Inhibition of the Mitochondrial Permeability Transition by Protein Kinase A in Rat Liver Mitochondria and Hepatocytes

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Abbreviations used: AMPPNP, 5′-adenyl-γ, β-imidodiphosphate; DETA-NONOate, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; DT-3, PKG inhibitor peptide; DTT, dithiothreitol; I/R, ischemia/reperfusion; KRH, Krebs-Ringer-Hepes buffer; MPT, mitochondrial permeability transition; NO, nitric oxide; ODQ, 1H-1,2,4-oxadiazole[4,3-a]quinoxalin-1-one; PKA, cAMP-dependent kinase; PKG, cGMP-dependent kinase; PKI, PKA inhibitor peptide; PT, permeability transition; TMRM, tetramethylrhodamine methylester; VDAC, voltage dependent anion channel.
ABSTRACT
NO and cGMP administered at reperfusion after ischemia prevent injury to hepatocytes mediated by the mitochondrial permeability transition (MPT). To characterize further the mechanism of protection, the ability of hepatic cytosol in combination with cyclic nucleotides to delay onset of the calcium-induced MPT was evaluated in isolated rat liver mitochondria. Liver cytosol plus cGMP or cAMP dose-dependently inhibited the MPT, required ATP hydrolysis for inhibition, and did not inhibit mitochondrial calcium uptake. Specific peptide inhibitors for protein kinase A (PKA), but not protein kinase G (PKG), abolished cytosol-induced inhibition of MPT onset. Activity assays showed a cGMP- and cAMP-stimulated protein kinase activity in liver cytosol that was completely inhibited by PKI, a PKA peptide inhibitor. Size exclusion chromatography of liver cytosol produced a single peak of cGMP/cAMP-stimulated kinase activity with an estimated protein size of 180-220 kDa. This fraction was PKI-sensitive and delayed onset of the MPT. Incubation of active catalytic PKA subunit directly with mitochondria in the absence of cytosol and cyclic nucleotide also delayed MPT onset, and incubation with purified outer membranes led to phosphorylation of a major 31 kDa band. After ischemia, administration at reperfusion of membrane-permeant cAMPs and cAMP-mobilizing glucagon prevented reperfusion injury to hepatocytes. In conclusion, PKA in liver cytosol activated by cGMP or cAMP acts directly on mitochondria to delay onset of the MPT and protect hepatocytes from cell death after ischemia/reperfusion.
Ischemia-reperfusion injury (I/R) is a primary cause of graft dysfunction and failure after liver transplantation (25). Identification of pathways that ameliorate this injury might lead to improved viability and function after liver transplantation and other types of hepatic surgery. An important mechanism leading to cell death in I/R injury to liver is onset of the mitochondrial permeability transition (MPT) (28;44;49;52). The MPT is characterized by opening of high conductance permeability transition (PT) pores that allow molecules of up to 1500 Da to diffuse freely through the mitochondrial inner membrane (22). The net result of PT pore opening is mitochondrial depolarization and uncoupling of oxidative phosphorylation. After uncoupling, impairment of cellular ATP supply is further exacerbated by hydrolysis of ATP by the mitochondrial F1F0-ATPase (21;24;40). After PT pore opening, mitochondrial swelling occurs driven by the colloid pressure of proteins in the matrix. This swelling leads to rupture of the mitochondrial outer membrane and release of proapoptotic proteins from the intermembrane space, such as cytochrome c, Smac/Diablo, and others (4;19;38;55). After the MPT, either necrosis or apoptosis ensues depending on cellular ATP. If ATP decreases profoundly, ATP-requiring caspase activation is blocked and necrosis develops. If ATP levels remain above 10-15%, then necrosis is prevented, and caspase-dependent apoptosis occurs instead (30;35;43).

Previously, we showed that hepatocytes subjected to I/R injury were protected from cell death by either nitric oxide (NO) or cGMP administered at the time of reperfusion (29). Confocal microscopy demonstrated that NO donors and cell permeable cGMP analogs prevented onset of the MPT after reperfusion. Moreover in a cell-free reconstituted system, liver cytosol in the presence of ATP and cGMP suppressed onset of the MPT in isolated rat liver mitochondria (29). This protection against the MPT was abolished by heat inactivation of cytosol and by the cGMP-dependent protein kinase (PKG) inhibitor, KT5823. However, KT5823, an ATP analog, may also inhibit other kinases (8). Thus, the goal of the present study was to characterize further the mechanism of cyclic nucleotide-dependent inhibition of the MPT in isolated rat liver mitochondria and cultured hepatocytes. Here, we show that liver cytosol contains a PKA activity activated by both cGMP and cAMP that delays onset of the Ca2+-induced MPT in isolated rat mitochondria. Moreover, since membrane permeant cAMPS decreased MPT-dependent cell death in hepatocytes subjected to I/R, PKA rather than PKG is likely responsible for cytoprotection by NO and cGMP.
MATERIALS AND METHODS

Materials. cAMP-dependent protein kinase catalytic subunit (PKA) was obtained from New England Biolabs (Ipswitch, MA), inhibitor peptide of PKA (PKI) from Upstate Biotechnology (Lake Placid, NY), [γ-32P] ATP from Perkin Elmer (Boston, MA), and DT-3, cGMP, cAMP and all cyclic nucleotide analogs from BioLog/Axxora (San Diego, CA). cGMP-dependent protein kinase (PKG-α) and other reagent grade chemicals were obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

Isolation of rat liver mitochondria. Animals were humanely treated using protocols approved by the Institutional Animal Use and Care Committee. Liver mitochondria from overnight-fasted male Sprague-Dawley rats (200-300 g) were isolated as previously described (3). Briefly, livers were excised and minced in Buffer A (250 mM sucrose, 0.5 mM EGTA, and 2 mM K+-HEPES buffer, pH 7.4), followed by homogenization on ice with 4 strokes of a Teflon Potter-Elvehjem tissue grinder loose-fitting pestle A and 1 stroke of pestle B at ~400 rpm. Homogenates were diluted to 10% (weight/volume) with Buffer A and centrifuged at 600g for 15 min at 4ºC. The resultant supernatants were centrifuged at 9750g for 10 min at 4º C, and the pellets were resuspended in buffer A. Centrifugation and resuspension were repeated twice with resuspension in Buffer A (250 mM sucrose, 2 mM K+-HEPES, pH 7.4). The final pellet was resuspended in Buffer B to a protein concentration of 50 mg/ml.

