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Alcohol inhibits the activation of NAD-linked dehydrogenases by calcium in brain and heart mitochondria

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Abstract

The effect of ethanol on the Ca^{2+}-dependent activation of mitochondrial dehydrogenases in rat brain and heart mitochondria was investigated. ADP-stimulated respiration of isolated brain and heart mitochondria (state 3) was stimulated further by submicromolar concentrations of free calcium when respiring on non-saturating concentrations of NAD-linked substrates. The stimulation of oxidative phosphorylation by Ca^{2+} was correlated with an increase of the mitochondrial matrix free calcium concentration ($[Ca^{2+}]_m$), as measured by fura-2, and with an increased reduction of the mitochondrial NAD(P) pool, indicating an activation of Ca^{2+}-dependent dehydrogenases. Sodium inhibited Ca^{2+}-dependent stimulation of state 3 respiration and NAD(P) reduction as a result of stimulation of Ca^{2+} efflux through the Na^+/Ca^{2+} antiporter which reduced the steady-state value of $[Ca^{2+}]_m$. Ethanol stimulated the Na^+/Ca^{2+} antiporter both in brain and heart mitochondria. As a result of this stimulation, ethanol, at pharmacological concentrations (50–300 mM), enhanced the sodium-dependent reduction of $[Ca^{2+}]_m$, and thus attenuated the activation of NAD-linked dehydrogenases and the stimulation of oxidative phosphorylation, by submicromolar concentrations of Ca^{2+}, both in brain and heart mitochondria. This pharmacological effect of ethanol, on brain and heart mitochondria, may be responsible, in part, for the acute and chronic effects of ethanol on brain and heart function and metabolism.

Keywords: Alcohol; Mitochondrion; Calcium; Oxidative phosphorylation; NAD-linked dehydrogenase

1. Introduction

Ethanol intoxication impairs mostly brain function [1], although high blood concentrations of ethanol also impair heart and muscle function [2]. Chronic alcoholism results in an extensive damage to the brain, heart, skeletal muscle, liver, the immune system and other organs [3–6]. Prenatal exposure to low concentration of ethanol results in a specific developmental impairment known as fetal alcohol syndrome [7]. The mechanisms of the acute and chronic effects of ethanol are largely unknown.

Ethanol inhibits force generation in heart and skeletal muscle, but the mechanism of this effect is not understood [8]. Ethanol effects on brain function vary, depending on the concentration, from mood alterations and anxiety relief (10–30 mM), to impairment of memory and motor control (> 30 mM), and to narcosis (> 80 mM) [9]. In chronic alcoholics, who are tolerant to alcohol, these effects are observed at much higher concentrations.

On the cellular level most of the effects of ethanol are limited to membrane systems (e.g., receptors, channels, transporters and membrane enzymes) [1,10,11]. In brain synaptic plasma membranes the GABA-Cl^- channel is stimulated while the NMDA-Ca^{2+} channel is inhibited by very low concentrations of ethanol. Higher concentrations of ethanol also inhibit other channels such as voltage-activated Ca^{2+} channels, and Ca^{2+}-activated K^+ channels [1,10,11].

Recently, we have found that in brain mitochondria the Na^+/Ca^{2+} antiporter, which ejects calcium from the mitochondrial matrix, is stimulated by ethanol [12,13]. Alcohol had no effect on the K_m of the antiporter for either Na^+ or Ca^{2+} but increased the V_{max}, apparently by modulating the dielectric constant of the membrane surface (13). This effect, which was observed at pharmacological concentra-

Abbreviations: BSA, bovine serum albumin; NAD(P), total pool of NAD and NADP; NAD(P)H, total pool of NADH and NADPH.

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tions of ethanol (50–300 mM), may contribute to the acute and chronic effects of ethanol, in vivo.

There are several calcium transport systems in mitochondria which transport calcium in and out of the matrix [14]. The free calcium concentration in the matrix, $[Ca^{2+}]_m$, is maintained in a steady-state when the efflux rate matches the uptake rate. Under physiological conditions calcium uptake into the matrix is driven by membrane potential through the electrogenic uniporter [15]. In brain and heart mitochondria calcium efflux from the matrix is driven mostly by a sodium gradient, through a Na^+/Ca^{2+} antiporter [16]. The maximal rate of the uniporter is at least two orders of magnitudes higher than the maximal rate of the Na^+/Ca^{2+} antiporter. However, because of the relatively low affinity for Ca^{2+} of the uniporter, under physiological conditions, the rate of uptake is very slow at low cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) [14]. Since calcium uptake by the uniporter is not affected by pharmacological concentrations of ethanol [12,13], while the efflux through the Na^+/Ca^{2+} antiporter is stimulated, it was predicted that ethanol would reduce the steady-state concentration of the mitochondrial matrix free calcium, $[Ca^{2+}]_m$.

Several NAD-linked dehydrogenases of the mitochondrial matrix, namely, the pyruvate dehydrogenase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase are activated by elevation of $[Ca^{2+}]_m$ [17,18]. The activation constant ($K_{0.5}$) for $[Ca^{2+}]_{ex}$, in vitro, ranges from 0.2 to 0.8 μM, depending on the free concentrations of Mg^+, spermine, pH, Na^+ and other factors [17–21]. For $[Ca^{2+}]_m$ to be a physiologically relevant regulator of mitochondrial metabolism, the mitochondrial calcium system must be able to maintain $[Ca^{2+}]_m$ at submicromolar levels. Under conditions that are near-physiological, at low submicromolar $[Ca^{2+}]_i$, $[Ca^{2+}]_m$ of heart mitochondria is lower than $[Ca^{2+}]_i$ [19,22–24]. Under these conditions $[Na^+]_i$ may modulate dehydrogenase activity through its effect on the rate of Na^+/Ca^{2+} exchange and hence on steady-state $[Ca^{2+}]_m$ [25].

It is now well established that the control of the rate of oxidative phosphorylation is exerted by several steps in the pathway [26,27]. One mechanism of stimulation of oxidative phosphorylation is through an increase in the cytoplasm free calcium, $[Ca^{2+}]_i$, which brings about a corresponding increase in $[Ca^{2+}]_m$ resulting in dehydrogenase activation and stimulation of ATP synthesis [17,21,28,29]. Activation of Krebs cycle should also enhance other related metabolic pathways, e.g., neurotransmitters synthesis.

Since the enhancement of Na^+/Ca^{2+} exchange inhibits the Ca^{2+}-dependent activation of the dehydrogenases [25] and ethanol stimulates Na^+/Ca^{2+} exchange [12,13], it was suggested that ethanol may inhibit the Ca^{2+}-dependent activation of the dehydrogenases (and oxidative phosphorylation) under near-physiological condition [12]. The purpose of this study was to test this suggestion. In addition, we wanted to find out whether the ethanol effect on Na^+/Ca^{2+} exchange and on the Ca^{2+}-dependent activation of NAD-linked dehydrogenases, which we observed in brain mitochondria, is also observed in heart mitochondria.

