Ca^{2+} – Induced Inhibition of Sodium Pump: Effects on Energetic Metabolism of Mouse Diaphragm Tissue

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Abstract. Tissues of mouse diaphragms were incubated in Liley solution containing 2, 4, 6 and 10 mmol/l calcium. When diaphragm tissue was incubated in 10 mmol/l calcium, an increase of intracellular calcium concentration from 314±28 to 637±26 nmol/l was estimated by fluorescent Ca^{2+} indicator Fura-2/AM. Moreover, incubation of the tissue in 10 mmol/l Ca^{2+} led to complete inhibition of electrogenic activity of the sodium pump, as measured by intracellular microelectrodes in a single muscle cell. This inhibition was fully reversible after 5 min washing with Liley solution containing 2 mmol/l $CaCl_2$. The Ca^{2+}-induced blocking effect on electrogenic activity of the sodium pump was accompanied by inhibition of glucose incorporation into the muscle tissue. Calcium at concentrations of 6 and 10 mmol/l in bath medium significantly inhibited both CO_2 production and O_2 consumption. A continual decrease of respiration (CO_2/O_2) quotient was observed under increasing concentrations of calcium. Moreover, an exponential decrease of ATP tissue levels was observed at increasing concentrations of calcium in the bath medium. On the other hand, massive acceleration of anaerobic glycolysis induced by incubation of the tissue in a medium containing high calcium concentration is improbable. This may be deduced from the fact that only about an 50% increase of lactate content in muscle tissue was observed when diaphragms were incubated for 30 min in medium containing calcium ions at 6 and 10 mmol/l as compared with the control tissue incubated for the same time in the medium containing 2 mmol/l $CaCl_2$.

In conclusion it could be stressed that increase of Ca^{2+} concentration in bath

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medium induced in diaphragm muscle tissue an elevation of intracellular Ca^{2+} concentration accompanied by a depression of sodium pump electrogenic activity and a depression of energy metabolism. These changes may be involved in pathology of muscle tissue during the Ca^{2+} overload.

Key words: Sodium pump — Intracellular calcium — Glucose uptake — Respiration

Introduction

Intracellular concentration of calcium ions is known to regulate numerous processes that are crucial for animal cells (Račay and Lehotský 1996; Račay et al. 1996; Maco et al. 1997; Sobol and Nesterov 1997; Stroffekova and Heiny 1997). Ca^{2+} ions (in millimolar concentrations) were previously found to inhibit the ATP hydrolytic activity of (Na^+/K^+)-ATPase (Lindenmayer and Schwartz 1975; Huang and Askari 1982; Vrbjar et al. 1986; Breier et al. 1998) in isolated preparations of the plasma membrane. Moreover, high extracellular Ca^{2+} was found to inhibit the transport or electrogenic activity of sodium pump in isolated cardiac myocytes (Hagane et al. 1989) and in mouse diaphragm tissue (Stankovičová et al. 1995). Effective inhibitory concentrations of Ca^{2+} can be shifted from millimolar to micromolar level by modulation with calmodulin and/or "calnactin" (Yingst 1983, 1988; Yingst and Marcovitz 1983; Yingst and Polasek 1985; Yingst et al. 1986, 1992). The latter represents putative Ca^{2+} modulating protein proposed to modulate Ca^{2+} induced inhibition of sodium pump. Recently, Okafor at al. (1997) have shown that calmodulin-dependent phospholipase A_2 may be involved in Ca^{2+}-induced inhibition of sodium pump at micromolar Ca^{2+} concentrations. All above indicated that, Ca^{2+} at concentrations that may be achieved in intracellular space during depolarization phase of muscle cells, may induce inhibition of (Na^+/K^+)-ATPase. This inhibition may take part as regulation of cation movements across the sarcolemma during its depolarization. When mouse diaphragm tissue was incubated in medium containing increased Ca^{2+} concentrations, an elevation of intracellular Ca^{2+} concentration was observed (Stankovičová et al. 1995). In these conditions, total depression of electrogenic activity of (Na^+/K^+)-ATPase was observed. Electrogenic activity of the enzyme was measured as K^+-induced hyperpolarization of the sarcolemma of myocytes previously loaded by Na^+ in the absence of K^+ (Kernan 1962). The electrogenic activity of sodium pump could be prevented against Ca^{2+} induced inhibition by calcium entry blockers like flunarizine, diltiazem and verapamil. However, any of these drugs did not exert a direct effect on ATP hydrolytic activity or on Ca^{2+}-induced inhibition of ATP hydrolytic activity of (Na^+/K^+)-ATPase in preparations of rat heart sarcolemmal fraction or dog kidney enzyme (Stankovičová et al. 1995). Therefore, the Ca^{2+} induced depression