Isolation of mitochondrial outer membranes. Mitochondrial outer membranes were isolated from mitoplasts, as described (46). Briefly, livers (~30 g) from 3-4 rats were homogenized, as described above, in 70 ml of H-medium (220 mM D-mannitol, 70 mM sucrose, 2 mM K+-HEPES buffer, and 0.5 mg/L bovine serum albumin, pH 7.4). The homogenate was further diluted to 10% and centrifuged for 15 min at 660g. The supernatant was then centrifuged for 15 min at 6,780g, and the resulting pellet resuspended in H-medium and centrifuged for 15 minutes at 9,770g. This last centrifugation was repeated, and the pellet was resuspended to exactly 100 mg/ml. Using continuous stirring with a magnetic bar, 3 ml of resuspended mitochondria (300 mg) were mixed with 3 ml of 1.45% digitonin, 0.5 mg/ml BSA in H-medium on ice. After 15 min, the suspension was diluted 4-fold into H-medium and centrifuged for 10 min at 10,800g. The yellow supernatant containing mitochondrial outer membranes was removed and centrifuged for 1 h at 144,000g at 4ºC. The resultant pellet was resuspended in a few drops of H-medium and contained approximately 3 mg of purified mitochondrial outer membranes. Contamination of isolated outer membranes with inner membrane was assessed by measurements of succinate dehydrogenase (SDH) protein and enzymatic activity. SDH protein and enzyme activity were not detectable (data not shown).

Hepatocyte isolation, culture and incubation. Hepatocytes were isolated and cultured as previously described (29). I/R in cultured hepatocytes was simulated by anoxia at pH 6.2 in Krebs-Ringer-Hepes buffer (KRH) followed by reoxygenation at pH 7.4, as previously described (44). Cell death was monitored by propidium iodide fluorometry, as described (39).
**Laser scanning confocal microscopy.** Hepatocytes plated on glass coverslips were co-loaded in KRH with 200 nM tetramethylrhodamine methylester (TMRM) and 1 μM calcein-AM for 15 min at 37°C (30). To simulate ischemia, hepatocytes were incubated at pH 6.2 in KRH containing 100 nM TMRM and 3 μM propidium iodide in an anoxic chamber for 4 h. During the last 15 min of anoxic incubation, cells were re-loaded with 0.5 μM calcein-AM to improve cellular calcein loading. Inside the anoxic chamber, hepatocyte-containing coverslips were mounted in a gas-tight Perfusion Open Closed-Reduced (POC-R) chamber (Zeiss, Jena, Germany). After 4 h, the sealed chamber was mounted on the microscope stage, and reperfusion was initiated by infusion of aerobic KRH at pH 7.4 containing 200 nM TMRM and 3 μM propidium iodide. In some experiments, glucagon (10 nM) was added for the last 20 min of ischemia and during reoxygenation. The green fluorescence of calcein and red fluorescence of TMRM and propidium iodide were imaged with a Zeiss LSM 510NLO META inverted laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) using a Zeiss N.A. 1.4 63X planapochromat objective lens, as previously described (30).

**Preparation of liver cytosolic extract:** Rat livers were excised and minced, as described above, and then washed three times in ice-cold phosphate-buffered saline (PBS, mM: 2.67 KCl, 1.47 KH2PO4, 138 NaCl, and 8.06 Na2HPO4, pH 7.2) supplemented with protease inhibitors (2.08 μM 4-[2-aminoethyl]-benzenesulfonyl fluoride, 1.6 μM aprotinin, 80 μM bestatin, 30 μM pepstatin A, 28 μM E-64, and 40 μM leupeptin). After washing, 3 g of liver were added to 20 ml of PBS with protease inhibitors and homogenized on ice at ~400 rpm with 10 strokes of Potter-Elvehjem tissue grinder pestle A followed by 3 strokes with pestle B. The homogenate was centrifuged for 10 min at 10,000xg, and the resultant supernatant was centrifuged again for 1 h at 100,000xg. The fatty top layer was aspirated, and the remaining supernatant was stored at on ice until used or frozen in 20% glycerol at -20°C for use within 48 h. Protein concentration averaged 10-12 mg/ml.

**Protein Assay:** Protein concentration in cytosolic extracts was measured with a BioRad Protein Assay Kit (BioRad, Hercules, Ca). Protein concentration of purified mitochondria was measured by a biuret method (17).

**Mitochondrial Respiration:** Respiration was measured with a Clark-type oxygen electrode (Hansatech, Norfolk, UK) in respiratory buffer (150 mM sucrose, 7.5 mM KH2PO4, 5 mM MgCl2, 5 mM succinate, 25 mM Hepes buffer, and 1 μM rotenone, pH 7.4, 23°C) containing 1 mg/ml rat liver mitochondria. Respiration was stimulated with 300 μM ADP. Mitochondria were not used if the ratio of ADP-simulated respiration (State 3) to unstimulated respiration (State 4) was less than 5.

**Ca2+-induced mitochondrial swelling:** For swelling assays, mitochondria (0.5 mg protein/ml) were incubated in 96-well (0.3 ml per well) or 24-well (0.5 ml/well) microtiter plates in swelling buffer (200 mM sucrose, 20 μM EGTA, 5 mM succinate, 2 μM rotenone, 1 μg/ml oligomycin, 20 mM Tris, 20 mM HEPES, and 1 mM KH2PO4, pH 7.3, 23°C), as described (3). Swelling was assessed by decreased absorbance at 540 nm using a ThermoMax 96-well plate reader.
(Molecular Devices, Sunnyvale, CA) or FLUOstar fluorescence plate reader (BMG, Germany). In some experiments, 1 µM Fluo5N (Molecular Probes, Eugene, Or) and 1 µM TMRM (Molecular Probes, Eugene, Or) were included to assess extra-mitochondrial Ca\(^{2+}\) and mitochondrial polarization, respectively, as described (3). Induction of the MPT was initiated with 200-300 µM CaCl\(_2\). Swelling traces are representative of 3 or more experiments.

**Measurement of cGMP and cAMP:** Hepatocellular cGMP and cAMP were determined using commercial kits (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer’s instructions. cGMP and cAMP were harvested from PBS-washed cells in 1 ml of cold 70 % ethanol. Protein precipitates were removed by centrifugation at 14,000xg for 5 min at 4ºC. Supernatants were dried under vacuum and resuspended in 300 µL of EIA buffer from the kit.

**Fractionation of liver cytosol and kinase assays.** Liver cytosol was prepared, as described above, and separated by FPLC (GE Healthcare, Piscataway, NJ) on a calibrated 150 ml Sephacryl S200 size exclusion column using S200 buffer (50 mM Tris pH 8.0, 250 mM NaCl, 5 mM dithiothreitol [DTT], and 2.5% (w/v) glycerol). Fractions were collected and stored at 4ºC for kinase activity assays (18). Briefly, 5 µL of FPLC fractions were assayed for kinase activity in 45 µL of kinase buffer (20 mM Tris, 10 mM DTT, 20 µM Mg·ATP, 5 µM sp-cGMP or sp-cAMP, and 1 µCi of [γ-32P] ATP with a specific activity of 4000 Ci/mM [Perkin Elmer, Boston, Ma]) with 30 µM Kemptide substrate (27). After 10 min, 20 µL of reaction were spotted onto p81 paper (Whatman, Boston, MA), washed 3 times in 0.75% phosphoric acid and rinsed once in 100% ethanol. After air-drying, samples were counted with a scintillation counter (Beckman Coulter, Fullerton, Ca). The PKA peptide inhibitor (PKI) was used at 4 µM unless otherwise stated. Fractions possessing kinase activity were pooled and concentrated with 50 kDa cut off Centricon concentrators (Millipore, Boston, Ma) for use in swelling assays.

