Cytosolic Ca²⁺ and Protein Kinase C α Couple Cellular Metabolism to Membrane K⁺ Permeability in a Human Biliary Cell Line

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Abstract

Cholangiocytes represent an important target of injury during the ischemia and metabolic stress that accompanies liver preservation. Since K⁺ efflux serves to minimize injury during ATP depletion in certain other cell types, the purpose of these studies was to evaluate the effects of ATP depletion on plasma membrane K⁺ permeability of Mz-ChA-1 cells, a model human biliary cell line. Cells were exposed to dinitrophenol (50 µM) and 2-deoxyglucose (10 mM) as the standard model of metabolic injury. Whole-cell and single K⁺ channel currents were measured using patch clamp techniques; and intracellular [Ca2+] ([Ca2+]i) was estimated by calcium green-1 fluorescence. Metabolic stress increased $[Ca^{2+}]_i$, and stimulated translocation of the α isoform of protein kinase C (PKCa) from cytosolic to particulate cell fractions. The same maneuver increased membrane K⁺ permeability 40–70-fold as detected by (a) activation of K^+ selective whole cell currents of 2,176 \pm 218 pA (n = 34), and (b) opening of a pamin-sensitive K^+ channels with a unitary conductance of 17.0 \pm 0.2 pS. PKC α translocation and channel opening appear to be related since stress-induced K⁺ efflux is inhibited by chelation of cytosolic Ca²⁺, exposure to the PKC inhibitor chelerythrine (25 µM) and downregulation of PKC by phorbol esters. Moreover, K⁺ currents were activated by intracellular perfusion with recombinant PKC α in the absence of metabolic inhibitors. These findings indicate that in biliary cells apamin-sensitive K⁺ channels are functionally coupled to cell metabolism and suggest that cytosolic Ca^{2+} and PKC α are selectively involved in the response. (J. Clin. Invest. 1997. 99:2890-2897.) Key words: ATP • cholangiocyte • ion channel • liver

Introduction

Intrahepatic bile ducts form an extensive network within the liver and contribute importantly to bile formation through absorption and secretion of fluid and electrolytes. While the cholangiocytes that form these ducts represent only 2–5% of the nuclear mass of the liver, they are metabolically active, and

J. Clin. Invest. © The American Society for Clinical Investigation, Inc. 0021-9738/97/06/2890/08 \$2.00 Volume 99, Number 12, June 1997, 2890–2897 ductular secretion accounts for up to 40% of bile volume in humans (1). Unlike hepatocytes, which extract most of their oxygen and nutrients from the portal venous system, intrahepatic bile ducts are perfused by branches of the hepatic artery containing oxygen-rich blood. Consequently, duct cells are more susceptible to injury during periods of ischemia and hypoxia. Preservation injury to intrahepatic ducts occurs in 11– 24% of patients receiving orthotopic liver transplantation and is a significant cause of increased morbidity and mortality (2, 3). Cold storage solutions are partially effective in prevention of injury (3), but longer storage times are associated with an increase in ischemic damage (4). Comparatively little is known regarding the adaptive responses of duct cells that might prolong cell survival and minimize later complications.

Many of the deleterious effects of ischemia result from hypoxia, which inhibits oxidative phosphorylation and depletes cellular ATP stores. Pharmacological agents including 2,4-DNP, which uncouples oxidative metabolism, and 2-deoxy-D-glucose (2-DG),¹ which impairs glycolysis, have been useful models of ATP depletion because in liver cells they produce biochemical changes similar to those caused by hypoxia (5, 6).

At the cellular level, one of the earliest effects of oxidative stress is dissipation of transmembrane cation gradients (7) and release of K^+ ions (8). In isolated liver, hypoxia stimulates an increase in the concentration of K^+ in the perfusate from 5.6 to 13.1 mM (9). K^+ efflux appears early, preceding release of aminotransferases, and correlates closely with the fall in cellular ATP levels (9). Net loss of K^+ ions is related in part to diminished Na⁺/K⁺ pump activity (6, 7). In addition, metabolic stress can stimulate release of K^+ through opening of ion channels in the plasma membrane of liver, vascular, and other cell types (10, 11). In cardiovascular tissues, channel opening represents an important adaptive response leading to membrane hyperpolarization, vasodilation, and restoration of blood flow (11).

