells were incubated for 2 hours in the absence (*solid bars*) or presence (*open bars*) of 500 nmol/L calphostin C, in KRH-glc or buffers containing low sodium ($[Na^+]_e = 0$) or low calcium ($[Ca^{2+}]_e = 0.02$ μmol/L). Data are mean ± SEM.[4-5] *$P < .05$ compared with control. NS, not significantly different from control by analysis of variance.

replacement of Na^+ with *N*-methyl-D-glucamine (88 ± 1[72]% inhibition).

As calphostin C has been shown to induce Ca^{2+}-dependent apoptosis,[34] we next searched for the presence of two phenomena associated with this type of cell death: phosphatidylserine externalization (by annexin V binding) and DNA fragmentation (by TUNEL analysis). After 4-hour exposure to calphostin C, most of the few cells that remained attached to the substrate were stained by both annexin V and propidium iodide (PI), suggestive of necrosis (Fig. 6). Consistently with the trypan blue and LDH results, coincubation with flufenamic acid or Gd^{3+} greatly diminished PI staining. However, NSCC blockers had no clear effect on annexin V staining (Fig. 6). When PI-negative cells were examined using FACScan analysis, it became clear that most cells that remained viable in the presence of calphostin C or menadione had become annexin V positive (Fig. 7A-B). In the case of menadione-treated cells, annexin V binding was partially inhibited by flufenamic acid and Gd^{3+} (not shown), evidenced as a leftward shift in the average cell staining intensity (Fig. 7E). As evidenced by TUNEL analysis, all cells exposed to calphostin C were undergoing DNA fragmentation (Fig. 6L), even

