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Atrial Natriuretic Peptide Attenuates Elevations in Ca^{2+} and Protects Hepatocytes by Stimulating Net Plasma Membrane Ca^{2+} Efflux*

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Elevations in intracellular Ca^{2+} concentration and calpain activity are common early events in cellular injury, including that of hepatocytes. Atrial natriuretic peptide is a circulating hormone that has been shown to be hepatoprotective. The aim of this study was to examine the effects of atrial natriuretic peptide on potentially harmful elevations in cytosolic free Ca^{2+} and calpain activity induced by extracellular ATP in rat hepatocytes. We show that atrial natriuretic peptide, through protein kinase G, attenuated both the amplitude and duration of ATP-induced cytosolic Ca^{2+} rises in single hepatocytes. Atrial natriuretic peptide also prevented stimulation of calpain activity by ATP, taurolithocholate, or Ca^{2+} mobilization by thapsigargin and ionomycin. We therefore investigated the cellular Ca^{2+} handling mechanisms through which ANP attenuates this sustained elevation in cytosolic Ca^{2+}. We show that atrial natriuretic peptide does not modulate the release from or re-uptake of Ca^{2+} into intracellular stores but, through protein kinase G, both stimulates plasma membrane Ca^{2+} efflux from and inhibits ATP-stimulated Ca^{2+} influx into hepatocytes. These findings suggest that stimulation of net plasma membrane Ca^{2+} efflux (to which both Ca^{2+} efflux stimulation and Ca^{2+} influx inhibition contribute) is the key process through which atrial natriuretic peptide attenuates elevations in cytosolic Ca^{2+} and calpain activity. Moreover we propose that plasma membrane Ca^{2+} efflux is a valuable, previously undiscovered, mechanism through which atrial natriuretic peptide protects rat hepatocytes, and perhaps other cell types, against Ca^{2+}-dependent injury.

A rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a common early event in cellular injury (1–6). Harmful increases in $[Ca^{2+}]_i$ can result from increased Ca^{2+} influx, sustained Ca^{2+} release from intracellular stores, or a reduced rate of Ca^{2+} extrusion across the plasma membrane (1, 7–8).

In hepatocytes, increases in $[Ca^{2+}]_i$ are associated with cytotoxicity (9) arising from diverse agents such as carbon tetrachloride (10, 11), paracetamol (12–14), extracellular ATP (15–19), and hydrophobic bile acids (20). Elevated $[Ca^{2+}]_i$ also injures hepatocytes in several pathophysiological liver conditions, including ischemia-reperfusion injury (6, 21), endotoxaemia (22), and hemorrhagic shock (23).

The $[Ca^{2+}]_i$ rise activates Ca^{2+}-dependent enzymes including calpain, a neutral cysteine protease (24) that plays a key role in initiation of cellular injury and subsequent necrosis or apoptosis (6, 25–30). In hepatocytes, calpain promotes hepatocyte plasma membrane blebbing (31), induces the mitochondrial permeability transition in hepatocyte necrosis (32), and contributes to hepatocyte necrosis in anoxia (33). Calpain has been implicated in hepatotoxicity in ischemia-reperfusion injury (34, 35), hemorrhagic shock (36), and bile acid toxicity (20).

In the search for cytoprotective agents and therapeutic interventions, those which suppress injurious $[Ca^{2+}]_i$ rises are considered highly promising (2–6, 30). Natriuretic peptides attenuate elevations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) in many cell types (37–50), including rat hepatocytes (51). Atrial natriuretic peptide (ANP)[3] is a circulating hormone released mainly by atrial myocytes (52) in response to stress conditions such as volume expansion or cardiac hypoxia. In addition to its hypotensive, vasodilatory, and natriuretic effects in the cardiovascular and renal systems (53), increasing evidence supports a more widespread role for ANP in several other organs (54), including the liver. ANP elevates cGMP in rat hepatocytes (51, 55–56) through the guanylyl cyclase A receptor (57), and is cytoprotective in both isolated hepatocytes (51, 55, 58) and perfused liver (57, 59–62). An early preliminary study showed that ANP attenuated both oxidant-induced cell injury and the accompanying $[Ca^{2+}]_i$ rise (51), suggesting that the hepatoprotective effects of ANP may be mediated by mechanisms involved in Ca^{2+} homeostasis (51, 55, 57). We have since examined the effects of ANP on *physiological* alterations in $[Ca^{2+}]_c$; we showed that ANP, through protein kinase G (PKG), attenuates $[Ca^{2+}]_c$ oscillations, dramatically increases the *basal* rate of plasma membrane Ca^{2+} efflux and modestly inhibits *basal* Ca^{2+} influx in rat hepatocytes (56).

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[3] The abbreviations used are: ANP, atrial natriuretic peptide; $[Ca^{2+}]_c$, cytosolic concentration of free Ca^{2+}; PKG, protein kinase G; TLC, taurolithocholate; SNP, sodium nitroprusside; WME, Williams' medium E; AVP, [Arg8]vasopressin; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis acetoxymethyl ester; R_p-8-*p*CPT-cGMPS, 8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphorothioate; Boc-Leu-Met-CMAC, *t*-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin; ECB, extracellular buffer; PBS, phosphate-buffered saline; PMCA, plasma membrane Ca^{2+}-ATPase; PIPES, 1,4-piperazinediethanesulfonic acid.

Although it has been demonstrated that ANP prevents oxidant-induced rises in hepatocyte $[Ca^{2+}]_i$ (51), how ANP regulates *pathophysiological* elevations in hepatocyte $[Ca^{2+}]_c$ has been very little studied. Cellular Ca^{2+} homeostasis is maintained through a complex interaction of Ca^{2+} fluxes between the cytosol, the intracellular stores of the endoplasmic reticulum and mitochondria and the extracellular milieu. A single early study based on $^{45}Ca^{2+}$ flux measurements suggested the involvement of a decrease in Ca^{2+} influx in the hepatoprotective effect of ANP (55). However, this was not a true uni-directional measurement of Ca^{2+} influx and possible effects of ANP on other Ca^{2+} handling mechanisms were not investigated. Further investigation of the effects of ANP on pathophysiological $[Ca^{2+}]_c$ and Ca^{2+} fluxes is therefore warranted.

Here we examine the effects of ANP on sustained rises in both cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) and calpain activity and reduction in cell viability induced by extracellular ATP in single rat hepatocytes. ATP is released from cells, including hepatocytes, in response to stress, anoxia, or injury (15, 63). At low, close-to-physiological, concentrations, extracellular ATP serves as a signaling molecule, inducing oscillations in cytosolic concentration of free Ca^{2+} ($[Ca^{2+}]_c$) (56, 64, 65) and modulating physiological and metabolic processes in hepatocytes (66). In contrast, ATP at higher concentrations induces sustained elevations in $[Ca^{2+}]_i$ (16–18, 64) that can lead to hepatocyte injury (18, 19) and death by necrosis and apoptosis (15–17). Ca^{2+}-mediated injury to hepatocytes by extracellular ATP has been revealed by calpain activation (18) and plasma membrane blebbing (19), both early events in the initiation of cell injury (6). Rosser *et al.* (18) have shown that ATP increases hepatocyte calpain activity concomitantly with an increase in $[Ca^{2+}]_i$, suggesting that ATP stimulates calpain activity through elevation of $[Ca^{2+}]_i$.

We show here that ANP attenuates ATP-induced sustained rises in $[Ca^{2+}]_c$. We also show that ANP protects hepatocytes against elevations in calpain activity and decreases in cell viability mediated by both ATP, and the hydrophobic bile acid, taurolithocholate (TLC). We therefore investigate the cellular Ca^{2+} handling mechanisms through which ANP attenuates the sustained elevation in $[Ca^{2+}]_c$. We show that ANP does not modulate the release from or re-uptake of Ca^{2+} into intracellular stores but, through PKG, stimulates plasma membrane Ca^{2+} efflux from and inhibits ATP-stimulated Ca^{2+} influx into hepatocytes. We therefore propose that stimulation of *net* plasma membrane Ca^{2+} efflux is the key process through which ANP protects hepatocytes against harmful rises in $[Ca^{2+}]_c$.

EXPERIMENTAL PROCEDURES

Materials

Collagenase was from Roche Applied Sciences. Williams' medium E (WME) was from Invitrogen. ATP, [Arg^8]vasopressin (AVP), ANP (rat atrial natriuretic factor 1–28), ionomycin, propidium iodide, sodium taurolithocholate (TLC), digitonin, BAPTA-AM, sodium nitroprusside (SNP), and 8-bromo-cGMP were from Sigma. R_p-8-*p*CPT-cGMPS (TEA salt), thapsigargin, and MDL 28170 were from CN Biosciences. FMC SeaPlaque-agarose and FMC SeaPrep-agarose were from Flowgen Instruments Ltd., Sittingbourne, UK. Fura-2 pentapotassium salt, fura-2 dextran (10 kDa), *t*-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (Boc-Leu-Met-CMAC) and Alexa Fluor 594-conjugated donkey anti-goat IgG (H+L; 2 mg/ml) were from Molecular Probes. Caloxin 1b1 peptide was prepared by custom synthesis by Dalton Pharma Services, Toronto, ON, Canada. Primary anti-PKG antibodies were from Santa Cruz Biotechnology, Inc. Citifluor was from Agar Scientific Ltd., Essex, UK. All other chemicals (VWR International Ltd., Lutterworth, UK) were of the purest grade available. For preparation of the efflux medium, ultrapure water was prepared using the Milli-Q RG system (Millipore).

