Amino Acids (2009) 36:449–456
DOI 10.1007/s00726-008-0099-5

ORIGINAL ARTICLE

Ca^{2+}-independent effects of spermine on pyruvate dehydrogenase complex activity in energized rat liver mitochondria incubated in the absence of exogenous Ca^{2+} and Mg^{2+}

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Received: 1 April 2008 / Accepted: 29 April 2008 / Published online: 24 May 2008

Abstract In the absence of exogenous Ca^{2+} and Mg^{2+} and in the presence of EGTA, which favours the release of endogenous Ca^{2+}, the polyamine spermine is able to stimulate the activity of pyruvate dehydrogenase complex (PDC) of energized rat liver mitochondria (RLM). This stimulation exhibits a gradual concentration-dependent trend, which is maximum, about 140%, at 0.5 mM concentration, after 30 min of incubation. At concentrations higher than 0.5 mM, spermine still stimulates PDC, when compared with the control, but shows a slight dose-dependent decrease. Changes in PDC stimulation are very close to the phosphorylation level of the $E_{1\alpha}$ subunit of PDC, which regulates the activity of the complex, but it is also the target of spermine. In other words, progressive dephosphorylation gradually enhances the stimulation of RLM and progressive phosphorylation slightly decreases it. These results provide the first evidence that, when transported in RLM, spermine can interact in various ways with PDC, showing dose-dependent behaviour. The interaction most probably takes place directly on a specific site for spermine on one of the regulatory enzymes of PDC, i.e. pyruvate dehydrogenase phosphatase (PDP). The interaction of spermine with PDC may also involve activation of another regulatory enzyme, pyruvate dehydrogenase kinase (PDK), resulting in an increase in $E_{1\alpha}$ phosphorylation and consequently reduced stimulation of PDC at high polyamine concentrations. The different effects of spermine in RLM are discussed, considering the different activities of PDP and PDK isoenzymes. It is suggested that the polyamine at low concentrations stimulates the isoenzyme PDP_2 and at high concentrations it stimulates PDK_2.

Keywords Pyruvate dehydrogenase · Mitochondria · Spermine · $E_{1\alpha}$ phosphorylation · Pyruvate dehydrogenase kinase · Pyruvate dehydrogenase phosphatase

Abbreviations

$\Delta\Psi$	Membrane potential value
E_1	Pyruvate dehydrogenase
E_2	Dihydrolipoamide transacetylase
E_3	Dihydrolipoamide dehydrogenase
EGTA	Ethylene glycol tetraacetic acid
FCCP	Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PDP	Pyruvate dehydrogenase phosphatase
Pi	Phosphate
ROS	Reactive oxygen species
RLM	Rat liver mitochondria
SPM	Spermine
TPP^+	Tetraphenylphosphonium

Introduction

The mammalian pyruvate dehydrogenase complex (PDC) catalyses the irreversible physiological step of carbohydrate

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fuels (Reed and Hackert 1990; Patel and Roche 1990; Behal et al. 1993). It consists of three enzymes organized in a high-molecular-mass complex: pyruvate dehydrogenase (E_1), dihydrolipoamide transacetylase (E_2) and dihydrolipoamide dehydrogenase (E_3); the catalytic components of these complexes are held together by non-covalent bonds. Besides these catalytic components, mammalian PDC also contains a structural component, E_3 binding protein (De Marcucci et al. 1986), and two regulatory enzymes, pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP) (Linn et al. 1969). The activities of these two enzymes determine the proportion of PDC in its active dephosphorylated state, control the flux through the complex, and contribute to the overall control of aerobic oxidation of carbohydrate fuels (Randle 1986).

In the 1980s and 1990s, PDC was the subject of intensive studies because it appears that, in many circumstances, changes in the phosphorylation state of the complex correlate directly with the existence of multiple isoenzymes of PDK and PDP (Popov et al. 1994; Gudi et al. 1995; Rowles et al. 1996; Huang et al. 1998). These isoenzymes are responsible for tissue-specific regulation of PDC activity (Bowler-Kinley et al. 1998; Sugden et al. 1998; Wu et al. 1998).