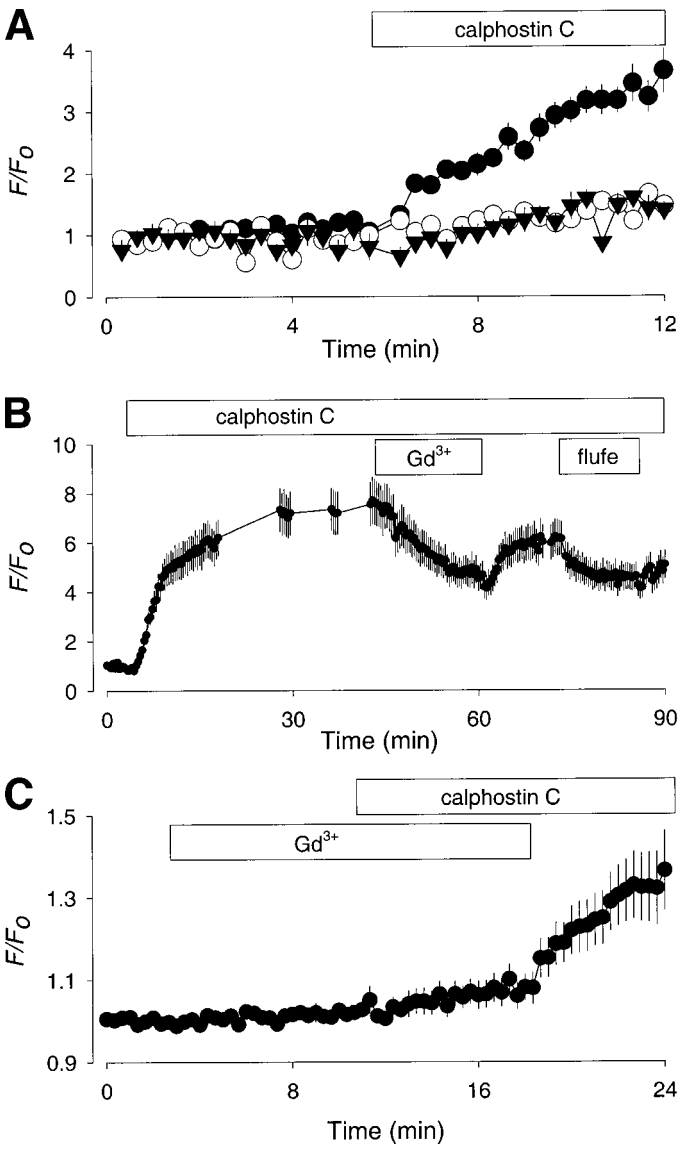

FIG. 4. Inhibition of calcium overload by NSCC blockers. (A) Fluo-3–loaded cells were exposed to 500 nmol/L calphostin C in buffer only (*solid circles*), 20 μmol/L Gd^{3+} (*open circles*), or 100 μmol/L flufenamic acid (*triangles*). (B) 20 μmol/L Gd^{3+} and 100 μmol/L flufenamic acid was added in the continuous presence of calphostin C. (C) Cells were exposed to 500 nmol/L calphostin C in the presence of 20 μmol/L Gd^{3+}. Cells were then washed and exposed to calphostin C only. Data are mean ± SEM [8].

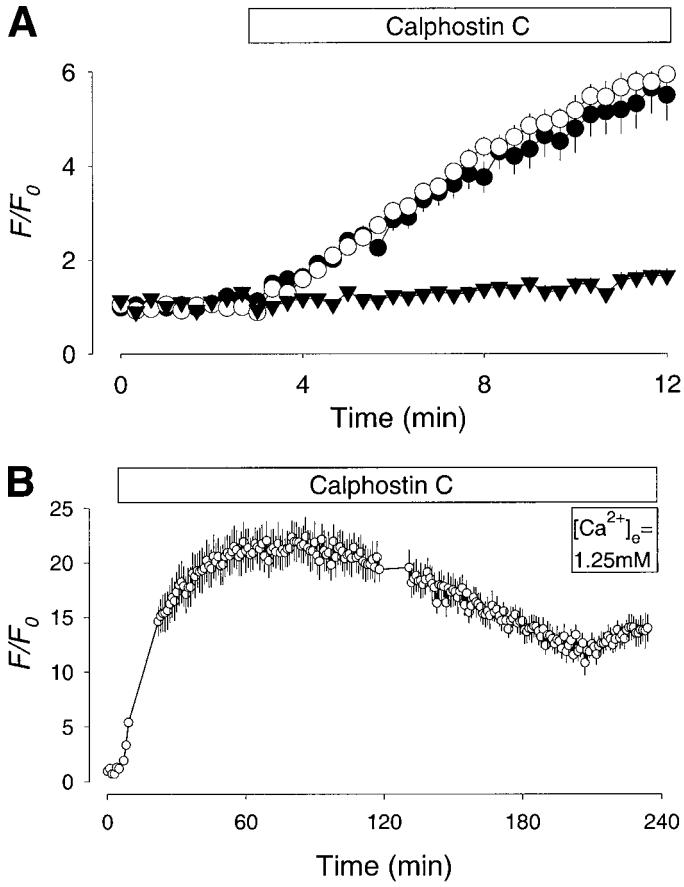

FIG. 5. Inhibition of calcium overload by extracellular sodium removal. (A) Fluo-3–loaded cells were exposed to 500 nmol/L calphostin C in buffer control (*solid circles*), 20 nmol/L free calcium buffer (*open circles*), or sodium-free buffer (*triangles*). Data are mean ± SEM [8]. (B) Cells were exposed to 500 nmol/L calphostin C in 20 nmol/L free calcium buffer. At the end of the experiment (t ≈ 210 minutes), the cells were bathed in high calcium buffer in the presence of calphostin C. Data are mean ± SEM [8].

though many were PI-negative (not shown). TUNEL staining was fully inhibited by NSCC blockage (Fig. 6). The more evident effect of NSCC blockers on TUNEL staining as compared with annexin V staining may relate to the late occurrence of DNA fragmentation as compared with phosphatidylserine exposure in the course of apoptosis.[45,46] In conclusion, both techniques suggest the occurrence of apoptotic events in Clone 9 cells exposed to free radicals. However, in the absence of complementary tests such as measurement of caspase activity, the data should be interpreted as only preliminary. If confirmed, they would mean that in Clone 9 cells, exposure to free radicals triggers both apoptosis and necrosis: in the presence of NSCC blockers, necrosis is blunted and apoptosis becomes evident. This scenario would be consistent with the prevailing notion that apoptosis often occurs in cells exposed to subnecrotic insults.