2. Materials and methods

2.1. Preparation of mitochondria

Rat brain free (non-synaptic) mitochondria were prepared essentially as described by Lai and Clark [30] with some modifications. For most experiments, except those in which $[Ca^{2+}]_m$ was determined (Figs. 6 and 7), the following procedure was used: the forebrains of three male Sprague-Dawley rats weighing 150–250 g were rapidly removed and rinsed in ice-cold SME medium (250 mM sucrose, 10 mM Mops-Tris, 1 mM EGTA-Tris (pH 7.3), containing 1% (w/v) BSA (fatty acids free). The tissue was minced and gently homogenized (12 strokes) in a Dounce homogenizer. The homogenate was centrifuged at $2000 \times g$ for 3 min. The supernatant was saved and the pellet was resuspended in fresh medium, homogenized again (6 strokes), and centrifuged at $2000 \times g$ for 3 min. The supernatants from the 2 centrifugation steps were combined and centrifuged at $12\,500 \times g$ for 10 min. The white, loose, upper layer of the pellet was carefully removed with a soft brush, whereas the dark-brown layer was resuspended, diluted with the fresh ice cold medium, and centrifuged at $12\,500 \times g$ for 10 min. The brown pellet was finally resuspended in a small volume (less than 1 ml) of SME and kept on ice until use. The protein concentration was determined by the biuret method in the presence of 0.33% deoxycholate and using BSA as standard. The yield was regularly 20–25 mg protein. The respiratory control values were 10.6 ± 0.84 S.D. ($n = 7$) with pyruvate (+malate) as substrate, 6.5 ± 0.35 ($n = 4$) with glutamate (+malate), 5.1 ± 0.41 ($n = 6$) with 2-oxyglutarate, and 4.0 ± 0.4 ($n = 4$) with succinate.

During the isolation of brain mitochondria a significant amount of Ca^{2+} is accumulated in the matrix (approx. 15–20 nmol Ca^{2+}/mg protein). This saturates the effect of Ca^{2+} on dehydrogenase activity, state 3 respiration and $[Ca^{2+}]_m$ measurements. To deplete the matrix Ca^{2+}, isolated brain mitochondria were incubated in 250 mM sucrose, 10 mM Mops, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM ADP, 10 mM NaCl, 0.5% BSA at 25° C for 30 min under moderate agitation. Thereafter, the mitochondrial suspension was diluted 15 times with fresh ice cold SME medium and centrifuged at $12\,500 \times g$ for 8 min. After this treatment submicromolar $[Ca^{2+}]_{ex}$ elicited the responses of brain mitochondria as described in detail in Results. For $[Ca^{2+}]_m$ measurements Ca^{2+} depletion and fura-2/AM loading were carried out simultaneously (see below). This preparation, while exhibiting high respiratory control, contained significant contamination of synaptosomes which interfered with the measurement of $[Ca^{2+}]_m$. Therefore, for

these measurements we purified the mitochondria further by a Percoll gradient as follows: the forebrains from 4 rats were minced in a cold medium of 320 mM sucrose, 1 mM Tris-EDTA, 0.25 mM DTT, pH 7.4. The minced brains were homogenized by hand (15 strokes) and suspended in 60 ml. The suspension was centrifuged at $1000 \times g$ for 3 min., the supernatant removed and the pellet resuspended, homogenized again (5 strokes), and centrifuged again. The combined supernatant was layered (7 ml per tube) on top of a percoll gradient (3%/10%/15%/23% percoll in the isolation medium at pH 7.4) and centrifuged at $32\,500 \times g$ for 6 min. The mitochondria was collected from the bottom pellet resuspended in SME medium (sucrose 250 mM, Tris-Mops 10 mM, Tris-EGTA 1 mM, pH 7.2) with 1% BSA (FA-free), and centrifuged at $15\,000 \times g$ for 15 min. The pellet was resuspended in 3 ml SME and loaded with fura-2 (see below).

Rat heart mitochondria were prepared as described previously [31] after incubation with 2–4 mg proteinase XXVII (Nagarse, Sigma) for 4–6 min.

2.2. Electron transport

The rate of oxygen uptake was measured with a Clark-type oxygen electrode. Mitochondria (1.5 mg protein) were incubated in 1.5 ml of an air-saturated medium that contained 120 mM KCl, 25 mM Mops-Tris (pH 7.2), 0.5 mM EGTA-Tris, 5 mM Tris-phosphate, 0.1% BSA, at 25° C with different substrates as described in Results. ADP (0.46 mM) was added to initiate state 3 respiration. The amount of dissolved oxygen was taken to be 508 ng atoms/ml medium at 25° C.

2.3. Na^+-dependent Ca^{2+} efflux

Mitochondria (2 mg protein) were incubated in 2.5 ml of a medium that contained 120 mM KCl, 20 mM Mops-Tris (pH 7.2), Tris-phosphate (1 mM), 1 mM $MgCl_2$, 0.5 mM ADP, 50 μM arsenazo III, 1 μM cyclosporine A, and ethanol as indicated, at 25° C. Then 5 mM succinate, 1 μM rotenone (dissolved in DMSO), and 100 nmol Ca^{2+} were added to load the mitochondria, after which 0.6 μM Ruthenium red were added. Na^+-dependent Ca^{2+} efflux was initiated by the addition of 20 mM NaCl. The rate of Ca^{2+} efflux was determined by following the rate of change in the absorbance difference at 685–675 nm, with an Aminco-DW2c dual-wavelength spectrophotometer under gentle stirring. The added Ca^{2+} served as calibration of the signal. We included cyclosporine A in many of our assays to prevent activation of the mitochondrial permeability transition (14). Although this does not occur normally, under our assay conditions, and thus there was little difference in the results with or without cyclosporine, occasionally, prolong incubation at high temperature resulted in significant activation and this was prevented by the inclusion of cyclosporine A [32].

2.4. Intramitochondrial NAD(P)H level

The activities of the mitochondrial NAD-linked dehydrogenases were measured by following the fluorescence of NAD(P)H. Mitochondria (2 mg protein for brain and 3 mg protein for heart) were incubated in 3 ml of a medium composed of 120 mM KCl, 25 mM Mops-Tris, 0.5 mM EGTA, 1 mM $MgCl_2$, pH 7.2, at 25° C. Other additions are indicated in the corresponding figures. The fluorescence of the mitochondrial suspension was measured at 460 nm with the excitation wavelength at 340 nm.

2.5. Intramitochondrial free Ca^{2+} concentration

Mitochondria were loaded with fura-2/AM and $[Ca^{2+}]_m$ was estimated from the fluorescence of fura-2-loaded mitochondria as described previously [33,20]. Loading of fura-2/AM into brain mitochondria and Ca^{2+} depletion was as follows: 15–20 mg mitochondrial protein were incubated in 3 ml of SME medium which also contained 1% BSA, 1 mM $MgCl_2$, 1 mM ADP, 10 mM NaCl and 5 μM fura-2/AM (Molecular Probes), at 30° C, for 20 min. Then the mitochondrial suspension was diluted 10–15 times with fresh ice cold SME medium (+1% BSA) and centrifuged at $9500 \times g$ for 10 min. After resuspension in 1 ml SME, mitochondria were kept on ice until used.

Heart mitochondria were loaded with fura-2/AM as described above except that 50 mg protein were incubated, in the same medium but without NaCl, for 15 min, at 25° C.

Fura-2-loaded brain mitochondria (0.5–1.0 mg protein) were incubated in 3 ml of an O_2-saturated medium, at 30° C. The medium contained 120 mM KCl, 25 mM Tris-Mops (pH 7.2), 0.5 mM Tris-EGTA, 0.2 mM Tris-phosphate, 0.5 mM Tris-ADP, 0.5 mM $MgCl_2$, 0.2% BSA, 1 μM cyclosporine A, with substrates, Na^+, Ca^{2+} and other reagents as indicated. Fluorescence was followed with a Fluorolog spectrophotometer at 510 nm emission with the excitation wavelengths at 340 and 380 nm under 100% O_2 gassing and gentle stirring. The fluorescence ratio at 340 nm/380 nm was used to calculate the matrix Ca^{2+} concentration using a fura-2 K_d of 210 nM for brain, as determined in this study (see results), and 149 nM for heart [26]. With heart mitochondria a ratio maximum (R_{max}) was attained by adding, at the end of each experiment, the Ca^{2+} ionophore Br-A23187 and a controlled excess of $CaCl_2$. This procedure was not satisfactory in brain mitochondria that are contaminated with synaptosomes which take up a significant fraction of fura-2. Therefore, in brain mitochondria the R_{max} was obtain simply after maximal uptake of calcium, from high Ca^{2+} medium (2 mM), by the energized mitochondria. A ratio minimum (R_{min}) was generated by the addition of excess EGTA and enough NaOH to raise the pH to a value higher than 7.4. Calculation of $[Ca^{2+}]_m$ from the fluorescence signal using the ratio method was according to Ref. [33].