Hepatocytes

Single hepatocytes were isolated from 150 to 250-g male Wistar rats by collagenase digestion as described previously (67). For all experiments on populations of hepatocytes, cells were counted using a hemocytometer then stored in suspension in WME on ice.

Measurement of cGMP

Isolated rat hepatocytes, at a cell density of 10^6/ml, were incubated in suspension in extracellular buffer (ECB) of composition: 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 1.8 mM $CaCl_2$, 11 mM glucose, pH 7.2, in a shaking water bath at 37 °C. ATP and ANP were added for the periods indicated. Cyclic GMP levels were measured using the acetylation method in a cGMP enzyme immunoassay system (Biotrak assays, GE Healthcare).

Measurement of $[Ca^{2+}]_c$ in Single Hepatocytes

Preparation of Single Cells for Microinjection—After harvesting, the hepatocytes were incubated at 37 °C at low density (~10^3 cells/ml) in 1.7% SeaPrep-agarose in WME. Single hepatocytes were transferred to 0.1-mm path length microslides containing 1.2% FMC SeaPlaque-agarose in WME, which was subsequently gelled at 4 °C for 2 min. The cells were then held at 37 °C under a layer of liquid paraffin.

Fura-2 Dextran Preparation—Fura-2 dextran (10 kDa) was dissolved in (in mM): KCl 150, PIPES 1, pH 7.2, to make a 5 mM stock solution that was stored at −70 °C. For microinjection, a small aliquot of 5 mM fura-2 dextran was held as a droplet under liquid paraffin.

Microinjection and Data Acquisition—Freshly pulled pipettes were filled with fura-2 dextran by dipping the tip for a few seconds in the fura-2 dextran droplet. Individual hepatocytes were injected to ~0.5% of the cell volume as described previously (68). The fura-2 dextran, thus localized in the cytoplasmic compartment, reports $[Ca^{2+}]_c$ only. Injected cells of healthy appearance were transferred within microslides to a heated (37 °C) stainless steel perfusion chamber on the stage of a Nikon Diaphot inverted epifluorescence microscope. Cells were superfused with ECB to which ATP, ANP, R_p-8-*p*CPT-cGMPS, and AVP were added as indicated. Cells were alternately excited at 340- and 380-nm wavelengths by means of a PTI D101 dual excitation light source. The 510-nm emission was detected by an ICCD camera (Photonic Science). $[Ca^{2+}]_c$ was calculated by calibrating the fura-2 signal *in situ* as described previously (69).

Measurement of Calpain Activity in Hepatocytes

Calpain activity of intact rat hepatocytes in suspension was measured by the rate of generation of the fluorescent cleavage

product of the calpain substrate Boc-Leu-Met-CMAC according to a method modified from those of Rosser *et al.* (18) and Han *et al.* (70). Boc-Leu-Met-CMAC is a non-fluorescent, membrane-permeant compound that diffuses readily into hepatocytes, where it is conjugated to glutathione. Intracellular calpain activity cleaves the bond between the methionine and the MAC-thiol groups, liberating and unquenching the highly fluorescent, membrane-impermeant MAC-thiol moiety within the cell. The slope of the fluorescent change with respect to time represents the intracellular calpain activity (18, 70). Before each assay, ~10^5 cells were washed 3 times in ECB. The cells were then resuspended in 1 ml of ECB at 37 °C, to which Boc-Leu-Met-CMAC (20 μM final concentration) was added. Fluorescence was measured, at 37 °C with constant stirring, using a PTI DeltaRam spectrofluorimeter, exciting at 380 nm and collecting emission at 460 nm.

Cell Viability

Cell viability was determined by propidium iodide fluorescence according to a method modified from that of Gores *et al.* (71). Before each assay, ~10^5 cells were washed 3 times in ECB. The cells were then incubated with 1 μM propidium iodide in ECB containing 0.2% bovine serum albumin for 10 min at 37 °C. 1 ml of the cell suspension was transferred to a fluorimeter cuvette and fluorescence was measured continuously (at 37 °C with constant stirring) using a PTI DeltaRam spectrofluorimeter, exciting at 515 nm and collecting emission at 610 nm. Agonists were added after 5 min (F_0) and fluorescence was recorded for a further 15 min (F_{15}). At the end of each experiment, digitonin (375 μM final concentration) was added to allow propidium iodide to enter cells and, after 20 min, produce maximum fluorescence (F_{max}). The % non-viable cells after 15 min agonist exposure was calculated using equation: % non-viable cells = $100 \times (F_{15} - F_0)/(F_{max} - F_0)$.

Measurement of Ca^{2+} Efflux from Hepatocytes

Before each assay, ~10^6 cells were washed 3 times in ice-cold efflux medium, of composition: 150 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 11 mM glucose, pH 7.2. Ca^{2+} efflux from the intact rat hepatocytes was monitored by measuring the concentration of Ca^{2+} in the extracellular medium ($[Ca^{2+}]_o$) using fura-2 as extracellular Ca^{2+} reporter. Thus the cells were resuspended in 1 ml of efflux medium at 37 °C, to which the membrane-impermeant pentapotassium salt of fura-2 (10 μM final concentration) was added. Fluorescence was measured at 37 °C with constant stirring, using a PTI DeltaRam spectrofluorimeter, exciting alternately at 340 and 380 nm and collecting emission at 510 nm. $[Ca^{2+}]_o$ was calculated by calibrating the photometric 340/380 nm ratio signal as described previously (56). $[Ca^{2+}]_o$ never rose above 1.5 μM, ensuring a true, unidirectional, Ca^{2+} efflux measurement.

Measurement of Mn^{2+} Influx into Single Hepatocytes

Single hepatocytes, microinjected with fura-2 dextran (10 kDa), were superfused with 50 μM $MnCl_2$ in ECB to which ATP, ANP, and R_p-8-pCPT-cGMPS were added as indicated. The fura-2 dextran, thus localized in the cytoplasmic compartment, reports Mn^{2+} influx into the cytosol only. Cells were alternately excited at 340 and 359 nm wavelengths by means of a PTI D101 dual excitation light source. The 510-nm emission was detected by an ICCD camera (Photonic Science).

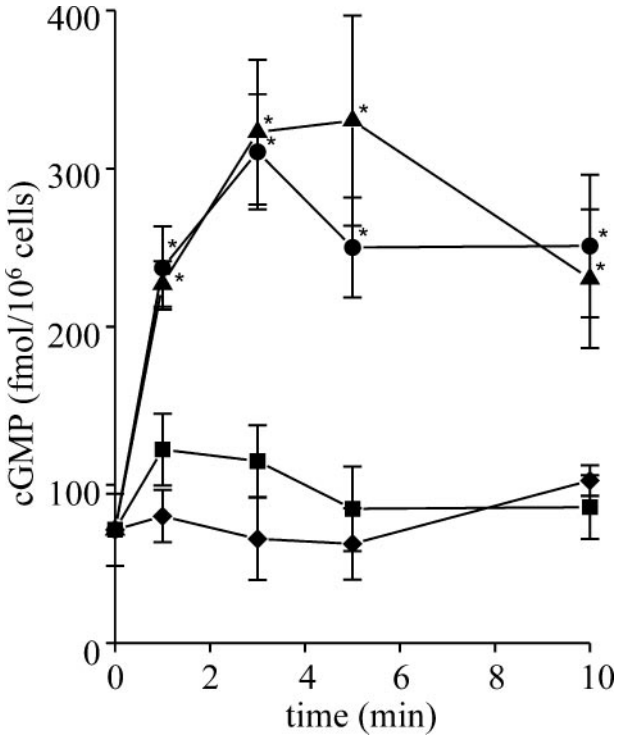

FIGURE 1. **ANP stimulates cGMP production in rat hepatocytes.** Isolated rat hepatocytes were incubated at 37 °C in the presence of no treatment (■), 25 μM ATP (◆), 200 nM ANP (▲), or 25 μM ATP + 200 nM ANP (●) for the periods indicated. Total cGMP levels were measured by enzyme immunoassay. *, significantly different from both control (no treatment) and ATP-treated hepatocytes at each corresponding time point.

Immunocytochemistry

Primary Antibodies—The following primary anti-PKG antibodies (Santa Cruz) were used: cGKIα (N-16), goat polyclonal, targeted against a 16-amino acid sequence near the N terminus of PKG Iα; cGKIβ (L-16), goat polyclonal, targeted against a 16-amino acid sequence near the N terminus of PKG Iβ; cGKIα/β (T-19), goat polyclonal, targeted against a 19-amino acid sequence near the C terminus of PKG Iα and PKG Iβ; cGKII (T-18), goat polyclonal, targeted against a 18-amino acid sequence near the N terminus of PKG II.

Immunolabeling—Hepatocytes were allowed to attach to glass coverslips for 6 and 24 h in WME at 37 °C and then fixed in 4% paraformaldehyde. Nonspecific binding was prevented by blocking for 1 h at 25 °C in phosphate-buffered saline (PBS) + 0.01% Triton X-100 containing 10% normal rabbit serum. Following three 5-min washes with PBS, cells were incubated at 4 °C overnight with the corresponding primary antibody diluted (1:100) in PBS + 0.01% Triton X-100. Following antibody incubation, cells were washed with PBS and then incubated in Alexa Fluor 594-conjugated donkey anti-goat IgG (H+L) for 2 h at 25 °C. The secondary antibody was diluted 1:1000 in PBS + 0.01% Triton X-100. Cells were then washed 3 times for 10 min in PBS and coverslips were mounted in citifluor (glycerol/PBS solution) on glass slides. Cells were examined under oil immersion (×63) using a Leica DMRE upright laser scanning confocal microscope with a TCS SP2 scan head.