of the (Na^+/K^+)-ATPase activity was proposed to be mediated via a mechanism located in the intracellular space (Stankovičová et al 1995)

The depression of sodium pump activity may be connected with dysfunction of energetic metabolism under Ca^{2+} overload of myocytes (Breier et al 1990) Recently, Tribulová et al (1992), Bakker et al (1994), Šeboková et al (1996) and Ziegelhoffer-Mihalovičová et al (1997) have shown that modulation of mitochondrial creatine phosphokinase activity and subsequent formation of mitochondrial contact site in rat heart could be regulated by intracellular Ca^{2+} In the latter four studies, the increase of intracellular Ca^{2+} concentration was achieved by an increase of Ca^{2+} concentration in prefusate during Langendorff perfusion of rat heart Mitochondrial creatine phosphokinase is functionally coupled with ATP/ADP translocator, and thus may represent a sensitive label for consideration about the effectivity of ATP production by mitochondria Thus, the question has to be answered how elevation of intracellular Ca^{2+} concentration (induced by increase in external Ca^{2+} concentration) influenced the energetic metabolism of muscle cells

Materials and Methods

Experiments with mouse diaphragm tissue

Experiments were performed on female mice, weighing 21 ± 1 g The animals were killed by cervical dislocation and the diaphragms (50 80 mg wet weight) were removed and transferred into Liley solution (Liley 1956) containing (in mmol/l) Na^+ 149 8, K^+ 5 0, Ca^{2+} 2 0–10 0, Mg^{2+} 1 0, HCO_3 12 6, H_2PO_4 1 0, glucose 11 0, pH 7 2 and equilibrated with 95% O_2 + 5% CO_2

Measurement of Na^+-pump induced hyperpolarization of sarcolemma

Muscle tissue was incubated for 4 6 h at the temperature of 21 °C in a K^+-free solution resulting in an increase of intracellular Na^+ to about 15 to 30 mmol/l This corresponded to loss of K^+ (Zemková et al 1982) and a decrease of the resting membrane potential (Vyskočil et al 1985) by 10–15 mV Application of 5 mmol/l K^+ induced temporary membrane hyperpolarization that can be explained by electrogenic sodium pump activity (Kernan 1962) The hyperpolarization was checked as an increase in resting membrane potentials (RMP) of superficial muscle fibers impaled with single glass microelectrodes filled with 3 mol/l KCl (7–15 MΩ) and connected to a high impedance preamplifier

Measurement of $^{45}Ca^{2+}$-uptake

$^{45}Ca^{2+}$-uptake was measured after 30 minutes of incubation of the tissue in Liley medium containing 2 or 10 mmol/l of $CaCl_2$ (activity 10,000 Bq per sample) in the absence or presence of verapamil (10 mmol/l) Samples were washed twice in modified Liley medium ($CaCl_2$ and NaCl were replaced by KCl and the medium

containing 1 mmol/l EGTA to eliminate superficially bound Ca^{2+}). The radioactivity of the tissue fragments was measured in Bray scintillation solution using 1214 Rackbeta liquid scintillation counter (LKB, Sweden). Differences in Ca^{2+} uptake observed in the absence and presence of verapamil were considered as verapamil sensitive portions of bulk Ca^{2+} uptake originating probably from verapamil blocking of Ca^{2+} entry.

