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EFFECT OF β-DIETHYLAMINOETHYL-DIPHENYLPROPYLACETATE HCl (SKF 525-A) ON CANINE HEART MITOCHONDRIAL FUNCTION*

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Abstract—The *in vitro* effect of β-diethylaminoethyl-diphenylpropylacetate HCl (SKF 525-A) on oxidative phosphorylation was examined using a canine heart mitochondrial preparation. The drug inhibited oxidative phosphorylation. The inhibition was dose- and protein-dependent. SKF 525-A at a concentration of less than 100 nmoles/mg of protein (100 μM) did not affect the state 3 rate, but increased the state 4 rate. At a concentration of more than 100 nmoles/mg of protein, SKF 525-A affected both state 3 and state 4 rates. Energy production was inhibited as evidenced by the decrease in the respiratory control index (RCI) and the ADP:O ratio. Energy utilization, assessed by substrate-dependent energy-linked Ca^{2+} uptake by mitochondria, was also inhibited. SKF 525-A affected the integrity of the mitochondrial membrane, since it stimulated ATPase activity and caused swelling. The effects of SKF 525-A on oxidative phosphorylation are discussed.

β-Diethylaminoethyl-diphenylpropylacetate HCl (SKF 525-A) has diverse biological actions. It has an inhibitory action on hepatic microsomal drug-metabolizing enzymes. Several theories have been put forward to explain the mechanism of inhibition. Netter [1] proposed that, because, the NADPH-dependent oxidase system is common to all oxidative reactions, SKF 525-A may act as an uncoupling agent. Brodie *et al.* [2] have suggested that SKF 525-A may modify the microsomal membrane which would alter drug permeability. Hollunger [3] has reported that SKF 525-A binds to the enzymes *per se* and inhibits them, rather than binding to the membranes. Lee *et al.* [4] found that drugs, at low concentrations, stabilized erythrocyte membranes against hypo-osmotic shock and postulated that this membrane effect might be involved in the alteration of enzyme activity.

SKF 525-A has been used as a tool to study contraction of vascular smooth muscle from rabbits and rats[5–7]. Kalsner *et al.* [5] and Krishnamurty [6] observed that potassium induced, but not norepinephrine induced contraction was inhibited by SKF 525-A. They concluded from their studies that SKF 525-A specifically inhibits the movement of extracellular and/or loosely bound calcium to the contractile proteins in response to membrane depolarization by potassium. However, it has little effect on the mobilization of firmly bound calcium which is utilized by norepinephrine.

Wei *et al.* [8] have shown decreased calcium uptake by plasma membranes and endoplasmic reticulum of normotensive and spontaneously hypertensive rat mesenteric arteries in the presence of SKF 525-A (100 μM).

We have observed complete inhibition of respiratory substrate dependent calcium uptake by dog heart mitochondria in the presence of SKF 525-A, 320–400 nmoles/mg of protein (400 μM). Since calcium uptake by mitochondria is an energy-dependent process, energy being supplied by either ATP hydrolysis or oxidation of the respiratory substrates [9], we studied the effect of SKF 525-A on energy coupling in dog heart mitochondria.

METHODS

Preparation of dog heart mitochondria. Dogs of either sex, weighing between 15 and 24 kg, were anesthetized with sodium pentobarbital (30 mg/kg, intravenously administered). A left thoracotomy was performed, the pericardium was opened and the heart was removed immediately. A portion (10–15 g) of the right ventricle was quickly dissected out and washed with ice-cold Hepes‡ buffer without Nagarse enzyme. About 10 g of tissue were finely minced and incubated for 15 min at 0° in 50 ml solution containing 250 mM sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA and 50 mg Nagarse. After incubation, 50 ml of the above solution without Nargase were added, and the mixture was homogenized in a Potter–Elvehjem homogenizer fitted to an electric motor.

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‡ Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; BSA, bovine serum albumin; TCA, trichloroacetic acid; and 2,4-DNP, 2,4-dinitrophenol.