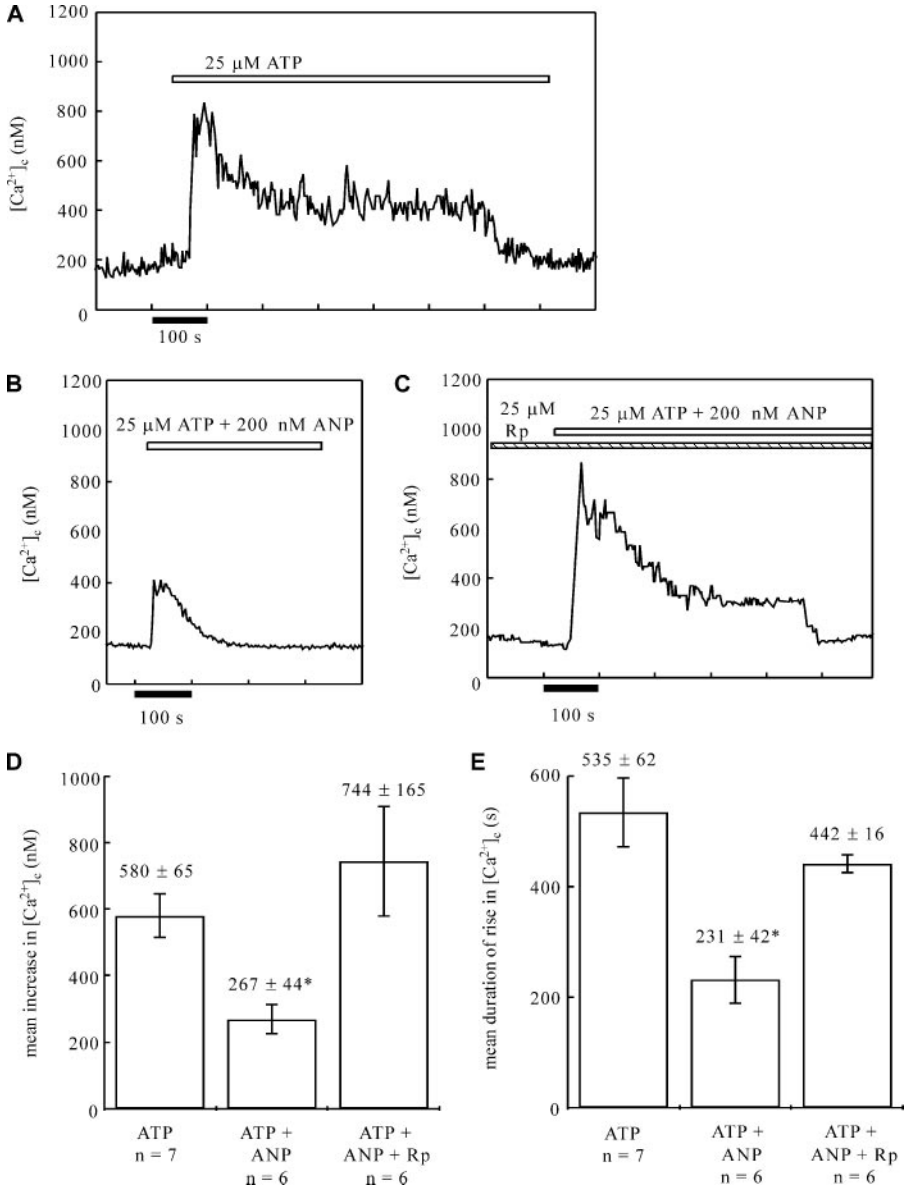

FIGURE 2. **ANP attenuates ATP-induced $[Ca^{2+}]_c$ rises in single rat hepatocytes.** Single, fura-2 dextran-injected rat hepatocytes responding to 25 μM ATP (*A*) with a rise in $[Ca^{2+}]_c$ were co-supplied with 200 nM ANP (*B*) or 25 μM R_p-8-*p*CPT-cGMPS (*Rp*) and 200 nM ANP (*C*) for the periods indicated by the *bars*. The traces shown in *A–C* are representative of several experiments. *D* and *E* show, respectively, the calculated mean increase in amplitude (nM) above resting $[Ca^{2+}]_c$ and the duration (*s*) of the $[Ca^{2+}]_c$ rise above resting $[Ca^{2+}]_c$ for each treatment. Calculated values are shown *above* each corresponding column. *, significantly different from control.

Statistics

Differences between means were compared by Student's *t* test using a level of significance of $p < 0.05$. Data were expressed as mean ± S.E. All experiments are from at least three independent hepatocyte preparations.

RESULTS

ANP Stimulates cGMP Production in ATP-treated Rat Hepatocytes—Fig. 1 shows that 25 μM ATP does not elevate hepatocyte cGMP levels above those detected in control, untreated rat hepatocytes. ANP (200 nM) stimulates cGMP production in isolated rat hepatocytes both in the presence and absence of ATP.

ANP, through PKG, Attenuates ATP-induced Elevations in $[Ca^{2+}]_c$ in Single Rat Hepatocytes—A single early study showed that ANP prevented oxidant-induced elevation of $[Ca^{2+}]_i$ in *populations* of isolated rat hepatocytes (51). Here we examine the effect of ANP on harmful elevations in $[Ca^{2+}]_c$ in *single* rat hepatocytes. Single rat hepatocytes responded to 25 μM ATP by the elevation of $[Ca^{2+}]_c$ (Fig. 2*A*, representative of 7/7 hepatocytes; Fig. 2, *D* and *E*, mean data). Co-application of 200 nM ANP caused a decrease in both the amplitude and duration of the ATP-induced $[Ca^{2+}]_c$ rise (Fig. 2*B*, representative of 6/6 hepatocytes; Fig. 2, *D* and *E*, mean data). In the presence of the membrane-permeant PKG inhibitor, R_p-8-*p*CPT-cGMPS, however, ANP did not significantly attenuate either the amplitude or duration of the ATP-induced rise in $[Ca^{2+}]_c$ (Fig. 2*C*, representative of 6/6 hepatocytes; Fig. 2, *D* and *E*, mean data). ANP applied alone did not alter resting $[Ca^{2+}]_c$ levels (5/5 hepatocytes; results not shown).

ANP, through PKG, Prevents ATP- and TLC-induced Stimulation of Calpain Activity in Rat Hepatocytes—Rosser *et al.* (18) have shown that extracellular ATP, through a rise in $[Ca^{2+}]_i$, stimulates a concomitant rise in calpain activity in rat hepatocytes. Here we examine whether ANP modulates the ATP- and Ca^{2+}-dependent rise in calpain activity. Calpain activity of intact rat hepatocytes in suspension was measured by the accumulation of the fluorescent proteolytic product of the calpain substrate Boc-Leu-Met-CMAC. Fig. 3*A* shows a typical spectrofluorimetric recording of basal calpain activity following the addition of Boc-Leu-Met-CMAC to hepatocytes in suspension. Following the addition of Boc-Leu-Met-CMAC, there is an initial lag of ~60 s duration before a linear increase in fluorescence intensity is recorded. This observation is consistent with previous studies (18, 70). Following this initial lag, the rate of increase in fluorescent intensity is a measure of intracellular calpain activity (18, 70).

Basal calpain activity, in the absence of any Ca^{2+}-elevating agent (Fig. 3*A*), was found to be consistent with previous estimates (18, 70). Fig. 3*B* shows that addition of ATP (25 μM) stimulated a rapid, mean 1.6-fold (Fig. 3*E*) elevation in