Previous reports indicate that the polyamine spermine is an important regulator of calcium transport in mitochondria, with consequent activation of Ca^{2+}-sensitive dehydrogenase, including PDC (Nicchitta and Williamson 1984; Votyakova et al. 1990).

Naturally occurring polyamines (putrescine, spermidine, spermine), polyvalent cations widely distributed in nature, are involved in the control of cellular growth and differentiation processes (Heby 1981; Pegg and McCann 1982; Pegg 1984) and also in many crucial physiopathological processes (Agostinelli et al. 2004). In addition to these effects, spermine has a remarkable protective action not only on DNA structure, but also on mitochondrial functions and membrane permeability, as it is a reactive oxygen species (ROS) scavenger (Philips and Chaffee 1982; Lapidus and Sokolove 1992, 1993, 1994; Tassani et al. 1995a, 1996; Sava et al. 2006) (for reviews on polyamine and mitochondria, see Toninello 2001; Toninello et al. 2004).

Although polyamines are synthesized in the extramitochondrial space, they are also present within mitochondria (Mancon et al. 1990). We have previously demonstrated that they are transported bidirectionally across the inner membrane in rat liver (Toninello et al. 1988, 1992a, 2000), heart (Toninello et al. 1990) and brain mitochondria (Tassani et al. 1995b). The influx, which occurs electrophoretically, depends on a high transmembrane potential ($\Delta\Psi$) and exhibits a non-linear current/voltage relationship (Toninello et al. 1992b). Therefore, the reversible transport of polyamines may be of remarkable physiological importance in modulating PDC activity.

Several laboratories report that spermine is able to stimulate E_1 activity and consequently PDC activity, in both purified enzymes (Thomas and Denton 1986; Damuni et al. 1984) and isolated adipose tissue, liver and heart mitochondria (Thomas and Denton 1986; Rutter et al. 1992; Moreno-Sanchez and Hansford 1988). Most of these observations state that this stimulation is Ca^{2+}- and Mg^{2+}-dependent, as Ca^{2+} decreases the Ka of PDP for Mg^{2+}, which activates this enzyme. It should also be noted that a further but distinct effect of spermine, which may also lead to the activation of PDP, is the activation of Ca^{2+} uptake into mitochondria (Nicchitta and Williamson 1984).

It is stressed here that the mitochondria used in the above-mentioned experiments were always de-energized and frequently disrupted by hypotonic media, organic solvents or detergents. The aim of this work is to ascertain if, in intact liver mitochondria—in the absence of exogenous Mg^{2+} and Ca^{2+} and in the presence of EGTA, to exclude any activation of the complex by contaminating cations—spermine is able to reach the inner mitochondrial compartment, where PDC is located, and activate it. The presence of the chelating agent, EGTA, minimizes the free Ca^{2+} concentration and excludes possible PDC activation by spermine-stimulated Ca^{2+} transport. In these conditions, the enzymatic complex can only use endogenous Mg^{2+} and Ca^{2+}.

Experimental procedures

[1-^{14}C]-Pyruvate was purchased from NEN^{TM} Life Science products, Inc. [γ-^{32}P]ATP was from Amersham. PDH-$E_{1\alpha}$(9H9) mouse monoclonal antibody was purchased from Sant Cruz Biotechnology, Inc. All other reagents were of the highest purity commercially available.

Rat liver mitochondria were isolated in 250 mM sucrose and 5 mM Hepes (pH 7.4) by conventional differential centrifugation (Schneider and Hogeboom 1950). Mitochondrial protein was assayed by a biuret method with bovine serum as a standard (Gornall et al. 1949).

Incubations were carried at 20°C with 1 mg of mitochondrial protein/ml suspended in the following standard medium: 200 mM sucrose, 10 mM Hepes-Cl (pH 7.4), 5 mM pyruvate, 5 mM malate, 2 mM phosphate (Pi), 1 mM EGTA. Spermine concentrations and other additions are indicated in the descriptions of specific experiments.

Membrane potential ($\Delta\Psi$) was measured in an open, thermostatically controlled, stirred vessel, by monitoring the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a selective electrode, prepared according to published procedures (Kamo et al. 1979; Affolter and Siegel 1979), and an Ag/AgCl reference electrode. Measurements were corrected as proposed by Jensen et al. (1986).