Centrifugation was carried out according to the procedure of Tyler and Gonze [10]. The mitochondrial pellet obtained after centrifugation was washed twice in 250 mM sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA and 0.05% BSA, and finally suspended in a small volume of the above solution at a protein concentration of 30–40 mg/ml. Protein was estimated by the method of Lowry *et al.* [11] using crystalline BSA as the standard.

Measurement of oxidative phosphorylation. Mitochondrial oxidative phosphorylation was studied by a polarographic technique using a Gilson oxygraph at 25° [12]. Calculations of the respiratory control index (RCI), state 3 and state 4 rates, and ADP:O were done according to Estabrook [12]. The assay medium (2 ml) contained 0.25 M sucrose, 10 mM KH_2PO_4, 10 mM Tris HCl (pH 7.4) and 5 mM substrate. The substrates used in the studies were either glutamate or succinate. Whenever succinate was used, rotenone (5 μM) was used to block NADH oxidation [13]. State 3 respiration was initiated by the addition of ADP.

Mitochondrial ATPase. Mitochondrial ATPase was assayed according to the procedure of Holton *et al.* [14]. The incubation mixture contained 50 mM Tris–HCl (pH 7.4), 75 mM KCl, 50 mM sucrose, 5 mM $MgCl_2$, 2.5 mM ATP, and 0.32 mg protein, in a final volume of 1 ml. Other additions are mentioned in Table 3. The mixture was incubated at 37° for 5 min. The reaction was stopped by the addition of an equal volume of cold 10% TCA. Phosphate in the supernatant fraction was assayed by the method of Ames and Dubin [15].

Mitochondrial swelling. Energy-dependent low amplitude mitochondrial swelling was monitored by changes in absorbancy at 520 nm using a Beckman J25 spectrophotometer equipped with a recorder. The reaction mixture contained 10 mM Tris–HCl (pH 7.4), 5 mM KH_2PO_4 buffer (pH 7.4), 0.25 M sucrose, 5 mM Na-succinate, and 8.4 mg of mitochondrial protein, in a total volume of 4 ml.

Materials. The following materials were obtained from the Sigma Chemical Co., St. Louis, MO: ADP, L-glutamic acid, BSA, succinic acid, oligomycin and 2,4-DNP persisted. When SKF 525 A, 320–Kline & French Co., Philadelphia, PA. It was dissolved in water. When added to the reaction mixture, no change in pH was noted. Nagarse was purchased from the Enzyme Development Corp., Pennplaza, NY. Beef heart submitochondrial particles were provided by Dr. Werringloer of our institute.

RESULTS

Effect of SKF 525-A on respiration-dependent Ca^{2+} uptake by dog heart mitochondria. Figure 1 shows respiration-dependent Ca^{2+} uptake by tightly coupled dog heart mitochondria. When oligomycin was added to the assay medium, as expected, ADP-stimulated state 3 respiration was abolished, but the Ca^{2+} uptake and the uncoupling property of 2,4-DNP persisted. When SKF 525 A, 320–400 nmoles/mg of protein (400 μM), was added to the assay medium, ADP-stimulated state 3 respiration, Ca^{2+} uptake and the uncoupling action of 2,4 DNP were abolished.