2.6. Ca^{2+}/EGTA buffers

The calculation of $[Ca^{2+}]_{ex}$ concentrations stabilized by Ca^{2+}/EGTA buffers was made using a published computer program [34].

3. Results

The activation of oxidative phosphorylation by submicromolar concentrations of calcium, and its modulation by Na^+ and ethanol can be monitored by their effects on the rate of ADP-stimulated electron transport (state 3). To stimulate the rate of oxidation by submicromolar Ca^{2+} concentrations, it was necessary to use non-saturating concentrations of substrates.

Fig. 1A shows the dependence of the stimulation of state 3 respiration, in brain mitochondria respiring on low concentration of pyruvate (0.15 mM), on $[Ca^{2+}]_{ex}$. In the presence of physiological concentration of Mg^{2+}, higher Ca^{2+} concentrations were required for stimulation of state 3 respiration as expected from a competitive inhibition of Ca^{2+} uptake by Mg^{2+} [35]. Fig. 1B shows that increasing Na^+ concentrations inhibited the Ca^{2+} stimulation of state 3 respiration, as expected from a decrease in $[Ca^{2+}]_m$ induced by activation of Na^+/Ca^{2+} exchange [25]. Fig. 1C shows that ethanol inhibited state 3 respiration in the presence of Na^+ and Ca^{2+}, but was without effect, in the absence of Na^+ and Ca^{2+}, up to 400 mM. At higher concentrations of ethanol, state 3 respiration was inhibited, regardless of the sodium or calcium concentrations [36].

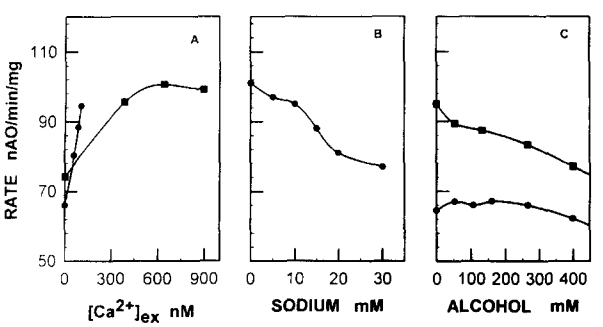

Fig. 1. The dependence of the rate of pyruvate oxidation by brain mitochondria (state 3 respiration) on the concentrations of Ca^{2+}, Na^+, and ethanol. The protocol and conditions are described in Materials and methods. Substrate was pyruvate (0.15 mM, with 1 mM malate) with Ca^{2+}, Mg^{2+}, Na^+, and ethanol concentrations as indicated. (A) The effect of Ca^{2+} on state 3 respiration. EGTA-Ca^{2+} mixtures were added to obtain the indicated $[Ca^{2+}]_{ex}$ concentrations [31]. The medium was either without Mg^{2+} (circles) or with 1 mM Mg^{2+} (squares). (B) The effect of $[Na^+]_{ex}$ on state 3 respiration. The medium was the same as in Fig. 1A, except for Ca^{2+}-EGTA (638 nM $[Ca^{2+}]$) and added NaCl as indicated. (C) The effect of ethanol on state 3 respiration. Medium was the same as in Fig. 1A except for the indicated ethanol concentrations, with Ca^{2+}-EGTA to obtain 624 nM $[Ca^{2+}]_{ex}$ and 10 mM NaCl. In control (circles) both Na^+ and Ca^{2+} were omitted (with 0.5 mM EGTA).

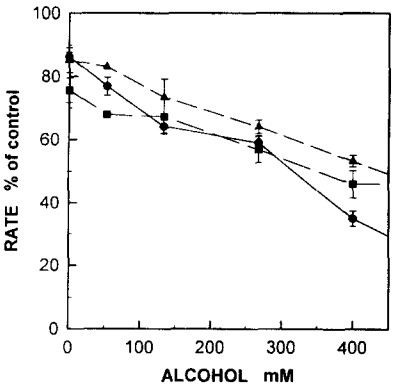

Fig. 2. Ethanol inhibition of the Ca^{2+}-dependent stimulation of state 3 respiration, with different NAD-linked substrates, in brain mitochondria. The medium was the same as in Fig. 1 except for the use of different substrates: 1 mM 2-oxoglutarate and 5 mM malate (squares); 2 mM glutamate and 5 mM malate (triangles); 0.15 mM pyruvate and 1 mM malate (circles). These results are the averaged inhibition by ethanol obtained with several preparations and are therefore normalized to the respiration rate obtained in the absence of ethanol. Also, because each preparation maintained somewhat different $[Ca^{2+}]_m$, after Ca^{2+} depletion, it was necessary to adjust $[Ca^{2+}]_{ex}$ (between 225 nM and 498 nM) and $[Na^+]_{ex}$ (between 6 and 10 mM) to obtain comparable stimulation of state 3 respiration (~ 80%) in the absence of ethanol. The rates of state 3 respiration in the presence of external Ca^{2+} and Na^+ but in the absence of ethanol were 41.3 ± 1.96 nAO/min per mg ($n = 8$) with 2-oxoglutarate (squares); 58.2 ± 3.97 nAO/min per mg ($n = 3$) with glutamate (triangles) and 75.5 ± 3.07 nAO/min per mg ($n = 10$) with pyruvate (circles). Statistical analysis of the results indicates that with pyruvate as substrate the inhibition by alcohol was significant ≥ 53 mM ($P < 0.05$) and highly significant ≥ 127 mM ($P < 0.01$). With other substrates the inhibition was highly significant ($P < 0.01$) ≥ 127 mM ethanol.

Since the inhibition at low ethanol concentration was dependent on the presence of both Na^+ and submicromolar $[Ca^{2+}]_{ex}$ it would appear that an enhancement of Na^+-dependent Ca^{2+} efflux was involved. Moreover, Diltiazem, a specific inhibitor of the mitochondrial Ca^{2+}/Na^+ antiporter, prevented the inhibition of respiration by both Na^+ and ethanol indicating the involvement of the antiporter. Ethanol was also without effect on respiration at saturating concentrations of substrates (not shown, but see Fig. 9 for similar finding in heart mitochondria).

The Na^+-dependent inhibition by ethanol of Ca^{2+}-stimulated state 3 respiration on pyruvate, was reproducible, and statistically significant, at alcohol concentrations ≥ 53 mM (Fig. 2). The inhibition was also observed, to a slightly lesser extent, with the NAD-linked substrates 2-oxoglutarate (+malate), and glutamate (+malate) as shown in Fig. 2.

To test the interpretation of the experiments shown in Figs. 1 and 2, namely, that ethanol inhibits the stimulation of state 3 respiration by Ca^{2+}, by inhibiting the activation of NAD-linked dehydrogenases, we measured the effect of ethanol on the steady-state level of NAD(P)H.

