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SHORT COMMUNICATIONS

Effects of calcium antagonists on glycolysis of rat brain synaptosomes

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Abstract—The effects of calcium antagonists nimodipine, nicardipine and flunarizine on lactate production and specific activities of some enzymes regulating glycolytic flux have been evaluated in synaptosomes isolated from rat whole brain and submitted to *in vitro* chemical hypoxia induced by rotenone, an inhibitor of mitochondrial respiration. The following enzymes have been tested; hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1), phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) and pyruvate kinase (ATP: pyruvate 2-*O* phosphotransferase, EC 2.7.1.40). The results show that rotenone increases by about eight times the production of lactate; nicardipine and nimodipine, starting from a concentration of 10^{-4} M, were able to counteract the rotenone-induced stimulation of glycolysis, but flunarizine was without effect. The dihydropyridines but not flunarizine decreased the maximum activity of phosphofructokinase. This effect was already detectable at a concentration of 10^{-5} M. Neither hexokinase nor pyruvate kinase were affected by any of the drugs studied.

Calcium antagonist drugs at high concentrations have been found to interact with sites different from the classical voltage-operated calcium channels [1]. These properties might account for some pharmacological and therapeutical actions which cannot be ascribed solely to their calcium-blocking activity.

In a recent study, utilizing rat brain synaptosomes, we observed a strong reduction of lactate production in the presence of high concentrations (90 μM) of nicardipine, both in basal conditions and when glycolysis was stimulated by the rotenone-induced inhibition of mitochondrial respiration [2]. This effect appears to be independent from the calcium antagonist activity, to which its anti-ischemic action was ascribed [3], in fact in the same conditions neither diltiazem nor verapamil showed this action [2], on the other hand the very high concentration utilized could induce quite unspecific effects.

Nevertheless, a drug able to slow down the massive lactate production in ischemia might potentially exert, in the brain, a protection against this pathological condition [4]. Therefore, the purpose of this study was to investigate the possibility that nicardipine, in a range of concentrations similar to that reached in the brain at therapeutical doses, still shows a braking action on lactate over-production. We also extended the investigation to another 2,4-dihydropyridine (DHP*), nimodipine which is considered to be specific for the cerebral compartment [5], and a drug from a different class of calcium antagonists, flunarizine (a diphenyl-piperazine), that also was claimed to exert an anti-ischemic action in brain [6]. The drugs' effects have been studied at concentrations of 10^{-3}–10^{-7} M both on lactate production under stimulated glycolysis and on the potential (maximum) activity of regulatory enzymes of glycolysis.

Materials and Methods

Isolation and incubation of synaptosomes. Male Sprague–Dawley rats, aged about 2 months, were utilized throughout. The animals were killed by decapitation, the brain was rapidly removed and immersed in ice-cold isotonic isolation medium (IM: 0.32 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4). Forebrains were homogenized in the same medium (1:10 w/v) by 14 strokes in a Dounce homogenizer with a glass pestle. Synaptosomes were isolated by differential and Ficoll-sucrose density gradient centrifugation [7]. The final pellet was suspended in KHH, at a final millimolar concentration of: 140 NaCl, 5 KCl, 1.3 $MgSO_4$, 5 $NaHCO_3$, 1 $NaHPO_4$, 10 Tris–Hepes, 1.2 $CaCl_2$, 10 glucose, pH 7.4) at 3–4 mg protein/mL.

Lactate production measurement. After 10 min pre-incubation at 30°, under shaking in KHH. for the recovery of membrane potential, synaptosomal suspensions were supplemented with the drugs at different concentrations (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} 10^{-3} M) or with an equivalent volume of vehicle (DMSO for nicardipine and nimodipine; cyclodextrin for flunarizine), in basal conditions or in the presence of 10 μM rotenone. After 30 min incubation under the same conditions, synaptosomal suspensions were deproteinized by rapid mixing with cold perchloric acid (4% w/v final concentration) to stop lactate production; the clear supernatant neutralized with triethanolamine/K_2CO_3 was used for the determination of lactate concentration by an enzymatic method [8] in a spectrophotometer at $\lambda = 340$ nm. In preliminary experiments we observed no interference due to the addition of the vehicles alone in which the drugs and rotenone were dissolved, except for cyclodextrin in the quantity used to obtain the highest concentration of flunarizine (10^{-3} M) which represented 10% of the total volume, while in all other cases the volume added did not exceed 1% of the total.