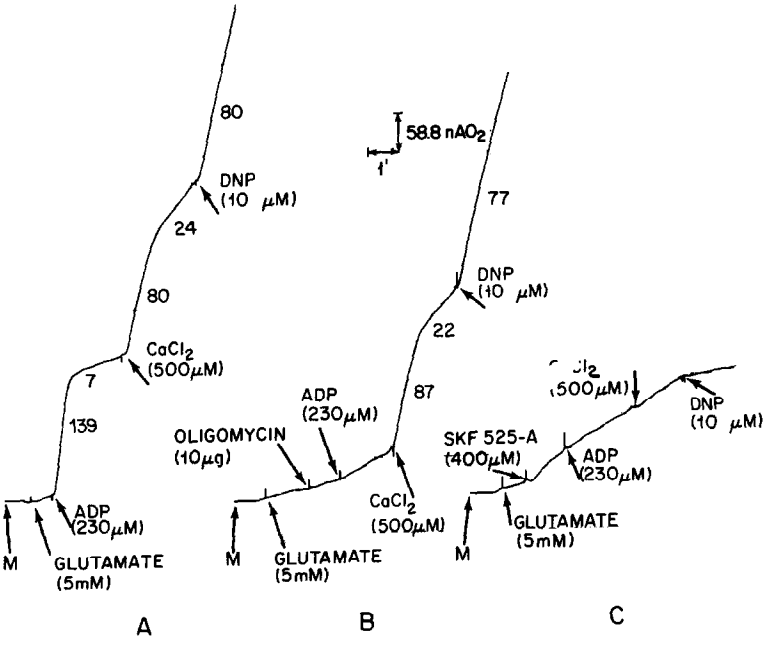

Fig. 1. Effects of SKF 525-A on mitochondrial oxidative phosphorylation and energy-dependent Ca^{2+} uptake. In each test the assay medium (see Methods) contained about 2–2.5 mg protein in a total volume of 2 ml. Glutamate (5 mM) was used as a substrate. SKF 525-A, oligomycin, 2,4-DNP and calcium were added where indicated. The numbers next to the traces indicate the rate of oxygen consumption in n-atoms/min/mg of mitochondrial protein. M = mitochondria. (A) Oxygen electrode tracing showing normal ADP, $CaCl_2$ and 2,4-DNP-induced oxygen consumption. (B) Oxygen electrode tracing following oligomycin addition. (C) Oxygen electrode tracing following SKF 525-A addition.

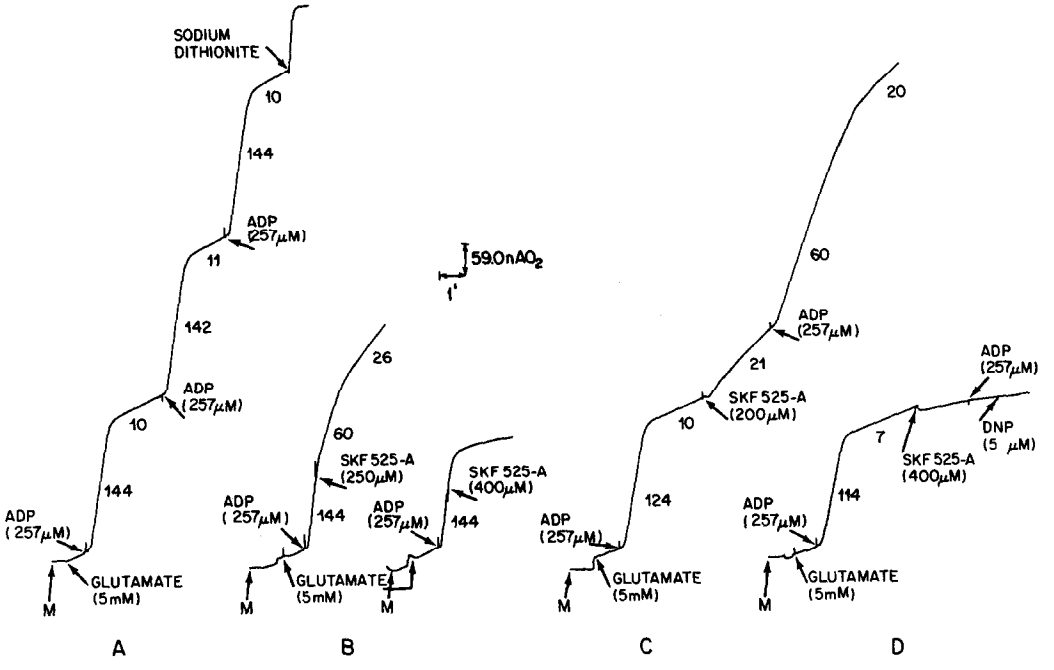

Fig. 2. Effects of SKF 525-A on mitochondrial oxidative phosphorylation. In each test the assay medium (see Methods) contained about 2–2.5 mg protein in a total volume of 2 ml. SKF 525-A was added where indicated. Glutamatate (5 mM) was used as a substrate. The numbers next to the traces indicate the rate of oxygen consumption in n-atoms/min/mg of mitochondrial protein. M = mitochondria. (A) Oxygen electrode tracing showing normal ADP-induced mitochondrial respiration. (B) Oxygen electrode tracing following SKF 525-A addition during ADP-induced state 3 respiration. (C) and (D) Oxygen electrode tracing following SKF 525-A addition during ADP-induced state 4 respiration.