Measurement of intracellular Ca^{2+} concentration

Concentration of free Ca^{2+} was measured on a Perkin Elmer LS-5 luminescence Spectrometer using Fura-2/AM as the intracellular Ca^{2+} indicator (Tsien et al. 1985). The ratio of fluorescent intensities (505 nm) obtained under excitation at 340 nm (Fura-2 + Ca^{2+} complex) and at 380 nm (Fura-2 free) was used to calculate free intracellular Ca^{2+}. The ratios of fluorescent intensities were converted to Ca^{2+} values using a calibration curve obtained from Fura-2 in complex buffer of known Ca^{2+} concentration and overall viscosity comparable to that of the cytoplasm (Tsien and Poenie 1986). K_d value for Fura-2 + Ca^{2+} complex was estimated according to Uto et al. (1991).

Diaphragm tissue was incubated for 10 minutes in Liley solution containing 2 and 10 mmol/l $CaCl_2$ in the presence and absence of verapamil (10 mmol/l) before estimation of intracellular Ca^{2+} concentrations. Strips of the diaphragm were attached on a glass frame and immersed into 1.0 × 1.0 cm cuvettes containing 2.5 ml of oxygenated Liley solution, Fura-2/AM was dissolved in DMSO and added to the Liley solution with the muscle strips (final concentration 4mmol/l, DMSO concentration 0.25%) for 60–90 min. Excess dye was removed by washing the tissue twice with 2.5 ml of oxygenated Liley solution (1.5 min at the room temperature each). There was no detectable unhydrolyzed Fura-2/AM present after the washing

[U-^{14}C]-glucose incorporation

The tissue was incubated for 2–30 minutes at 37°C under shaking in Liley solution in the presence of 2–10 mmol/l Ca^{2+} and [U-^{14}C]-glucose (2 MBq/mmol, 11 mmol/l). The tissue fragments were removed after these time intervals from the incubation medium and rapidly washed in glucose free medium. In control experiments, the tissue was incubated for 5 seconds and immediately washed. The radioactivity of the tissue fragments was measured in Bray scintillation solution using 1214 Rackbeta liquid scintillation counter (LKB, Sweden).

Respiration experiments

For respiration experiments, the incubation of diaphragm tissue at 37°C in Liley solution containing of $CaCl_2$ (2–10 mmol/l) was carried out directly in Warburg manometric vessels, and both CO_2 production and O_2 consumption were monitored during 20 min.

Estimation of metabolite contents

After 30 minutes of incubation (37°C) in Liley medium containing 2–10 mmol/l Ca^{2+} chloride, diaphragm tissue was homogenized by ultraturax knife homogenizer (8000 rpm), and metabolites were extracted by ice cold perchloric acid. Samples were centrifuged (3000 × *g*), supernatants were neutralized by 150 mmol/l triethanolamine buffer solution containing 10% K_2CO_3 pH 9.8, and recentrifuged. ATP contents were estimated by luciferin luciferase test in the type 2115 Luminometer (LKB). Lactate and pyruvate contents were measured by Boehringer Manheim tests.

Results and Discussion

In mouse diaphragm tissue previously loaded by Na^+ in the absence of K^+, potassium ions induced hyperpolarization of the sarcolemma membrane potential of about −18 mV (Stankovičová et al. 1995). This hyperpolarization could be fully abolished by ouabain (0.1 mmol/l, Stankovičová et al. 1995), and may therefore be considered as a result of electrogenic activity of sodium pump (Kernan 1962; Dlouhá et al. 1981). Similar behavior was observed in the present work (see Table 1). Total depression of K^+ – induced hyperpolarization of the sarcolemma could also be observed when Ca^{2+} concentration in bath medium was increased from 2 to 10 mmol/l. The latter effect was found to be fully reversible and could be removed by 5 min lasting additional incubation of the tissue in Liley solution containing 2 mmol/l Ca^{2+} (Stankovičová et al. 1995). Inhibition of K^+-induced hyperpolarization of plasma membrane by Ca^{2+} could be fully prevented by Ca^{2+} entry blocking agents like verapamil (10 mmol/l, Table 1). Similar results were obtained when other Ca^{2+} entry blockers, diltiazem or flunarizine were used (Stankovičová et al. 1995).