Fig. 3 shows the effects of glutamate, $[Ca^{2+}]_{ex}$, Na^+ and ethanol on the steady-state level of NAD(P)H in brain mitochondria. The stimulation of the NAD-linked dehydro-

genases activity by addition of glutamate (in the presence of Ca^{2+}) led to an increase in the steady-state level of NAD(P)H (trace 1). When rotenone was added, NADH oxidation is inhibited and all the NAD(P) is reduced to NAD(P)H. Addition of Na^+ (1 mM) reduced the steady-state level slightly (trace 2) and this inhibition was enhanced by ethanol at 133 mM (trace 3) and further enhanced by 267 mM ethanol (trace 4). Ruthenium red, a specific inhibitor of Ca^{2+} uptake by the uniporter, when added in the presence of Na^+ and Ca^{2+}, not only inhibited the generation of NAD(P)H by added glutamate but also caused a reversal of the initial, attenuated rise, most likely due to the Na^+-dependent efflux of Ca^{2+} from the matrix, which is driven by the sodium gradient that is generated by respiration on glutamate (trace 6).

Fig. 4 shows the dependence of the steady-state levels of NAD(P)H on glutamate concentration, $[Ca^{2+}]_{ex}$, sodium concentration and alcohol concentration in brain mitochondria. As expected, increasing glutamate concentrations (in the presence of 569 nM Ca^{2+}) increased the steady-state level but only up to half maximal value (Fig. 4A). Increasing $[Ca^{2+}]_{ex}$ further enhanced the steady-state level of NAD(P)H, almost to its maximal value (4B). Notice also the parabolic dependence on $[Ca^{2+}]_{ex}$ that correspond to the Ca^{2+} activation of the dehydrogenases [19]. As expected Na^+, in the presence of Ca^{2+}, greatly reduced the steady-state level (Fig. 4C) and ethanol, in the presence of both Ca^{2+} and Na^+ also reduced the steady-state level of NAD(P)H (Fig. 4D). This inhibition by ethanol was also dependent on the presence of both Ca^{2+} and Na^+ (results not shown).

The deactivation of the dehydrogenases by ethanol was reproducible and significant with both glutamate and pyruvate, as shown in Fig. 5. However, It was significantly stronger with the substrate glutamate (+malate) than with pyruvate (+malate).

To test whether the inhibition of Ca^{2+}-dependent activation of the dehydrogenases by ethanol is the result of reduced $[Ca^{2+}]_m$ we measured the effect of ethanol on $[Ca^{2+}]_m$ in fura-2-loaded brain mitochondria. An example of the protocols of these experiments is shown in Fig. 6, which shows the fluorescence ratio (340:380) during the course of these experiments. The mitochondria were incubated in the presence of EGTA and different concentrations of ethanol (0 mM trace 1, and 217 mM trace 2). The initial ratio was close to R_{min} (0.78) indicating very low $[Ca^{2+}]_m$. After addition of substrates (Tris-succinate in Fig. 6A and Tris-pyruvate + Tris-malate in Fig. 6B), Ca^{2+}/EGTA buffer was added to bring $[Ca^{2+}]_{ex}$ to the desired concentration (668 nM). This resulted in increased

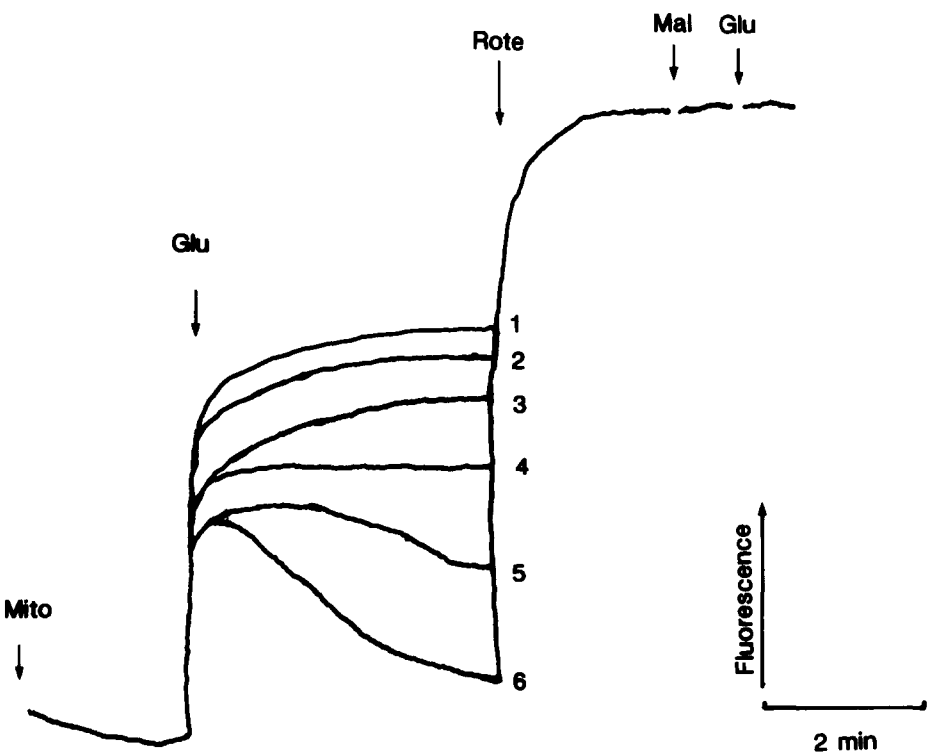

Fig. 3. The effect of $[Ca^{2+}]_{ex}$, Na^+ and ethanol on NAD(P) reduction by glutamate in brain mitochondria. The protocol and assay conditions are as described in Materials and methods except for the addition of 0.02% BSA, 5 mM P_i-Tris, 5 mM malate and cyclosporine A (1 μM) to the incubation medium. Other additions are : trace 1 with 531 nM $[Ca^{2+}]_{ex}$; trace 2 with 531 nM $[Ca^{2+}]_{ex}$ plus 1 mM Na^+; trace 3 and 4 with 133 and 267 mM ethanol in the presence of 531 nm $[Ca^{2+}]_{ex}$ and 1 mM Na^+; trace 5 without Ca^{2+} and Na^+; Trace 6 with 531 nM $[Ca^{2+}]_{ex}$ plus 10 mM Na^+, 2 mM Mg^{2+} and 0.5 μM Ruthenium red. All experiments were started by the addition of 1 mM glutamate, second addition was 2 μM rotenone, third addition was 1.7 mM malate and last addition 5 mM glutamate. Calibration of the fluorescence signal was made assuming 0% NAD(P)H as the value reached before addition of substrate and 100% NAD(P)H as the value generated after adding rotenone and excess glutamate plus malate.

$[Ca^{2+}]_m$ which reached a steady state after about 30 min (624 nM with succinate and 844 nM with pyruvate). Then, Na^+ (10 mM) was added which initiated Ca^{2+} efflux reducing $[Ca^{2+}]_m$ to a lower steady-state. The reduction of $[Ca^{2+}]_m$ was larger in the presence of ethanol than without ethanol; with succinate (6A) Na^+ reduced $[Ca^{2+}]_m$ from 624 to 488 nM (22%) without ethanol and to 432 nM (31%) with ethanol; with pyruvate (6B) Na^+ reduced $[Ca^{2+}]_m$ from 844 to 689 nM (19%) without ethanol and to 604 nM (28%) with ethanol. Then a very high concentration of Ca^{2+} was added (2 mM) which increased the ratio to a very high value. The maximum value of the ratio after the addition of high calcium was taken as the R_{max} of the mitochondrial trapped fura-2, since mitochondria can accumulate Ca^{2+} to μM concentration which would bind all the available fura-2. Addition of ionomycin further increased the ratio (up to 7.2) and this is attributed to fura-2 trapped in contaminating synaptosomes. It is not possible to determine precisely how much of the fura-2 is trapped in synaptosomes because a small fraction of the fluorescence signal, even before the addition of ionomycin, is contributed by the synaptosomes. However, the manipulations employed to change $[Ca^{2+}]_m$ (e.g., substrates, nM Ca^{2+} concentrations, Na^+, alcohol) have little effect on fura-2 signal trapped in purified synaptosomes (Li and Rottenberg, unpublished data). Therefore, the relative changes induced by these agents are only slightly attenuated by the presence of synaptosomal bound fura-2. In the presence of Ruthenium red, which inhibit mitochondrial Ca^{2+} uptake, only a small increase in the fluorescence ratio was induced by high Ca^{2+} indicating that the amount of external dye is less than 5% of the total. No significant changes in the fluorescence ratio were detected in mitochondria which were not loaded with fura-2 under the experimental conditions of Fig. 6. Thus, the increase in the fluorescence ratio of fura-2-loaded mitochondria by the addition of a Ca^{2+}/EGTA buffer is largely the result of an increase in $[Ca^{2+}]_m$. The amount of trapped fura-2 in the mitochondrial matrix was calculated to be 80–95 pmol/mg protein. The loading procedure caused a relatively small change in the functional quality of the mitochondrial preparation: the respiratory control ratio with glutamate plus malate as substrate was 7.42 ± 0.59 (S.D., $n = 6$) and 10.7 ± 0.15 (S.D. $n = 6$) for loaded and unloaded mitochondria, respectively.