Effect of SKF 525-A *on mitochondrial oxidative phosphorylation.* Figure 2 shows tightly coupled oxidative phosphorylation of dog heart mitochondria in the presence of glutamate. When SKF 525-A at a concentration of 200–250 nmoles/mg of protein (250 μM) was added during state 3 respiration, state 3 respiration decreased (58 percent), the state 4 rate increased (160 percent) and ADP:O decreased (24 percent). Addition of 320–400 nmoles SKF 525-A/mg of protein (400 μM) completely abolished oxidative phosphorylation. Similarly, the addition of 160–200 nmoles SKF 525-A/mg of protein (200 μM) during state 4 respiration increased the state 4 rate (100 percent), decreased the state 3 rate (52 percent) and decreased the ratio of ADP:O (35 percent). The addition of 320–400 nmoles SKF 525-A/mg of protein (400 μM) during state 4 respiration completely abolished oxidative phosphorylation as before.

In Figs. 1 and 2, the inhibition of glutamate oxidation by SKF 525-A was compared with the effects of oliogomycin and DNP. Oligomycin inhibited state 3 respiration and the inhibition was reversed by 2,4-DNP (Fig. 1B). The addition of 320–400 nmoles SKF 525-A/mg of protein (400 μM) caused an increased rate of respiration, which is consistent with an uncoupling effect (Fig. 1C); however, the subsequent addition of ADP, Ca^{2+} or 2,4-DNP to SKF 525-A-treated mitochondria had no effect on the respiratory rate, suggesting inhibition of the respiratory chain directly (Figs. 1C and 2D). Also, the addition of 2,4-DNP to mitochondria previously inhibited with 400 μM SKF 525-A caused further inhibition of respiration. These results suggest that SKF 525-A may act somewhat differently from a typical energy-transfer inhibitor (oligomycin), and that its blocking site is located on the respiratory chain side of the 2,4-DNP sensitive site which causes uncoupling of oxidative phosphorylation at low levels and respiratory chain inhibition at high levels. Similarly, in Figs. 2B (first trace) and 2C, the addition of 160–250 nmoles SKF 525-A/mg of protein (200–250 μM) caused an increase in the state 4 respiratory rate. Thus, uncoupling seems to be involved.

Effect of SKF 525-A *on the mitochondrial oxidative phosphorylation: dose dependence.* Table 1 shows the dose-dependent effect of SKF 525-A on mitochondrial oxidative phosphorylation utilizing glutamate as the substrate. SKF 525-A up to 100 nmoles/mg of protein (100 μM) had no inhibitory action on state 3 respiration, but markedly increased state 4 respiration. When the concentration was increased above 100 nmoles/mg of protein (100 μM), the state 3 rate decreased and the state 4 rate increased further. The ADP:O were significantly diminished by SKF 525-A at concentrations above 150 nmoles/mg of protein (150 μM). SKF 525-A at a concentration above 240 nmoles/mg of protein (300 μM) completely abolished the oxidative phosphorylative process.