Modulation of membrane K^+ permeability represents an important point for regulation of a broad range of biliary cell functions, including maintenance of the driving force for Cl⁻ secretion, electrogenic uptake of solutes, and regulation of cell volume (12, 13). These processes are impaired during ischemia, and little is known regarding the cellular response to ATP depletion or the channels and regulatory mechanisms involved. Consequently, the purpose of these studies of a model human biliary cell line was to evaluate the relationship between cellular metabolism and membrane K⁺ permeability and to characterize the K⁺ channels and signaling pathways involved as a guide for possible pharmacologic modification of the cellular response to injury.

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^{1.} *Abbreviations used in this paper:* 2-DG, 2-deoxy-D-glucose; PKC, protein kinase C.

Methods

Cell culture. All studies were performed in Mz-ChA-1 cells, a model biliary cell line derived from human cholangiocarcinoma (12, 14). These express cytokeratin 19 and γ -glutamyl transpeptidase as well as receptors and ion channels similar to primary cholangiocytes isolated from rat intrahepatic ducts (12, 15). Cells were passaged at weekly intervals and maintained in HCO₃⁻-containing CMRL-1066 media (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 µg/ml) as previously described (12).

Metabolic inhibition. Cells in culture were exposed to 2,4-dinitrophenol (DNP, 50 μ M) and 2-deoxyglucose (2-DG, 10 mM) as the standard model for metabolic inhibition, and cellular toxicity was assessed by propidium iodide fluorescence as previously described (10). Propidium iodide (50 μ g/ml) is thought to penetrate only damaged membranes to form intercalation complexes with double-stranded DNA with an amplification of nuclear fluorescence (excitation at 530 nm and an emission filter of 620 nm) (16). In subconfluent cell monolayers, the number of positively staining nuclei was measured at timed intervals in standard buffer and in buffer containing DNP/2-DG in the same concentrations used for the electrophysiologic studies.

Measurement of K⁺ *currents.* Membrane K⁺ *currents were measured using whole-cell and cell-attached patch clamp recording techniques (17) as previously described (18, 19). Cover slips containing cells were mounted in a chamber (volume ~ 400 \mul) and were perfused at 4–5 ml/min with a standard extracellular solution containing (mM): 140 NaCl, 4 KCl, 1 CaCl₂, 2 MgCl₂, 1 KH₂PO₄, 10 glucose, and 10 Hepes/NaOH (pH ~ 7.40). The standard intracellular (pipette) solution for whole cell recordings contained (mM): 130 KCl, 10 NaCl, 2 MgCl₂, 10 Hepes/KOH, 0.5 CaCl₂, and 1 EGTA (pH 7.3), corresponding to a free [Ca²⁺] of ~ 100 nM (20). Movement of positive charge out of the pipette is shown as an upward deflection of the current trace.*

In the whole-cell configuration, outward currents carried by K⁺ (I_K) were measured at a test potential of 0 mV to minimize the contribution of currents carried by Cl⁻ or cations (E_{Cl} and E_{cation} ~ 0 mV) (18). Current–voltage (I-V) relations were measured between –120 mV and +100 mV in 20 mV increments (400 ms duration, 2 s between test potentials). Currents were analyzed using pCLAMP version 5.5 programs (Axon Instruments, Burlingame, CA) as previously described (19, 21). Single channel currents were digitized at 5 kHz and filtered at 2 kHz for storage on a computer. The number of channels present in each patch was determined by the open levels on an all points amplitude histogram, and channel open probability (P_o) was measured during one or more 10-s sample periods using pCLAMP programs where currents > 0.5 of the fully open level are considered open.

Intracellular Ca²⁺ concentration. Changes in intracellular [Ca²⁺] were estimated using the fluorescent membrane-permeant probe calcium green-1. Cells were loaded in 5 µM calcium green-1 in the presence of 0.2% Pluronic F127 (Molecular Probes, Inc., Eugene, OR) for 30 min at room temperature, washed with the standard buffer to remove extracellular dye, and then placed on the stage of an inverted microscope (Carl Zeiss, Inc., Thornwood, NY). Studies were performed during continuous perfusion using a gravity-fed system at a flow rate of 10 ml/min. A multibarrelled pipette (Adams and List, Westbury, NY) was placed \sim 50 μ m from the cell for delivery of test reagents. Each barrel was controlled by a valve, with a solution flow rate of 6 µl/s and a dead space of 0.5-1.5 µl. This allowed delivery of test reagents to the cell within 80-250 ms depending on the dead space and distance from the cell. A 1% neutral density filter was used to attenuate the excitation light emitted by the mercury lamp of the microscope, and fluorescence was excited at 510 nm. Fluorescence was imaged using an IC-100 camera (Photon Technology, Santa Clara, CA) interfaced with a DT2867 image processor system. Data were expressed as a change in fluorescence percentage $(F-F_0)/F_0$ for each cell, where F refers to the peak fluorescence from a cell body after exposure to test reagents, and F_0 refers to basal fluorescence. If peak fluorescence saturated the dynamic range of the camera, a region within the same cell of less intense fluorescence was selected. Usually two cells were close enough to the perfusion system to be simultaneously analyzed by the image processing system.