**Mitochondrial phospholabeling and electrophoresis.** Mitochondria (150 µg) in 100 µL of swelling buffer were incubated at 23ºC with catalytic PKA subunit (50 units/µL), 20 µM Mg·ATP and 1 µCi of [γ-32P] ATP with a specific activity of 4000 Ci/mM (Perkin Elmer, Boston, Ma). One unit of PKA was defined as that phosphorylating 1 pmol of Kemptide substrate in 1 min at 30ºC. After 10 min, the mitochondrial suspension was centrifuged at 9000xg for 1 min at 4ºC. The pellet was resuspended in 90 µL Lamelli buffer, and 30 µL was subjected to polyacrylamide gel electrophoresis (PAGE) on a 4-12% gel (Invitrogen, Ca). For phosphorylation of mitochondrial outer membranes, 20 µg of purified mitochondrial outer membranes were incubated for 10 min at 23ºC with 100 units/µL catalytic PKA subunit, 20 µM Mg·ATP and 1 µCi of [γ-32P] ATP. The reaction was stopped by the addition of 2X Lamelli buffer. Half of the stopped reaction (25 µL) containing 10 µg of outer mitochondrial membrane protein was subjected to PAGE. The resultant gels were fixed for 1 h in fix/destain solution (10% methanol, 7% glacial acetic acid) and stained overnight in stain solution (0.08%, Coomassie blue G-250 [Bio-Rad, Hercules, CA], 8% ammonium sulphate, 1% phosphoric acid, 20% methanol). The gels were destained in destain solution until bands were not visible, transferred to storage solution (1%
acetic acid) for 30 min, and exposed overnight to X-OMAT AR film (Kodak, Rochester, NY).

**Protein digestion and mass spectrometry:** The exposed film was overlaid on the stained gel and Coomassie visible bands were excised, minced into <1-mm pieces and placed in siliconized microfuge tubes. Gel fragments were immersed and shaken in destain solution (12 mM ammonium bicarbonate, 40% acetonitrile) for 15 min at room temperature. If the gel pieces still had Coomassie blue stain after removing the destain solution, the destain procedure was repeated. Upon removal of destain solution, the tubes were put into a speed vac and dried down. The dried gels were rehydrated in 25 mM ammonium bicarbonate (pH 8.0) and incubated overnight at 37 °C with 0.2 μg of tosyl phenylalanyl chloromethyl ketone-modified sequencing grade trypsin (Promega, Madison WI). After centrifugation at 14,000xg for 5 min, supernatants were transferred to new siliconized centrifuge tubes, and the trypsinized gels were washed and shaken for 15 min in 50 μL of 60% acetonitrile/5% formic acid solution. After centrifugation for 5 min at 14,000xg, the second supernatants were combined with the first supernatants, dried in a speed–vac, resuspended in 0.1% trifluoroacetic acid and purified with C18 ZipTips (Millipore, Billerica, MA) according to the manufacturer’s instructions. Using 10 μL of elution buffer (0.1% trifluoroacetic acid, 70% acetonitrile and 1% α-cyano-4-hydroxycinnamic acid), peptides were eluted directly onto a matrix-assisted laser desorption/ionization (MALDI) plate and allowed to dry. The peptides were then analyzed with an Applied Biosystems 4700 tandem mass spectrometer (Life Technologies, Carlsbad, CA).

**Statistics.** Differences between groups were analyzed by the student’s t-test. A p value of less than 0.05 was considered significant. Data shown are means ± S.E.M.
RESULTS

Inhibition of Ca^{2+}-induced mitochondrial swelling by liver cytosol. In previous work, we showed that liver cytosol delays onset of the MPT in a cGMP-dependent manner (29). To characterize further the requirements of liver cytosol to delay onset of the Ca^{2+}-induced MPT, we examined the dose dependence of liver cytosol in delaying onset of the MPT. When mitochondria were incubated in the presence of ATP (20 µM) and 8-pCPT-cGMP (100 µM), no swelling occurred over 30 min (Fig. 1, trace a). By contrast, swelling occurred promptly after addition of 250 µM Ca^{2+}, as evidenced by a large decrease in absorbance (Fig. 1, trace b). Relative to the Ca^{2+} only control, 25 µg/ml of liver cytosol (Fig 1, trace c) delayed swelling by approximately 12 min, and 50 µg/ml of liver cytosol delayed swelling by approximately 22 min (Fig. 1, trace d). Addition of 100 µg/ml of liver cytosol delayed swelling only slightly longer than 50 µg/ml cytosol (Fig. 1, trace e). Swelling in this assay represents onset of the MPT. To insure that cGMP had no effect on the Ca^{2+}-induced MPT, mitochondria in the absence of cytosol were treated with membrane-permeant cGMP (0–300 µM). The cGMP treatment of mitochondria had no effect on the MPT following 250 µM Ca^{2+} (data not shown). Thus, liver cytosol delayed the Ca^{2+}-induced MPT in a dose-dependent fashion in the presence of ATP and 8-pCPT-cGMP. Additionally, cAMP could replace cGMP as the cyclic nucleotide required for cytosol-dependent inhibition of the MPT (data not shown).

Requirement of ATP for cytosolic-induced delay of MPT onset. Mitochondria were incubated with 0 to 20 µM ATP in the presence of a constant amount of cytosol (25 µg/ml) and 8-pCPT-cGMP (100 µM). In the absence of ATP, Ca^{2+} induced prompt mitochondrial swelling (Fig. 2A, trace a). The addition of 10 and 20 µM ATP together with cytosol and 8-pCPT-cGMP delayed onset of the Ca^{2+}-induced MPT in a dose-dependent fashion (Fig. 2A, traces c and e). To determine whether delayed MPT onset was a direct effect of ATP, 20 µM ATP (with 8-pCPT-cGMP) was added in the absence of cytosol, but Ca^{2+}-induced swelling was not delayed (Fig. 2A, trace d) and was identical to Ca^{2+}-induced swelling in the absence of ATP with and without cytosol plus 8-pCPT-cGMP (Fig. 2A, traces a and b). Since higher concentrations of ATP (>50 µM) partially delayed the MPT in the absence of cytosol and 8-pCPT-cGMP (data not shown), such higher concentrations were not used in the present work. Since ADP formed by ATP hydrolysis might act to inhibit MPT onset, the mitochondrial F_{1}F_{0}-ATPase inhibitor, oligomycin, was routinely included in the swelling buffer. Moreover, ADP (30 and 60 µM) in the presence of diadenosine pentaphosphate (10 µM) to inhibit adenylate kinase did not block the Ca^{2+}-induced MPT (data not shown).