Verapamil present during the incubation of muscle tissue in Liley medium containing 2 or 10 mmol/l $^{45}CaCl_2$ was found to significantly depress Ca^{2+} uptake (Fig. 1). This depression may be considered as a result of the verapamil blocking effect on Ca^{2+} entry. An increase of Ca^{2+} concentration in bathing medium from 2 mmol/l to 10 mmol/l induced an increase of both the total and the verapamil-sensitive portion of the Ca^{2+} uptake (Fig. 1, Panels *A* and *B*). Moreover, the intracellular Ca^{2+} concentration measured by the intracellular Ca^{2+} indicator Fura-2/AM was found to increase about twofolds when Ca^{2+} concentration in bath medium was changed from 2 to 10 mmol/l (Fig. 1, Panel *C*). Verapamil (10 μmol/l) proved to depress the observed increase of intracellular Ca^{2+} concentration induced by the higher Ca^{2+} concentration in bath medium. Thus, the increases of Ca^{2+} concentration in bath medium caused an increase of intracellular Ca^{2+} level. During prolonged depolarization calcium ions may enter the muscle cells through calcium

Table 1. Effect of increased Ca^{2+} concentration in bath solution on K^+-evoked hyperpolarization of plasma membrane in mouse diaphragm

Incubation	Membrane potential		Difference
	A K^+-free [mV]	B K^+ [5 mmol/l] [mV]	B–A [mV]
Control			
2 mmol/l Ca^{2+}	61 53 ± 0 83	79 89 ± 0 97	18 36 ± 1 80
Effect of ouabain			
0 01 mmol/l	61 26 ± 1 23	67 29 ± 1 47	6 03 ± 2 70
0 1 mmol/l	64 58 ± 0 73	65 34 ± 0 85	0 76 ± 1 58
Effect of increased Ca^{2+} concentration			
10 mmol/l Ca^{2+} washout with	62 43 ± 0 98	64 39 ± 1 25	1 96 ± 2 23
2 mmol/l Ca^{2+}	63 37 ± 1 15	79 40 ± 1 26	16 03 ± 2 41
10 mmol/l Ca^{2+} and 10 μmol/l verapamil	63 52 ± 1 27	80 25 ± 1 18	16 73 ± 2 45

The values are given as means of RMP ± S E M for 23–48 independent measurements Difference B–A represents K^+-evoked ouabain-sensitive hyperpolarization of plasma membrane, and may be considered as a product of electrogenic activity of sodium pump (Kernan 1962, Dlouhá 1981)

ion channels Probably, there are L-type channels that are less inactivated than other types in this condition (for review see Fisher et al. 1976). It is generally accepted that Ca^{2+} entry blockers such as verapamil inhibit predominantly Ca^{2+} influx through this type of channel Calcium ions may additionally enter the myocytes by Na^+/Ca^{2+}-exchanging system in conditions of Na^+ loaded tissue under prolonged depolarization (Gillis 1985; Schwartz et al. 1985). Thus, Na^+/Ca^{2+}-exchanging system could be at least partly responsible for the observed increase of cytosolic Ca^{2+} concentration in diaphragm tissue under the above conditions. Verapamil, particularly in massive doses (Hosey and Lazdunski 1988), may depress the Ca^{2+} influx through (Na^+/Ca^{2+})-exchanger during the long-lasting depolarization when muscle cells are loaded by Na^+. Thus, it is hard to conclude yet if the structure responsible for this enhancement of intracellular Ca^{2+} concentration is L-type of calcium channels alone or if Na^+/Ca^{2+}-exchanger is partially involved in the calcium influx under the described conditions. Nevertheless, the application

