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Effect of Calcium Ions on Ethanol Oxidation and Drug Glucuronidation in Isolated Hepatocytes

By Bo ANDERSSON, Dean P. JONES and Sten ORRENIUS
Department of Forensic Medicine, Karolinska Institutet, S-104 01 Stockholm, Sweden

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The effect of extracellular Ca^{2+} concentration on ethanol oxidation and drug metabolism was studied in isolated rat hepatocytes. Both ethanol oxidation and drug glucuronidation showed similar dependence upon Ca^{2+}, which was a stimulation of activity as Ca^{2+} was increased to physiological concentration, and inhibition at higher concentration.

Reports by Otto and Ontko (Otto & Ontko, 1974, 1978; Ontko *et al.*, 1975) demonstrate Ca^{2+}-induced alterations in the oxidation–reduction state in isolated mitochondria and hepatocytes. The implications of their observations are very important with regard to the overall function of cells, since many metabolic activities are coupled to the cellular redox pools. Among these are ethanol oxidation, which appears to be rate-limited by the reoxidation of NADH (Meijer *et al.*, 1975), and drug metabolism by the glucuronidation pathway, which requires NAD^+ for synthesis of UDP-glucuronic acid (cf. Dutton, 1966). The interrelationship of these two processes has been demonstrated by Moldéus *et al.* (1978*a*), who found that ethanol oxidation markedly inhibits glucuronidation reactions in intact hepatocytes.

Recognition of variations in plasma Ca^{2+} concentration under physiological and pathological conditions (Hald & Eisenman, 1937; Snyder & Katzenelbogen, 1942; Paloyan *et al.*, 1973; Thomas, 1976) and evidence for Ca^{2+}-induced increase in ethanol-induced sleeping time (Erickson *et al.*, 1978) prompted us to examine the effect of added Ca^{2+} on ethanol oxidation and drug glucuronidation in isolated hepatocytes.

As a model substrate for glucuronidation, we used harmol. This substance is also metabolized to a sulphate conjugate in an energy-requiring process that is not dependent upon available NAD^+, and therefore serves as a control for Ca^{2+}-induced changes in glucuronidation.

Materials and Methods

Male Sprague–Dawley rats (200–250g) were used for hepatocyte preparations. Isolation and characterization of cells were performed as described previously (Moldéus *et al.*, 1978*b*). Trypan Blue and NADH exclusions of 90–100% were observed immediately after cell preparation.

Hepatocytes were washed with Ca^{2+}-free Krebs–Henseleit buffer (pH 7.4), containing 25mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], before incubation. With the use of 200μM-harmol as substrate, incubation in Ca^{2+}-free Krebs–Henseleit buffer and analytical procedures were carried out as reported by Andersson *et al.* (1978). Ca^{2+} concentrations are expressed as total Ca^{2+} added to the incubation mixtures.

The oxidation of ethanol was measured by the method of Grundin (1975), with the following modifications. Hepatocytes (3×10^6/ml) were suspended in Ca^{2+}-free Krebs–Henseleit buffer (pH 7.4) containing 25mM-Hepes, gassed with O_2/CO_2 (19:1) and incubated for 30min in 25ml Erlenmeyer flasks covered with Parafilm. No decrease in hepatocyte viability was observed during these experiments. Blank incubations, which contained no hepatocytes, were used to correct for ethanol evaporation. Samples (0.2μl) were analysed on a Beckman GC72-5 gas chromatograph (FID) with the use of a glass column (3.5m×2.5mm internal diam.) packed with Carbopack c (80–100 mesh)/0.2% carbowax 1500. The column temperature was 125°C, and N_2 flow was 20ml/min.

Harmol hydrochloride was purchased from Aldrich-Europe, Beerse, Belgium. Collagenase (grade II) was obtained from Boehringer/Mannheim G.m.b.H., Mannheim, Germany, and Carbopack c was from Supelco, Bellefonte, PA, U.S.A. All other chemicals used were at least of reagent quality and purchased locally.

Results and Discussion

Addition of Ca^{2+} to isolated hepatocytes, which had been washed to decrease extracellular Ca^{2+} (to less than 10μM), resulted in a Ca^{2+}-dependent increase in the rate of ethanol oxidation, at least until the physiological extracellular Ca^{2+} concen-

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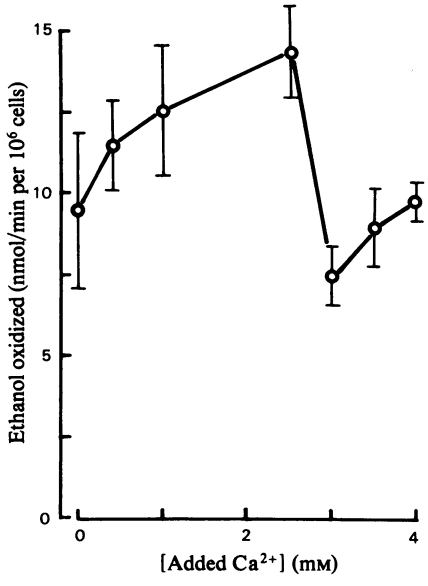

Fig. 1. *Effect of Ca^{2+} on ethanol oxidation in isolated hepatocytes*

Ethanol at a concentration of 6mM was used, and incubations were performed as described in the Materials and Methods section. Values are means ± S.E.M. for two to five different hepatocyte preparations.