Table 2 shows the dose-dependent effect of SKF 525-A on mitochondrial oxidative phosphorylation utilizing succinate as the substrate. The ADP-depen-

Table 1. Effect of SKF 525-A on mitochondrial oxidative phosphorylation*

Addition	Concn (μM)	O_2 (n-atoms min^{-1} mg $protein^{-1}$) State 3	State 4	RCI (State 3/State 4)	ADP:O
None		149	11	13.5	2.96
SKF 525-A	50	149	18	8.3	2.96
	100	149	20	7.5	2.99
	150	108	23	4.7	2.88
	200	77	23	3.3	2.70
	250	62	27	2.3	2.26
	300	14	14	1.0	0
	350	7	7	1.0	0
	400	4	4	1.0	0

* Oxygen uptake was assayed as described under Methods, using glutamate as the substrate. The protein concentration was 2–2.5 mg/2 ml assay volume. State 4 respiration refers to the respiratory rate in the absence of ADP, while state 3 respiration refers to the respiratory rate in the presence of ADP.

dent state 3 rate for succinate was unaffected by up to 100 nmoles SKF 525-A/mg of protein (100 μM), whereas the state 4 rate was significantly increased. When the concentration of SKF 525-A was increased above 100 nmoles/mg of protein (100 μM), the state 3 rate decreased and the state 4 rate increased further. The ADP:O ratios were significantly diminished by SKF 525-A at concentrations above 100 nmoles/mg of protein (100 μM).

Inhibition of state 3 respiration by SKF 525-A: protein dependence. The inhibition of state 3 respiration by SKF 525-A was protein-dependent. This was assessed by varying the protein concentration in the assay medium. Four different protein concentrations, 0.45, 1.11, 2.23 and 4.46 mg per 2 ml, were used. The inhibition of state 3 respiration by SKF 525-A decreased on increasing the protein concentration in the assay medium (Fig. 3). SKF 525-A concentrations for half-maximal inhibition of state 3 respiration were 300, 210, 140 and 60 μM when the assay medium (2 ml) contained 4.46, 2.23, 1.11 and 0.45 mg of protein respectively. Figure 3 also indicates that the minimum effective concentration of the drug depends on the protein concentration in the assay medium. For example, the minimum effective concentration of the drug was 200 μM when the protein concentration of the assay medium was 4.46 mg/2 ml assay medium.

NADH oxidation. Figure 4 shows the effects of different concentrations of SKF 525-A on aerobic oxidation of NADH by submitochondrial particles of beef heart. NADH oxidation was inhibited by SKF 525-A in a dose-dependent manner. Fifty percent inhibition was seen with approximately 152 nmoles SKF 525-A/mg of protein (175 μM).

Mitochondrial swelling. Figure 5 shows the effects of SKF 525-A on low amplitude energy-dependent swelling of mitochondria. Ninety-five nmoles SKF 525-A/mg of protein (200 μM) in the presence of phosphate and succinate induced rapid substantial swelling of mitochondria.

ATPase activity. Table 3 shows the effects of SKF 525-A on intact mitochondrial ATPase activity. ATPase activity was increased by 140 percent by

Table 2. Effect of SKF 525-A on mitochondrial oxidative phosphorylation

Addition	Concn (μM)	O_2 (n-atoms min^{-1} mg $protein^{-1}$) State 3	State 4	RCI (State 3/State 4)	ADP:O
Control		148	50	3.0	1.95
SKF 525-A	50	146	64	2.3	1.91
	100	147	75	2.0	1.87
	150	130	81	1.6	1.49
	250	101	101	1.0	0
	400	91	91	1.0	0

* Oxygen uptake was assayed as described under Methods, using succinate as the substrate. The protein concentration was 2–2.5 mg/2 ml assay volume.

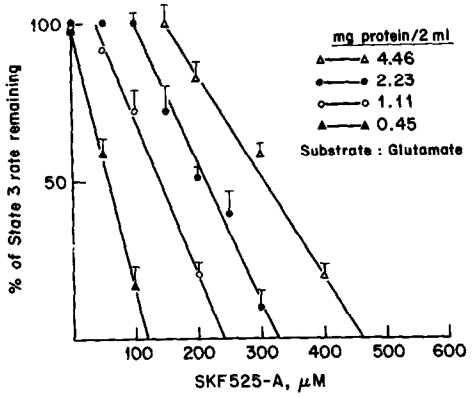

Fig. 3. Effect of SKF 525-A on mitochondrial oxidative phosphorylation: protein dependence. In each test the assay medium (see Methods) contained various amounts of protein (0.45–4.46 mg) in a total volume of 2 ml. The oxygen content of the medium was 1040 n-atoms. Glutamate (4 mM) was used as a substrate. Vertical bars represent (±) standard deviation from 4–5 experiments.