Uptake of [C^{14}]spermine was determined by a previously described centrifugal filtration method (Toninello et al. 1988).

Efflux of Ca^{2+} and Mg^{2+} was measured by absorption spectroscopy of the supernatant with a centrifugal filtration method (Toninello et al. 1988).

PDC activity was estimated on the basis of $^{14}CO_2$ production by PDC, by supplying [1-C^{14}]-pyruvate (0.05 μCi/ml) to standard medium. The incubation mixture was placed in Stopper Top Gross vials (Kontes) and maintained in agitation. The $^{14}CO_2$ produced during incubation was trapped by hyamine hydroxide placed in an appropriate basket in a vial with filter paper. At the times indicated in the experiments, the reaction was blocked with TCA 50%. After 30 min all samples, or filter papers soaked in hyamine hydroxide, were transferred to mini-vials together with 4.5 ml of scintillator liquid, and radioactivity was assayed on a Beckman counter.

To evidence the relationship between PDC activity and its degree of phosphorylation, we compared, in the same conditions, the results of $^{14}CO_2$ production by the complex (with increasing concentrations of polyamine) with its phosphorylation pattern in intact rat liver and heart mitochondria. We therefore carried out experiments in which Ser-phosphorylation of pyruvate dehydrogenase complex was achieved by incubating 1 mg of mitochondrial protein in 1 ml of incubation medium, as previously described (Clari et al. 1990), at 20°C for 30 min. The reaction mixture (pH 7.4) contained 12 μM ATP and γ-[P^{32}]-ATP (24 μCi/ml). The samples differed in their concentrations of polyamine spermine, as indicated in the experiments (see below). The reactions were stopped by the addition of 2% SDS and 1% 2-mercaptoethanol, followed by a 5' treatment at 100°C, as described in Clari et al. (1990). The solubilized mitochondria were analysed by SDS/PAGE, essentially according to Laemmli (1970), and transferred to nitrocellulose membranes. After treatment with 3% bovine serum albumin at 4°C overnight, the membranes were incubated with anti-$E_{1\alpha}$ antibody for 2 h, and then with an appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Bound antibodies were detected by the ECL system (Amersham, Bioscience). The amount of ^{32}P incorporated into $E_{1\alpha}$ was quantified on a Packard Instant Imager or by autoradiography and scintillation counting of the identified radiolabelled bands.

Results

As also previously reported in different experimental conditions (Toninello et al. 1988, 2000), Fig. 1a shows that RLM, energized by the oxidation of pyruvate plus malate in the presence of Pi and EGTA, can take up about 30 nmol/mg prot of spermine in 30 min of incubation, an amount far less than that of energization with succinate plus rotenone and in the absence of EGTA (Toninello et al. 1988). In the absence of Pi, spermine uptake is greatly reduced to 16–17 nmol/mg prot; when the de-energizing agent FCCP is also present, uptake almost completely involves initial membrane binding (≈ 6–7 nmol/mg prot), with negligible transport. The observed different extents of spermine accumulation in