DISCUSSION

The present report presents 3 complementary lines of evidence that suggest that Na^+ influx through NSCCs mediates necrotic death in Clone 9 cells. (1) A flufenamate-sensitive NSCC was rapidly activated in response to oxidative stress. (2) Free radical–induced Ca^{2+} overload, cell swelling, and cell death were inhibited by NSCC blockers. (3) Free radical–induced Ca^{2+} overload, cell swelling, and cell death were inhibited by extracellular Na^+ removal. Our results are consistent with a recent study in HTC rat hepatoma cells.[27] In those cells, volume recovery from shrinking induced by free-radicals (H_2O_2 or D-alanine plus a D-amino acid oxidase) was associated with an approximately 100-fold increase in membrane cation permeability. The conductance, characterized as whole cell currents was ohmic and displayed similar selectivity for Na^+ and K^+, features that resemble the channel described in the present report. Interestingly, the same group observed NSCCs of approximately 18 and 28 pS in HTC cells[35] whereas hepatocytes displayed NSCCs of 16 and 30 pS.[16] It is tempting to speculate that the channel in Clone 9 cells corresponds to the lower conductance channel identified in hepatocytes and hepatoma cells. Because HTC cells exposed to free radicals did not show signs of damage, as assessed by PI exclusion and LDH release, it was suggested that activation of NSCC and the consequent regulatory volume increase response was homeostatic, at least in the time frame studied (<15 minutes). This was found to be consistent with a previous study in hepatocytes, which had shown that regulatory volume increase in response to an osmotic shock is mediated by the Na^+/H^+ exchanger, the $Na^+/K^+/2Cl^-$ cotransporter, and a conductive pathway, with the latter being predominant.[47] Notwithstanding obvious differences between the studies (*i.e.,* cell systems, free radical generating systems, time frame studied, electrophysiologic configurations), our results support the hypothesis advanced by Schlenker et al.[27] in that NSCC activation may be an initially protective mechanism that leads to cell damage in the longer term. Also, the differences between reports may relate to oxidant concentrations or cell sensitivity to free radicals. We favor the latter because the data suggest that Clone 9 cells are much more sensitive to calphostin C toxicity than several other cell lines, where the compound has been used routinely as a PKC inhibitor. On the other hand, transformed cell lines may be relatively resistant to oxidative stress as compared with their nontransformed counterparts.[48] It will be most informative to test these possibilities in both cell types under comparable experimental conditions.

The nature of the signal(s) linking free radical production and channel activation is a matter for speculation. A 28-pS NSCC has been shown to be directly activated by oxidized glutathione in endothelial cells exposed to free radical donors,[24] whereas a larger NSCC (70 pS), which can also be activated during oxidative stress by high $[Ca^{2+}]_i$, low reduced glutathione, and high NAD^+, has been described in the insulin-secreting cell line CRI-G1.[22] Moreover, in the HTC cell study discussed above, the effects of H_2O_2 were completely inhibited by dialysis of the cell interior with glutathione and were markedly enhanced by inhibition of glutathione oxidase.[27] ATP, a known modulator of NSCCs may also play a role in its activation by free radicals because its concentration has been shown to plummet in menadione-treated hepatocytes.[14] As ATP is essential for Na^+ and Ca^{2+} extrusion, and its depletion may be both cause and effect of NSCC activation, the link ATP-NSCC may constitute a positive feedback loop that leads cells to necrosis.

Ca^{2+} is a known activator of NSCC channels in many systems and thus a candidate to mediate their activation by oxidative stress. However, in Clone 9 cells the calcium rise was abolished when the NSCC channel was inhibited either phar-