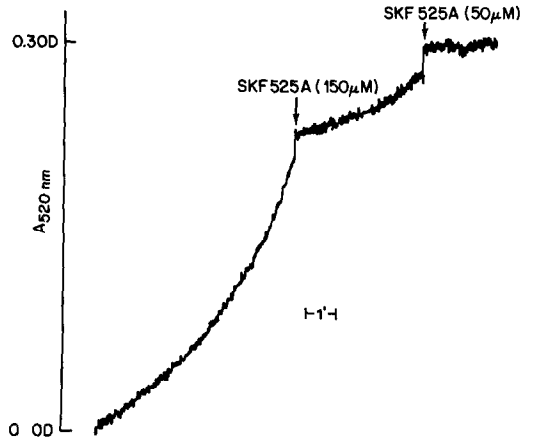

Fig. 5. Effect of SKF 525-A on energy-dependent low amplitude mitochondrial swelling. The assay medium contained 10 mM Tris–HCl (pH 7.4), 5 mM KH_2PO_4 buffer (pH 7.4), 0.25 M sucrose, 5 mM Na-succinate, and 8.4 mg mitochondrial protein, in a total volume of 4 ml. The ordinate represents the O.D. of the suspension at 520 nm. SKF 525-A was added where indicated.

Mg^{2+}. On inclusion of 2,4-DNP in the incubation medium, ATPase activity was stimulated by 46 percent in the presence of Mg^{2+} and by 274 percent in the absence of Mg^{2+}. This activity was inhibited completely by sodium azide. SKF 525-A stimulated ATPase activity by 33 percent in the presence of Mg^{2+} and by 54 percent in the absence of Mg^{2+}. Stimulation was blocked by oligomycin.

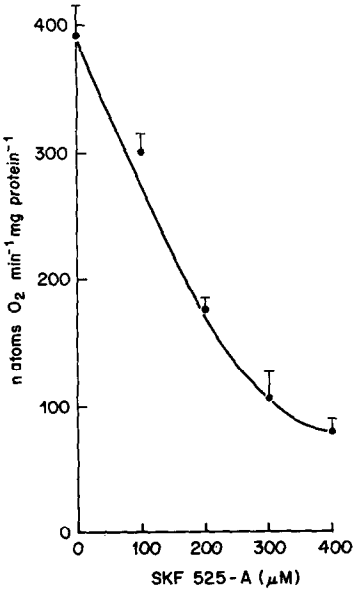

Fig. 4. Effect of SKF 525-A on NADH oxidation by beef heart submitochondrial particles. The assay medium contained 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), 8.5 mM KH_2PO_4 buffer (pH 7.4), and 2.3 mg protein, in a total volume of 2 ml. Oxygen uptake was initiated by the addition of 0.5 mM NADH. The oxygen content of the medium was 1040 n-atoms. Mean values (± S.D.) from four experiments are shown.

DISCUSSION

SKF 525-A has an inhibitory action on extracellular or loosely bound calcium movement to the contractile proteins [5–7]. Thus, it inhibits the potassium induced contraction of vascular smooth muscle more than the norepinephrine-induced contraction. This agent also inhibits calcium uptake by the plasma membranes and the endoplasmic reticulum of rat mesenteric arteries [8]. In the present communication, we have reported the inhibitory action of SKF 525-A on mitochondrial respiratory substrate-dependent calcium uptake. Since the uptake of Ca^{2+} by mitochondria is an energy-dependent reaction, the energy for which is derived either from ATP hydrolysis or substrate oxidation [9], the inhibition of mitochondrial Ca^{2+} uptake by SKF 525-A is probably due to the inhibition of substrate oxidation.