To examine the importance of ATP hydrolysis for inhibition of the MPT, ATP was compared to AMP-PNP, a nonhydrolyzable ATP analog. In the presence of 8-pCPT-cGMP but absence of cytosol, Ca^{2+} addition induced rapid swelling (Fig. 2B, trace a). Similarly, rapid Ca^{2+}-induced swelling occurred in the presence of 25 µM ATP and 8-pCPT-cGMP but no cytosol (Fig. 2B, trace b) and of 60 µg/ml cytosol plus 8-pCPT-cGMP without ATP (Fig. 2B, trace c). As in Fig. 2A, the combination of cytosol, ATP and 8-pCPT-cGMP delayed onset of the Ca^{2+}-
induced MPT (Fig. 2B, trace d). However, when 25 µM PNP-AMP or 500 µM PNP-AMP replaced ATP in the presence of cytosol plus 8-pCPT-cGMP, the Ca\textsuperscript{2+}-induced MPT was not prevented (Fig. 2B, traces e and f).

**Effects of cytosol on Ca\textsuperscript{2+} uptake and mitochondrial membrane polarization.** To measure Ca\textsuperscript{2+} uptake, mitochondria were incubated with the membrane-impermeant Ca\textsuperscript{2+}-indicating green-fluorescing fluorophore, Fluo-5N (1 µM), whose fluorescence increases with increasing extramitochondrial free Ca\textsuperscript{2+} concentration. To monitor mitochondrial membrane polarization, the membrane potential-indicating red-fluorescing fluorophore, TMRM (1 µM), was also added to the medium whose fluorescence decreases (quenches) with increasing membrane potential. Using a fluorescence plate reader, green and red fluorescence was monitored together with absorbance to assess mitochondrial swelling (Fig. 3).

Mitochondria incubated with ATP but without cytosol or 8-pCPT-cGMP (Fig. 3A, trace a) or with cytosol without ATP or 8-pCPT-cGMP (Fig. 3A, trace b) rapidly accumulated exogenous Ca\textsuperscript{2+} during the first minute, which occurred even before the first measurements could be made. Subsequently, mitochondria rapidly released the accumulated Ca\textsuperscript{2+} as the MPT occurred, which also mostly occurred before the first measurement could be made. By contrast, when cytosol was combined with ATP and 8-pCPT-cGMP, Fluo5N fluorescence initially remained low because onset of the MPT was delayed, and Ca\textsuperscript{2+} was taken up and retained in mitochondria (Fig 3A, trace c). Subsequently after many minutes, Fluo5N fluorescence began to increase, which signified Ca\textsuperscript{2+} release from mitochondria at onset of the MPT. In the presence of cyclosporin A, a PT pore inhibitor, mitochondria did not release Ca\textsuperscript{2+} over the course of the incubation, and Fluo5N fluorescence remained low (Fig. 3A, trace d).

Polarized mitochondria take up TMRM electrophoretically. As TMRM becomes concentrated inside mitochondria, quenching of TMRM fluorescence occurs. Upon depolarization, mitochondria release TMRM, and unquenching of TMRM fluorescence occurs (3). In parallel to Ca\textsuperscript{2+} release measured by Fluo5N fluorescence (Fig 3A), Ca\textsuperscript{2+} addition produced depolarization of mitochondria in the presence of ATP without cytosol or 8-pCPT-cGMP (Fig 3B, trace a) or with cytosol without ATP or 8-pCPT-cGMP (Fig 3B, trace b), as shown by an increase of TMRM fluorescence. By contrast, mitochondria incubated with the combination of ATP, 8-pCPT-cGMP and cytosol remained polarized much longer after Ca\textsuperscript{2+} addition (Fig 3B, trace c). After a delay of many minutes, mitochondria then began to depolarize in parallel with Ca\textsuperscript{2+} release. Cyclosporin A completely prevented this increase of TMRM fluorescence (Fig 3B, trace d). Swelling monitored by absorbance closely paralleled mitochondrial Ca\textsuperscript{2+} release and depolarization, confirming that cyclosporin A-sensitive Ca\textsuperscript{2+} release and depolarization were consequences of the MPT (Fig 3C). Taken together, these data show that cytosol plus ATP and 8-pCPT-cGMP do not prevent mitochondrial Ca\textsuperscript{2+} uptake as the mechanism of inhibition of the MPT.

**Involvement of protein kinase A in the cytosol-induced delay of MPT onset.** In previous work, the protein kinase G (PKG) inhibitor, KT-5823, abrogated inhibition of the MPT by cytosol, ATP and 8-pCPT-cGMP (29).
However, KT-5823 may inhibit other protein kinases, particularly PKA (8). To address if cytosol-dependent MPT inhibition was mediated by PKA or PKG, peptide inhibitors for PKA (PKI) (7) and PKG (DT-3) (10) were tested for their ability to abolish cytosol-dependent inhibition of the Ca\textsuperscript{2+}-induced MPT. When mitochondria were incubated in 8-pCPT-cGMP (100 µM) and ATP (25 µM) in the absence of Ca\textsuperscript{2+}, no MPT occurred unless Ca\textsuperscript{2+} was added (Fig. 4A, traces a and b). When mitochondria were incubated with cytosol (60 µg/ml), 8-pCPT-cGMP (100 µM) and ATP (25 µM), onset of the Ca\textsuperscript{2+}-induced MPT was delayed (Fig. 4A, trace c). PKI (10 µM), the PKA inhibitor, completely abolished the cytosol-dependent delay of MPT onset (Fig. 4A, trace d). By contrast, DT-3 (1 µM), a PKG peptide inhibitor, had no effect (Fig. 4A, trace e). Both inhibitors were used at concentrations at least 10 times higher than their reported IC\textsubscript{50} concentration to assure kinase inhibition would occur.

**Delay of the MPT by cGMP- and cAMP-stimulated kinase activity in liver cytosol.** To characterize cyclic nucleotide-stimulated kinase activity in liver cytosol, fractionation of liver cytosol was performed with a calibrated size exclusion S-200 FPLC column. Kinase assays were performed on the fractions in absence (control) and presence of the phosphodiesterase (PDE)-resistant cyclic nucleotides, sp-cGMP and sp-cAMP. Cyclic nucleotide-stimulated kinase activity was greatest in fractions 14 through 17, corresponding to a molecular weight range of 180 to 220 kDa (Fig 4B). In pooled fractions 14-17, cGMP/cAMP-stimulated kinase activity was abolished by the PKA inhibitor, PKI (4 µM) (Table I). By contrast, the PKG peptide inhibitor, DT-3 (1 µM), did not inhibit kinase activity (Table I). Nonetheless, cGMP stimulated the kinase activity of pooled fractions 14-17 with half maximal stimulation at 3-4 µM (Fig. 4C).

Pooled fractions 14-17 were assayed for the ability to delay onset of the calcium-induced MPT. The pooled fractions were first concentrated 40-fold by 50 kDa centrifugal filtration, as described in MATERIALS AND METHODS. In the presence of 8-pCPT-cGMP and ATP, no swelling of mitochondria was observed during the course of the experiment (Fig. 4D, trace a), unless Ca\textsuperscript{2+} was added in which case swelling was rapid (data not shown). The concentrated pooled fractions delayed Ca\textsuperscript{2+}-induced swelling in the presence of 8-pCPT-cGMP and ATP (Fig. 4D, trace b), and PKI (4 µM), a peptide inhibitor of PKA, abolished this delay (Fig. 4D, trace c). The peptide inhibitor of PKG, DT-3 (1 µM), had no effect on the observed delay (Fig. 4D, trace d).