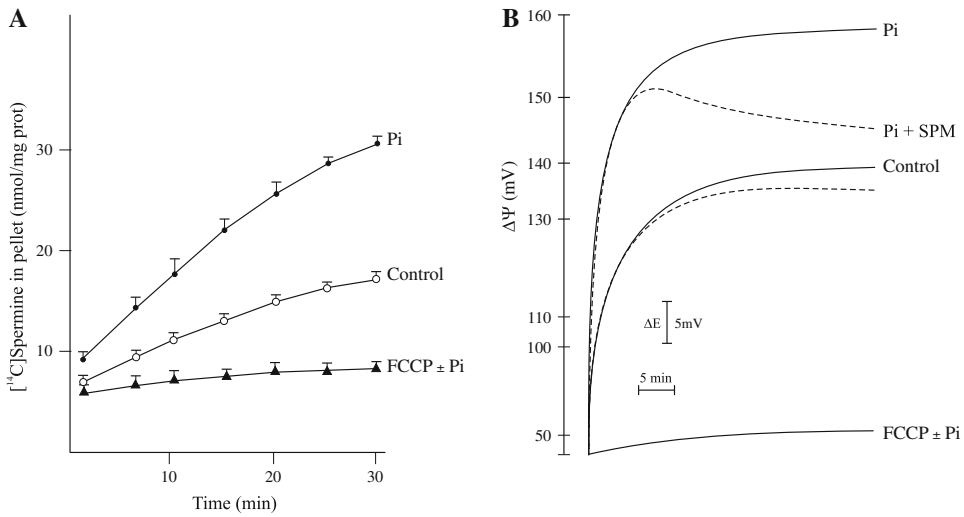

Fig. 1 Time-dependent spermine uptake by RLM at various ΔΨ values imposed by Pi and FCCP (**a**). Measurements of corresponding ΔΨ values (**b**). RLM were incubated in standard medium in conditions indicated in "Experimental procedures". **a** 0.5 mM [^{14}C]spermine (0.5 μCi/ml). When indicated, 2 mM Pi and 0.1 μM FCCP were added. Mean values ± SD of three different experiments are reported. **b** Where indicated, 0.5 mM spermine (SPM), 2 mM Pi and 0.1 μM FCCP were present. 1 μM TPP^+ was added for ΔΨ measurement. ΔE = electrode potential. Four different experiments gave almost identical results

RLM are due to the different ΔΨ values of the organelles in the various experimental conditions. As shown in Fig. 1b, the presence of Pi imposes a ΔΨ value of about 160 mV, whereas in its absence this value falls to about 140 mV. The presence of FCCP, in fact, causes ΔΨ to fall to about 40–50 mV, both with and without Pi. These observations account for the electrophoretic transport of spermine, exhibiting an apparent exponential trend with increasing ΔΨ values (Toninello et al. 1992a, 2000). This force/flux relationship is clearly evidenced by the gradual drop in ΔΨ, when a large amount of polyamine is accumulated in the presence of Pi. In its absence, this effect is almost negligible (see dashes in Fig. 1b). Figure 2 shows the movements of endogenous cations depending on experimental conditions. When RLM are incubated in standard medium deprived of EGTA, they accumulate the amount of Ca^{2+} present in the medium as a contaminant (≈2–5 nmol/ml) and maintain it throughout the experiment. In the presence of EGTA, which chelates contaminant Ca^{2+} and blocks Ca^{2+} cycling, a rapid Ca^{2+} efflux is induced which is further enhanced, with a slight dose-dependent effect, by spermine (Fig. 2a). Incubation of RLM in the absence or presence of EGTA releases 4–5 nmol/mg prot of Mg^{2+}, corresponding to the amount present in the intermembrane space. Also in this case the presence of spermine induces a very low, dose-dependent increase in Mg^{2+} efflux, most probably coming from the inner compartment (Fig. 2b), as also observed in a previous paper (Salvi and Toninello 2003).

The results represented in Fig. 3a show that RLM, incubated in the presence of Pi, produces 62 nmol/mg prot of CO_2 in 30 min of incubation. In the presence of 0.5 mM spermine, this production increases up to 150 nmol/mg prot (140% stimulation). If the medium is deprived of Pi (see control in Fig. 2a), CO_2 production is about 60 nmol/mg prot. In this condition, the presence of spermine only induces a slight increase, to about 75 nmol/mg prot.

Figure 3b shows the dose-dependent effect of spermine on CO_2 generation after 30 min of incubation. Spermine induces a gradual, almost linear, concentration-dependent stimulation of CO_2 production, up to 0.5 mM. Higher concentrations also stimulate CO_2 production, but with a dose-dependent, slight, gradual decrease. In the absence of Pi, spermine only induces a slight increase in CO_2 production, compared with the control (absence of spermine) (Fig. 3a). The maximum increase obtained in this condition, with 0.5–1 mM spermine, was 78 nmol/mg prot (25% stimulation), which also remained constant at the higher concentrations (Fig. 3b). The results (Fig. 3b, inset) show the dose-dependent net spermine uptake (subtraction of initial membrane binding) by RLM in the presence or absence of Pi, demonstrating that, in the latter condition, spermine transport is greatly reduced.