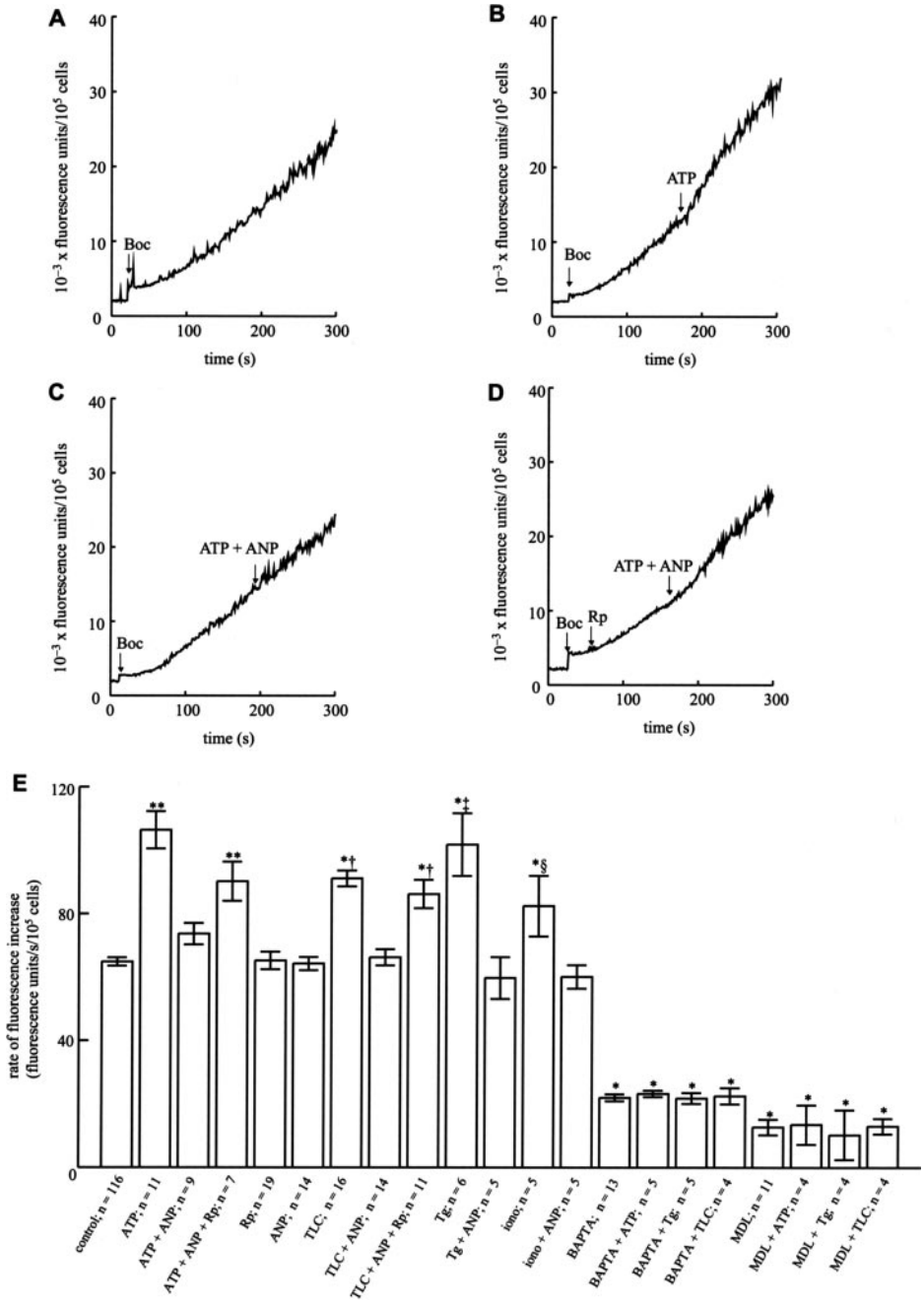

FIGURE 3. **ANP attenuates the ATP-induced elevation in calpain activity in rat hepatocytes.** Calpain activity in populations of intact rat hepatocytes was measured at 37 °C, using the fluorigenic calpain substrate Boc-Leu-Met-CMAC. The *traces* shown in *A–D* are representative of several experiments. 20 μM Boc-Leu-Met-CMAC (*BOC*), 25 μM ATP, 200 nM ANP, or 25 μM R_p-8-*p*CPT-cGMPS (*Rp*) were added where indicated by the *arrows*. *E* shows calculated mean rates of increase in fluorescence (fluorescence units/s/10^5 cells) from several experiments. In the presence of 20 μM Boc-Leu-Met-CMAC, populations of intact rat hepatocytes were exposed to 25 μM ATP, ATP + 200 nM ANP, ATP + ANP + 25 μM R_p-8-*p*CPT-cGMPS (*Rp*), R_p alone, ANP alone, 200 μM TLC, TLC + ANP, TLC + ANP + R_p, 1 μM thapsigargin (*Tg*), Tg + ANP, 2 μM ionomycin (*iono*), iono + ANP; 20 μM BAPTA (30 min preincubation), BAPTA + ATP, BAPTA + Tg, BAPTA + TLC, 20 μM calpain inhibitor MDL 28170 (*MDL*), MDL + ATP, MDL + Tg, and MDL + TLC during continuous spectrofluorimetry. *, significantly different from control; **, significantly different from both control and ATP + ANP; *†, significantly different from both control and TLC + ANP; *‡, significantly different from both control and Tg + ANP; *§, significantly different from both control and iono + ANP.

calpain activity. Co-application of ANP (200 nM) prevented any rise in calpain activity above basal levels (Fig. 3*C*). We also show that the hydrophobic bile acid TLC stimulated a similar elevation in calpain activity (Fig. 3*E*), which was prevented upon co-application of ANP (Fig. 3*E*). TLC elevates hepatocyte Ca^{2+} (72–74) through release from intracellular stores by directly increasing store Ca^{2+} permeability (73).

In the presence of the membrane-permeant PKG inhibitor, R_p-8-*p*CPT-cGMPS (25 μM), however, ANP was without significant effect on either the ATP- (Fig. 3*D*) or TLC-stimulated elevation in calpain activity (Fig. 3*E*). Neither ANP nor R_p-8-*p*CPT-cGMPS applied alone altered basal calpain activity (Fig. 3*E*).

Fig. 3*E* shows that preincubation (30 min) of hepatocytes with the intracellular Ca^{2+} chelator BAPTA-AM (20 μM) inhibited calpain activity to ~35% of basal and prevented an increase in calpain activity in the presence of either ATP or TLC. (Preincubation of hepatocytes for 30 min with the Me_2SO vehicle did not alter basal calpain activity; results not shown.) The endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (1 μM) and the Ca^{2+} ionophore ionomycin (2 μM), well characterized agents that elevate $[Ca^{2+}]_c$, both increased calpain activity in hepatocytes (Fig. 3*E*). The magnitude and the time scale of the ATP-, thapsigargin-, and ionomycin-induced rises in hepatocyte calpain activity observed here are very similar to the findings of Rosser *et al.* (18). These data, taken together, suggest that both the basal calpain proteolytic activity and the elevated proteolysis in the presence of ATP or TLC are dependent on $[Ca^{2+}]_c$.

Fig. 3*E* also shows that, in the added presence of ANP, neither thapsigargin nor ionomycin elevated calpain activity above basal levels. This finding lends further support to our hypothesis that ANP prevents the ATP-induced elevation in calpain activity by attenuating the rise in $[Ca^{2+}]_c$.

The specificity of the assay for calpain activity was assessed by measuring the effect of the membrane-permeant calpain inhibitor MDL 28170, which inhibits calpain activity in many cell types including hepatocytes (75). Fig. 3*E* shows that MDL 28170 (20 μM) inhibited basal generation of the fluorescent proteolysis product to ~20% of basal. MDL 28170 also prevented

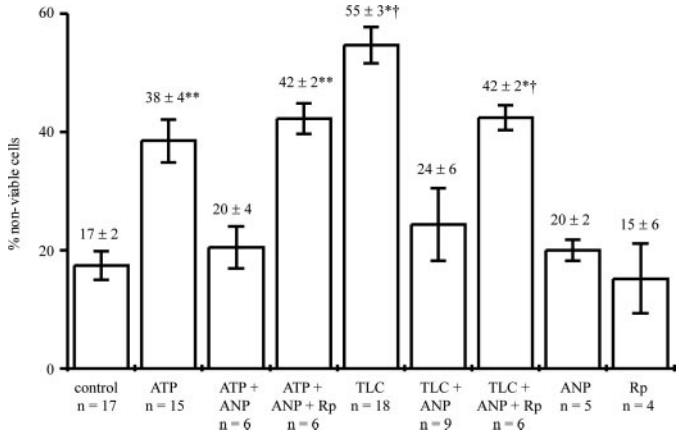

FIGURE 4. **ANP protects hepatocytes from ATP- and TLC-induced cell death.** Cell viability of populations of rat hepatocytes was measured by propidium iodide fluorimetry. In the presence of 1 μM propidium iodide, populations of rat hepatocytes were exposed to 25 μM ATP, ATP + 200 nM ANP, ATP + ANP + 25 μM R_p-8-*p*CPT-cGMPS (*Rp*), 200 μM TLC, TLC + ANP, TLC + ANP + R_p, R_p alone, and ANP alone for 15 min at 37 °C during continuous fluorimetry. The figure shows calculated mean % changes in number of non-viable cells (calculated from changes in propidium iodide fluorescence) from several experiments. The mean values are shown *above* each corresponding column. *, significantly different from control; **, significantly different from both control and ATP + ANP; *†, significantly different from both control and TLC + ANP.

increases in proteolytic activity by ATP, TLC, and thapsigargin (Fig. 3*E*).

ANP, through PKG, Prevents the ATP- and TLC-induced Decline in Hepatocyte Viability—Fig. 4 shows that exposure of hepatocytes to either ATP (25 μM) or TLC (200 μM) for 15 min significantly increased the percentage of non-viable cells in the population. Co-addition of ANP protected hepatocytes; in the presence of ANP, neither ATP nor TLC elevated the % of non-viable hepatocytes above control levels. The PKG inhibitor, R_p-8-*p*CPT-cGMPS, prevented hepatoprotection by ANP; in the presence of R_p-8-*p*CPT-cGMPS (25 μM), ANP was without significant effect on either the ATP- or TLC-induced hepatocyte death. Neither ANP nor R_p-8-*p*CPT-cGMPS alone significantly altered hepatocyte viability compared with control levels. These data suggest that the hepatoprotective effect of ANP is mediated by PKG.