**Inhibition of the MPT by purified protein kinase A.** To test directly if PKA inhibits CaCl\textsubscript{2}-induced swelling of isolated mitochondria, rat liver mitochondria were treated with the increasing amounts of a constitutively active catalytic subunit of PKA. In the absence of PKA, mitochondria did not swell (Fig 5A, trace a) unless CaCl\textsubscript{2} was added (Fig 5A, trace b). CaCl\textsubscript{2}-induced swelling occurred identically in mitochondria exposed to ATP alone (Fig. 5A, trace c) and to PKA alone (Fig. 5A, trace d). By contrast, treatment with the catalytic subunit of PKA in the presence of ATP inhibited Ca\textsuperscript{2+}-induced swelling in a dose-dependent fashion (Fig 5A, traces e and f).

Since PKA and PKG phosphorylate similar sites (42), we evaluated the effect of purified PKG I\textalpha on mitochondrial swelling. As previously, mitochondrial
swelling did not occur with cGMP and ATP alone (Fig. 5B, trace a) but occurred rapidly after Ca\(^{2+}\) addition (Fig. 5B, trace b). By contrast, PKG \(\lambda\) in the presence of cGMP and ATP inhibited swelling after Ca\(^{2+}\) (Fig. 5B, trace c).

**Phosphorylation of proteins of intact mitochondria and purified mitochondrial outer membranes by the constitutively active catalytic subunit of protein kinase A.** To determine phosphorylation of mitochondrial proteins, mitochondria were incubated with \(\gamma\text{-}^{32}\text{P}\) ATP. When whole mitochondria were incubated with \(\gamma\text{-}^{32}\text{P}\) ATP without PKA, only two phosphorylated bands were visible with estimated molecular weights 40 kDa and 31 kDa (Fig. 5C, lane 1). By contrast when mitochondria were incubated with \(\gamma\text{-}^{32}\text{P}\) ATP plus PKA, the regions around the 40 kDa and 31 kDa bands intensified, and additional strong bands of estimated molecular weights of 47 kDa and 18 kDa became visible plus weaker bands of estimated molecular weights of 170 kDa and 100 kDa (Fig. 5C, lane 2). When the PKA inhibitor, PKI, was present with PKA, only the two phosphorylated bands (40 kDa and 31 kDa) observed in the absence of PKA were present (data not shown). When catalytic PKA was incubated with \(\gamma\text{-}^{32}\text{P}\) ATP in the absence of mitochondria, only a 40 kDa band was visible (Fig. 5C, lane 3). This band is likely the PKA catalytic subunit, since PKA is a 41 kDa protein that autophosphorylates (6).

Unlike whole mitochondria where 40 kDa and 31 kDa phosphorylated bands were visible in the absence of PKA (Fig 5C lane 1), no phosphorylated bands were visible when mitochondrial outer membranes were incubated with \(\gamma\text{-}^{32}\text{P}\) ATP without PKA (Fig. 5D, lane 1). When purified outer membranes were incubated with \(\gamma\text{-}^{32}\text{P}\) ATP and PKA, strong phosphorylated bands became present at 40 kDa and 31 kDa with weaker bands at 100 kDa, 49 kDa, 17 kDa and 15 kDa (Fig. 5D lane 2). Furthermore, when mitochondrial outer membranes were incubated with \(\gamma\text{-}^{32}\text{P}\) ATP and PKA in the presence of PKI, all bands disappeared with the exception of a faint 40 kDa band of autophosphorylated PKA (Fig. 5D, lane 3).

The 40 kDa phosphorylated bands in both whole mitochondrial and outer membrane extracts presumably included autophosphorylated PKA. Attempts to sequence the 31 kDa band from whole mitochondria (Fig. 5C lane 2) were unsuccessful because of the large number of inner membrane and matrix proteins present in the extracts. However, the phosphorylated 31 kDa band from outer membranes (Fig. 5D, lane 2) was identified by MALDI-TOF mass fingerprinting to include VDAC1 (SwissPro accession # Q9Z2L0) with a Protein Prospector MOWSE score of 1.31 x 10\(^{11}\). The recovered peptides represented 77% (217/282AA's) of full length VDAC1. Efforts to identify the weak 100 kDa, 49 kDa, 17 kDa and 15 kDa phosphorylated bands from outer membranes were unsuccessful.

**Membrane-permeant cAMP protects hepatocytes from ischemia/reperfusion-induced cell death.** Since catalytic PKA delayed onset of the MPT in isolated mitochondria, we assessed whether PKA activation by membrane permeant cAMP analogs protects hepatocytes against MPT-dependent I/R injury. I/R was simulated by anoxia at pH 6.2 for 4 h followed by reoxygenation at 7.4, as described (29). After reperfusion at pH 7.4, cell death
detected by propidium iodide fluorometry increased progressively to about 60% after 2 h (Fig. 6A, open circle). pH less than 7 inhibits the MPT, and reperfusion at pH 6.2 prevented most cell death, as observed previously (Fig. 6A, filled circle). The membrane permeant PKA activator, 8-pCPT-cAMP, added just prior to reperfusion at pH 7.4 also decreased cell killing in a dose-dependent fashion. After 2 h of reperfusion, cell killing decreased from 61% without 8-pCPT-cAMP to 55%, 35% and 22%, respectively, with 10, 100 and 200 µM 8-pCPT-cAMP (Fig 6A). Since 8-pCPT-cAMP can also activate PKG (45), we assessed sp-cAMP, a more selective PKA activator, for protection against I/R injury. After 2 hours of reperfusion, cell death decreased from 63% without sp-cAMP to 58, 53 and 43%, respectively, with 10, 100 and 200 µM sp-cAMP (Fig. 6B).

Glucagon prevents the mitochondrial permeability transition and cell death after ischemia/reperfusion to hepatocytes. In hepatocytes, glucagon activates adenylate cyclase and increases intracellular cAMP (15). Accordingly, we evaluated the effect of this physiological cAMP-mobilizing agonist on MPT onset and cell death after simulated I/R. To assess the effect of glucagon on MPT onset, the cytosol of hepatocytes was loaded with calcein, a 623 Da solute that does not ordinarily permeate through the mitochondrial inner membrane (41). TMRM was also present to monitor mitochondrial polarization. Confocal microscopy of green-fluorescing calcein at the end of ischemia just prior to reperfusion revealed numerous dark voids in the cytoplasm representing individual mitochondria excluding the fluorophore, whereas mitochondrial TMRM fluorescence was dim indicating depolarization during ischemia (Fig. 7A). After reperfusion, the voids of calcein fluorescence began to fill within 15 min and disappeared entirely after about 30 min. Additionally, mitochondria did not accumulate TMRM, indicating an absence of mitochondrial repolarization. Mitochondrial inner membrane permeabilization and sustained depolarization after reperfusion signified onset of the MPT. Subsequently after inner membrane permeabilization occurred, cell viability was lost in both cells in the field within 45 min, as indicated by loss of virtually all green cytosolic calcein fluorescence and nuclear labeling with propidium iodide (Fig. 7A, far right panels). By contrast, when hepatocytes were treated with glucagon, mitochondrial dark voids did not fill with green calcein fluorescence after reperfusion (Fig. 7B). Instead, mitochondria repolarized in a sustained fashion as shown by uptake of red-fluorescing TMRM and calcein fluorescence was retained in the cytoplasm out to 2 h (Fig. 7B, far right panels). Thus, glucagon prevented MPT onset and cell death after I/R.