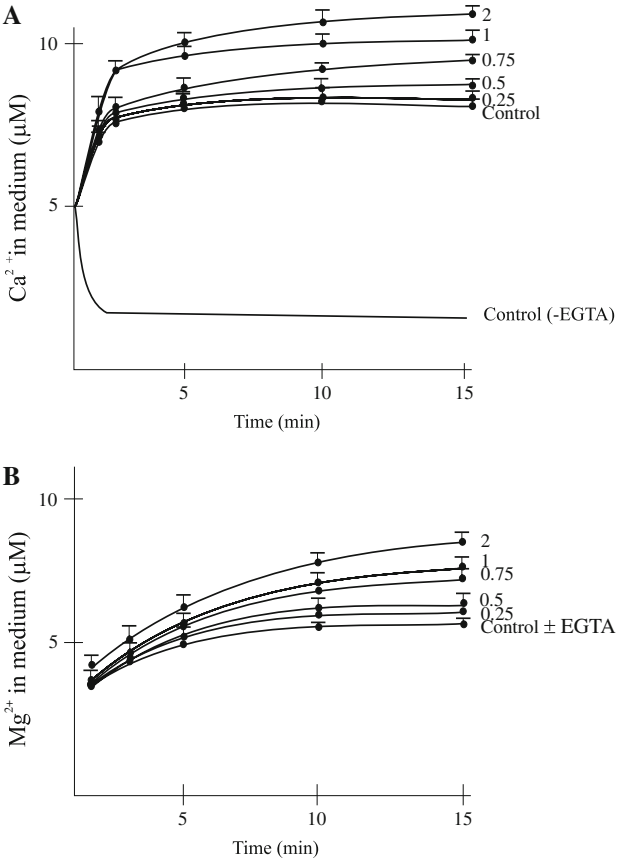

Fig. 2 Efflux of Ca^{2+} (**a**) and Mg^{2+} (**b**) from RLM in presence of varying spermine concentrations. RLM were incubated in standard medium in conditions indicated in "Experimental procedures". Spermine was present at various μM concentrations, as indicated at sides of traces. Contaminant Ca^{2+} was 5 μM; its mitochondrial content was 6.5 nmol/mg prot. Endogenous Mg^{2+} was 21.4 nmol/mg prot

Figure 4a shows autoradiographic phosphorylation of the $E_{1\alpha}$ subunit of PDC (41-kDa labelled bands, normally phosphorylable/dephosphorylable by PDP/PDK in physiological conditions; Randle et al. 1986) in liver mitochondria, and corresponding to the activity reported above (Fig. 3b). Lane 1, in panel a, shows the radioactive incorporation of mitochondrial proteins corresponding to the $E_{1\alpha}$ subunit of PDC obtained in the absence of spermine (control). All the other lanes show the labelled mitochondrial proteins, again corresponding to the $E_{1\alpha}$ subunit, obtained with spermine. Incubation with increasing amounts of spermine, up to 0.5 mM, induces a gradual decrease of this phosphorylation (see lanes 2–3 in panel a). Further increases in spermine concentration, up to 3 mM, cause slight enhancement of phosphorylation (lanes 4–6 in panel a). Lane 7, in panel a, shows radioactive incorporation by purified PDC, isolated from porcine heart and used for purposes of comparison. Panel b in Fig. 4 shows a quantitative measurement of $E_{1\alpha}$ phosphorylation, as the percentage of the control obtained in