ANP, through PKG, Stimulates Ca^{2+} Efflux from ATP-treated Rat Hepatocytes—As shown in Fig. 5, *A* and *G*, a basal rate of Ca^{2+} efflux was recorded from intact rat hepatocytes in the absence of agonist. This observation is consistent with the findings of our previous studies (56, 76). ATP (25 μM) did not alter the basal rate of Ca^{2+} efflux (Fig. 5, *B* and *G*). Fig. 5, *C* and *G*, show that ANP (200 nM) stimulated a large, approximate 2.7-fold, increase in the rate of Ca^{2+} efflux from ATP-treated hepatocytes. Stimulation of Ca^{2+} efflux by ANP was prevented by the PKG inhibitor, R_p-8-*p*CPT-cGMPS; in populations of hepatocytes to which R_p-8-*p*CPT-cGMPS (25 μM) had previously been added, addition of ANP and ATP did not significantly increase Ca^{2+} efflux above the basal rate (Fig. 5, *D* and *G*). Fig. 5*G* also confirms that R_p-8-*p*CPT-cGMPS did not, itself, alter the basal rate of Ca^{2+} efflux from hepatocytes.

In contrast, but consistent with our previous findings (56), co-addition of ATP and SNP (100 μM), an NO donor that activates soluble guanylyl cyclase and elevates hepatocyte cGMP (56), did not significantly alter the basal rate of Ca^{2+} efflux (Fig. 5*G*). In further agreement with our previous observations, a moderate stimulation of Ca^{2+} efflux was achieved by co-application of ATP and 8-bromo-cGMP (100 μM), a membrane permeant analogue of cGMP (Fig. 5*G*).

Fig. 5, *E* and *G*, confirm that the basal Ca^{2+} efflux recorded here is predominantly due to plasma membrane Ca^{2+}-ATPase (PMCA) activity; caloxin 1b1 peptide, a specific PMCA inhibitor (77), inhibited basal Ca^{2+} efflux by ~65%. This observation is consistent with our previous demonstration, using a similar assay, of basal hepatocyte Ca^{2+} efflux inhibition by the PMCA inhibitors carboxyeosin and mini-glucagon (76). Moreover, neither ATP (Fig. 5*G*), nor ATP and ANP (Fig. 5, *F* and *G*) stimulated Ca^{2+} efflux in the presence of caloxin 1b1 peptide, indicating that the ANP-stimulated Ca^{2+} efflux is PMCA-mediated.

ANP, through PKG, Inhibits ATP-induced Cation Influx into Single Rat Hepatocytes—The quench by Mn^{2+} of cytoplasmic fura-2 fluorescence was used to estimate relative rates of Ca^{2+} influx into single hepatocytes. Fig. 6, *A* and *E*, show that addition of 50 μM Mn^{2+} to single unstimulated hepatocytes promptly caused a quenching of the fura-2 signal at both 340 nm and the Ca^{2+}-insensitive wavelength, 359 nm. This observation of continuous basal Mn^{2+} influx into unstimulated hepatocytes is consistent with previous studies of both our laboratory (56, 69) and others (78, 79).

Fig. 6, *B* and *E*, show that addition of ATP (25 μM) increased the rate of Mn^{2+} influx into single hepatocytes, a finding consistent with previous reports (80). Although we have previously shown that ANP partially inhibits *basal* Mn^{2+} influx into hepatocytes (56), its effect on *ATP-stimulated* Mn^{2+} influx, which occurs through a distinct channel (79, 81), has not previously been examined. Co-addition of ANP (200 nM) completely prevented the stimulation of Mn^{2+} influx by ATP (Fig. 6, *C* and *E*). In the presence of the PKG inhibitor R_p-8-*p*CPT-cGMPS, however, ANP was without effect; Fig. 6, *D* and *E*, show that R_p-8-*p*CPT-cGMPS (25 μM) prevented any inhibition of ATP-stimulated Mn^{2+} influx by ANP.

ANP Does Not Modulate the Release from or Re-uptake of Ca^{2+} into Intracellular Stores—We have shown here that ANP attenuates $[Ca^{2+}]_c$ rises, inhibits ATP-stimulated Ca^{2+} influx, and stimulates Ca^{2+} efflux in the presence of ATP. However, it is not known whether the attenuation of the $[Ca^{2+}]_c$ rise by ANP is entirely attributable to its observed effects on plasma membrane Ca^{2+} fluxes. It is conceivable that ANP may also modulate intracellular Ca^{2+} handling by altering the release from and/or re-uptake of Ca^{2+} into intracellular stores. The aim of this set of experiments was to determine whether, in the absence of plasma membrane Ca^{2+} fluxes, ANP is still effective in modulating the agonist-evoked $[Ca^{2+}]_c$ rise.

ATP could not be used as the Ca^{2+} mobilizing agonist in this set of experiments, because it forms a precipitate with La^{3+}. Instead, hepatocytes were stimulated with 50 nM AVP. AVP and ATP have similar effects on hepatocyte $[Ca^{2+}]_i$; they release the same pool of intracellular Ca^{2+} (82), the $[Ca^{2+}]_c$ response to AVP, like that to ATP, is attenuated by ANP (results not shown), they stimulate Ca^{2+} influx through a common channel (81) and 50 nM AVP, like 25 μM ATP, does not alter basal Ca^{2+}

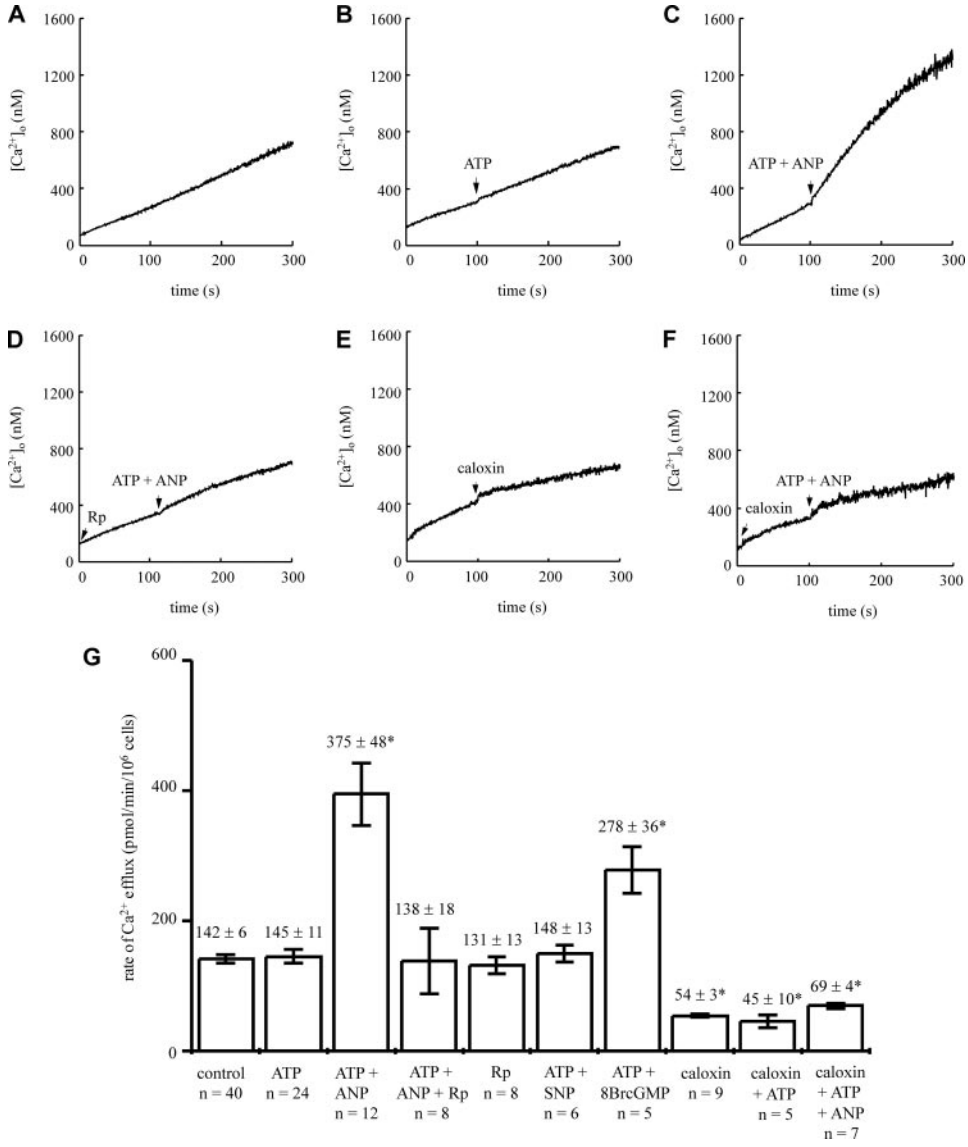

FIGURE 5. **Effects of ANP on the rate of Ca^{2+} efflux from ATP-treated rat hepatocytes.** Ca^{2+} efflux from populations of intact rat hepatocytes was measured at 37 °C, using fura-2 in the extracellular medium. The *traces* shown in *A–F* are representative of several experiments. 25 μM ATP, 200 nM ANP, 25 μM R_p-8-*p*CPT-cGMPS (R_p), or 300 μM caloxin 1b1 (*caloxin*) were added where indicated by the *arrows*. Calculated rates of Ca^{2+} efflux are shown in *G*; the mean Ca^{2+} efflux values are shown *above* each corresponding column. *, significantly different from control.

efflux (results not shown). In normal Ca^{2+}-containing ECB in the absence of La^{3+}, the $[Ca^{2+}]_c$ rise induced in single fura dextran-injected hepatocytes by 50 nM AVP was similar, in terms of both mean increase in $[Ca^{2+}]_c$ (592 ± 36 nM; $n = 3$) and duration (548 ± 66 s; $n = 3$), to that evoked by 25 μM ATP (Fig. 2, *A*, *D*, and *E*). A typical recording is shown in Fig. 7*A*.