Enzyme activity evaluation. Maximum rate of enzyme activities (specific activity expressed as nmol/min/mg of protein) was measured by spectrophotometric techniques using pyridin-nucleotide oxidizing/reducing coupled reactions; the following enzyme assays were carried out: hexokinase [9], phosphofructokinase [10] and pyruvate kinase [11]. To unmask all the compartmentalized enzyme molecules, Triton X-100 was added to the reaction mixtures at non-inhibiting concentrations (0.1–0.2% v/v). The vehicle or drugs were also added in the cuvette at different concentrations (10^{-6}, 10^{-5}, 10^{-4} M). We omitted the 10^{-3} and 10^{-7} M concentrations utilized for experiments of lactate determination because the highest one is probably toxic and without pharmacological relevance *in vivo* and the lowest one did not affect lactate production.

Other measurements. Protein content was evaluated by the biuret method [12] with bovine serum albumin as a standard. Statistical analysis was performed using one- or

* Abbreviations: KHH, Krebs–Henseleit–Hepes buffer; DMSO, dimethyl sulfoxide; DHP, dihydropyridine; HK, hexokinase; PFK, phosphofructokinase; PK, pyruvate kinase.

two-factor analysis of variance, and Dunnett's test for comparisons with the control groups.

Chemicals. Nicardipine, nimodipine, flunarizine, Ficoll 400 DL, DMSO and cyclodextrin were from the Sigma Chemical Co. (St Louis, MO, U.S.A.); enzymes, co-enzymes and substrates were from Boehringer Mannheim (Germany). Other reagents were of the highest degree of purity commercially available. Bidistilled water further purified by a Milli-Q (Milipore, Bedford, MA, U.S.A.) apparatus was utilized.

Results and Discussion

Total lactate levels measured in synaptosomal suspensions after 30 min were 30.3 ± 2.6 nmol/mg synaptosomal protein (N = 16) in basal unstimulated condition (no rotenone addition); 10 μM rotenone induced an eight-fold stimulation: 234.6 ± 9.0 nmol/mg synaptosomal protein (N = 16).

Nicardipine (Fig. 1) in the basal condition did not affect lactate production at any concentration tested. The drug was able to inhibit 83% and 55% of the rotenone-induced stimulation of lactate production at the highest concentrations 10^{-3} (47.0 ± 5.7, N = 6; $P < 0.01$) and 10^{-4} M (118.7 ± 26.8, N = 6; $P < 0.01$), respectively, while at 10^{-5} M showed only a slight 11% (234.2 ± 17.2, N = 6; statistically non-significant) contrast of rotenone-induced increase in lactate production (262.8 ± 18.2 nmol/mg protein, N = 6).

Nimodipine (Fig. 1) in the unstimulated condition did not affect lactate production at any of the concentrations tested. The drug was able to inhibit 73% and 23% of the rotenone-induced stimulation of lactate production at the highest concentrations 10^{-3} (68.6 ± 7.9, N = 4; $P < 0.01$) and 10^{-4} M (176.3 ± 11.6, N = 6; $P < 0.01$), respectively, while at 10^{-5} M showed no inhibition of the rotenone-induced increase in lactate production (228 ± 12.1 nmol/mg protein, N = 6).

Flunarizine (Fig. 1) exerted an effect only at the highest concentration of 10^{-3} M, increasing lactate production in the unstimulated condition (137.5 ± 18.5 vs 33.3 ± 6.0 nmol/mg protein; N = 4), an effect probably due to the higher concentration of vehicle utilized, and decreasing the rotenone-induced stimulation by 31% (148.2 ± 12.7 vs 215.1 ± 10.6 nmol/mg protein; N = 5).

The results concerning the enzyme activities evaluated are shown in Table 1. It can be observed that HK and PK specific activities were not affected by any concentration of any drug tested (Table 1). PFK specific activity was reduced by 10^{-4} M nimodipine to 60% of basal value (72.8 ± 7.5 vs 109.9 ± 5.4 nmol/min/mg protein; $P < 0.05$, N = 6); 10^{-5} M nimodipine showed only a trend in this direction (100.1 ± 8.4 vs 109.9 ± 5.4 nmol/min/mg protein, N = 6). Only the highest concentration of nicardipine induced a decrease in PFK activity by about 18% (91.4 ± 7.1 vs 111.5 ± 5.7 nmol/min/mg protein, N = 6), although statistical significance was not reached.

The results obtained in this investigation show that in spite of the fact that nicardipine is mostly utilized as a peripheral vasodilator in our model it shares with the other DHP nimodipine the ability to affect lactate production at concentrations around 0.1 mM, nicardipine being more potent than nimodipine; flunarizine even at the highest concentration utilized was unable to counteract rotenone-stimulated lactate production, reproducing in this way a behaviour shown by both diltiazem and verapamil [2].