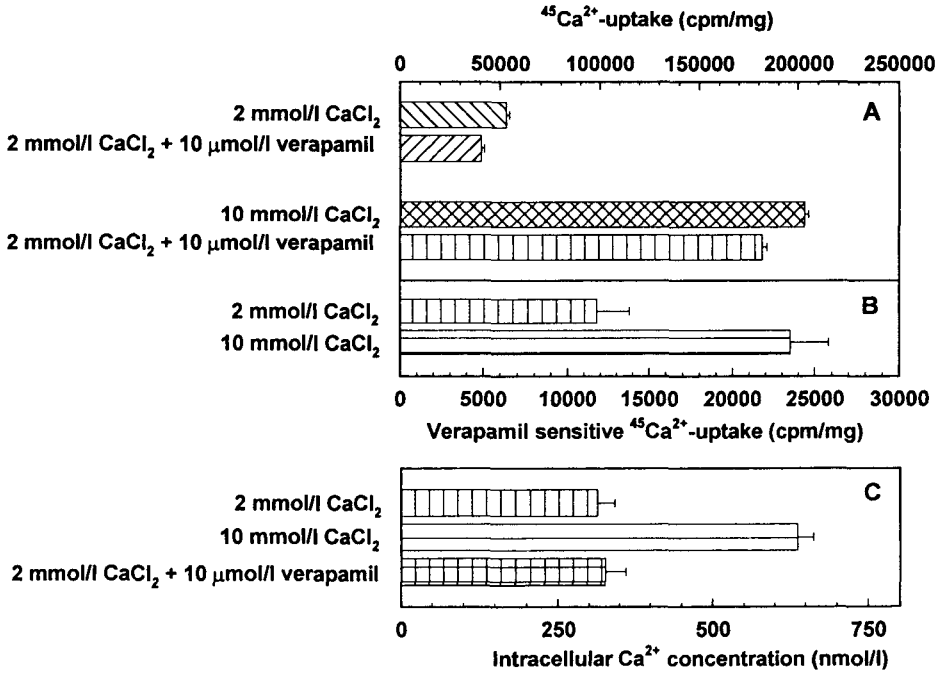

Figure 1. $^{45}Ca^{2+}$-uptake (total – panel *A* and verapamil sensitive – panel *B*) and intracellular Ca^{2+} concentrations (panel *C*) measured in mouse diaphragm tissue. Ca^{2+} uptake was measured after 30 minutes lasting incubation of diaphragm tissue in Liley solution containing 2 or 10 mmol/l $^{45}CaCl_2$ in the absence or presence of verapamil. Intracellular Ca^{2+} concentrations were estimated using the intracellular Ca^{2+} indicator Fura-2/AM. For details see Materials and Methods. Data represent mean ± S.E.M. from 6 independent experiments.

of 10 mmol/l of Ca^{2+} ions into bath medium caused further elevation of cytosolic Ca^{2+} concentration and concomittant inhibition of the electrogenic activity of sodium pump, and both these processes were prevented by verapamil.

An increase of Ca^{2+} concentration in the bath medium during incubation of the tissue from 2 to 4, 6, and 10 mmol/l induced change in the shape of the time course of glucose incorporation from linear into hyperbolic (Fig. 2*A*). The initial rate of [U-^{14}C]-glucose incorporation was increased with the increasing Ca^{2+} concentration (Fig. 2*C*). Nevertheless, the hyperbolic shape of the glucose uptake time dependencies (at increased Ca^{2+} concentration in bath medium, Fig. 2*A*) indicated a stepwise decrease of glucose incorporation rate in relation to time of incubation. The rate of glucose incorporation during 30 minutes of incubation exponentially decreased with the increasing concentrations of Ca^{2+} in bath medium (Fig. 2*C*).