Cyclic GMP and cyclic AMP levels after ischemia/reperfusion. Previously, we showed that NO donors protect against I/R injury in hepatocytes (29). To determine changes of cGMP and cAMP after reperfusion with an NO donor, cyclic nucleotides were measured during the first hour of reperfusion in the presence of the NO donor, DETA NONOate. In the first 5 minutes of reperfusion, cGMP increased 400% compared to normoxic incubation and remained elevated through the first hour of reperfusion (Fig. 8A). By contrast, cAMP decreased more than 80% in the first 5 minutes of reperfusion compared to normoxia (Fig. 8B). Subsequently, cAMP recovered partially, but after 60 min
of reperfusion, cAMP was still only 30% of levels in untreated normoxic hepatocytes. Thus, cGMP rather than cAMP is most likely stimulating protein kinase after reperfusion in the presence of NO donors.
DISCUSSION

In previous work, nitric oxide protected hepatocytes from MPT-dependent I/R injury, which led us to hypothesize that a cGMP-activated protein kinase exists in the cytosol which delays onset of the MPT and protects hepatocytes from cell death (29). In the present study, we provide evidence that this cGMP-activated protein kinase is PKA and that PKA in hepatic cytosol acts directly on mitochondria to inhibit MPT onset and protect hepatocytes from cell death after I/R injury.

In our earlier study, ODQ, a soluble guanylate cyclase inhibitor, abrogated NO protection against cell death. This finding implies that NO-stimulated formation of cGMP is in the cytoprotective pathway. In support, Br-cGMP, a cGMP analog, protected hepatocytes from cell death after I/R. Moreover, NO increased cGMP in hepatocytes during reperfusion. In cultured hepatocytes, confocal microscopy showed that both NO and Br-cGMP inhibited the MPT after reperfusion, and in isolated rat liver mitochondria, liver cytosol inhibited onset of the MPT in a cGMP- and ATP-dependent manner, an effect abrogated by the PKG inhibitor, KT5823. Although these findings pointed to PKG as mediating cytoprotection by NO and cGMP, other evidence indicates that hepatocytes do not express any known form of PKG (32). Accordingly, the goal of this investigation was to better characterize the cyclic nucleotide-stimulated kinase activity in liver cytosol that is responsible for inhibiting the MPT. Our evidence is consistent with the conclusion that the cytoprotective kinase is PKA rather than PKG.

Enzymatic and pharmacological experiments indicated the presence of a highly active cyclic nucleotide-dependent protein kinase activity in liver cytosol that inhibited calcium-induced onset of the MPT. Cytosolic extract was prepared by high-speed centrifugation of a homogenate of 3 g of liver in 20 ml of buffer. Assuming an approximate 80% intracellular volume fraction for liver and a 60% volume fraction for cytosol within the intracellular space (37), then our liver cytosolic extract represented a 16-fold dilution of the cytosol. In the presence of cyclic nucleotide and ATP, cytosolic extract inhibited onset of the MPT with a maximum effect at about 100 µg/ml, corresponding to a 400-fold dilution of cytosol (Fig. 1). This titration signifies high kinase activity in the cytosol and implies specific phosphorylation target sites on mitochondria that inhibit MPT onset.

Inhibition of the MPT determined with a swelling assay was confirmed by showing that mitochondrial depolarization and release of calcium associated with the MPT were also blocked by cytosolic extract plus ATP and cyclic nucleotide (Fig. 2). One possible mechanism for inhibition of the calcium-induced MPT is blockade of electrogenic mitochondrial calcium accumulation, as reported for the cytoprotectant and MPT inhibitor, minocycline (52). However, using Fluo-5N as a probe for extramitochondrial calcium, we found that cytosolic extract did not prevent mitochondrial calcium uptake, the necessary first step in the calcium-induced onset of the MPT (Fig. 2).

The ATP requirement for cytosol to inhibit the MPT may indicate that ATP is needed as a regulatory molecule rather than as a phosphate donor. However,
AMPPNP, a nonhydrolyzable analog of ATP, could not replace ATP in promoting cytosol- and cyclic nucleotide-dependent MPT inhibition, which is consistent with a phosphate donor role for ATP in the inhibition (Fig. 3).

In ATP dose-response experiments, the observed half maximal ATP concentration to inhibit the MPT in the presence of cytosolic extract and cyclic nucleotide was between 10 and 20 µM. The $K_m$ for ATP by PKA and PKG is 3.1 µM and 7.1 µM, respectively (11), which are both close to values observed in the MPT assay. A likely reason that more ATP was needed to inhibit the MPT is that both mitochondria and cytosol possess ATPases that decrease the effective concentration of ATP.

Previously, the PKG inhibitor, KT-5823, was shown to abrogate cyclic nucleotide- and cytosol-dependent protection against the MPT (29). A possible role for PKG in blocking the MPT is also supported in the present work by the observation that purified recombinant PKG-I α delayed onset of the calcium-induced MPT (Fig. 5). Nonetheless, recent reports question the specificity and use of KT-5823 for PKG (1;14;53). Furthermore, northern blots and PCR of liver mRNA fail to reveal expression of any known form of PKG (PKG Iα/β or PKG II) (32;50). Additionally, in the present work, the highly specific PKG I peptide inhibitor (DT-3; $K_i = 25$ nM) (10) did not block cytosol-dependent inhibition of the MPT (Fig. 4). Thus, PKG is most likely not the kinase in liver cytosol that delays onset of the MPT.

Since KT-5823, a staurosporine analog, inhibits a number of protein kinases, including PKA (1;5;8;23), we examined the effects of highly specific peptide inhibitors of PKA and PKG to address further the possible involvement of PKG and PKA in cytosol-dependent inhibition of the MPT. PKI, a peptide inhibitor of PKA (16), prevented protection against the MPT by cytosol, cyclic nucleotide and ATP, whereas DT-3, a PKG I inhibitory peptide (10) did not (Fig. 4). PKI and DT-3 were chosen because they are widely recognized to be potent and extremely specific inhibitors of their respective kinases with no cross inhibition. These findings indicated that PKA and not PKG is the likely effector kinase responsible for the cytosol-induced delay of the MPT.

