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Anoxia/Reoxygenation Injury in Hepatocytes Is Not Prevented By Calcium Channel Blockers

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SEVERAL investigators have reported that calcium channel blockers may protect the liver from anoxic injury or from other hepatotoxic agents.[1,2] Because the putative protective effects of calcium channel blockers have been obtained in perfused whole liver, it is impossible to determine which cells within the liver were actually the target of the calcium antagonists: vascular smooth muscle cells, endothelial cells, Kupffer cells, or parenchymal cells. In addition, most of the protective effects observed have occurred after reoxygenation and not during anoxia, suggesting that calcium antagonists act to prevent reperfusion injury and not anoxic injury. The objective of the present experiments was to determine whether calcium channels are involved in the damage induced by anoxia in perfused isolated rat hepatocytes. Specifically the studies described were designed to determine whether the massive rise in cytosolic-free calcium (Ca_i^{2+}) and the increase in loss of cytosolic lactate dehydrogenase (LDH) evoked by anoxia can be blocked by specific Ca^{2+} channel blockers. Three classes of Ca^{2+} antagonists (nifedipine, verapamil, and diltiazem) were studied. Each was studied at his K_i (10^{-7}) and at concentrations 10 and 100 times above K_i.

MATERIALS AND METHODS

Hepatocytes

Freshly isolated hepatocytes were prepared from adult male Sprague-Dawley rats weighing between 200 and 250 g. The cells were isolated from fed animals using collagenase (Sigma type IV, Sigma Chemicals, St Louis, Mo) by the perfusion method described previously.[3] Cell viability assessed by trypan blue exclusion averaged 90%. The cells were embedded in agarose threads and were perfused at a rate of 0.6 mL/min with standard Krebs-Henseleit bicarbonate buffer (KHB) at 37°C. In all experiments the hepatocytes were first perfused for 1 hour in control KHB saturated with 95% O_2–5% carbon dioxide (CO_2). During the 2 hours of experimental anoxia, the cells were perfused with KHB saturated with 95% N_2–5% CO_2. After 2 hours of anoxia, the cells were reperfused with oxygenated KHB for 1 hour. In the experimental studies, the cells were exposed, during the 2 hours of anoxia, to KHB containing 10^{-7}, 10^{-6}, 10^{-5} mol/L verapamil, nifedipine, or diltiazem.

Cytosolic Ionized Calcium

Ca_i^{2+} was measured with the Ca^{2+}-sensitive photoprotein aequorin[4] incorporated into the hepatocytes by gravity-loading. The aequorin-loaded cells were imbedded in agarose gel threads, placed in the cuvette of an aequorin luminescence photometer, and continuously perfused with KHB.

Lactate Dehydrogenase

Cell injury was monitored by measuring LDH release from the cells into the perfusate effluent before, during and after the anoxic period. Because the concentration of perfused cells imbedded in the agarose threads varied slightly between individual experiments, LDH release was expressed as the percent increase above the control value measured during the 1-hour control period at the beginning of each experiment.

RESULTS

Under control conditions, the Ca_i^{2+} was 126 ± 10 nmol/L. In the control group anoxia increased Ca_i^{2+} in two distinct phases, reaching a maximum value of 1,215 ± 78 nmol/L after 1 hour. Lactate dehydrogenase release increased sixfold during the second hour of anoxia. During reoxygenation, Ca_i^{2+} and LDH returned to control levels within 45 minutes. To determine whether the source of Ca^{2+} responsible for the increase in Ca_i^{2+} during anoxia was intra- or extracellular these experiments were repeated in the absence of extracellular Ca^{2+} (Ca_o^{2+}) during the 2 hours of anoxia. The initial smaller peak in Ca_i^{2+} seen previously in the first 10 minutes of anoxia was present even in the absence of Ca_o^{2+}. This suggests that the source of Ca^{2+} responsible for the initial Ca_i^{2+} peak is probably intracellular. The second major Ca_i^{2+} peak, previously observed 1 hour after the initiation of anoxia, was completely abolished in the absence of extracellular Ca^{2+}, indicating that this second increase in Ca_i^{2+} was due to an influx of Ca^{2+} from the perfusate to the cytosol. Verapamil, nifedipine, and diltiazem (10^{-7} to 10^{-5} mol/L) added to the perfusate during the anoxic period did not inhibit the biphasic rise in Ca_i^{2+}. Furthermore, the calcium antagonists, at all three concentrations used, did not protect hepatocytes from anoxic injury measured by the release of LDH in the perfusate effluent.

DISCUSSION

These experiments demonstrate that anoxia causes a biphasic increase in Ca_i^{2+}. The second surge in Ca_i^{2+} is totally abolished when Ca^{2+} is removed from the perfus-

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ate, indicating that the source of Ca^{2+} responsible for the second increase in Ca_i^{2+} is the extracellular fluid. The lack of an effect on Ca_i^{2+} observed with the three Ca^{2+} channel blockers verapamil, diltiazem, and nifedipine in concentrations up to 10^{-5} mol/L strongly suggests that pathways other than L-type voltage-operated Ca^{2+} channels are involved in the influx of Ca^{2+} responsible for the increase in Ca_i^{2+} evoked by anoxia. Furthermore, concentrations of verapamil, diltiazem, and nifedipine ranging from 10^{-7} to 10^{-5} mol/L did not block the LDH release induced by anoxia. Thus, Ca^{2+} antagonists do not protect liver parenchymal cells against anoxic injury. The reported cytoprotective effects of Ca^{2+} channel blockers seen in ischemic perfused whole liver must occur as a result of an effect on cells other than hepatocytes (ie, endothelial cells or smooth muscle cells of the liver vasculature).

REFERENCES

1. Landon EJ, Naukam RJ, Rama Sastri BV: Biochem Pharmacol 35:697, 1986
2. Takei Y, Marzi I, Kauffman FC, et al: Transplantation 50:14, 1990
3. Studer RK, Borle AB: J Biol Chem 257:7987, 1982
4. Borle AB, Snowdowne KW: Methods Enzymol 124:90, 1986