Agonists were applied to single fura dextran-injected hepatocytes in the presence of 0.5 mM La^{3+} in nominally Ca^{2+}-free ECB to eliminate both Ca^{2+} influx (79, 81) and Ca^{2+} efflux (82–84). Fig. 7*B* shows that stimulation of single fura dextran-injected rat hepatocytes with AVP under these conditions evoked a rise in $[Ca^{2+}]_c$, but that the amplitude of AVP-induced $[Ca^{2+}]_c$ rise was considerably decreased compared with that seen in normal Ca^{2+}-containing ECB in the absence of La^{3+} (Fig. 7*A*). This observation is consistent with several previous studies that have shown that the amplitude of the hepatocyte $[Ca^{2+}]_c$ rise evoked by several agonists, including AVP and ATP, is significantly reduced when Ca^{2+} influx is prevented (85–88). Under these conditions (0.5 mM La^{3+} in nominally Ca^{2+}-free ECB), however, *i.e.* in the absence of plasma membrane Ca^{2+} fluxes, any effect of ANP on the rates of rise or decay of the AVP-induced $[Ca^{2+}]_c$ elevations will be attributable to modulation of stored Ca^{2+} release or re-uptake, respectively (89). Fig. 7, *B–G*, show that neither the rates of rise (Fig. 7*D*) or decay (Fig. 7*E*) nor the mean amplitude (Fig. 7*F*) or duration (Fig. 7*G*) of the AVP-induced $[Ca^{2+}]_c$ response was altered by the co-addition of ANP (Fig. 7*C*).

PKG Isoforms Iα and Iβ Are Expressed in Rat Hepatocytes—Two mammalian isotypes of PKG have been described: type I, consisting of an α and a β isoform, splice variants of a single gene, and type II (90).

Immunoreactivity, using the commercially available antibodies cGKIα (N-16) and cGKIβ (L-16), demonstrated expression of PKG isoform Iα and PKG isoform Iβ (visualized using Alexa 594, red secondary) in isolated rat hepatocytes seeded onto coverglass for 6 (results not shown) or 24 h (Fig. 8). No staining was observed using the Alexa 594 secondary antibody alone. Both isoform Iα and isoform Iβ appear localized to hot spots at the plasma membrane and at a region adjacent to the nucleus, with lesser diffuse staining throughout the cytosol (Fig. 8). Similar immunostaining was also achieved using antibody cGKIα/β (T-19), targeted against a 19-amino acid sequence near the C terminus of PKG Iα and PKG Iβ. We have therefore demonstrated positive immunostaining for PKG isotype I using antibodies raised against both the N and C termini. In contrast, an absence of staining using antibody cGKII (T-18) suggested a lack of PKG isoform II expression (results not shown).

DISCUSSION

Extracellular ATP induces potentially harmful $[Ca^{2+}]_i$ elevations in rat hepatocytes. We have shown here that ANP, through PKG, attenuates both the amplitude and duration of the ATP-induced rise in $[Ca^{2+}]_c$, the accompanying Ca^{2+}-dependent elevation in calpain activity and the decline in cell viability. Our study has then characterized the mechanism underlying the hepatoprotective effect of ANP against Ca^{2+}-

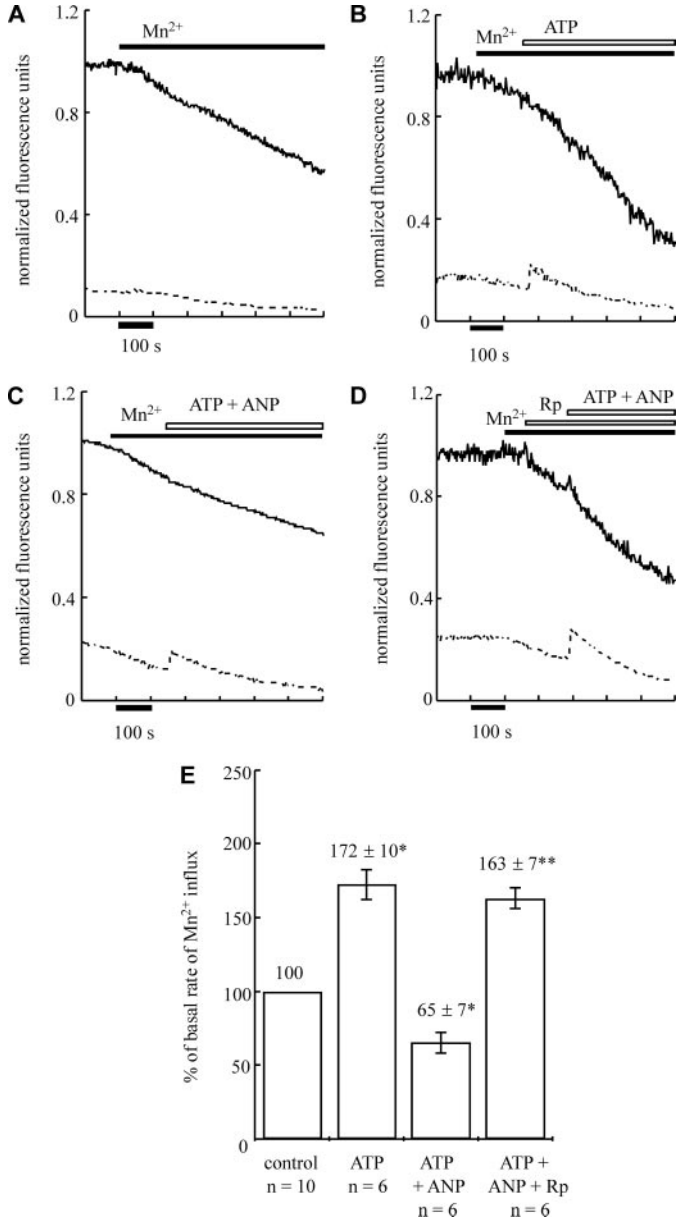

FIGURE 6. **ANP, through PKG, attenuates ATP-stimulated Mn^{2+} influx into single rat hepatocytes.** Single, fura-2 dextran-injected, rat hepatocytes were superfused with 50 μM Mn^{2+} (*A*) and 25 μM ATP (*B*), 25 μM ATP + 200 nM ANP (*C*), or 25 μM ATP + 200 nM ANP + 25 μM R_p-8-*p*CPT-cGMPS (R_p; *D*) for the periods indicated by the *bars*. Changes in fluorescence were recorded at the Ca^{2+}-insensitive wavelength, 359 nm. Fluorescence was normalized to the maximum fluorescence units. The *traces* shown in *A–D* are representative of several experiments. Calculated changes in Mn^{2+} influx are shown in *E*. For each cell, the rate of Mn^{2+} quench following addition of agonist(s) was expressed as a percentage of its basal rate of Mn^{2+} quench; the mean percentage values from several cells were then calculated and are shown *above* each corresponding column. *, significantly different from control; **, significantly different from both control and ATP + ANP.

dependent injury (51). We have shown that ANP both stimulates Ca^{2+} efflux from and inhibits Ca^{2+} influx into ATP-treated hepatocytes. Under experimental conditions where plasma membrane Ca^{2+} fluxes are eliminated, ANP is without effect on $[Ca^{2+}]_c$ rises, suggesting that it does not modulate the release from or re-uptake of Ca^{2+} into intracellular stores. The data thus suggest that stimulation of net plasma membrane Ca^{2+} efflux (to which both Ca^{2+} efflux stimulation and Ca^{2+} influx inhibition contribute) is the key process through which ANP attenuates $[Ca^{2+}]_c$ rises and consequent hepatocyte injury.

It has been estimated that basal calpain activity is present when hepatocyte $[Ca^{2+}]_i$ is 100–200 nM and increases significantly at $[Ca^{2+}]_i$ between 400 and 1000 nM (18). Our data agree well with this estimation; we show here that ATP elevated $[Ca^{2+}]_c$ from ~150–200 to ~800 nM (Fig. 2*A*) and stimulated a 1.6-fold increase in calpain activity (Fig. 3, *B* and *E*). Furthermore, we demonstrate here, for the first time, that ANP prevents the ATP-induced elevation in calpain activity. This effect is entirely consistent with our observation that in the added presence of ANP, ATP elevated $[Ca^{2+}]_c$ to only ~400 nM (Fig. 2*B*). Together with our demonstration that ANP also prevents stimulation of calpain activity by either thapsigargin or ionomycin, this suggests that ANP prevents the ATP-induced elevation in calpain activity by attenuating the rise in $[Ca^{2+}]_c$.

We also show that ANP prevents elevation of calpain activity and diminished cell viability induced by the hydrophobic bile acid TLC. Hydrophobic bile acids are classic hepatotoxicants *in vivo*, causing cholestatic liver injury. Numerous studies have demonstrated that hydrophobic bile acids, including TLC, elevate hepatocyte Ca^{2+} (72–74, 91–93) and calpain activity (20), which play a key role in bile acid hepatotoxicity (20, 72–74, 91–93).