Protein kinase C detection and translocation. Intact cells in subconfluent culture were washed twice with ice-cold phosphate-buffered saline, then lysed with boiling Laemmli sample buffer (250 µl/ 100-mm dish). 25 μ l of each sample containing \sim 100 μ g protein was separated on SDS-PAGE gradient gels (4-20%) (Novex Corp., San Diego, CA), then transferred to PVDF membranes by a semi-dry blot method (22). Membranes were preincubated in 130 mM NaCl, 10 mM Tris (pH 7.4), supplemented with 3% defatted milk, then with anti-PKCα rabbit antibody raised against peptide SYVNPQFVHPILQSAV (23) at 1:2,000 dilution for 2 h at ambient temperature. Membranes were washed four times in the same buffer without serum, then incubated for 30 min with goat anti-rabbit peroxidase conjugate (Sigma Chemical Co., St. Louis, MO) at 1:7,500 dilution. The membrane was washed thrice in buffer supplemented with milk, then thrice in unsupplemented buffer. Immunoreactive bands were visualized by an enhanced chemiluminescence technique (ECL; Amersham Corp., Arlington Heights, IL) on preflashed film. Typical exposures were for ~ 1 min.

For translocation assays, cells were washed twice with ice-cold PBS, then scraped into hypotonic lysis buffer consisting of 10 mM NaCl, 10 mM Hepes, 5 mM EDTA, 5 mM EGTA, and 5 μ l each of leupeptin, bacitracin, PMSF, and soybean trypsin inhibitor. Lysates were centrifuged at 500,000 g for 20 min. Cytosolic supernatants were harvested after centrifugation, concentrated with Centricon-10 concentrators (Amicon, Danvers, MA), assayed for protein content and mixed with 2× Laemmli sample buffer. Particulate fractions were resuspended in lysis buffer then centrifuged at 300 g for 30 min. The pellets were discarded, and the supernatant fraction was again centrifuged at 300 g for 30 min. The pellets were again discarded, and the supernatant centrifuged at 30,000 g for 30 min, then resuspended in lysis buffer, assayed for protein, and diluted with 2× Laemmli buffer. Cytosolic and membrane extracts were run side by side after normalization and immunoblotted as described above.

Purification of protein kinase $C\alpha$ from insect cells. Sf9 cells were infected with a baculovirus construct for the alpha isoform of protein kinase C (PKC) at a multiplicity of infection of 5-10 (24, 25). After 60-72 h, cells were gently pelleted (200 g for 15 min) and washed twice by resuspension and centrifugation in PBS. For purification of PKCa, cell pellets were resuspended in ice-cold homogenization buffer (containing mM: Tris 20 (pH 8), EDTA 5, EGTA 5, benzamidine 5, supplemented with protease inhibitors and 0.3% 2-mercaptoethanol), homogenized, and centrifuged at 100,000 g for 60 min. The supernatant (4°C) was loaded onto a 15×2 cm TSK-DEAE-5PW column preequilibrated with (mM) Tris 20 (pH 7.5), EDTA 2, benzamidine 10, and 0.3% 2-mercaptoethanol. The column was eluted with a linear gradient of NaCl (0-500 mM). Fractions were assessed for PKC activity using histone as a substrate and averaged 150 nmol $PO_4 \cdot min^{-1}$ at 30°C/mg PKCa (25). The stock solution of PKCa contained 2.65 mg protein/ml and was added to the pipette solution in the dilutions indicated.

Statistics. Results are presented as the mean \pm standard error, with *n* representing the number of cells for patch clamp studies and the number of culture plates or repetitions for other assays. Student's *t* test was used to assess statistical significance as indicated.