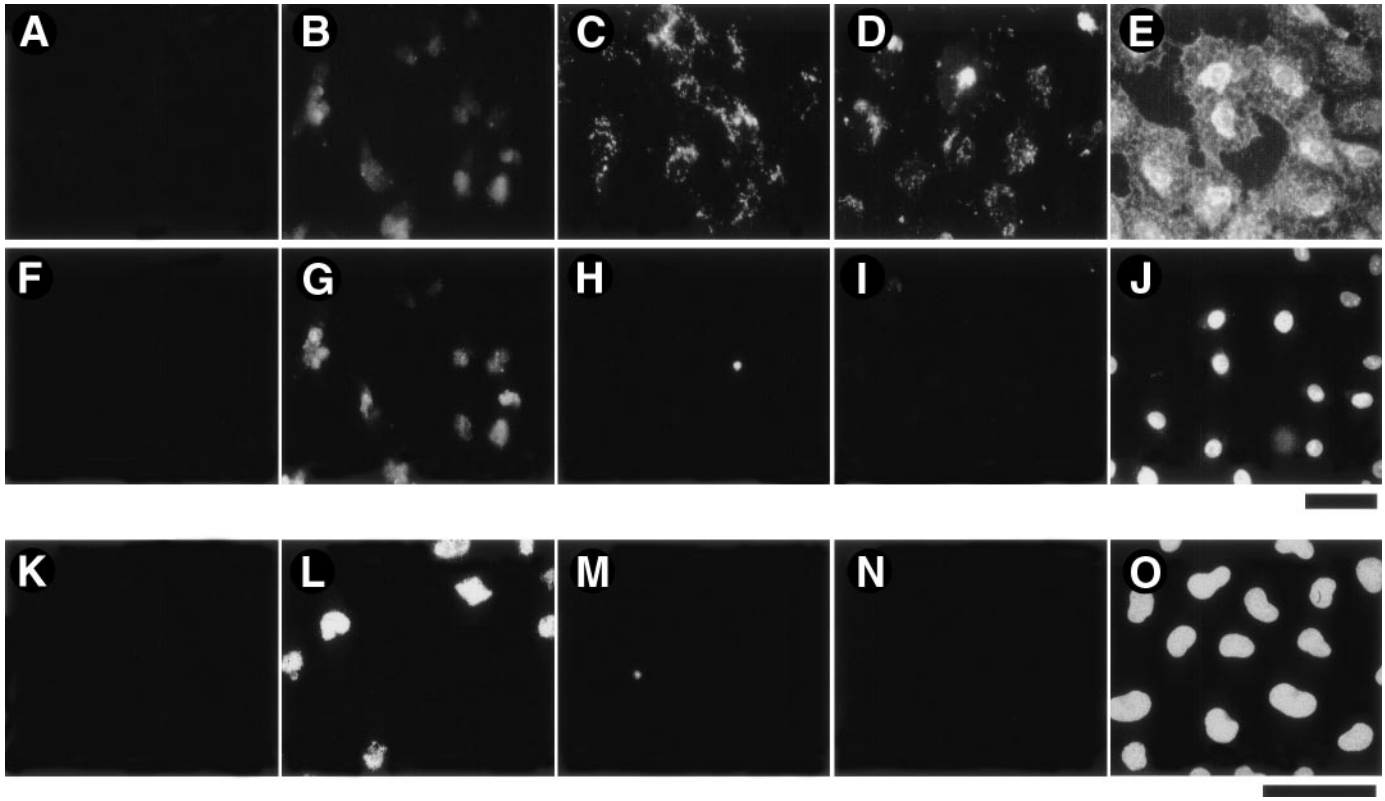

FIG. 6. Effects of NSCC blockers on phosphatidylserine externalization and DNA fragmentation. Epifluorescence microscopy. Cells were exposed to experimental conditions for 4 hours and then double-stained with annexin V-FITC (A-E) and PI (F-J). Other cells were subjected to TUNEL analysis (K-O). Conditions were as follows: controls (A, F, and K), 500 nmol/L calphostin C (B, G, and L), 500 nmol/L calphostin C plus 100 μmol/L flufenamic acid (C, H, and M), 500 nmol/L calphostin C plus 20 μmol/L Gd^{3+} (D, I, and N). E and J show positive controls permeabilized with 100 μmol/L digitonin. O depicts a positive control for the TUNEL assay obtained with DNAse I (1 μg/mL). Images are representative of at least 3 experiments performed in duplicate for each condition. All images show confluent monolayers (approximately 15 to 20 cells) except B, G, and L where fewer cells remained attached to the coverslip. Control experiments showed that Clone 9 cells exposed to flufenamic acid or Gd^{3+} in the absence of free-radical donors were negative for annexin V, PI, and TUNEL staining (not shown). Bars are 50 μm.