To calculate $[Ca^{2+}]_m$ from the fura-2 fluorescence ratio it is necessary to know the apparent K_d value of the fura-2-Ca^{2+} complex in the matrix of brain mitochondria. We determined this value by measuring the ratio, in the presence of excess 4-Br-A23187, as a function of $[Ca^{2+}]_{ex}$ in an incubation medium lacking oxidizable substrates and in the presence of rotenone. This protocol should not greatly disturb the matrix viscosity, osmolarity, or ionic strength. A value of 210 nM was calculated from this titration which is in reasonable agreement with the range of values reported by others under similar experimental conditions for mitochondria [19–24]. However, this procedure does not discriminate between the mitochondrial fura-2 and the fura-2 trapped in the contaminating synaptosomes. The effect of Na^+ and ethanol on $[Ca^{2+}]_m$ were similar whether succinate or pyruvate were used as sub-

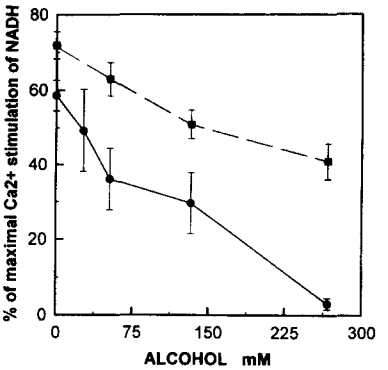

Fig. 5. The inhibition by ethanol of the Ca^{2+}-induced activation of NAD-linked dehydrogenases, with different substrates, in brain mitochondria. Conditions were as described in Fig. 3, with either pyruvate (0.15 mM) plus 1 mM malate (squares, $n = 6$), or glutamate (1 mM) plus 5 mM malate (circles, $n = 5$) and ethanol as indicated. Statistical analysis shows significant inhibition by ethanol with both substrates ≥ 53 mM ethanol ($P < 0.05$).

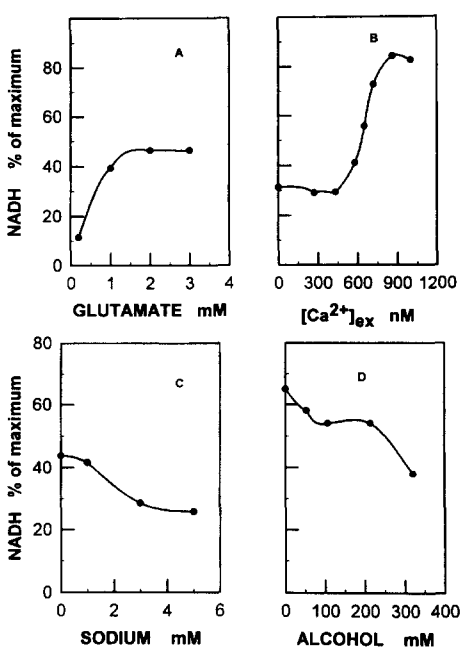

Fig. 4. The dependence of NAD(P)H steady-state level in brain mitochondria on glutamate, Ca^{2+}, Na^+, and ethanol concentrations. Conditions were as described in Fig. 3 except for the glutamate concentrations, Ca^{2+}, Na^+, and ethanol as indicated. (A) The effect of the glutamate concentration (with 569 nM Ca^{2+}). (B) The effect of $[Ca^{2+}]_{ex}$. Conditions were as in Fig. 4A, except for the presence of 1 mM glutamate and different amount of Ca^{2+}-EGTA buffer to obtain $[Ca^{2+}]_{ex}$ values as shown. (C) The effect of sodium. Conditions were as in Fig. 4B, except for $[Ca^{2+}]_{ex}$ which was 516 and Na^+ concentration as indicated. (D) The effect of alcohol. Conditions were as in Fig. 4B, except for $[Ca^{2+}]_{ex}$ which was 695 nM, Na^+ which was 2 mM and ethanol as indicated.

strate. However, because changes in NAD(P)H fluorescence and absorbance during pyruvate respiration (see Fig. 3) may interfere with fura-2 fluorescence, we conducted further measurements of $[Ca^{2+}]_m$ with succinate (+rotenone) as substrate.

The magnitude of $[Ca^{2+}]_m$ in brain mitochondria as a function of $[Ca^{2+}]_{ex}$ and $[Na^+]_{ex}$ is shown in Fig. 7A. It was previously shown for heart mitochondria (cf. [19]), and is confirmed here (Fig. 11), that the Ca^{2+} gradient ($[Ca^{2+}]_m/[Ca^{2+}]_{ex}$) is inverted (i.e., < 1), in the presence of 2 mM P_i, 1 mM Mg^{2+} and 5–10 mM Na^+ (pH 7.2). This also appears to be the case with brain mitochondria at low $[Ca^{2+}]_{ex}$, in the presence of 0.2 mM P_i, 0.5 mM Mg^{2+} and with 7–20 mM Na^+ (pH 7.2). However, considering the uncertainties in calculating $[Ca^{2+}]_m$, which result from the contaminating synaptosomes, this conclusion is tentative. Nevertheless, these results are compatible with the suggestion, that in the brain, as in the heart, the mitochondrial Ca^{2+} transport systems control $[Ca^{2+}]_m$ in a range that allow control by both $[Ca^{2+}]_i$ and $[Na^+]_i$ of the rate of NADH generation and hence oxidative phosphorylation. Fig. 7B shows that ethanol further reduced $[Ca^{2+}]_m$, in the presence of Na^+, thus supporting the suggestion that the inhibition by ethanol of oxidative phosphorylation results from stimulation of Na^+-dependent Ca^{2+} efflux. There was no effect of ethanol on $[Ca^{2+}]_m$ in the absence of Na^+, and diltiazem inhibited the ethanol effect in the presence of Na^+ (not shown).

Similar to brain mitochondria, in heart mitochondria, Na^+/Ca^{2+} exchange is the dominant Ca^{2+} efflux pathway. Therefore, it was of great interest to see whether the effect of ethanol on Na^+/Ca^{2+} exchange and the resulting inhibition of the Ca^{2+}-dependent activation of NAD-linked dehydrogenases can also be observed in heart mitochondria.