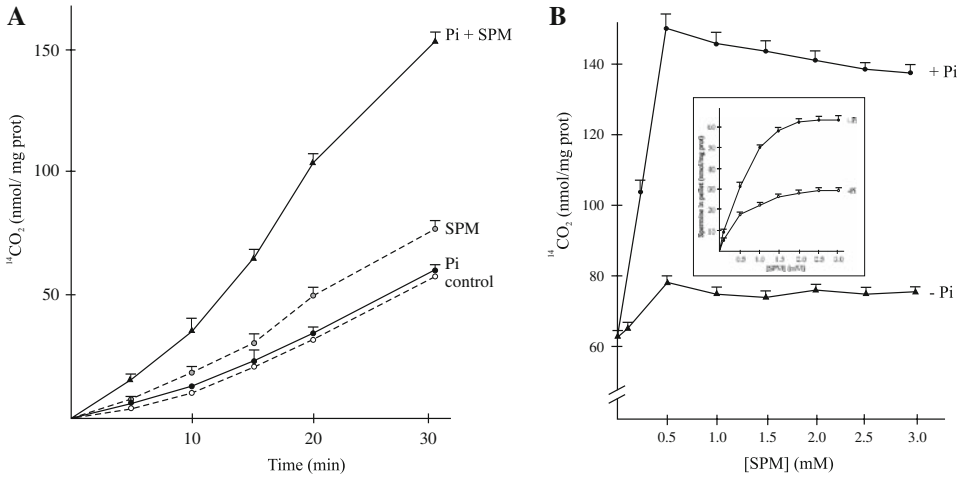

Fig. 3 Time-dependent (**a**) and dose-dependent (**b**) effects of spermine on CO_2 production by RLM. RLM were incubated for 30 min in standard medium, as described in "Experimental procedures", in presence of 1-[^{14}C]pyruvate (0.5 μCi/ml). **a** Where indicated, 0.5 mM spermine (SPM) and 2 mM Pi were present. **b** Spermine was present at various concentrations, as indicated. When present, Pi was 2 mM. *Inset*: spermine transport depends on exogenous concentration

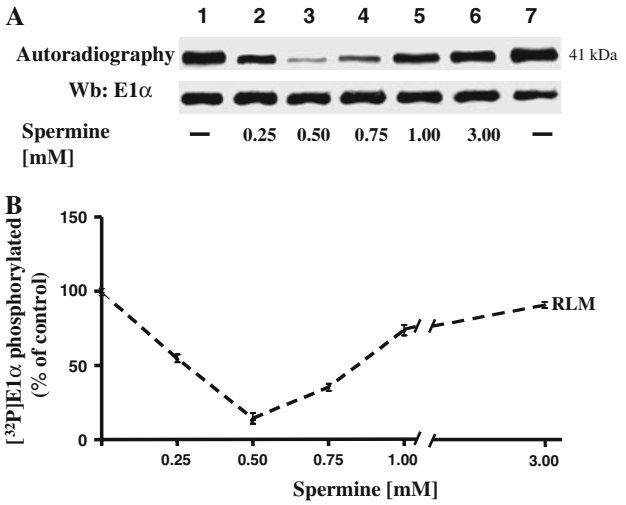

Fig. 4 Dose-dependent effect of spermine on phosphorylation of $E_{1\alpha}$ subunit of PDC in RLM (**a**). Quantitative measurement of $E_{1\alpha}$ phosphorylation (**b**). **a** RLM and purified PDC were incubated as in experiments of Fig. 3b, in presence of 1 mM [γ-^{32}P]-ATP (0.05 μCi/mol), 5 μM oligomycin, and 2 mM Pi. After 30 min of incubation, mitochondria were analysed by SDS/PAGE or immunoblotting with anti-$E_{1\alpha}$ antibody. *Lane 1*, control; *lanes 2–6*, spermine at indicated concentrations; *lane 7*, purified PDC (5 ng). Wb: Western blot analyses of $E_{1\alpha}$ for RLM. **b** RLM were treated as described in **a**. Radioactivity incorporated into $E_{1\alpha}$ was evaluated either by analysis on a Packard Instant Imager or by autoradiography and scintillation counting of identified radiolabelled bands. [^{32}P]-$E_{1\alpha}$ phosphorylation is expressed as percentage of control values obtained in absence of spermine. A total of eight preparations of RLM were analysed. Representative results are shown

the same conditions as panel a. It should be emphasized that, if we consider the conversion of [^{32}P]ATP to ADP, due to PDK activity, possible resynthesis of ATP with cold phosphate must be excluded, due to the presence of oligomycin which blocks ATP-synthase activity. So, the labelled bands represent the real phosphorylation of the $E_{1\alpha}$ subunit.