Concerning the molecular target in the glycolytic pathway, it appears that the maximum activity of PFK is specifically affected by the DHPs (Table 1). In this case nimodipine is more potent than nicardipine, but flunarizine is again ineffective. The discrepancy between the results on lactate production and enzyme activity, as far as relative drug potency is concerned could be due to their different liposolubility [13] that can affect both the solubility in the incubation medium, the rate of passing through synaptosomal plasma membrane, and the nature of interactions with the enzyme proteins. These results obtained *in vitro* might be related to the observation of several authors who found *in vivo* DHPs, in particular nimodipine, active in slowing down lactate production and acidosis induced by both ischemia [14] in hyperglycemic

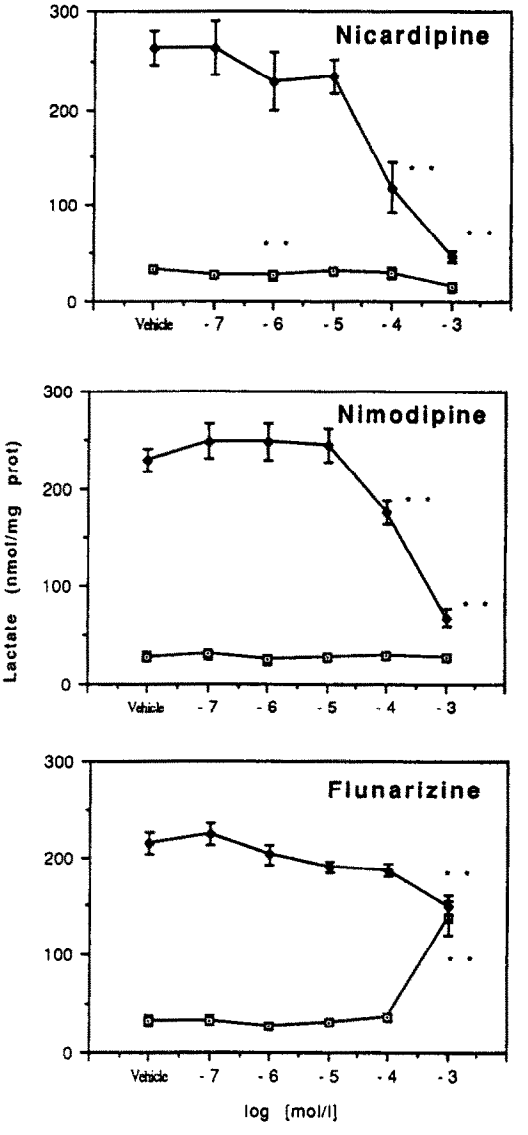

Fig. 1. Effect of scalar concentrations of nicardipine, nimodipine and flunarizine on lactate production in rat brain synaptosomes incubated in basal (-□-) conditions or with 10 μM rotenone (-◆-). Lactate concentrations shown (nmol/mg protein), are measured by standard spectrophotometric techniques at the end of a 30 min incubation at 30°, as described in Materials and Methods, in standard medium supplemented with 10 mM glucose and 1.27 mM $CaCl_2$. Data represent the mean values (with standard error bars) from five separate experiments, each performed on a single animal. Statistical analysis was performed using two-factor analysis of variance (factor A, rotenone presence; factor B, drug concentration) and Dunnett's test for comparisons with the control (vehicle, inside the group of the same factor A). ** $P < 0.01$.

Table 1. Effect of scalar molar concentrations of nicardipine, nimodipine and flunarizine on specific activities of the enzymes HK, PFK and PK, evaluated on rat brain synaptosomes

Enzyme	Concentrations (M) 0	10^{-6}	10^{-5}	10^{-4}
Nicardipine				
HK	128.10 ± 9.5	140.10 ± 6.9	133.60 ± 8.0	135.80 ± 7.7
PFK	111.51 ± 5.7	118.89 ± 10.4	113.62 ± 8.8	91.37 ± 7.1
PK	587.23 ± 42.5	646.90 ± 42.6	609.81 ± 45.9	531.40 ± 52.1
Nimodipine				
HK	146.60 ± 9.1	143.20 ± 12.7	147.30 ± 8.2	146.50 ± 9.6
PFK	109.95 ± 5.4	114.66 ± 10.5	100.12 ± 8.4	72.83 ± 7.5*
PK	593.46 ± 83.6	620.08 ± 89.7	592.01 ± 94.8	545.17 ± 92.6
Flunarizine				
HK	136.00 ± 14.0	137.80 ± 13.3	135.00 ± 11.8	133.50 ± 9.0
PFK	124.44 ± 5.4	132.49 ± 7.5	122.66 ± 5.3	121.96 ± 2.4
PK	585.46 ± 106.5	639.61 ± 84.6	606.31 ± 76.2	632.24 ± 143.3

Enzymatic activities were evaluated in the synaptosomal fraction as described in Materials and Methods, and expressed as specific activities (nmol/min/mg protein). The values are the mean ± SE of N = 4 experiments, each performed using a single animal.