The stimulation by ethanol of the rate of Na^+/Ca^{2+} exchange in heart mitochondria is shown in Fig. 8. The rate of Na^+-induced Ca^{2+} efflux from heart mitochondria that accumulated Ca^{2+} and then were inhibited by Ruthenium red was measured with Arsenezo III as described previously [12,32]. Spermine appears to attenuate the alcohol effect at high concentrations. This is similar to its effect in brain mitochondria [12], and appears to result from the inhibitory effect of spermine of the sodium-independent Ca^{2+} efflux [32].

Fig. 9 shows the effect of ethanol on state 3 respiration,

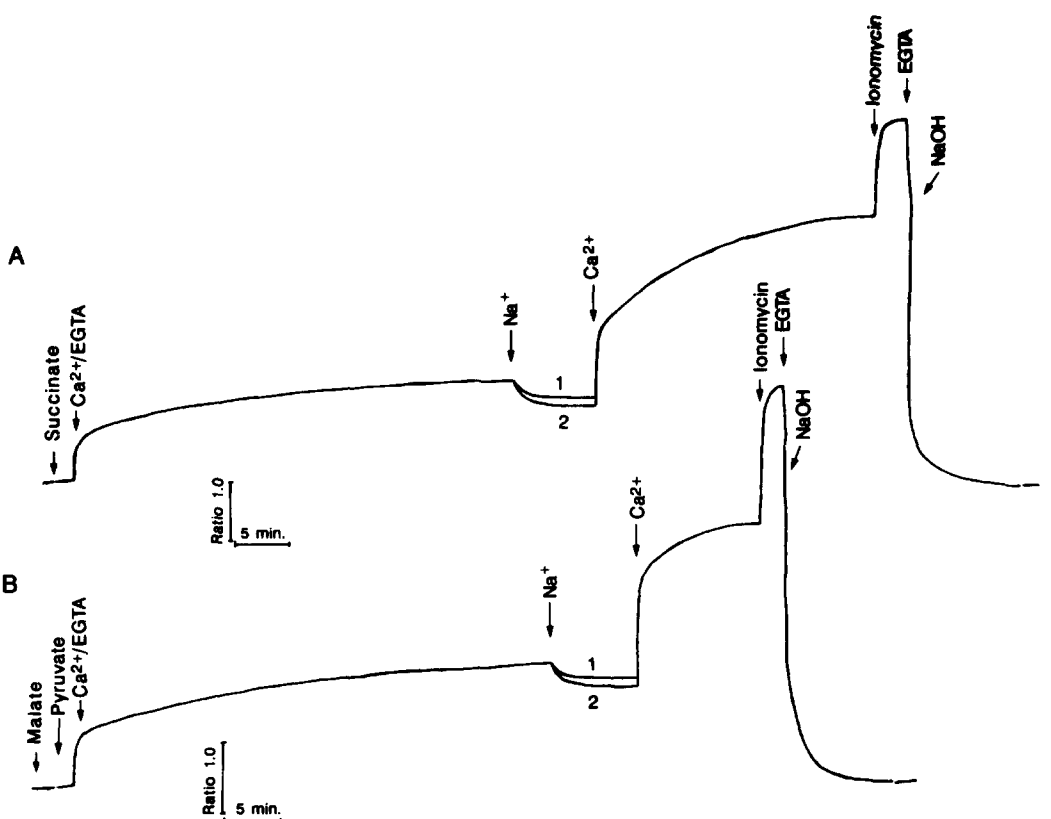

Fig. 6. The effects of $[Ca^{2+}]_{ex}$, Na^+ and ethanol on fura-2 fluorescence trapped in brain mitochondria. The protocol and conditions are described in Materials and methods. Top figure (A) is with 2 μM rotenone and 10 mM succinate as a substrate and bottom figure (B) is with 1 mM Tris-pyruvate and 1 mM Tris-malate. The experiment was started with the addition of EGTA/Ca^{2+} buffer to obtain $[Ca^{2+}]_{ex}$ = 668 nM. After steady state was established, Na^+ was added (10 mM). The signal was calibrated by addition of 2 mM Ca^{2+}, then 40 μg/mg protein ionomycin and finally 10 mM EGTA, and 16.7 μM NaOH. $R_{min} = 0.78$, $R_{max} = 7.2$. Trace 2 was with the same additions as 1 but pre-incubated with 214 mM ethanol.

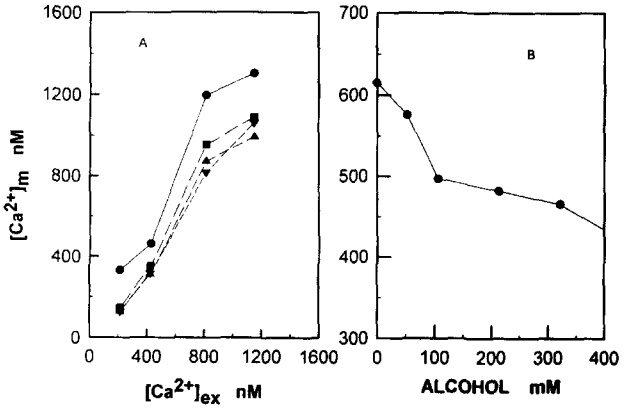

Fig. 7. The effect of Ca^{2+}, Na^+, and ethanol on $[Ca^{2+}]_m$ in brain mitochondria. $[Ca^{2+}]_m$ was determined as described in Materials and methods and as illustrated in Fig. 6A, with succinate (+rotenone) as substrate. (A) Dependence on $[Ca^{2+}]_{ex}$ and $[Na^+]_{ex}$. $[Ca^{2+}]_{ex}$ was added as indicated. Top curve (circles) was without Na^+, second curve (squares) with 7 mM Na^+, third curve (triangles) 13 mM Na^+, and bottom curve (inverted triangles) 20 mM Na^+. Values of $[Ca^{2+}]_m$ were calculated using a K_d value of 210 nM for the matrix fura-2-Ca^{2+} complex. (B) The effect of ethanol. Conditions were as in Fig. 7A, except that $[Ca^{2+}]_{ex}$ was 562 nM, and Na^+ was 10 mM.

in heart mitochondria, with 2-oxoglutarate as substrate, at submicromolar $[Ca^{2+}]_{ex}$ and in the presence of Na^+. Similar to what we observed in brain mitochondria the rate of state 3 respiration was inhibited by pharmacological concentrations of ethanol in the presence of Na^+ and Ca^{2+} at low substrate concentrations (1 mM, empty circles). Without Na^+ and Ca^{2+}, ethanol inhibited state 3 respiration significantly only above 300 mM (filled squares). Also at high substrate concentration (10 mM, filled circles) or in the presence of the Na/Ca antiporter inhibitor diltiazem (empty squares) there was no significant inhibition by

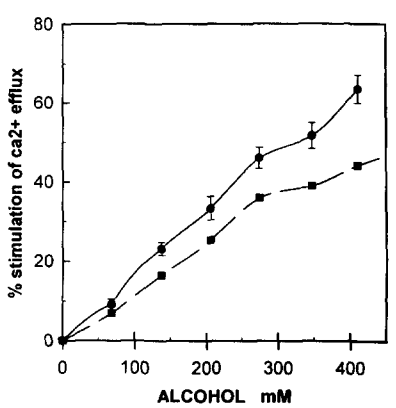

Fig. 8. Stimulation by ethanol of Na^+-dependent Ca^{2+} efflux in heart mitochondria. The protocols and conditions are described in Materials and methods except for the ethanol concentrations as shown. The effect of ethanol on the rate of Na^+-dependent Ca^{2+} efflux was tested with (squares) or without (circles) 0.5 mM spermine. The average rate of Na^+-dependent Ca^{2+} efflux, without ethanol and spermine was 10.1 ± 1.3 nmol/mg protein per min ($n = 5$).