SKF 525-A inhibited substrate oxidation, as well as phosphorylation (ATP synthesis) of dog heart mitochondria using either glutamate or succinate as the substrate. The inhibition was concentration- and protein-dependent. SKF 525-A at a lower concentration (< 100 nmoles/mg of protein) affected state 4 (resting) respiration, while at a high concentration (> 100 nmoles/mg of protein), both state 3 (energy-dependent) and state 4 respirations were affected.

There may be one or more sites of action of SKF 525-A on mitochondria. Inhibition of state 3 respiration and activation of state 4 respiration by SKF 525-A may be explained by a number of possible mechanisms. Since the oxidation of both glutamate (NAD^+-dependent) and succinate (flavoprotein-dependent) was affected by SKF 525-A, this agent may exert an inhibitory effect directly on the respiratory chain complex I (NADH-ubiquinone oxidoreductase) and complex III (reduced ubiquinone cytochrome oxidoreductase). However, inhibition of glutamate oxidation was more than that of suc-

Table 3. Effect of SKF 525-A on ATPase activity of intact mitochondria*

Addition	ATPase activity (μmoles phosphate mg protein^{-1} hr^{-1} at 37°)	
	With $MgCl_2$ (5 mM)	Withou $MgCl_2$
None	34.4	14.3
2,4-DNP (0.5 mM)	50.2	53.5
2,4-DNP (0.5 mM) + oligomycin (10 μg)	10.5	12.8
SKF 525-A (100 μM)	45.7	22.0
SKF 525-A (100 μM) + oligomycin (10 μg)	2.2	
Sodium azide (5 mM)	1.9	

* Enzyme was assayed as described under Methods, using 0.32 mg protein.

cinate oxidation [half-maximal inhibition of state 3 respiration for glutamate oxidation required 160–200 nmoles SKF 525-A/mg of protein (200 μM), compared to 480–600 nmoles SKF 525-A/mg of protein (600 μM) for succinate oxidation]; hence, inhibition of primary dehydrogenases may also be one of the factors for inhibition of substrate oxidation. Rapid turnover of the primary dehydrogenases is required during state 3 respiration [16]. Inhibition of enzyme activity may result in inhibition of oxygen consumption. The stimulation of state 4 respiration by SKF 525-A may indicate that other factor(s) may be involved. The effect of SKF 525-A on mitochondrial ATPase activity, a partial reaction of oxidative phosphorylation, was studied. SKF 525-A stimulated ATPase activity of intact mitochondria. However, the extent of stimulation was less when compared to the stimulation by 2,4-DNP (Table 3). Increased ATP hydrolysis to ADP and P_i by ATPase is possibly one of the factors responsible for the increased state 4 rate observed in the presence of SKF 525-A. ADP formed from ATP hydrolysis is recycled, consuming oxygen and thus increasing the state 4 rate.

Since intact mitochondria are impermeable to NADH, NADH oxidation was studied in submitochondrial particles. SKF 525-A inhibited NADH oxidation in a dose-dependent manner. Thus, inhibition of NADH oxidase would directly affect the electron flow through the respiratory chain, which in turn would decrease the state 3 rate of glutamate oxidation [17].

The observed increase in the state 4 rate, in spite of the decreased state 3 rate, may also be due to a permeability factor. It has been suggested that the rate of substrate oxidation is regulated by the uptake of substrate into the mitochondria [18–19]. Therefore, SKF 525-A may enhance the uptake of substrates to their sites of oxidation by altering membrane permeability. Figure 5 shows that SKF 525-A increases mitochondrial swelling measured by light scattering at 520 nm in the presence of succinate and phosphate, which suggests an alteration in membrane structure. Swelling of mitochondria in the presence of SKF 525-A may also be responsible for the observed decrease in the state 3 rate and uncoupled oxidative phosphorylation.

Thus, besides inhibiting hepatic drug-metabolizing enzymes and decreasing calcium uptake by plasma membranes and the endoplasmic reticulum, SKF 525-A also inhibits and uncouples oxidative phosphorylation and energy (respiratory substrate)-dependent calcium uptake by mitochondria.

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