macologically (flufenamic acid) or functionally (Na^+ depletion). This suggests that the calcium rise is not a requirement but a consequence of channel activity. Consistently, in HTC cells, whole cell cationic currents could still be elicited in the presence of only 100 nmol/L Ca^{2+} in the pipette.[27] As shown by its insensitivity to extracellular calcium chelation, the source of the calcium rise must be intracellular (*e.g.*, endoplasmic reticulum). As this release required extracellular Na^+, it seems unlikely that nonspecific damage of the stores by free radicals is the actual mechanism at work. In the light of studies in neurons,[13,49] two possible mechanisms by which a large Na^+ overload might cause or potentiate a $[Ca^{2+}]_i$ rise are (1) direct inhibition or reversal of the Na^+/Ca^{2+} exchanger by high cytosolic sodium, and (2) calcium pump inhibition by ATP depletion. We are currently investigating the contribution of these transport systems in our model of necrosis.

Ascertaining the significance of the gadolinium experiments will require further work. Used at low micromolar concentrations, the lanthanide was shown to be as good an inhibitor as flufenamic acid at inhibiting cell swelling, calcium increase, and cell necrosis. As the channel identified by patch-clamp was not inhibited by Gd^{3+}, it is then likely that a second NSCC plays a role in oxidative cell necrosis. However, as the presence of such channel was not shown in Clone 9 cells, and Gd^{3+} may affect other conductances such as calcium channels, the hypothesis of a role for stretch-activated NSCC in cell necrosis must remain speculative. Incidentally, Gd^{3+} is routinely used to protect liver cells from oxidative damage, an effect usually ascribed to its ability to block and destroy Kupffer cells. Our results suggest the possibility of an alternative mechanism, which is supported by the observation that Gd^{3+} can protect isolated hepatocytes from CCl_4 toxicity.[50] Moreover, the lanthanide was also protective in the brain, an organ devoid of Kupffer cells.[51]

Edema and necrosis-like cell death have been shown to result from abnormal constitutive activation of NSCCs (degenerins) in *Caenorhabditis elegans*.[52] As Ca^{2+} does not permeate through these channels, unchecked Na^+ influx may well be the main pathogenic mechanism at work. Taking together the data from nematodes and mammalian systems, a model may be proposed in which these channels, ubiquitous though largely inactive in healthy cells, are activated under oxidative conditions. Although possibly homeostatic under certain conditions, the influx of Na^+ through these channels would eventually cause generalized metabolic derangement, leading cells to their lysis. These results might relate to cell protective effects of fenamates that are not fully accounted for by their ability to inhibit prostanoid synthesis.[53]

Acknowledgment: The authors thank Martin Raff and Francisco V. Sepúlveda for helpful advice.