Results

Activation of K^+ currents by metabolic stress. Outward currents carried by K^+ (I_K) were measured at 0 mV in the whole-cell configuration since K^+ is the only ion with a reversal potential more negative than 0 mV. With glucose in the bath as a metabolic substrate, basal currents were ≤ 50 pA and remained sta-



Figure 1. Activation of macroscopic K⁺ currents by metabolic stress. (*A*) Membrane currents were measured in the whole cell configuration as described in Methods. From a holding potential of -40 mV, outward currents carried by K⁺ (I_K) were assessed by stepping to a test potential of 0 mV every 10 s; and inward currents were measured at a test potential of -80 mV (*inset*, 400 ms duration). Exposure to DNP/2-DG as indicated by the bar reversibly increased outward currents at 0 mV. (*B*) In the same cell, higher resolution currents measured between -120 mV and +100 mV in 20-mV increments (2 s between steps) are shown. Time-dependent inactivation of DNP/2-DG caused a large increase in outward currents at 0 mV and a negative shift in reversal potential toward the K⁺ equilibrium potential.

ble for > 30 min in the absence of intervention. Exposure to DNP/2-DG caused a rapid increase in I_K from 32±8 pA to 2,176 \pm 218 pA (n = 34, P < 0.0001), and a representative example is shown in Fig. 1. Current activation was associated with a negative shift in the average reversal potential to -68mV as anticipated for opening of a K⁺-selective conductance, and, in separate studies, the response to DNP/2-DG was completely inhibited by the nonselective K⁺ channel blocker Cs²⁺ (2 mM, 54 \pm 29 pA, n = 8, P < 0.001). Current amplitude was maximal within 0.5-1 min of exposure, and the effects were fully reversible upon removal of DNP/2-DG from the perfusate. During sustained exposures, currents gradually decreased over 2-5 min despite the continued presence of DNP/2-DG. When measured near the peak response, currents had a nearly linear current-voltage (I-V) relationship over the physiologic range of potentials. At depolarizing potentials above +60 mV, currents showed time-dependent inactivation (Fig. 1*B*).

In parallel studies, exposure to DNP/2-DG had no effect on cell viability as assessed with propidium iodide fluorescence. After a 1-h incubation, nuclear fluorescence was de-



Figure 2. Effect of K⁺ channel blockers on DNP/2-DG-activated currents. (*A*) Exposure to apamin (50 nM, arrow), an inhibitor of SK_{Ca} channels, inhibited I_K when added either after (*top tracing*) or before (*middle tracing*) exposure to DNP/2-DG (*arrow*). In contrast, charybdotoxin, an inhibitor of BK channels, had no effect (*bottom tracing*) compared with control values. (*B*) After activation of I_K by DNP/2-DG, currents measured 10–20 s after exposure to apamin (n = 10) or charybdotoxin (n = 5) are normalized to peak values measured in the same cells (I/I_{DNP/2-DG}). Exposure to apamin significantly inhibited I_K (P < 0.001). Because I_K responses are dynamic and transient, it is important to emphasize that similar results were obtained in cells pre-incubated with apamin or charybdotoxin as described in Results.

tected in 0.55 \pm 0.06% of cells in control buffer as compared to 0.43 \pm 0.13% of cells in the presence of DNP/2-DG (NS). Subsequent exposure to Triton X (0.1%) caused rapid appearance of fluorescence in all cells. These results demonstrate that metabolic stress causes activation of a K⁺-selective current before detectable cell or membrane injury.

Role of cytosolic [Ca²⁺]. Increases in intracellular [Ca²⁺] increase the K⁺ permeability of biliary cells (12, 15) and have been implicated in the response to metabolic stress in other cell types (26). In Mz-ChA-1 cells, exposure to DNP/2-DG increased calcium green-1 fluorescence to 50±7% above basal values (arbitrary fluorescence units, increase detected in 32:45 cells, P < 0.001). Consequently, the effects of apamin, an inhibitor of small conductance K^+ (SK_{Ca}) channels, and charybdotoxin, an inhibitor of large conductance K^+ (BK⁺) channels, were assessed (Fig. 2). When currents were first activated by DNP/2-DG, addition to the bath of apamin (50 nM) caused a rapid decrease in outward currents in each of 10 cells from 456 \pm 85 pA to 140 \pm 55 pA (P < 0.001). Since DNP-activated currents tended to decrease over time in the absence of blockers, additional studies were performed in cells preincubated with apamin for 1-2 min. Prior exposure to apamin completely prevented current activation by DNP/2-DG (n = 6). In con-