To characterize protein kinase activity in liver cytosolic extracts further, gel filtration was performed on a calibrated S-200 column. Using Kemptide as a substrate, enriched cGMP/cAMP-stimulated protein kinase activity was identified in fractions eluting at apparent molecular weights between 170 and 200 kDa. Kinase activity was stimulated by both cGMP and cAMP analogs but was inhibited by PKI, the PKA inhibitor, and not by DT-3, the PKG inhibitor (Table I). This finding is consistent with previous reports that both cAMP and cGMP are capable in activating PKA (36). The apparent molecular weight of 170-200 kDa for this PKA activity is also consistent with the expected size of the inactive PKA tetramer, composed of two catalytic (41 kDa) and two regulatory (45 kDa) subunits with a total molecular weight of 172 kDa (13).

Fractions eluting between 170 and 200 kDa were pooled, concentrated and assessed in the MPT swelling assay. The pooled fractions in the presence of cGMP delayed onset of the MPT (Fig. 4D trace b), an effect abrogated by PKI (Fig. 4D trace c). Taken together, these experiments support the conclusion that
PKA activity in liver cytosol underlies cyclic nucleotide-dependent inhibition of the MPT. The molecular weight of this activity also would appear to rule out contributions to MPT inhibition by MAP kinases and JNK kinases whose molecular weights are less than 50 kDa (9;47).

To address if additional cytosolic proteins are required for PKA-dependent inhibition the calcium-induced MPT, we evaluated the effects of the recombinant purified, constitutively active catalytic subunit of PKA (Fig. 5A). Like cytosolic extract, the catalytic subunit of PKA delayed onset of the calcium-induced MPT in a dose-dependent fashion (Fig. 5A, traces e and f). ATP was required for this PKA catalytic subunit-dependent inhibition (Fig. 5A, trace c). This evidence indicates that PKA acts directly on mitochondria rather than by a cascade of reactions involving other cytosolic proteins.

While PT pore components remain in dispute, proposed models often include VDAC as one of the components (20). However, recent work shows that the calcium-induced MPT still occurs in VDAC deficient mitochondria (31). Although VDAC may not be an integral part of PT pores, VDAC may still regulate MPT onset. In support of this hypothesis, we found that VDAC1 was present in the major phosphorylated 31 kDa band separated by PAGE from purified outer membranes after exposure to PKA, as determined by MALDI-TOF mass spectrometry fingerprint analysis. Previous work has also shown that purified VDAC1 is a substrate for PKA in vitro (2), that PKA phosphorylation of VDAC blocks or inhibits association of VDAC with other proteins, such as Bax and tBid, and that PKA-dependent VDAC phosphorylation decreases VDAC conductance (2). Since association of Bax and tBid with mitochondria promotes cell death signaling, decreased Bax and tBid association with mitochondria may represent another mechanism by which PKA activation is protective during reperfusion. Nonetheless, a protein of similar molecular weight other than VDAC may be the real target of PKA. For example, a 32 kDa protein in VDAC deficient mitochondria appears to be the target of MPT inhibition by Ro 68-3400 (31).

NO administered during reperfusion of ischemic hepatocytes increases cGMP, an effect blocked by ODQ, a guanylate cyclase inhibitor (29). However, PKA activation might also occur due to increased cAMP after reperfusion with NO donors. To address which cyclic nucleotide, cGMP or cAMP, activates PKA after NO-treatment during reperfusion, cAMP and cGMP were measured during the first hour of reperfusion in NO-treated hepatocytes (Fig. 7A). Whereas cGMP increased more than 4-fold compared to normoxic controls, cAMP decreased 4-fold. These results suggest that decreased cAMP with a consequent decrease of PKA activity may be a factor promoting MPT onset during reperfusion, which NO-dependent cGMP formation acts to counteract. In support of this concept, the membrane-permeant cAMP agonists, 8-pCPT-cAMP and sp-cAMP, deceased cell killing after reperfusion dose-dependently (Fig. 6). Similarly, the cAMP-mobilizing hormone glucagon also protected against cell killing after I/R, and protection was associated with prevention of the MPT in the reperfused hepatocytes (Fig. 7). These data are consistent with the conclusion that PKA activation by cAMP and its analogs protect hepatocytes from I/R injury.
Other reports show cGMP activation of PKA both in vivo (12;33) and in vitro (34). Elevated cGMP may activate PKA due to the degeneracy of the PKA-cyclic nucleotide binding sites (13). In the present study, cGMP increased kinase activity of pooled FPLC fractions of cytosol with half-maximal stimulation at approximately 3 µM (Fig. 4C). This activation by cGMP is similar to that previously described for cGMP-activation of type II PKA (34), which is expressed in the liver (48).

Other protein kinases that may regulate onset of the MPT after I/R include protein kinase C epsilon (PKCε), glycogen synthase kinase-3 beta (GSK-3β) and c-jun nuclear kinase (JNK). PKCε is a cytosolic serine/threonine kinase that translocates to mitochondria to delay onset of the MPT, whereas GSK-3β and JNK, particularly the JNK-2 isoform, promote the MPT (26;51;54). However, these protein kinases are insensitive to PKI and KT5823 and have molecular weights well below the 170-200 kDa range of the protein kinase identified in liver cytosolic extract that causes cyclic nucleotide-dependent inhibition of MPT onset. Thus, the kinase in liver cytosol that modulates onset of the MPT in the presence of cGMP and cAMP is unlikely to be PKCε, GSK-3β or JNK-2.

In conclusion, PKA activation by cGMP and cAMP at reperfusion protects ischemic hepatocytes from MPT-dependent cell death after reperfusion. Protection by PKA seems to be a direct effect on mitochondria that does not require other proteins and is not related to inhibition of mitochondrial calcium uptake. Specific PKA peptide inhibitors like PKI, but not PKG peptide inhibitors, block protection. The molecular targets of phosphorylation by PKA that confer cytoprotection remain to be determined. Future progress in identifying critical PKA phosphorylation sites for cytoprotection may lead to a better understanding of the exact molecular composition of PT pores.

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FIGURE LEGENDS

Fig. 1. Inhibition of the mitochondrial permeability transition by liver cytosol. Mitochondria were incubated in swelling buffer in the presence of ATP (20 µM), 8-pCPT-cGMP (100 µM) and 0 (a,b), 25 µg/ml (c), 50 µg/ml (d), 100 µg/ml (e) of liver cytosol, as described in Materials and Methods. In traces b-e, 250 µM Ca²⁺ was added at the arrow. Absorbance at 540 nm was measured. Data are representative of at least three separate experiments.

Fig. 2. Inhibition of the mitochondrial permeability transition requires hydrolysable ATP. Mitochondria were incubated in swelling buffer plus 8-pCPT-cGMP (100 µM), as described for Fig. 1, and absorbance was measured after addition of 250 µM Ca²⁺ (arrows). In A, additions to the incubation medium were (a) none, (b) cytosol (60 µg/ml), (c) 10 µM ATP and cytosol (60 µg/ml), (d) 20 µM ATP, (e) 20 µM ATP and cytosol (60 µg/ml). In B, additions were: (a) none, (b) ATP (25 µM), (c) liver cytosol (60 µg/ml), (d) ATP (25 µM) and cytosol, (e) cytosol and AMPPNP (25 µM), and (f) cytosol and AMPPNP (500 µM).