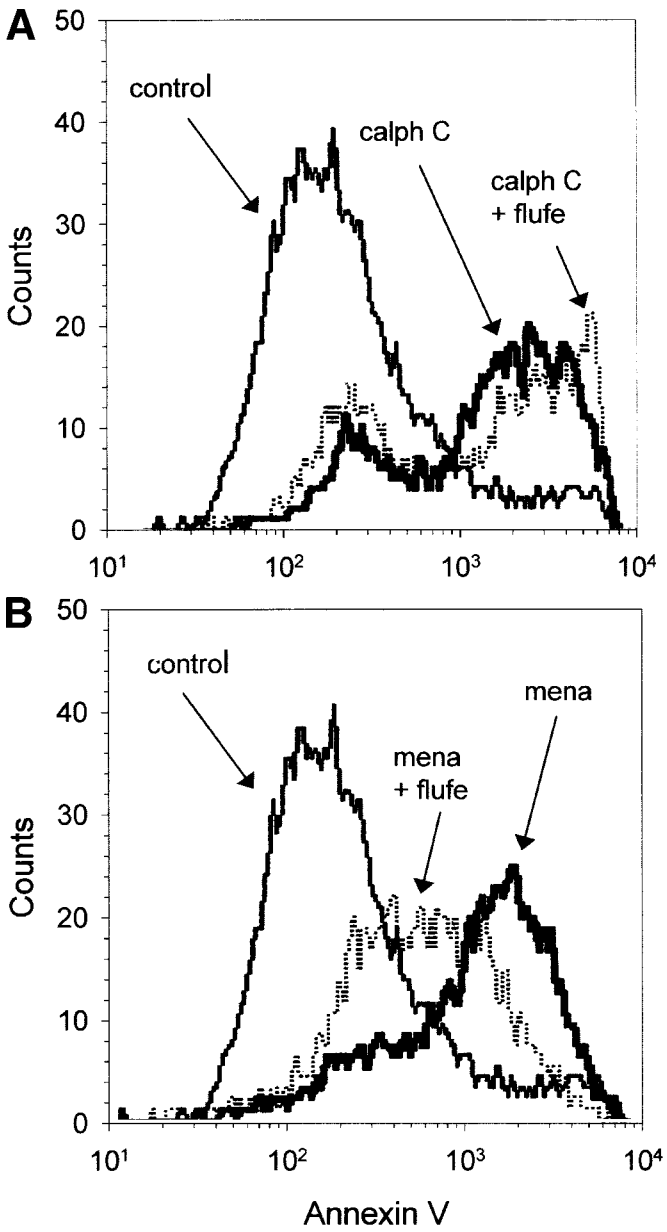

FIG. 7. Effect of flufenamic acid on phosphatydilserine externalization. FACS analysis. After exposure to experimental conditions for 4 hours, those cells that remained attached to the substrate were harvested by trypsinization, double-stained with annexin V-FITC and PI, and then analyzed by flow cytometry. Only PI-negative cells (*i.e.* viable) are represented. Calphostin C (A) and menadione (B) were used at 500 nmol/L and 100 μmol/L, respectively. Flufenamic acid was used at 100 μmol/L (A and B).