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Figure 3. Activation of K⁺-selective channels by metabolic inhibition. (A) Single ion channel currents were measured in the cell-attached configuration; the composition of the pipette solution is shown at the left of each tracing. Exposure to DNP/2-DG (perfusion artifact) caused rapid appearance of channels carrying inward membrane current with KCl in the pipette (top); and outward membrane current with NaCl in the pipette (bottom). Channel opening was inhibited by inclusion of apamin in the pipette (50 nM, middle). Currents are shown at the resting potential (Vp 0 mV) except where indicated. The presence of multiple current levels in each patch was characteristic. (B)Representative currents measured at different pipette potentials. The pipette contained the KCl-rich solution, and the closed level is indicated by the arrow. The transmembrane potential is not measured in this configuration, and more positive values of Vp represent relative hyperpolarization of the patch from the resting potential. (C) Currentvoltage relations. With physiologic NaCl solutions in the bath and pipette, channels carried outward membrane current at the resting potential (0 mV, closed circles). Increasing the concentration of K⁺ in the pipette from 5 to 140 mM caused a positive shift in reversal potential, the direction anticipated for currents through K⁺-selective channels (open circles).

trast, charybdotoxin (CTX, 500 nM, n = 5) had no effect on DNP/2-DG-activated currents (939±283 pA in control cells versus 812±162 pA in the presence of CTX). These studies indicate that the increase in membrane K⁺ permeability is related at least in part to opening of apamin-sensitive SK_{Ca} channels.

Identification of single channel currents. To identify the channels responsible for K⁺ efflux, single channel currents were measured in cell-attached membrane patches. In this configuration, the cell membrane and signaling pathways remain intact and are not altered by intracellular dialysis (17). In glucose-containing buffer, spontaneous opening of channels carrying outward membrane current was rare. Openings were present in < 5% of > 100 cells, and in each case the open probability (P_0) was < 0.01. In contrast, exposure to DNP/2-DG caused rapid opening of channels within 0.5–3 min in 23 out of

25 cells as shown in Fig. 3. Typically, patches contained multiple channels; in studies where P_0 could be quantitated (\leq 3 open levels), average values increased from 0 to 0.14 \pm 0.08 (n = 5).

With physiologic solutions (140 mM Na⁺ and 5 mM K⁺ in the bath and pipette), channels carried outward membrane current (downward deflection of the current trace) at the resting membrane potential and had an average conductance of 17.0 ± 1.6 pS (n = 15) and reversal potential of -18 ± 4 mV (Fig. 3 C). With these solutions, outward currents are likely to reflect movement of K⁺ out of the cell. Several additional observations support this conclusion. First, increasing the concentration of K⁺ in the pipette from 5 mM to 140 mM caused a positive shift in the reversal potential to $+54\pm5$ mV and increased the conductance for inward currents to 25.0 ± 1.2 pS (n = 10). This corresponds to a change of ~ 50 mV per 10-fold increase in [K⁺]_o, close to values predicted for selective perme-



Figure 4. Effect of cytosolic $[Ca^{2+}]$ and [ATP] on response to DNP/2-DG. The composition of the pipette (intracellular) solution was modified by increasing $[Ca^{2+}]$ to 300 nM (0.7 mM Ca²⁺, 1 mM EGTA); decreasing $[Ca^{2+}]$ to < 10 nM (0 Ca²⁺, 2 mM EGTA); removal of ATP (apyrase 3 U/ml); or addition of ATP (5 mM) as indicated. Representative recordings are shown in A, and average results from 5 to 8 cells are shown in *B*. Increasing intracellular $[Ca^{2+}]$ caused transient activation of I_K (*first recording*); and chelation of Ca²⁺ inhibited activation of currents by DNP/2-DG (*arrow*, *third recording*). Removal of ATP with apyrase failed to activate currents (*second recording*); and inclusion of supplemental ATP in the pipette failed to prevent activation by DNP/2-DG (*arrow*, *lower recording*).

ability to K⁺ using the Goldman-Hodgkin-Katz equation. Second, channel opening was unaffected by inclusion in the pipette of the Cl⁻ channel blocker NPPB (100 μ M, 16±3.3 pS, n = 5), but was completely inhibited by apamin in concentrations that inhibited macroscopic K⁺ currents (25 or 50 nM, $P_0 = 0.0, n = 4$). Thus, these channels are likely to contribute to the increase in membrane K⁺ permeability observed in intact cells.