Our data suggest that the observed effects of ANP on hepatocyte Ca^{2+} homeostasis, calpain activity, and viability are mediated by cGMP and PKG. We have shown that ANP, at the concentration used throughout this study, stimulates cGMP production in rat hepatocytes in either the presence or absence of ATP. The PKG inhibitor, R_p-8-*p*CPT-cGMPS, prevents attenuation by ANP of the ATP-induced rise in both hepatocyte $[Ca^{2+}]_c$ and calpain activity and also its hepatoprotective effect. R_p-8-*p*CPT-cGMPS also prevents the ANP-mediated modulation of both Ca^{2+} efflux and Ca^{2+} influx recorded in the presence of ATP.

Several previous studies, in diverse cell types, have demonstrated PKG-mediated inhibition of Ca^{2+} influx by natriuretic peptides (38, 89, 94–97). In contrast, considerably fewer studies have demonstrated plasma membrane Ca^{2+} efflux stimulation by natriuretic peptides: ANP has been shown to stimulate Na^+-dependent Ca^{2+} efflux from rat cardiomyocytes (98). ANP and 8-bromo-cGMP stimulate Na^+-independent Ca^{2+} efflux from vascular smooth muscle cells (99). We have previously shown that ANP and cGMP, through PKG, stimulate *basal* Ca^{2+} efflux from rat hepatocytes (56), and we show here that ANP also dramatically stimulates Ca^{2+} efflux from *ATP-treated* hepatocytes. Consistent with our observations, however, are several reports of Ca^{2+} efflux stimulation by cGMP. cGMP, through PKG, stimulates Ca^{2+} efflux from endothelial cells (100) and platelets (101). Furthermore, cGMP regulates $[Ca^{2+}]_c$ in smooth muscle cells through stimulation of the PMCA (102); a similar role for cGMP in pancreatic acinar cells has also been suggested (103). It is possible, therefore, that future studies may subsequently reveal the involvement of Ca^{2+} efflux stimulation in the ability of natriuretic peptides to attenuate elevations in $[Ca^{2+}]_c$ in other cell types, as we have demonstrated here in rat hepatocytes.

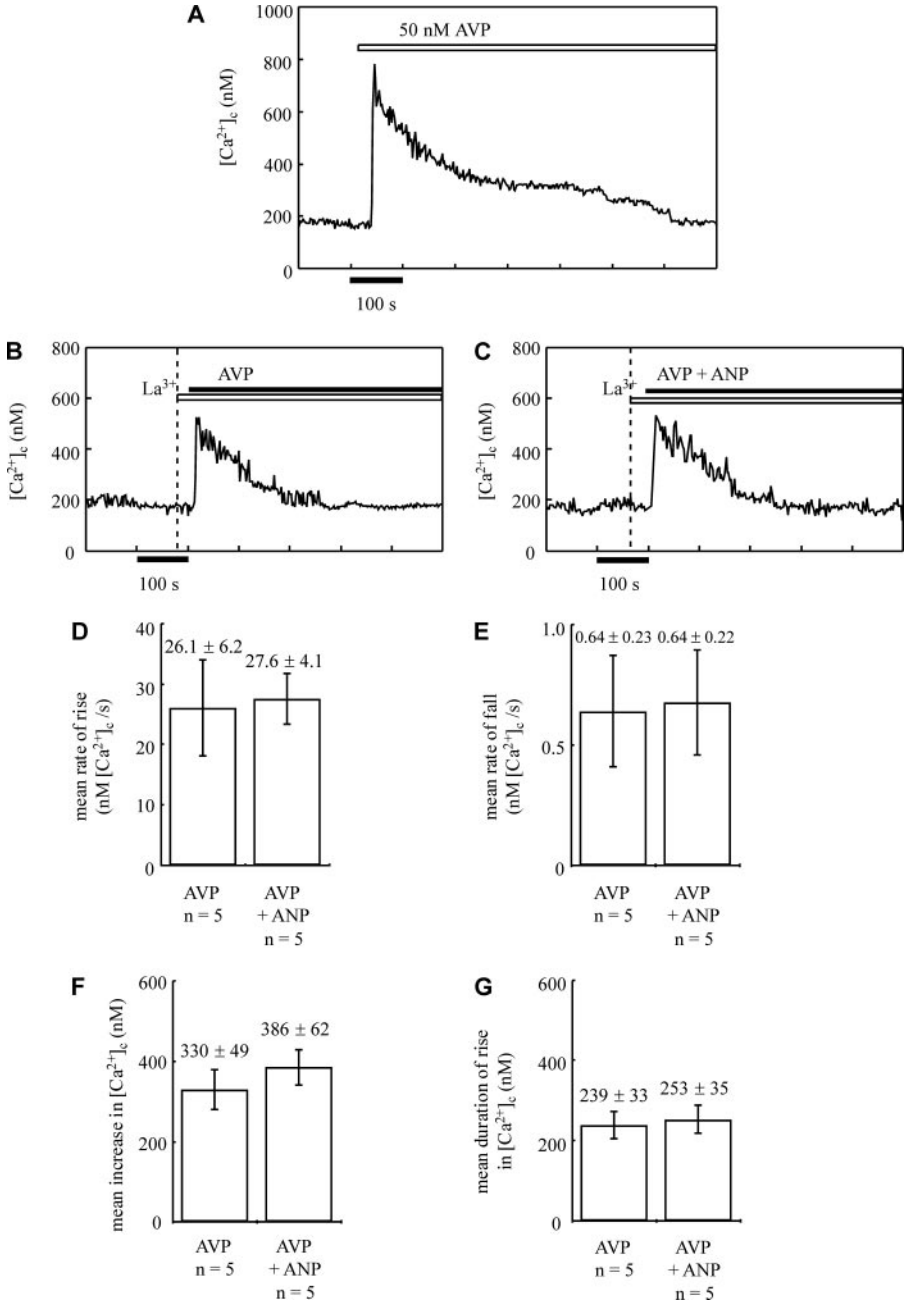

FIGURE 7. **In the presence of La^{3+}, ANP does not alter the agonist-induced rise in hepatocyte $[Ca^{2+}]_c$.** Single fura-2 dextran-injected rat hepatocytes were supplied with 50 nM AVP (*A*), 0.5 mM La^{3+} and 50 nM AVP (*B*), or 0.5 mM La^{3+} and AVP + 200 nM ANP (*C*) for the periods indicated by the *bars*. The *dashed lines* indicate the time at which the superfusion medium was changed to nominally Ca^{2+}-free ECB. The *traces* shown in *A–C* are representative of several experiments. *D–G* show, respectively, the calculated mean rate of rise (nM $[Ca^{2+}]_c$/s), rate of fall (nM $[Ca^{2+}]_c$/s), increase in amplitude (nM) above resting $[Ca^{2+}]_c$ and duration (*s*) of the $[Ca^{2+}]_c$ rise above resting $[Ca^{2+}]_c$ for each treatment. Calculated values are shown *above* each corresponding column.

Ca^{2+} efflux from rat hepatocytes is achieved by the PMCA; the predominant PMCA isoform in adult rat liver and isolated hepatocytes is PMCA1, whereas PMCA2 is also present at much lower levels (104). Na^+/Ca^{2+} exchange does not play a role in the regulation of $[Ca^{2+}]_c$ in this cell type (105). Whereas we have demonstrated previously (76), and confirm here, that basal Ca^{2+} efflux from hepatocytes is predominantly PMCA-mediated, we now also show that ANP-stimulated hepatocyte Ca^{2+} efflux is mediated by the PMCA. Stimulation of PMCA by PKG has been shown in intact cells (99, 101), crude membrane preparations (102, 106), and partially purified enzyme preparations (107, 108). In both smooth muscle preparations (102) and intact platelets (101), PKG increased the V_{max} of the PMCA without affecting its K_m. PKG stimulated smooth muscle PMCA activity by up to 4-fold; this stimulation was independent of, and additive to, the approximate 2-fold stimulation by calmodulin (102). Intriguingly, however, several studies have demonstrated that PMCA itself is not a substrate for PKG (107–109). Of the many PMCA isoforms identified, only one, PMCA1b, contains the consensus sequence for phosphorylation by cyclic nucleotide-dependent protein kinases (110). However, it has been demonstrated that PMCA1b is not phosphorylated by PKG, but that direct phosphorylation of PMCA is not required for its stimulation by PKG (108). Indeed, rather than PMCA stimulation by direct PKG phosphorylation, two alternative mechanisms for PKG-mediated PMCA stimulation were proposed. First, it was suggested that PKG may stimulate the PMCA indirectly via phosphorylation of a phosphatidylinositol kinase, thus generating phosphatidylinositol 4-phosphate that binds and activates the PMCA (107). Alternatively, it was postulated that PKG phosphorylates an intermediate 240-kDa protein, later identified as the type I inositol, 1,4,5-trisphosphate receptor (111), which is responsible for PMCA activation (108). However, the same group has since demonstrated that whereas PKG does indeed stimulate the PMCA without phosphorylating it, PKG-dependent phosphorylation of the 240-kDa protein/inositol 1,4,5-trisphosphate receptor is not an essential step (112).

We have demonstrated here, by immunocytochemistry, the presence of PKG isoforms Iα and Iβ in primary rat hepatocytes. High levels of PKG type I are found in vascular smooth muscle cells, endothelial cells, and platelets, where it is thought to attenuate elevations in $[Ca^{2+}]_c$ (90). It has been shown that PKG Iα, but not PKG Iβ, stimulates the partially purified porcine aorta PMCA without any detectable PMCA phosphorylation (112). We therefore propose that PKG Iα mediates ANP-induced stimulation of the PMCA in rat hepatocytes.