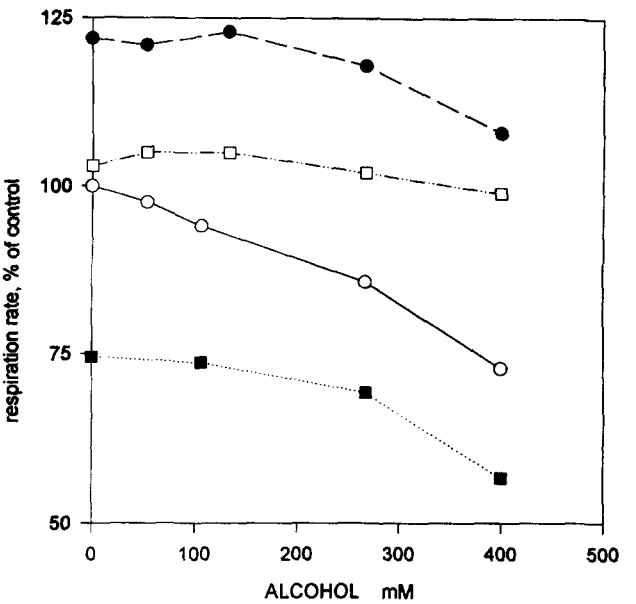

Fig. 9. The inhibition by ethanol of the Ca^{2+}-dependent stimulation of state 3 respiration in heart mitochondria. The protocols and conditions are described in Materials and methods. Substrate was 2-oxoglutarate (1 mM with 5 mM malate) with 1 mM Mg^+ and ethanol concentrations as indicated. Ethanol inhibition was observed in the presence of 1064 nM $[Ca^{2+}]_{ex}$ and 5 mM Na+ (empty circles); as controls dilitazem (20 μM) was added (empty squares), or 2-oxyglutarate was increased to 10 mM (full circles), or both Ca^{2+} and Na^+ were omitted (full squares). The rate of state 3 respiration with 1 mM 2-oxoglutarate in the presence of Ca^{2+} and Na^+ was taken as reference in each experiment (100%).

ethanol up to 300 mM. These results indicate that alcohol inhibits the Ca^{2+}-dependent activation of respiration by its effect on the Ca^{2+}/Na^+ antiporter.

Similar to brain mitochondria, Ca^{2+} increased the steady-state reduction of NAD(P) which was reduced by

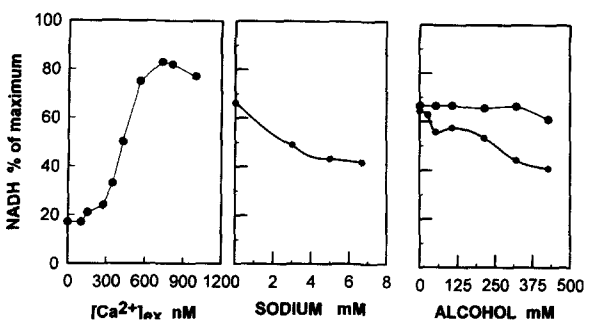

Fig. 10. The effect of $[Ca^{2+}]_{ex}$, Na^+, and ethanol on the steady-state reduction of NAD(P)H in heart mitochondria. The protocols and conditions are as described in Materials and methods except for the presence of 1 mM ADP, 0.5 mM 2-oxoglutarate and 0.5 mM malate and $[Ca^{2+}]_{ex}$, Na^+, and alcohol concentration as indicated. (A) The effect of $[Ca^{2+}]_{ex}$ (in the absence of Na^+, and ethanol). (B) The effect of Na^+. Conditions were as in Fig. 10A except for $[Ca^{2+}]_{ex}$ which was 556 nM and the indicated Na^+ concentrations. C: The effect of ethanol. Conditions were as in Fig. 10B except for $[Ca^{2+}]_{ex}$ which was 695 nM, and Na^+ which was either 10 mM (small circles) or 0 mM (large circles) and the indicated ethanol concentrations.

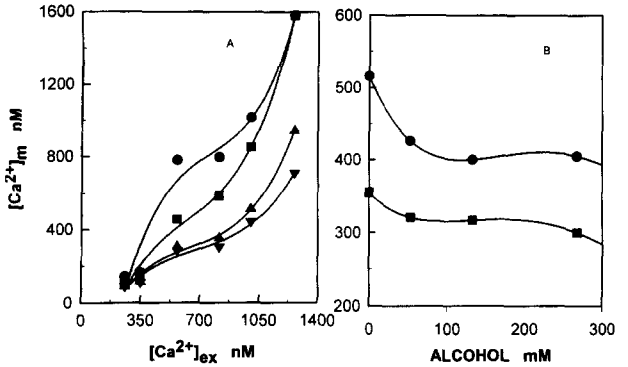

Fig. 11. The effects of $[Ca^{2+}]_{ex}$, Na^+ and ethanol on $[Ca^{2+}]_m$ in heart mitochondria. The protocol and conditions are as described in Materials and methods except that ADP was omitted and Pi was increased to 2 mM. The substrate was succinate (10 mM) with 2 uM rotenone. (A) $[Ca^{2+}]_m$ as function of $[Ca^{2+}]_{ex}$ at different Na^+ concentrations. Top curve (circles) 1 mM Na+; second curve (squares) 2 mM Na+; third curve (triangles) 5 mM Na+; and bottom curve (inverted triangles) 10 mM Na^+. (B) The effect of ethanol on $[Ca^{2+}]_m$. Condition were in Fig. 11A, except for $[Ca^{2+}]_{ex}$ which was 561 nM, the ethanol concentrations as indicated and Na^+ which was either 3 mM (circles) or 5 mM (squares).

Na^+ and ethanol. The left panel of Fig. 10 shows the dependence on $[Ca^{2+}]_{ex}$ of NAD(P)H steady-state levels in heart mitochondria which saturates above 0.9 μM (in the absence of Na^+). The middle panel of Fig. 10 shows the dependence of NAD(P)H steady-state level (at 556 nM Ca^{2+}) on $[Na^+]_{ex}$. These results are in agreement with previous observations in heart mitochondria [19] and are very similar to the results obtained with brain mitochondria (Fig. 4). The half-maximal concentration for $[Ca^{2+}]_{ex}$ was about 400 nM in fair agreement with previous studies [19]. At concentrations below 300 mM, ethanol induced a decrease in the steady-state level of NAD(P)H in the presence but not in the absence of Na^+ (Fig. 10, right panel), adding further support to the suggestion that ethanol inhibits Ca^{2+}-dependent activation of NAD-linked dehydrogenases by stimulating the Na^+/Ca^{2+} exchange.

Fig. 11 shows the effects of $[Ca^{2+}]_{ex}$, $[Na^+]_{ex}$ and ethanol on $[Ca^{2+}]_m$ in heart mitochondria, as estimated from the fluorescence ratio of fura-2 loaded mitochondria. Fig. 11A shows $[Ca^{2+}]_m$ as a function of $[Ca^{2+}]_{ex}$ in the presence of different Na^+ concentrations (1–10 mM). The magnitude of the inverted Ca^{2+} gradient ($[Ca^{2+}]_m/[Ca^{2+}]_{ex}$) in heart mitochondria was well below unity at external Ca^{2+} concentrations of 1 μM or lower (Fig. 10A) as previously reported [19]. The addition of Na^+ induced a decrease in the steady-state level of $[Ca^{2+}]_m$ at values higher than 350 nM; the lack of effect by Na^+ at lower $[Ca^{2+}]_m$ may be related to the affinity of the Na^+/Ca^{2+} antiporter for matrix Ca^{2+}. Similarly ethanol enhanced the Na^+-dependent decrease in $[Ca^{2+}]_m$ much more strongly when $[Ca^{2+}]_m$ was above 500 nM than under condition where $[Ca^{2+}]_m$ was 350 nM (Fig. 11B).