For statistical analysis one-way analysis of variance was performed, and data confronted with the control values (no drug) using Dunnett's test. * $P < 0.05$.

rats [15] and in thiamine-deprived rats [16]. Although these effects of nimodipine were not found by other authors utilizing different experimental models of ischemia [17, 18], it is interesting to note that when the effects on blood flow are excluded, like in ischemia induced in the isolated brain model [14] or in a model of thiamine-deprivation-induced metabolic acidosis [16], a direct action of nimodipine on tissular lactate production becomes evident.

Before making a direct correlation between these results obtained *in vitro* on isolated nerve terminals and in *in vivo* ischemic conditions we need to know if the concentrations of the drugs present inside synaptosomes are of the same order of magnitude as those obtained *in vivo* with therapeutic doses of these drugs. Future experiments will be addressed to clarify this point.

In conclusion, the results obtained in this investigation suggest a new molecular mechanism for nimodipine and nicardipine consisting of a direct and specific action on neuronal glycolysis. This action, detectable on whole brain synaptosomes, agrees with others found for different calcium antagonists [1] which at high concentrations can interact with cellular and macromolecular targets different from L-type voltage-operated calcium channels.

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Increased sterigmatocystin-induced mutation frequency in *Saccharomyces cerevisiae* expressing cytochrome P450 CYP2B1

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Currently almost 25% of the world population will develop cancer. The DNA damage associated with exposure to environmental chemicals is likely to play a major role in this process [1]. Consequently, rapid and reliable tests to determine the carcinogenic/mutagenic potential of chemicals are required. The large majority of chemical carcinogens are only mutagenic when a suitable metabolic activation system is present [2]. Almost invariably these activation systems comprise hepatic subcellular fractions containing cytochrome P450 isoenzymes [3–6]. This is a limitation in the various predictive tests that are in use since short-lived or highly reactive species will be formed outside the cell and may, therefore, not interact with the DNA to elicit a detectable response [7]. There is currently a need for new mutation tests with additional properties including endogenous activation [8]. This can be achieved by the expression of P450 isoenzymes in suitable recipient cells using the growing number of cDNAs now available. *Saccharomyces cerevisiae* was identified as a suitable organism for such studies due to its ease of manipulation and the presence of cytochrome P450 reductase which is required for a functional monooxygenase activity. The rat CYP2B1 (P450IIB1, the major phenobarbital (PB*)-inducible cytochrome P450 form) cDNA was expressed using a 2 μM-based yeast expression vector, pMA56 [9] and mutation assays carried out to establish whether the potent mycotoxin STC was activated to mutagenic products by this enzyme.

Materials and Methods

The details regarding the subcloning of the rat CYP2B1 cDNA have been outlined elsewhere [10]. The yeast strain used in these experiments, KY118 (α, trpΔ1, ade2-101°, ura3-52, his3-200, lys2-801^{am}) was chosen because of its extremely low (almost undetectable) P450 levels. The lithium acetate method [11] was used to introduce the expression vector into the yeast strain.

Protein levels were determined using the folin phenol reagent [12] and cytochrome P450 from the reduced carbon monoxide difference spectrum [13]. The O-dealkylation of benzyloxyresorufin was determined as described previously [10, 14].

Western blots were carried out [15–16] using polyclonal antisera to either CYP2B1 or CYP2C6 proteins. Mutation assays were carried out using L-canavanine resistance as a determinant of mutation frequency [10, 17–20].

Results and Discussion

The carcinogenic mycotoxin STC is produced by fungal species of the *Aspergillus, Bipolaris* and *Penicillium* genera [21]. This compound has been shown to induce hepatocellular carcinomas after oral or intraperitoneal administration as well as squamous cell carcinomas after repeated application to the skin [22]. Although the STC-producing fungi are widespread the health hazard to humans remains unclear as the presence of STC in foodstuffs has been found only rarely despite extensive surveillance programmes [23].

STC is of interest as a model compound in studies of the mechanism of carcinogenesis due to its structural homology with aflatoxin B_1. Although the DNA lesion produced by STC has been isolated and characterized [24] little information is available on the metabolic activation of STC. However, it has been demonstrated that mutation rates are increased by the use of PB-induced microsomes. Thus, this study was carried out to determine if CYP2B1 (the major rat PB-inducible cytochrome form) is involved in the activation of this compound.

The levels of CYP2B1 expression following transfection of yeast with pMA56 carrying the CYP2B1 insert are shown in Fig. 1. Densitometric analysis of the Western blots indicated that CYP2B1 represented between 0.1–0.2% of total yeast protein. This protein was not found in the control strain (56 par). When probed with antisera specific to CYP2C6 no cross-reacting bands were observed in either strain indicating that the CYP2B1 antisera is specific for

* Abbreviations: PB, phenobarbital; STC, sterigmatocystin.