 Ca^{2+} mobilization versus ATP depletion. In cardiovascular tissues, ATP depletion leads to K⁺ efflux through opening of glybenclamide-sensitive K_{ATP} channels (27, 28). To evaluate the relative roles of increasing intracellular [Ca²⁺] versus decreasing intracellular [ATP], additional studies were performed where the Ca²⁺ and ATP concentrations in the pipette solution were modified as shown in Fig. 4. Dialysis of cells with pipette solutions in which the [Ca²⁺] was increased to ~ 300 nM (0.7 mM Ca²⁺, 1.0 mM EGTA) resulted in transient activation of outward currents. Immediately after gaining access to the cell interior, I_K increased from initial values of 45±12 pA to 2,354±351 pA (n = 8) as the Ca²⁺-containing solution equilibrated with the cell interior. Currents recovered toward basal values within 2 to 5 min despite the continued presence of ~ 300 nM Ca²⁺. To evaluate whether [Ca²⁺]_i is involved in the response to DNP/2-DG, the Ca²⁺ buffering capacity of the intracellular (pipette) solution was increased (0 Ca²⁺, 2 mM EGTA). Under these conditions, exposure to DNP/2-DG failed to activate I_K (n = 5), consistent with a potential regulatory role for intracellular [Ca²⁺].

K_{ATP} channels are opened by falling cytoplasmic ATP concentrations and closed by ATP concentrations in the physiologic range. However, in Mz-ChA-1 cells, when ATP levels were depleted by inclusion of the phosphatase apyrase in the pipette solution (3 U/ml) no K⁺ currents were detected under basal conditions (n = 7), and the response to DNP/2-DG was completely inhibited (peak currents 51 ± 18 pA, n = 6). Inclusion of 5 mM ATP in the pipette solution had no consistent effect on the amplitude or duration of the response to DNP/ 2-DG (n = 5). These findings are opposite to those expected for K_{ATP} channels (Fig. 4). Because the volume of the pipette solution is large with respect to the cell, it is not likely that DNP/2-DG can deplete ATP under these conditions. Moreover, the K_{ATP} channel blocker glybenclamide (50 μ M) failed to inhibit the response. DNP/2-DG stimulated currents of 740 ± 180 pA (n = 6) in the presence of glybenclamide, values not different from control measurements on the same study days. In aggregate, these findings indicate that DNP/2-DGstimulated K⁺ efflux is not directly related to ATP depletion and is not likely to be mediated by opening of K_{ATP} channels.

Role of Ca²⁺-dependent kinases. The requirement for both cytosolic Ca²⁺ and ATP suggests a potential role for Ca²⁺dependent kinases in channel opening. This possibility was evaluated initially in whole cell recordings using pipette solutions supplemented with 1 mM ATP and two experimental paradigms. First, currents were activated by DNP/2-DG, and cells were exposed to putative kinase inhibitors (Fig. 5 A). These included H7, a nonspecific inhibitor and chelerythrine, a more specific inhibitor of PKC. Second, cells were preequilibrated with these inhibitors for 3-6 min before exposure to DNP/2-DG, and results were compared to controls performed on the same day (Fig. 5 B). After activation of currents by DNP/2-DG, addition of chelerythrine to the bath (25 μ M) caused rapid and nearly complete inhibition of IK from $1,139\pm209$ pA to 190 ± 101 pA (n = 10, P < 0.001), and H7 had similar effects. Similarly, prior exposure to chelerythrine or H7 inhibited current activation by DNP/2-DG, consistent with a regulatory role for PKC. To further assess this possibility, cells were incubated overnight with phorbol-12,13-myristate acetate (0.5 mM, 12–16 h) to downregulate phorbol-sensitive PKC isoforms. The response to DNP/2-DG was completely inhibited (4 \pm 20 pA, *n* = 7).

Metabolic inhibition stimulates translocation of PKC α . Probing of biliary cell lysates with isoform-specific antisera identified PKC α as the dominant Ca²⁺- and phorbol-sensitive isoform present; beta_I and beta_{II} isoforms were below the limit of detection, and gamma was present in trace amounts only (data not shown). If PKC α is involved in the response to DNP/ 2-DG, then metabolic inhibition would be anticipated to stimulate translocation of PKC α from cytosol to membrane as one measure of activation. For these studies, cells in subconfluent culture were maintained in serum-free media for 36 h. Under basal conditions, the majority (> 95%, densitometric reading in arbitrary units) of PKC α was in the cytosolic fraction. After exposure to DNP/2-DG, PKC α appeared in the membrane fraction within 1 min and increased to > 20% within 10 min (Fig. 6, n = 3).



Figure 5. Effect of kinase inhibitors on I_K. Cells were exposed to the kinase inhibitors chelerythrine (25 μ M) and H7 (50 μ M) either after (*A*) or before (*B*) exposure to DNP/2-DG. Representative recordings and average responses in 5–10 cells are shown; control values in *B* were measured in the absence of inhibitors on the same study day. Kinase inhibitors caused a significant decrease in activation of I_K by metabolic stress. In addition, downregulation of protein kinase C by exposure to DNP/2-DG.