In evaluating these results, we emphasized that, in our experimental conditions, the rates of PDC reaction, appraisable by CO_2 generation, are not at their maximum extent. This is because the respiratory chain is operating at a reduced rate, due to the lack of ADP in the medium ("respiratory state 4"). Actually, incubation of mitochondria in the presence of ADP ("state 3") or the uncouple FCCP, really augment the rate of CO_2 generation (results not reported). However, spermine does change the activity of ATP-synthase (Philips and Chaffee 1982; Solaini and Tadolini 1984), so that the presence of various spermine concentrations induces the synthesis of differing amounts of ATP and, consequently, different phosphorylation of the $E_{1\alpha}$ subunit. In other words, the extent of $E_{1\alpha}$ phosphorylation for each spermine concentration is not comparable with PDC activity. Also, incubation in the presence of FCCP cannot be used, as this uncouple activates mitochondrial ATP-ase, resulting in ATP hydrolysis and consequent hindrance of $E_{1\alpha}$ phosphorylation.

In conclusion, the experimental conditions used in this study were optimal in evaluating the effect of spermine on $E_{1\alpha}$ phosphorylation and PDC activity.

Discussion

The results of this study show for the first time that the polyamine spermine is able to stimulate the activity of PDC in isolated RLM in the absence of exogenous Ca^{2+} and Mg^{2+}. Indeed, another peculiarity of this study is that the observed effects of spermine are obtained in energized mitochondria—unlike other studies, also reporting stimulation of PDC by spermine, but using permeabilized mitochondria or the purified enzyme (Damuni et al. 1984; Thomas and Denton 1986; Moreno-Sanchez and Hansford 1988; Rutter et al. 1992). It is also noteworthy that the stimulation of PDC by spermine in energized mitochondria highlights the key role of polyamine transport in these organelles (Toninello et al. 1988, 1990, 2000).

As Fig. 3b shows, by entering the matrix space of liver mitochondria, spermine can activate PDC in a dose-dependent manner up to 0.5 mM. This effect may be due, as previously proposed (Kiechle et al. 1990), to spermine-dependent lowering of the K_m of PDP due to endogenous Mg^{2+}, which activates this enzyme. However, as Mg^{2+} was not present in the incubation medium and a large amount was released during incubation (Fig. 2b), its concentration in the matrix was far lower than in the above experiment, so that the possibility of direct activation by spermine of PDP cannot be discarded. In any case, the autoradiograph of Fig. 4a clearly shows that, up to 0.5 mM of spermine, the $E_{1\alpha}$ subunit of PDC is gradually dephosphorylated with respect to the control condition (see also Fig. 4b). It should be noted that, probably due to the particular metabolic conditions of the animals, the optimal concentration of spermine might be lower (0.35 mM) or higher (0.65 mM) than 0.5 mM. Concentrations above 0.5 mM also stimulate PDC activity, but a gradual slight decrease in stimulation is observed (from 140% at 0.5 mM to 100% at 3 mM) (see Fig. 3b). The results shown in Fig. 4 confirm the gradual re-phosphorylation of the $E_{1\alpha}$ subunit in the presence of spermine above 0.5 mM. These effects are closely connected with high $\Delta\Psi$ values ($\sim$160 mV in the presence of Pi) (Fig. 1b), which allow the transport of sufficient amounts of spermine (see Fig. 1a, and inset in Fig. 3b) and consequent dephosphorylation of the $E_{1\alpha}$ subunit of PDC. In the absence of Pi, RLM have a $\Delta\Psi$ value of about 140 mV during the oxidation of pyruvate in the presence of malate (Fig. 1b). This value, when the non-ohmic flux-voltage relationship of spermine transport in mitochondria is taken into account (Toninello et al. 1992), greatly reduces spermine uptake (Fig. 1a, and inset in Fig. 3b), and consequently PDC is activated to a far lower extent. Other artificial modulations of $\Delta\Psi$ by pulses of respiratory chain inhibitors (e.g. antimycin A or azide), which would allow the evaluation of a threshold $\Delta\Psi$, to obtain the maximum effect, cannot be used because these drugs strongly stimulate PDC by unknown mechanisms (data not reported). The effect of FCCP, instead, has been explained above.