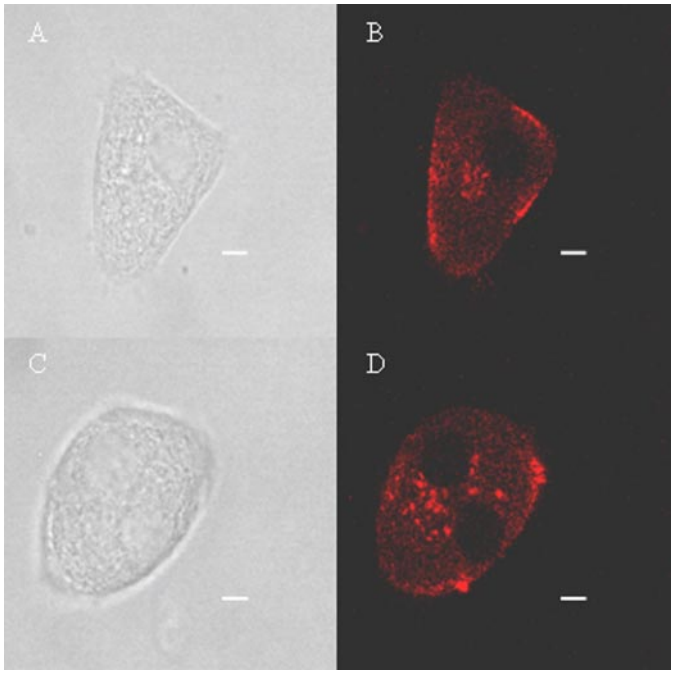

FIGURE 8. **PKG isoforms Iα and Iβ are expressed in rat hepatocytes.** Isolated rat hepatocytes were immunostained with either anti-PKG1α (cGKIα (N-16), *B*) or anti-PKG1β (cGKIβ (L-16), *D*). Alexa 594 anti-goat (*red*) was used to visualize immunoreactivity. Corresponding phase images demonstrate general cell morphology (*A* and *C*, respectively). The *scale bar* represents 10 μm and data are representative of three separate experiments.

Moreover, it has been demonstrated that ANP causes PKG isoform I to translocate to the plasma membrane, whereas SNP, an NO donor that activates soluble guanylyl cyclase, does not (113). This is entirely consistent with our previous finding, confirmed here, that SNP, unlike ANP, does not stimulate hepatocyte Ca^{2+} efflux. We previously proposed that their differing abilities to stimulate Ca^{2+} efflux may be related to the differing cellular location of their respective target guanylyl cyclases (56). Thus, ANP may, through plasma membrane guanylyl cyclase A receptor-mediated localized cGMP elevation, activate PKG Iα, causing it to stimulate the PMCA. Elevation of cGMP in the bulk cytosol by SNP may therefore, in contrast, be ineffective in stimulating the PMCA. Indeed, since we first demonstrated differential effects of soluble and particulate guanylyl cyclase activation on Ca^{2+} signaling (56, 89), others have reported similar effects in several cell types that have likewise been attributed to cGMP compartmentation (43, 115, 116).

The ability of ANP to attenuate pathophysiological elevations in $[Ca^{2+}]_c$ by stimulation of net Ca^{2+} efflux may be particularly valuable in cytoprotection of hepatocytes, because we also show here that ATP does not, itself, stimulate Ca^{2+} efflux from hepatocytes. This finding is consistent with our previous observation that the rate of basal Ca^{2+} efflux from hepatocytes is not altered by Ca^{2+}-mobilizing agonists at lower concentrations that stimulate $[Ca^{2+}]_c$ oscillations (56). This is in contrast to the ability of Ca^{2+}-mobilizing agonists to stimulate Ca^{2+} efflux from several other cell types (89, 117). This interesting observation deserves separate further investigation, but may be related to reports that the PMCA in rat hepatocytes, unlike other cell types, is not subject to dynamic regulation by calmodulin (110, 117). The classical model for calmodulin regulation of the PMCA involves calmodulin binding to the PMCA in a Ca^{2+}-dependent manner, stimulating its V_{max} and, more dramatically, decreasing its K_m (Ca^{2+}) by 1 order of magnitude from 10–20 mM to ~0.5 μM (110). In rat hepatocyte plasma membranes, however, the PMCA already exhibits high Ca^{2+} affinity ($K_m \sim 0.5$ μM) in the absence of exogenously added calmodulin (118). It has therefore been proposed that the rat hepatocyte PMCA may be permanently associated with calmodulin, which may thus maintain the PMCA in the high Ca^{2+} affinity form (110, 117). Although Ca^{2+} itself stimulates PMCA activity (110, 118), it is possible that, in rat hepatocytes, the PMCA is already close to Ca^{2+} saturation even in unstimulated cells, and thus may be insensitive to agonist-evoked $[Ca^{2+}]_c$ elevations. Indeed, it has been shown that subplasmalemmal $[Ca^{2+}]$ is higher than bulk $[Ca^{2+}]_c$ even in unstimulated cells (119). ANP in contrast, is effective in stimulating hepatocyte Ca^{2+} efflux because PKG-mediated PMCA stimulation increases V_{max} (101) and is, as discussed above, independent of, and additive to, stimulation by calmodulin (102).

Most attempts to avert or alleviate Ca^{2+}-dependent hepatocyte injury have involved prevention of plasma membrane Ca^{2+} influx, and have been met with some success (13, 120–122). However, inhibition of Ca^{2+} influx (123) or removal of extracellular Ca^{2+} (124) does not always prevent hepatocyte injury (123–125). In contrast, possible hepatoprotective effects of Ca^{2+} efflux stimulation alone have not been investigated. However, intracellular *chelation* of excess cytosolic Ca^{2+} has been shown to be highly effective in preventing and reversing hepatocyte injury (19, 126). It is possible that removal of excess Ca^{2+} from the cytosol could be more effective than prevention of Ca^{2+} influx, particularly when the injurious $[Ca^{2+}]_i$ rises originate from intracellular stores. It is probable that the modulatory effect of ANP on elevated $[Ca^{2+}]_c$ is determined by the *net* flux of Ca^{2+} across the plasma membrane, to which both increased efflux and decreased influx contribute. However, we propose that stimulation of Ca^{2+} efflux is a key valuable, previously undiscovered, mechanism in ANP-mediated hepatoprotection, rendering the hepatocyte able to actively clear Ca^{2+} overload, rather than simply preventing further $[Ca^{2+}]_i$ increases.

Through this and our previous study we have thus shown that ANP attenuates both physiological (56) and pathophysiological Ca^{2+} signals in hepatocytes. ANP may therefore serve an *in vivo* role as a circulating modulator of both physiological and pathophysiological elevations in hepatocyte $[Ca^{2+}]_c$. Moreover, mRNA coding for natriuretic peptides has been demonstrated in human liver tissue (127) suggesting that, in addition to circulating ANP, liver may be exposed to locally generated natriuretic peptides.

Ca^{2+}- and calpain-dependent injury is not unique to hepatocytes. Indeed, a rise in $[Ca^{2+}]_c$ is a common pivotal event in cellular injury and has been implicated in numerous disease processes, for example, pancreatitis (3), cardiovascular disease (4), and neurotoxicity (5). Similarly, calpain activation plays a key role in cellular injury in diverse pathologies, including oxidant-induced pancreatic acinar cell injury (27), myocardial ischemia-reperfusion injury (128), neurotoxicity (129), spinal cord injury (28), and endothelial dysfunction in type 2 diabetes

(29). Because natriuretic peptides act on several organs (54) and attenuate elevations in $[Ca^{2+}]_c$ in many cell types (37–50), it is unlikely that their cytoprotective effects are restricted to liver. Indeed, natriuretic peptides may have an *in vivo* role as circulating inhibitors of pathophysiological elevations in $[Ca^{2+}]_c$ and calpain activity.

Natriuretic peptides and their receptors may therefore be promising therapeutic targets to protect cells, including hepatocytes, against Ca^{2+}- and calpain-mediated injury. Natriuretic peptide therapy is already in development for the management of cardiovascular disorders (53, 130–132); clinical intervention with natriuretic peptides has been approved for treatment of heart failure (133). Of particular interest is a report that ANP infusion decreases portal pressure and increases hepatic blood flow in cirrhotic patients (134). Moreover, a recent study has demonstrated that inhibition of ANP degradation decreases intrahepatic vascular resistance, with little effects on systemic hemodynamics in cirrhotic rats (114). We predict that such treatment will also protect hepatocytes against Ca^{2+}- and calpain-dependent injury. Natriuretic peptide therapy may prove to have more widespread clinical utility for the prevention or alleviation of Ca^{2+}- and calpain-dependent cellular injury in several organs.

In conclusion, our data show that ANP attenuates ATP-induced elevations in $[Ca^{2+}]_c$ and calpain activity in rat hepatocytes by modulating plasma membrane Ca^{2+} fluxes. We demonstrate that stimulation of net plasma membrane Ca^{2+} efflux is the key process through which ANP protects hepatocytes. Moreover we propose that Ca^{2+} efflux stimulation is a valuable, previously undiscovered, mechanism in the protection of hepatocytes, and perhaps other cell types, against Ca^{2+}-dependent injury.

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Mechanisms of Signal Transduction:

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