Figure 6. Effect of metabolic inhibition on PKC α . PKC α was measured in cytosolic (*c*) and membrane (*m*, particulate) fractions using isotype-specific antisera as described in Methods. In quiescent cells, > 95% of activity was located in the cytosolic fraction. Exposure to DNP/2-DG resulted in translocation of PKC α to the membrane fraction.

Activation of I_K by PKC α . These findings support a potential role for PKC α in activation of I_K during metabolic stress. To evaluate this possibility more directly, cells were dialyzed with purified recombinant PKC α and the effects on I_K determined (Fig. 7). For these studies, the standard pipette (intracellular) solution was modified by increasing [Ca²⁺] to ~ 300 nM, adding 1 mM ATP and including PMA (50 nM). In the absence of PKC α , there was transient activation of outward currents due to the higher Ca²⁺ concentration, with recovery to basal values within 2–5 min. In contrast, inclusion of PKC α (10 µJ/250 µl solution; total activity of 3,975 pmol PO₄·min⁻¹ per µl) resulted in sustained activation of I_K (1,389±227 pA, n = 6, Fig. 7). In the same cells, PKC α also stimulated large inward currents at -80 mV, the reversal potential for K⁺ (-2,069±993 pA). These appeared after a brief



Figure 7. Activation of I_K by PKCα. Purified recombinant PKCα (10 μ l/250 μ l; total activity of 3,975 pmol PO₄·min⁻¹ per μ l) was included in the pipette solution, and membrane currents were measured at test potentials of 0 mV and -80 mV as described in Fig. 1. After gaining access to the intracellular space, the low access resistance (R_s < 10 MΩ) allows rapid exchange between the pipette solution and the cell interior. Intracellular dialysis with PKCα resulted in rapid and sustained activation of I_K (*top*) as well as inward currents at -80 mV. Average currents at 0 mV (I_K, *bottom left*) and current-voltage relations (*bottom right*) are shown. The pipette solution contained 300 nM free Ca²⁺, 50 nM PMA, and 1 mM ATP to support PKC-dependent phosphorylation; average values were measured 5 min or more after gaining access to the cell interior to allow inactivation of Ca²⁺dependent currents.

delay and their ionic basis has not been determined. Thus, purified PKC α appears to activate I_K directly in the absence of metabolic stress.

Discussion

Cholangiocytes play a central role in hepatobiliary secretion and bile formation but are susceptible to injury during periods of ischemia and metabolic stress. The principal findings of these studies of a model biliary cell line are that (*a*) metabolic stress stimulates a 40- to 70-fold increase in membrane K⁺ permeability that is mediated in part by opening of apamin-sensitive K⁺ (SK_{Ca}) channels; and (*b*) channel opening is dependent on cytosolic Ca²⁺ and ATP, and is associated with translocation of PKC α to the plasma membrane. These findings indicate the presence of dynamic interactions between cell metabolic status, protein kinase C, and K⁺ channels that may be relevant to the adaptive response of biliary cells to ischemia and other forms of injury.

At least three mechanisms for coupling cell metabolism to membrane K^+ permeability have been identified, including (a) glybenclamide-sensitive KATP channels where ATP itself is responsible for channel gating (11, 27, 29), (b) Ca²⁺-sensitive K⁺ channels in tissues where metabolic inhibition increases $[Ca^{2+}]_i$ (28, 30), and (c) regulatory phosphorylation of K⁺ channels or closely associated channel-regulatory proteins (10). These findings in a human biliary cell model are most consistent with channel opening through a Ca2+- and PKC-dependent mechanism. Several observations support these conclusions. First, DNP/2-DG caused an increase in calcium green-1 fluorescence in \sim 71% of cells, consistent with a rise in cytosolic [Ca²⁺]; and prevention of the increase in $[Ca^{2+}]$ by increasing the buffering capacity of the pipette solution completely inhibited the current response to DNP/2-DG. The lack of detectable calcium increases in some cells may be related in part to the limited range of the perfusion pipette resulting in failure to deliver the expected concentrations of DNP/2-DG. Second, intracellular ATP was necessary for current activation, but increasing the concentration of ATP in the pipette and/or exposure to glybenclamide failed to inhibit the response to DNP/2-DG. Thus, ATP depletion per se is not responsible for channel opening, and K_{ATP} channels are not likely to be involved since increasing intracellular ATP would be expected to close rather than open these channels. Finally, activation of I_K was inhibited by chelerythrine, or by PKC downregulation caused by prolonged exposure to phorbol esters. The concentration of chelerythrine of 25 μ M used for these studies is above the K_i for PKC of ~ 1 μM but below that required for inhibition of protein kinase A $(\sim 160 \,\mu\text{M})$ or calmodulin-dependent kinase (> 100 μ M) (31), suggesting a selective effect. The actual intracellular concentration of chelerythrine is likely to be lower because of the time required for diffusion into the cell (32). While the potential for nonspecific effects is acknowledged, in aggregate the studies support the concept that current activation represents a physiologic response to specific signaling events and not toxic or nonspecific effects of the different experimental reagents.