Fig. 3. Lack of inhibition mitochondrial calcium uptake by liver cytosol. Mitochondria were incubated in the presence of Fluo5N (1 µM), and TMRM (1 µM) in swelling buffer, as described in the Materials and Methods, before addition of 250 µM Ca²⁺ (arrows). Green fluorescence of Fluo5N (A), red fluorescence of TMRM (B) and absorbance at 540nm (C) were then measured.

Fig. 4. cGMP-stimulated PKA-like activities in cytosol and fractionated cytosol. (A) Isolated mitochondria were incubated in swelling buffer with 8-pCPT-cGMP (100 µM) and ATP (25 µM), and absorbance was measured, as described in Fig. 1. Other additions to the incubation buffer were (a,b) none, (c) cytosol (60 µg/ml), (d) cytosol plus PKI (10 µM) and (e) cytosol plus DT-3 (1 µM). In b-e, 250 µM Ca²⁺ was added at the arrow. (B) Liver cytosol was loaded onto an S-200 column and fractionated by FPLC, as described in Materials and Methods. Shown are kinase activities from 10 µl of each fraction measured in the presence of 5 µM sp-cAMP (dotted line), 5 µM sp-cGMP (solid line) or no activator (dashed line). (C) Phosphorylation of Kemptide substrate (30 µM) was measured in the presence of 6.4 µg of the pooled fractions 14-17 in the presence of 0-10 µM cGMP and 500 µM IBMX, as described in Materials and Methods. (D) Mitochondria were incubated with 100 µM 8-pCPT-cGMP and 30 µM Mg-ATP, as described in A, and absorbance was measured. Additions to the incubation buffer were (a) none, (b) concentrated fractions 14-17 (20 µg/ml), (c) concentrated fraction plus 4 µM PKI, and (d) concentrated fraction plus 1 µM DT-3. In b-d, 250 µM Ca²⁺ was added at the arrow.

Fig. 5. Direct effect of the catalytic subunit of PKA on the mitochondrial permeability transition and phosphorylation of mitochondrial proteins. (A) Mitochondrial swelling was measured by absorbance during incubation in swelling buffer, as described in MATERIALS AND METHODS. Additions were none (trace a), 250 µM Ca²⁺ (arrow) plus 25 µM ATP (trace b), 250 µM Ca²⁺ plus 17 units/µL PKA (trace d), 250 µM Ca²⁺, 25 µM ATP and 8 units/µL PKA (trace e), 250 µM Ca²⁺, 25 µM ATP and 17 units/µL PKA (trace f). (B) Mitochondrial swelling was measured with additions of none (trace a), 250 µM Ca²⁺ (arrow) plus 25 µM ATP (trace b), and 250 µM Ca²⁺, 25 µM ATP and 20
units/µL PKG (trace c). (C) Autoradiograms of phosphorylated proteins after incubation of 30 µM γ-labeled ATP were prepared with intact mitochondria (lane 1), mitochondria plus 17 units/µL catalytic PKA (lane 2), and PKA alone (lane 3), as described in MATERIALS AND METHODS. (D) Autoradiograms were prepared after incubation of mitochondrial outer membranes with γ-labeled ATP plus no further addition (lane 1), 17 units/µL catalytic PKA (lane 2), and PKA and 4 µM PKI (lane 3).

Fig. 6. Protection by cAMP analogs against cell death after simulated ischemia/reperfusion injury. Hepatocytes were incubated in anaerobic KRH buffer at pH 6.2 for 4 h to simulate ischemia. (A) Hepatocytes were reoxygenated with aerobic KRH at pH 6.2 (closed circles) or at pH 7.4 in the presence of 0 (open circles), 10 µM (upright triangles), 100 µM (squares), and 200 µM (inverted triangles) pCPT-cAMP, as described in Materials and Methods. Error bars represent SEM. (B) Hepatocytes were reoxygenated with aerobic KRH at pH 6.2 (closed circles) and at pH 7.4 in the presence of 0 (open circles), 10 µM (upright triangles), 100 µM (squares), and 200 µM (inverted triangles) sp-cAMP. Values are from 4 or more hepatocyte isolations for each group.

Fig. 7. Mitochondrial inner membrane permeabilization and depolarization after ischemia-reperfusion of hepatocytes: protection by glucagon. Overnight cultured rat hepatocytes were subjected to 4 h of anoxia at pH 6.2 to simulate ischemia followed by reoxygenation at pH 7.4 to simulate reperfusion, as described in Materials and Methods. Hepatocytes were ester-loaded with green-fluorescing calcein into the cytosol to monitor inner membrane permeability and reperfused with red-fluorescing TMRM and propidium iodide to monitor mitochondrial polarization and loss of cell viability, respectively. In A, glucagon (10 nM) was added 15 minutes prior to and then continuously after reperfusion. In A, note that mitochondrial voids in the green calcein fluorescence filled after reperfusion, which was followed by loss of nearly all cytoplasmic calcein fluorescence and nuclear labeling with propidium iodide after 40 to 45 min (arrows). Mitochondria did not take up TMRM. In panels marked “3x”, red fluorescence intensity was upwardly rescaled by a factor of 3 to illustrate better nuclear labeling with propidium iodide and the absence of mitochondrial TMRM uptake. In B in the presence glucagon, mitochondrial voids of calcein fluorescence were not lost, and mitochondria rapidly accumulated TMRM. Nuclear labeling with propidium iodide and loss of cytoplasmic calcein fluorescence did not occur even after 120 min of reperfusion. Each experiment is representative of 3 or more replicates.

Fig. 8. Cyclic AMP and cyclic GMP levels in NO-treated hepatocytes following simulated ischemia/reperfusion injury. Hepatocytes were incubated in anaerobic KRH buffer at pH 6.2 for 4 h to simulate ischemia. At reperfusion the hepatocytes were treated with DETA NONOate, and cAMP (A) and cGMP (B) were measured, as described in the Materials and Methods. Values represent the average of three separate cultures of 1 x 10⁶ hepatocytes.
Table I. Cyclic nucleotide-stimulated kinase activity and inhibitor sensitivity of pooled FPLC fractions 14-17. Liver cytosol was fractionated by FPLC, as described in Materials and Methods. As indicated, kinase activity of pooled fractions 14-17 was measured in the presence of 5 µM sp-cAMP or 5 µM sp-cGMP with and without 4 µM PKI and 1 µM DT-3. Activity (± S.E.M.) is expressed as the multiple of activity in the presence of added nucleotide only. *, p<0.001.

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<th>Nucleotide</th>
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<th>DT-3</th>
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<tr>
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<td>16.8 ± 0.91</td>
<td>0.98 ± 0.07*</td>
<td>17.1 ± 0.70</td>
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