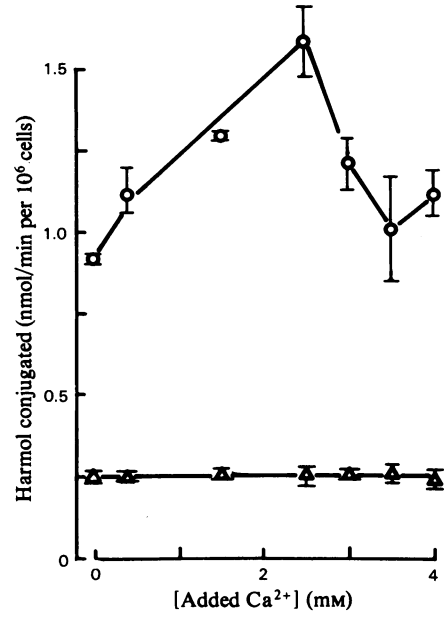

Fig. 2. *Effect of Ca^{2+} on the conjugation of harmol in isolated hepatocytes*

Incubations were performed for 20min as previously described (Andersson *et al.*, 1978). Values are means ± S.E.M. for two to four different hepatocyte preparations. △, Sulphate conjugates; ○, glucuronides.

tration was reached (Fig. 1). However, as Ca^{2+} concentration was further increased, a decrease to 50–60% of the maximal rate was observed. This decrease could be due to a variety of factors affecting the ethanol-oxidation rate. The lack of an inhibitory effect of Ca^{2+} on the purified alcohol dehydrogenase (results not shown) indicates that it is not a direct cationic inhibition. Rather, since ethanol oxidation is known to be rate-limited in intact tissues by the reoxidation rate of NADH (Meijer *et al.*, 1975), it appears that Ca^{2+} is somehow interfering with this reoxidation process. The initial effect of Ca^{2+} on the redox state in hepatocytes is an oxidation caused by the rapid stimulation of O_2 consumption after utilization of energy in Ca^{2+} uptake (Ontko *et al.*, 1975). This is followed by a change to a more reduced state, which is reflected in an increase in the [β-hydroxybutyrate]/[acetoacetate] ratio in the mitochondrial compartment (Otto & Ontko, 1974), and has been interpreted as being due to activation of mitochondrial fatty acid oxidation by Ca^{2+} (Otto & Ontko, 1978). The NADH generated by this system also serves as substrate for the mitochondrial electron-transport pathway and therefore could compete with reducing equivalents generated by oxidation of ethanol. Since ethanol oxidation is limited by reoxidation of NADH, this would result in inhibition of ethanol oxidation. Alternatively, Ca^{2+} could interfere with transport of reducing equivalents into mitochondria or indirectly interfere with alcohol oxidation through other Ca^{2+}-dependent enzymes involved in intermediary metabolism [e.g. glycerol 3-phosphate dehydrogenase (Wohlrab, 1977) and isocitrate dehydrogenase (Vaughan & Newsholme, 1969)].

Since ethanol oxidation inhibits glucuronidation of drugs, presumably by decreasing the available NAD^+ required for UDP-glucuronic acid synthesis (Dutton, 1966), we examined the effect of Ca^{2+} on glucuronidation. The effect of Ca^{2+} on harmol conjugation in hepatocytes is shown in Fig. 2. Formation of glucuronides was markedly enhanced by Ca^{2+} addition, and reached a maximum rate at a total concentration of 2.5mM. Further increases in Ca^{2+} concentration caused a more than 35% decrease in glucuronidation. In contrast, the sulphation of harmol was not affected by the increasing Ca^{2+} concentration (Fig. 2).

The total metabolism of harmine to harmol and

its conjugation products by hepatocytes showed no dependence upon Ca^{2+} (results not shown). Similar results have been obtained when metabolism of harmine and conjugation of harmol were studied in the presence of ethanol (Moldéus *et al.*, 1978*a*). Since glucuronidation of harmol does not proceed at a maximal rate when harmine is metabolized (Andersson *et al.*, 1978), the synthesis of UDP-glucuronic acid is not limiting under these conditions, and therefore this process is not affected by Ca^{2+}. However, at saturating concentrations of harmol, glucuronidation reaches a maximal rate, and consequently an effect of Ca^{2+} on the glucuronide synthesis is observed (Fig. 2).

These results demonstrate that two intracellular systems that are dependent upon NAD^+ concentration (or rate of NADH oxidation to NAD^+) respond similarly to changes in extracellular Ca^{2+} concentration. They confirm that Ca^{2+} penetrates the cell membrane of isolated hepatocytes (Dubinsky & Cockrell, 1975; Kleineke & Söling, 1976) and suggest that the previously reported effect on redox state may be important in modulating intracellular processes. The lack of an effect of Ca^{2+} on drug demethylation and sulphation indicates that the observed changes in ethanol oxidation and harmol glucuronidation (Figs. 1 and 2) were not a consequence of variations in a damaged-cell population, since these processes require maintenance of membrane integrity in order to prevent loss of required metabolites and cofactors.

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