It should be stressed that, to examine properly the effect of spermine interaction with PDC, mitochondria were incubated in a standard sucrose medium, not interfering with spermine transport, normally used in previous studies of this process (Toninello et al. 1988, 1990, 2000). A more physiological saline medium of high ionic strength reduces spermine uptake by causing a decrease in the percentage of PDC activation and a shift towards higher spermine concentrations (>1 mM) of the activation peak (data not reported). In this regard, entry into the matrix of a few nanomoles of spermine is sufficient to activate PDC (compare Fig. 3b, with its respective inset), thus confirming that the phenomenon is also reproducible in conditions contrasting spermine transport. The results reported here also clearly demonstrate that spermine, depending on its concentration, inhibits or stimulates the phosphorylation of the $E_{1\alpha}$ subunit in RLM (Fig. 4). These observations indicate that spermine can act on both regulatory enzymes of PDC, that is, on PDP and PDK. In order to explain the mechanism of these effects, it is necessary to recall the characteristics of both these enzymes and the particular experimental conditions used in this study. There are two Mg^{2+}-dependent isoenzymes of PDP, PDP1 and PDP2. PDP1 is composed of two subunits, PDP1c (catalytic) and PDP1r (regulatory) (Yan et al. 1996). PDP1 is normally stimulated by Ca^{2+}, but it should be emphasized that it is poorly expressed in liver (Huang et al. 2003). Taking into account these considerations together with the results of Fig. 2a, showing that endogenous Ca^{2+} is almost completely released during the experiment, it is proposed that PDP1 is not involved in the spermine effect. The isoform PDP2 is abundant in liver, is Ca^{2+}-insensitive, and also Mg^{2+}-independent, having an extremely high Km for Mg^{2+}. However, it can be regulated by spermine, which lowers this Km (Huang et al. 1998). These considerations indicate that spermine acts at the level of PDP2 by directly activating it or by lowering the Km for the remaining Mg^{2+} in the matrix (Fig. 2b).

If PDC stimulation is to be ascribed to spermine-dependent activation of PDP2, the explanation for the diminished stimulation of PDC by spermine concentrations higher than 0.5 mM must take into account possible activation of PDK by spermine. In fact, the increasing phosphorylation of the $E_{1\alpha}$ subunit, at spermine concentrations higher than 0.5 mM, and the consequent decrease in PDC activation may be due, not only to strong inhibition of PDP, but also to activation of PDK. Most probably there is a threshold concentration of spermine for PDK activation, around 0.5 mM, which begins to counteract the stimulated activity of PDP. As observed in Fig. 2b, spermine induces a dose-dependent release of Mg^{2+} although its effects are not so significant when compared to the control. However, at high spermine concentrations (>0.5 mM), the release of Mg^{2+} may exceed a threshold level at which PDP2 is completely inhibited. In these conditions, spermine activation of PDK may be proposed.

As previously reported (Bowker-Kinley et al. 1998), liver mitochondria normally contain PDK2 and smaller amounts of PDK4 isoenzymes. PDK2 appears to be abundantly expressed, and its kinetics are consistent with the idea that it is the major isoenzyme responsible for metabolic control of PDC activity in RLM (Popov et al. 1994; Bowker-Kinley et al. 1998). Instead, PDK4 normally has a specific activity one order of magnitude higher than that of PDK2, and greatly increases its activity after 48 h of starvation (Kerbey and Randall 1982; Priestman et al.

1994), suggesting that it contributes to regulating the adaptative response or long-term control of PDC activity (Priestman et al. 1994; Randle 1986). In this regard, as mentioned above, our experiments were usually performed with starved rats, a condition in which PDK4 activity may be more important. In conclusion, both these isoenzymes may be involved in the control of PDC activity.

These results highlight the very important physiological role played by polyamine transport in liver mitochondria in regulating the activity of PDC and consequently the energy metabolism of these organelles. Our results demonstrate that spermine acts in different ways at the level of the various PDP and PDK isoenzymes, and that it can substitute Ca^{2+} in activating PDC. As Ca^{2+} acts within intact mitochondria by enhancing the Mg^{2+}-sensitivity of PDP, it is proposed that spermine directly interacts with a specific site and, up to 0.5 mM concentration, can cause PDP activation even in the absence of added Mg^{2+}, most probably by using the residual endogenous amount of the cation. At higher concentrations, by a still unknown mechanism, spermine can also activate PDK isoenzymes by reducing PDP stimulation. Contrary to a previous statement proposing that spermine may mimic the effects of other yet unknown compounds (Huang et al. 1998), the physiological ligand for the site would be spermine, thus excluding other compounds, as reported elsewhere (Thomas et al. 1986).

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