The PKC family of protein-serine kinases has been implicated in regulation of a broad range of cellular functions. Exposure to DNP/2-DG caused rapid translocation of PKC α to the particulate (membrane) fraction. Moreover, intracellular perfusion with recombinant PKC α caused sustained activation of I_K in the absence of metabolic inhibition. The most direct interpretation of these findings is that PKC α stimulates I_K by phosphorylation of apamin-sensitive SK_{Ca} channels. However, these channels could not be evaluated in excised patches since they inactivated within seconds of excision. It is also possible that PKC acts more proximally to regulate Ca²⁺ release or other processes, and the involvement of intermediary phosphoproteins or other PKC isoforms present in amounts below the limits of detection cannot be excluded. It is notable that intracellular dialysis with recombinant PKCa stimulates sustained currents above those caused by 300 nM Ca2+ alone. Preliminary studies indicate that apamin and chelerythrine fail to block the K⁺ currents stimulated by thapsigargin-induced Ca²⁺ mobilization (our unpublished observations). Together, these findings suggest that the responses to Ca²⁺ mobilization and metabolic stress are likely to be mediated by separate K⁺ channel types and that PKC is required for stress-induced but not Ca2+-induced K+ efflux. Additional studies are required to establish the site(s) of action of PKC.

These results are consistent with recent findings in HTC hepatoma cells where metabolic stress is also associated with translocation of PKC (10). This similarity raises the possibility that K⁺ efflux represents a general process that is relevant to both hepatocytes and cholangiocytes, the two principal epithelial cell types of the liver. This common response is notable since the transport functions of these cells are dissimilar in other respects. Hepatocytes are endowed with a variety of mechanisms for transport and metabolism of organic anions, whereas cholangiocytes appear to function primarily as Clsecretory epithelia (1). Assuming that similar mechanisms are operative in primary liver cells, several additional questions remain to be addressed. First, the cellular signals responsible for Ca²⁺ mobilization and PKC activation are not clear. Generally, activation of PKC involves receptor stimulation, hydrolysis of phosphatidylinositol bisphosphate, and release of diacylglycerol and IP₃. This response to stress, however, occurs in the absence of an obvious receptor agonist implying that other signals are involved. Both translocation of PKCa and activation of I_K are detectable within 1 min, and precede any evidence of cell damage, consistent with a signaling event. Second, intracellular dialysis with PKCa does not exactly reproduce the effects of DNP/2-DG. Specifically, the predominant response to metabolic stress involves transient activation of I_{K} , followed after a brief delay by appearance of an inward current at -80 mV in approximately one third of cells. With the solutions used, the inward current is presumably carried by Cl⁻ ions. In contrast, the response to PKCα includes sustained activation of I_K and much larger inward currents in all cells tested. Thus, PKC α is not likely to account for all of the effects of metabolic stress on membrane ion permeability, and other stimulatory or inhibitory signals are presumably involved as well. However, PKC appears to be essential since DNP-induced activation of I_K is blocked completely by depletion of cytosolic Ca²⁺ or ATP, prolonged incubation with phorbol esters, or exposure to putative PKC inhibitors.

Finally, the physiologic implications of K^+ efflux during metabolic stress are not known. By analogy with vascular cells, opening of K^+ channels in biliary cells could be beneficial in the early stages of injury by stimulation of electrogenic uptake of substrates important for regeneration of ATP stores or regulation of cell volume (13). Over longer periods, however, sustained release of K^+ in the setting of Na⁺/K⁺ pump inhibition would contribute to dissipation of transmembrane cation gradients. In view of the fundamental importance of K^+ channels to different liver cell functions, it is attractive to speculate that hepatic and biliary SK_{Ca} channels represent potential targets for pharmacologic modification of the cellular response to injury and for coupling the metabolic state of cells to changes in membrane K^+ permeability.

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