REFERENCES

1. Thomson RK, Arthur MJ. Mechanisms of liver cell damage and repair. Eur J Gastroenterol Hepatol 1999;11:949-955.
2. Leist M, Nicotera P. The shape of cell death. Biochem Biophys Res Commun 1997;236:1-9.
3. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol 1995;146:3-15.
4. Yu SP, Yeh CH, Sensi SL, Gwag BJ, Canzoniero LM, Farhangrazi ZS, Ying HS, et al. Mediation of neuronal apoptosis by enhancement of outward potassium current. Science 1997;278:114-117.
5. Yu SP, Yeh C, Strasser U, Tian M, Choi DW. NMDA receptor-mediated K+ efflux and neuronal apoptosis. Science 1999;284:336-339.
6. Lee JM, Zipfel GJ, Choi DW. The changing landscape of ischaemic brain injury mechanisms. Nature 1999;399(Suppl):A7-A14.
7. Flores J, DiBona DR, Beck CH, Leaf A. The role of cell swelling in ischemic renal damage and the protective effect of hypertonic solute. J Clin Invest 1972;51:118-126.
8. Powell WJJ, DiBona DR, Flores J, Frega N, Leaf A. Effects of hyperosmotic mannitol in reducing ischemic cell swelling and minimizing myocardial necrosis. Circulation 1976;53(Suppl):I45-I49.
9. Powell WJJ, DiBona DR, Flores J, Leaf A. The protective effect of hyperosmotic mannitol in myocardial ischemia and necrosis. Circulation 1976;54:603-615.
10. Leaf A. Cell swelling. A factor in ischemic tissue injury. Circulation 1973;48:455-458.
11. Trump BF, Berezesky IK, Chang SH, Phelps PC. The pathways of cell death: oncosis, apoptosis, and necrosis. Toxicol Pathol 1997;25:82-88.
12. Cotran RS, Kumar V, Collins T, eds. Robbins Pathological Basis of Disease. 6 ed. Philadelphia: Saunders, 1999.
13. Itoh T, Itoh A, Horiuchi K, Pleasure D. AMPA receptor-mediated excitotoxicity in human NT2-N neurons results from loss of intracellular Ca2+ homeostasis following marked elevation of intracellular Na+. J Neurochem 1998;71:112-124.
14. Carini R, Autelli R, Bellomo G, Albano E. Alterations of cell volume regulation in the development of hepatocyte necrosis. Exp Cell Res 1999; 248:280-293.
15. Carini R, Bellomo G, Benedetti A, Fulceri R, Gamberucci A, Parola M, Dianzani MU, et al. Alteration of Na+ homeostasis as a critical step in the development of irreversible hepatocyte injury after adenosine triphosphate depletion. HEPATOLOGY 1995;21:1089-1098.
16. Lidofsky SD, Xie MH, Sostman A, Scharschmidt BF, Fitz JG. Vasopressin increases cytosolic sodium concentration in hepatocytes and activates calcium influx through cation-selective channels. J Biol Chem 1993;268: 14632-14636.
17. Lidofsky SD, Sostman A, Fitz JG. Regulation of cation-selective channels in liver cells. J Membr Biol 1997;157:231-236.
18. Chen WH, Yeh TH, Tsai MC, Chen DS, Wang TH. Characterization of Ca(2+)- and voltage-dependent nonselective cation channels in human HepG2 cells. J Formos Med Assoc 1997;96:503-510.
19. Fernando KC, Barritt GJ. Characterisation of the divalent cation channels of the hepatocyte plasma membrane receptor-activated Ca2+ inflow system using lanthanide ions. Biochim Biophys Acta 1995;1268:97-106.
20. Bear CE. A nonselective cation channel in rat liver cells is activated by membrane stretch. Am J Physiol 1990;258(Pt 1):C421-C428.
21. Eguiguren AL, Rios J, Riveros N, Sepúlveda FV, Stutzin A. Calcium-activated chloride currents and non-selective cation channels in a novel cystic fibrosis-derived human pancreatic duct cell line. Biochem Biophys Res Commun 1996;225:505-513.
22. Herson PS, Lee K, Pinnock RD, Hughes J, Ashford ML. Hydrogen peroxide induces intracellular calcium overload by activation of a non-selective cation channel in an insulin-secreting cell line. J Biol Chem 1999; 274:833-841.
23. Koivisto A, Klinge A, Nedergaard J, Siemen D. Regulation of the activity of 27 pS nonselective cation channels in excised membrane patches from rat brown-fat cells. Cell Physiol Biochem 1998;8:231-245.
24. Koliwad SK, Elliot SJ, Kunze DL. Oxidized glutathione mediates cation channel activation in calf vascular endothelial cells during oxidant stress. J Physiol (Lond) 1996;495:37-49.
25. Korbmacher C, Volk T, Segal AS, Boulpaep EL, Fromter E. A calcium-activated and nucleotide-sensitive nonselective cation channel in M-1 mouse cortical collecting duct cells. J Membr Biol 1995;146:29-45.
26. Orser BA, Bertlik M, Fedorko L, O'Brodovich H. Cation selective channel in fetal alveolar type II epithelium. Biochimica et Biophysica Acta 1991; 1094:19-26.
27. Schlenker T, Feranchak AP, Schwake L, Stremmel W, Roman RM, Fitz JG. Functional interactions between oxidative stress, membrane Na(+) permeability, and cell volume in rat hepatoma cells. Gastroenterology 2000;118:395-403.
28. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cell and cell-free membrane patches. Pflügers Arch 1981;391:85-100.
29. Barros LF. Measurement of sugar transport in single living cells. Pflugers Arch 1999;437:763-770.
30. Kao JP, Harootunian AT, Tsien RY. Photochemically generated cytosolic calcium pulses and their detection by Fluo-3. J Biol Chem 1989;264: 8179-8184.
31. Nadal A, Fuentes E, McNaughton PA. Albumin stimulates uptake of calcium into subcellular stores in rat cortical astrocytes. J Physiol (Lond) 1996;492:737-750.