4. Discussion

4.1. The effect of ethanol on the mitochondrial Na^+/Ca^{2+} antiporter

This study demonstrates that the Na^+/Ca^{2+} antiporter of heart mitochondria is stimulated by ethanol, similar to the previously reported stimulation in brain mitochondria [12,13]. There are some apparent differences between the effects of ethanol in the two systems. In particular, it appears that the stimulation of Ca^{2+} efflux require somewhat higher concentrations of ethanol in heart mitochondria (EC_{50} = 200 mM) than in brain (EC_{50} = 130 mM).

4.2. Ca^{2+} regulation of oxidative phosphorylation in the central nervous system

Modulation of dehydrogenase activity and oxidative phosphorylation by Ca^{2+} in the brain has been a topic of some controversy because of the contradictory findings of stimulation of respiration by Ca^{2+} in brain mitochondria and synaptosomes [37,38]. As shown in this work, in agreement with previous reports [38], a Ca^{2+} stimulation of mitochondrial metabolism can be observed in isolated, Ca^{2+}-depleted, brain mitochondria, at low NAD-linked substrates concentrations. Recently, we have also found a very pronounced $[Ca^{2+}]_i$-dependent stimulation of glucose oxidation in synaptosomes, and this stimulation was also inhibited by ethanol. At low ethanol concentrations this inhibition resulted from a stimulation of the mitochondrial Na^+/Ca^{2+} exchange, while at higher concentrations, the inhibition resulted from inhibition of the voltage-activated Ca^{2+} channels (Li, H.L. and Rottenberg, H., unpublished data).

It is usually assumed that the cellular Na^+ concentration is well above the saturating concentration of the antiporter (K_{50} = 7–8 mM) and is essentially constant. Recent measurement of myocytes $[Na^+]_i$ with fluorescent indicators cast doubts on the validity of this assumption [39]. Excitation of nerve cells is associated with a rapid transient rise in $[Ca^{2+}]_i$, and this should rapidly stimulate oxidative phosphorylation. However, prolonged excitation is associated with prolonged elevation of $[Ca^{2+}]_i$, which may overload $[Ca^{2+}]_m$. But, since such excitation is also associated with a slow rise of $[Na]_i$ [40], this may protect the mitochondria from Ca^{2+} overloading.

4.3. The effects of ethanol on $[Ca^{2+}]_m$, dehydrogenase activation and oxidative phosphorylation

The inhibitory effect of low concentrations of ethanol on respiration and NAD(P)H level was completely dependent on the presence of both mM concentrations of Na^+, and submicromolar concentrations of Ca^{2+}, and thus appears to be the result of the stimulatory effect on the

Na^+/Ca^{2+} antiporter. However, at high concentrations, ethanol inhibited pyruvate and glutamate oxidation independently of Na^+ and Ca^{2+}. This nonspecific effect, which we have reported previously [36], is largely irrelevant to the pharmacological effects of ethanol.

There are apparent differences between the extent and concentration-dependence of the inhibition of $[Ca^{2+}]_m$, NAD(P)H steady-state levels, and state 3 respiration by Na^+ and ethanol, both in heart and brain mitochondria. These differences partially arise from differences in the optimal assay conditions for each of the measured parameters. However, more importantly, these differences result from the fact that each preparation maintains somewhat different $[Ca^{2-}]_m$ and hence was modulated to a different degree by added Ca^{2+}, Na^+ and alcohol. As Fig. 11A shows, the magnitude of $[Ca^{2+}]_m$ greatly affects the magnitude of the effects of Na^+ and ethanol. When $[Ca^{2+}]_m$ is too low, Na^+/Ca^{2+} exchange can not be stimulated, and when it is too high, (above the $K_{0.5}$ for NAD-linked dehydrogenases activation), stimulation of the exchange does not reduce $[Ca^{2+}]_m$ sufficiently to deactivate the enzymes. Nevertheless, it is clear that in both heart and brain mitochondria, under near physiological conditions (e.g., mM concentrations of Mg^{2+}, Na^+, and P_i, submicromolar Ca^{2+}, non-saturating substrates concentration, and physiological pH (7.2)), ethanol, at pharmacological concentrations, can attenuate the excitation-induced Ca^{2+} signalling to mitochondria. In most of our experiments we observed significant effects of ethanol at 50 mM and above, concentrations which are frequently found in the blood of binge drinkers, and often maintained, for long periods of time, in the blood of chronic alcoholics. Thus it is likely that the effects on mitochondrial metabolism, described in this study, occurs, in vivo, and contribute to the acute and chronic effects of ethanol, on the brain, heart, skeletal muscle and other tissues, in which $[Ca^{2+}]_m$ is controlled by the Na^+/Ca^{2+} antiporter.

Since sodium-induced Ca^{2+} efflux significantly reduced Ca^{2+} content of brain mitochondria and alcohol enhanced this effect (32,12) it is clear that ethanol attenuates the Ca^{2+} buffering capacity of mitochondria, which may have important implication to cell Ca^{2+} homeostasis as well. It is generally believed that mitochondria do not contribute, significantly, to the regulation of the cell free Ca^{2+} concentration [41]. This conclusion, is well-established in regard to the resting, steady-state Ca^{2+} level, which is about 100 nM in most cells. However, during cell excitation, Ca^{2+} can rise locally and transiently, well above 1 μM, which is well within the buffering capacity of mitochondria [32]. Indeed, several recent studies with neurons, synaptosomes, pancreatic β-cells, and myocytes clearly demonstrate the ability of mitochondria to buffer cell calcium during excitation [42–46]. Thus, attenuation, by ethanol, of this capacity can modulate calcium signalling in susceptible cells. Moreover, occasionally, cells are subjected to abnormally high Ca^{2+} insult, as for example during hypoxia, ischemia/reperfusion, excitotoxicity, or drug withdrawal. It is believed that mitochondrial calcium metabolism affects the outcome of these events, although at present, the precise role of mitochondria in these processes is not clear. Nevertheless, the fact that ethanol impairs the buffering capacity of mitochondria suggest that it can modulate the outcome of these catastrophic events (cf. [47]).

4.4. Acute and chronic effects of ethanol on brain and heart and the ethanol effect on mitochondrial metabolism, $[Ca^{2+}]_m$ signalling, and $[Ca^{2+}]_m$ buffering

Ethanol inhibits glucose utilization in selected brain regions [48,49], and this inhibition may be mediated by the attenuation of calcium-dependent activation of oxidative phosphorylation. Chronic alcohol feeding was shown to cause profound disturbances of glucose metabolism in muscle [50] and to inhibit the excitation-dependent increase in ATP level in myocytes [51]. There is little information regarding the acute effect of ethanol on Ca^{2+}-dependent stimulation of oxidative phosphorylation in heart or brain, in vivo. However, our recent findings of strong inhibition by ethanol of Ca^{2+}-activated glucose oxidation in synaptosomes (Li, H.L. and Rottenberg, H., unpublished data) support the conclusion that this effect is physiologically relevant.

In conclusion, this study shows that ethanol induces a decrease in mitochondrial $[Ca^{2+}]_m$, in both brain and heart, and thus inhibits the Ca^{2+}-dependent stimulation of NAD-linked dehydrogenases and oxidative phosphorylation. This effect is the direct result of a stimulation by ethanol of the mitochondrial Na^+/Ca^{2+} antiporter. This observation may explain, in part, the inhibition by alcohol of glucose utilization, which is associated with inhibition of excitability, in the brain. It may also explain, in part, the inhibition by ethanol of force generation in muscle and the development of myopathies in chronic alcoholics.

Acknowledgements

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