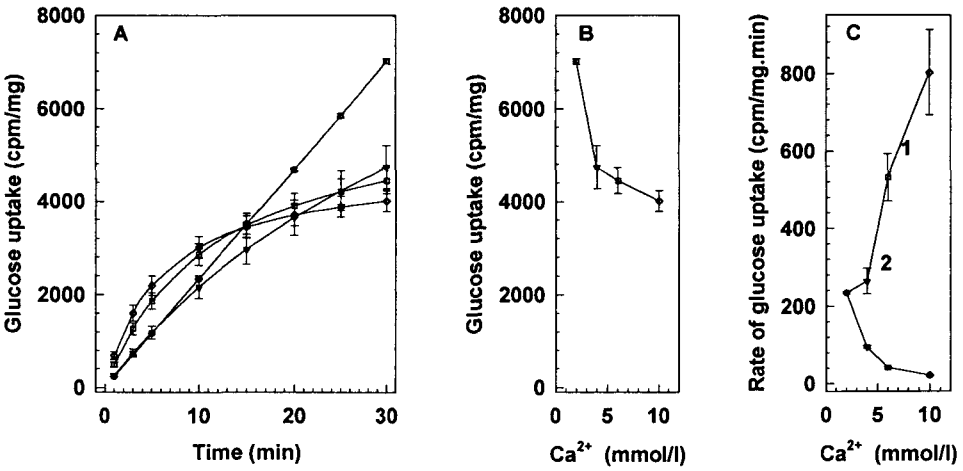

Figure 2. Effect of Ca^{2+} on [U-^{14}C]-glucose incorporation into mouse diaphragm tissue Glucose incorporation was established after incubation of the tissue in Liley solution with 2 (●) 4 (▼), 6 (■) and 10 (♦) mmol/l $CaCl_2$ Panel *A* Time-dependence of glucose incorporation Each point represents the mean from 6 independent measurements ± S E M The experimental data obtained in the presence of 2 mmol/l $CaCl_2$ were fitted by linear regression (slope 234 ± 2 cpm/mg min, r = 0 993) The experimental data obtained in the presence of 4–10 mmol/l $CaCl_2$ were fitted by nonlinear regression according to rectangular hyperbola equation The amounts of glucose incorporated in equilibrium were 11,811 ± 650, 6174 ± 45 and 4820 ± 180 cpm/mg for 4, 6, and 10 mmol/l $CaCl_2$, respectively The corresponding half times were 44 7 ± 3 6, 11 6 ± 1 0 and 6 0 ± 0 7 minutes Panel *B* total amount of glucose incorporation after 30 min lasting incubation as a function of Ca^{2+} concentration Panel *C* The initial rate of glucose incorporation (curve 1) and rate of glucose incorporation after 30 minutes of diaphragm tissue incubation (curve 2) as a function of Ca^{2+} concentration Rate values were computed by solution of the first derivation from the relationships depicted in panel A for t = 0 and t = 30 minutes respectively

This action of Ca^{2+} is responsible for the observed depression of the total amount of glucose incorporated during 30 minutes of incubation from 7300 cpm/mg wet tissue (measured in 2 mmol/l Ca^{2+}) to 4100 cpm/mg wet tissue (measured in 10 mmol/l Ca^{2+}, Fig. 2*B*). These data suggest that the higher concentration of Ca^{2+} in bath medium (at which the sodium pump is inhibited) induced a depression of glucose transport and/or utilization in muscle cells.

Ca^{2+} at 6 and 10 mmol/l in bath medium significantly inhibited both CO_2 production and O_2 consumption by mouse diaphragm muscle tissue (Fig. 3). The linear shapes of time dependencies of CO_2 formation and/or O_2 consumption observed during the tissue incubation in media containing 2 or 4 mmol/l of Ca^{2+} changed