32. Chen Q, Cederbaum AI. Menadione cytotoxicity to Hep G2 cells and protection by activation of nuclear factor-kappaB. Mol Pharmacol 1997; 52:648-657.
33. Sata N, Klonowski-Stumpe H, Han B, Haussinger D, Niederau C. Menadione induces both necrosis and apoptosis in rat pancreatic acinar AR4-2J cells. Free Radic Biol Med 1997;23:844-850.
34. Zhu DM, Narla RK, Fang WH, Chia NC, Uckun FM. Calphostin C triggers calcium-dependent apoptosis in human acute lymphoblastic leukemia cells. Clin Cancer Res 1998;4:2967-2976.
35. Fitz JG, Sostman AH, Middleton JP. Regulation of cation channels in liver cells by intracellular calcium and protein kinase C. Am J Physiol 1994; 266(Pt 1):G677-G684.
36. Zahrebelski G, Nieminen AL, al Ghoul K, Qian T, Herman B, Lemasters JJ. Progression of subcellular changes during chemical hypoxia to cultured rat hepatocytes: a laser scanning confocal microscopic study. HEPATOLOGY 1995;21:1361-1372.
37. Gray MA, Argent BE. Non-selective cation channel on pancreatic duct cells. Biochim Biophys Acta 1990;1029:33-42.
38. Gogelein H, Dahlem D, Englert HC, Lang HJ. Flufenamic acid, mefenamic acid and niflumic acid inhibit single nonselective cation channels in the rat exocrine pancreas. FEBS Lett 1990;268:79-82.
39. Weiser T, Wienrich M. Investigations on the mechanism of action of the antiproliferant and ion channel antagonist flufenamic acid. Naunyn Schmiedebergs Arch Pharmacol 1996;353:452-460.
40. Yang XC, Sachs F. Block of stretch-activated ion channels in Xenopus oocytes by gadolinium and calcium ions. Science 1989;243(Pt 1):1068-1071.
41. Caldwell RA, Clemo HF, Baumgarten CM. Using gadolinium to identify stretch-activated channels: technical considerations. Am J Physiol 1998; 275:C619-C621.
42. Nakano M, Kikuyama M, Hasegawa T, Ito T, Sakurai K, Hiraishi K, Hashimura E, et al. The first observation of O2- generation at real time in vivo from non- Kupffer sinusoidal cells in perfused rat liver during acute ethanol intoxication. FEBS Lett 1995;372:140-143.
43. Brass CA, Roberts TG. Hepatic free radical production after cold storage: Kupffer cell-dependent and -independent mechanisms in rats. Gastroenterology 1995;108:1167-1175.
44. Schlichter LC, Sakellaropoulos G. Intracellular Ca2+ signaling induced by osmotic shock in human T lymphocytes. Exp Cell Res 1994;215:211-222.
45. Chan A, Reiter R, Wiese S, Fertig G, Gold R. Plasma membrane phospholipid asymmetry precedes DNA fragmentation in different apoptotic cell models. Histochem Cell Biol 1998;110:553-558.
46. Clarke RG, Lund EK, Johnson IT, Pinder AC. Apoptosis can be detected in attached colonic adenocarcinoma HT29 cells using annexin V binding, but not by TUNEL assay or sub-G0 DNA content. Cytometry 2000;39: 141-150.
47. Wehner F, Tinel H. Role of Na+ conductance, Na(+)-H+ exchange, and Na(+)-K(+)-2Cl- symport in the regulatory volume increase of rat hepatocytes. J Physiol (Lond) 1998;506(Pt 1):127-142.
48. Bartoli GM, Piccioni E, Agostara G, Calviello G, Palozza P. Different mechanisms of tert-butyl hydroperoxide-induced lethal injury in normal and tumor thymocytes. Arch Biochem Biophys 1994;312:81-87.
49. Agrawal SK, Fehlings MG. Mechanisms of secondary injury to spinal cord axons in vitro: role of Na+, Na(+)-K(+)-ATPase, the Na(+)-H+ exchanger, and the Na(+)-Ca2+ exchanger. J Neurosci 1996;16:545-552.
50. Badger DA, Kuester RK, Sauer JM, Sipes IG. Gadolinium chloride reduces cytochrome P450: relevance to chemical- induced hepatotoxicity. Toxicology 1997;121:143-153.
51. Vaz R, Sarmento A, Borges N, Cruz C, Azevedo I. Effect of mechanogated membrane ion channel blockers on experimental traumatic brain oedema. Acta Neurochir (Wien) 1998;140:371-374.
52. Hall DH, Gu G, Garcia-Anoveros J, Gong L, Chalfie M, Driscoll M. Neuropathology of degenerative cell death in Caenorhabditis elegans. J Neurosci 1997;17:1033-1045.
53. Chen Q, Olney JW, Lukasiewicz PD, Almli T, Romano C. Fenamates protect neurons against ischemic and excitotoxic injury in chick embryo retina. Neurosci Lett 1998;242:163-166.