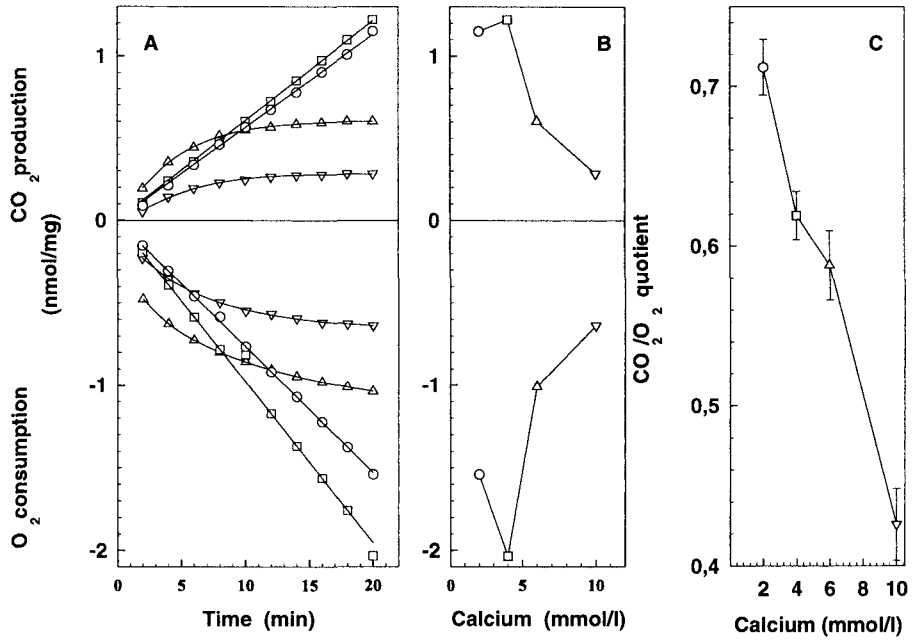

Figure 3. CO_2 production and O_2 consumption by mouse diaphragm tissue during incubation in media containing 2 (○), 4 (□), 6 (△), or 10 (▽) mmol/l $CaCl_2$ Panel *A* Time dependencies, Panel *B* Total CO_2 production and O_2 consumption after 20 minutes of diaphragm tissue incubation as a function of Ca^{2+} concentration in the bath medium Panel *C* Dependence of CO_2/O_2 quotient on Ca^{2+} concentration in the bath medium Each point represents the mean from 6 independent measurements The respective S E M never exceeded 6% of the mean

to hyperbolic shapes when Ca^{2+} was present at 10 mmol/l. An increase of Ca^{2+} concentration from 2 to 4 mmol/l caused a significant acceleration of O_2 consumption that was not followed by a similar expressive effect on CO_2 production. This suggested acceleration of respiration, i.e. acceleration of ATP production by mitochondria. This fact could be considered as being in agreement with the acceleration of ATP production and/or translocation by rat heart mitochondria under conditions of heart perfusion (Langendorff method) with a medium containing slightly increased Ca^{2+} (Tribulová et al. 1992; Bakker et al. 1994; Šeboková et al. 1996; Ziegelhöffer-Mihalovičová 1997). Calcium ions in each of the concentrations applied into bath medium was not able to change the energy metabolism from aerobic to anaerobic type; this is clearly documented by the fact that no prevalence of CO_2 production over O_2 consumption could be observed (Fig. 3). On the contrary, a continual decrease of CO_2/O_2 ratio (from 0.75 to 0.45) was observed upon increas-

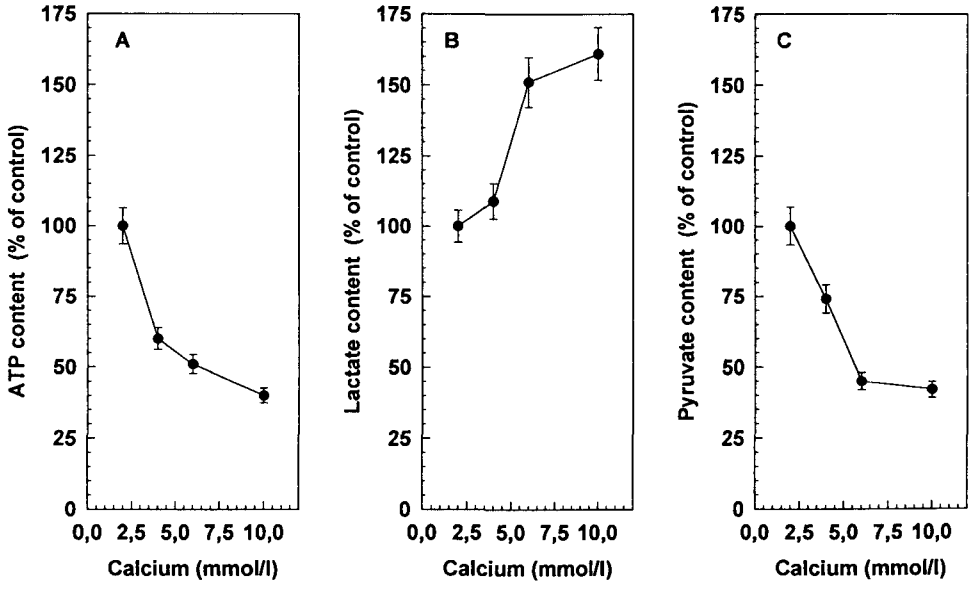

Figure 4. Effect of increased concentration of Ca^{2+} ions in bath medium on intracellular contents of ATP (panel *A*), lactate (panel *B*) and pyruvate (panel *C*) in diaphragm tissue after 30 min incubation Each point represents the mean ± S E M from 6 independent measurements Data were expressed as percents of the respective metabolite contents in control tissue (6 52±0 07, 2 83±0 11 and 0 071±0 005 mmol of ATP, lactate and pyruvate per gram wet tissue, respectively)

ing the concentration of calcium ions in bath medium from 2 to 10 mmol/l This suggested utilization of substrates in lower oxidative state other than sacharides by the oxidative pathway or acceleration of biosynthesis

An increase of Ca^{2+} concentration in bath medium caused a gradual depression of intracellular ATP and pyruvate levels (Fig 4) On the other hand, an increase of the intracellular levels of lactate by about 50% was observed upon increasing Ca^{2+} concentration from 4 to 6 mmol/l An increase of Ca^{2+} concentration from 2 to 4 as well as from 6 to 10 mmol/l had no significant effect on intracellular lactate levels This fact indicated that the transition of energy metabolism from aerobic to anaerobic glycolysis is not very effective because lactate levels, as an indicator of anaerobic metabolism, only increased about 50% (Fig 4) A similar behavior, i e a slight increase of lactate accompanied by decreased ATP and pyruvate tissue levels was observed in Ca^{2+} overloaded heart muscle tissue under calcium paradox (Ziegelhoffer et al 1989, Ravingerová et al 1991, 1993)

The above data suggest that at high concentrations of calcium in bath medium,

overloading of diaphragm myocytes by calcium should be expected. The increase of intracellular calcium concentration results in the inhibition of the electrogenic activity of the sodium pump. Under this condition, tissue respiration and glucose uptake were dramatically depressed. This mechanism is finally responsible for a low efficiency of ATP production insufficient to supply myocytes with energy.

The increase of intracellular Ca^{2+} concentration that induces inhibition of (Na^+/K^+)-ATPase will subsequently cause a decrease of transsarcolemmal Na^+ gradient Transsarcolemmal Na^+ gradient represents the driving force for the transport of Ca^{2+} and protons through (Na^+/Ca^{2+})-and (Na^+/H^+)-exchange systems (Aronson and Sackor 1975, Aronson 1984) Therefore, Ca^{2+}-induced inhibition of sodium pump will cause an additional increase of intracellular Ca^{2+} concentration and, in addition, raise intracellular proton concentration Ca^{2+} overload and thus induced acidosis may be responsible for metabolic disregulation that may induce subsequent depression of energy metabolism. This idea is supported by the assumption by Vrbjar et al. (1995) that inhibition of (Na^+/K^+)-ATPase by Ca^{2+} may be involved in ischemia - induced inhibition of heart sarcolemmal ATPases.

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