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Research Article

Long-chain fatty acid uptake by skeletal myocytes: a confocal laser scanning microscopy study

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Abstract. Studies of regulation of free fatty acid (FFA) utilization by skeletal muscles have focused on plasma FFA delivery and on intracellular factors affecting FFA metabolism. The present study was conducted to directly analyse the uptake process of fatty acids into single myocytes. Cells were isolated from the rat flexor digitorum brevis muscle. Confocal laser scanning microscopy was utilized to analyse the uptake of the fluorescent fatty acid derivative 12-NBD-stearate, which is not metabolized by muscle tissue. Uptake represented a saturable function of the unbound fatty acid concentration in the medium (K_m 366 ± 118 nM, V_{max} 2.1 ± 0.3 AU/s) and depended on the medium sodium concentration. Reduced buffer pH increased initial uptake rates, whereas lactate (10 mM) had no effect. Membrane hyper- and depolarization decreased uptake rates. This study demonstrates for the first time kinetic data from isolated myocytes with evidence for a carrier-mediated transport mechanism for long-chain fatty acids.

Key words. Isolated myocytes; free fatty acid uptake; 12-NBD-stearate; confocal laser scanning microscopy.

Skeletal muscles consume large amounts of unesterified long-chain fatty acids (FFAs) and the amount consumed increases during contractile activity [1]. Numerous studies on regulation of FFA utilization by muscles have focused on the role of FFA supply or on intracellular factors effecting their metabolism. However, little information is available how fatty acids enter myocytes. Uptake of palmitate into isolated perfused rat hindquarters revealed saturation kinetics, thus suggesting a carrier-mediated uptake process for this FFA. In addition, a fatty acid translocase protein has been found to be expressed severalfold higher in the rat soleus muscle (a skeletal muscle with high oxidative activity) compared with the extensor digitorum longus (a muscle with high glycolytic activity), suggesting an involvement of the protein in FFA transport across the plasma membrane [2]. Furthermore, a fatty acid-binding protein of 40 kD has been isolated from human muscle plasma membranes [3]. Unfortunately, studies on initial uptake kinetics in isolated myocytes were still lacking. The aim of the present study was therefore to examine cellular uptake of an FFA on a single myocyte level. To directly visualize the uptake process and the involved steps, a confocal laser scanning microscopy technique was employed. This technique has the advantage of a clear spatial definition of the cellular compartments under investigation. Thus differentiation between

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uptake into the plasma membrane or the cytosol is possible [4]. Prerequisite is the use of the fluorescent fatty acid derivative 12-NBD-stearate with the fluorescent moiety covalently linked to the C12 position. 12-NBD-stearate was shown in earlier studies to enter cells by the same uptake mechanism described for natural FFAs [4, 5]. Further characteristics include that 12-NBD-stearate binds tightly to the cytosolic fatty acid-binding protein with an affinity similar to that of FFAs, and that it is not metabolized in the liver [5].

Materials and methods

Chemicals. 12-(*N*-methyl)-*N*-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)octadecanoic acid (12-NBD stearic acid) and 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl]-amino)-hexanoic acid (NBD hexanoic acid) were obtained from Molecular Probes (Eugene, OR, USA). Fatty acid-free bovine serum albumin (BSA, fraction V) and collagenase type I were from Sigma (St. Louis, MO, USA). All other chemicals used were of the highest grade available. Matrigel was from Collaborative Research, Bedford, MA, USA.

Isolation of rat skeletal myocytes. Myocytes were isolated from male Wistar rats (90–120 g) that had free access to food and water and were obtained from the animal facilities of the University of Heidelberg, Germany. The animal experiments had been reviewed and permitted by a state board on animal experimentation. After pentobarbital sodium (50 mg/kg body weight, intraperitoneal) anaesthesia, both flexor digitorum brevis muscles were dissected and single cells were isolated as described [6]. The muscles were gently shaken for 4 h at 37 °C in bicarbonate-free Dulbecco's modified Eagle Medium (DMEM) containing 25 mM Hepes, pH 7.4, and 0.15% collagenase type I. After washing, the muscles were transferred to DMEM containing 10% fetal calf serum, and single myocytes were liberated by gentle trituration using a pipette. The cells were plated on 60-mm culture dishes precoated with Matrigel and incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. After the incubation the myocytes were viable, as judged by trypan blue exclusion, had distinct striations and adhered to the Matrigel tightly enough for microscopic uptake studies.

Uptake studies. Fatty acid uptake studies were performed as previously described [4]. Briefly, the cells were washed twice with the appropriate prewarmed uptake buffer and superfused for at least 5 min at a rate of 40 ml/min prior to fatty acid uptake. The standard uptake buffer composition was 135 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.8 mM Na_2HPO_4 and 5 mM glucose in 10 mM Hepes, pH 7.4. Hyperpolarization of the plasma membrane was achieved by adding 10 μM valinomycin, a K^+ ionophore, to the uptake buffer 30 s before fatty acid uptake started. Depolarization of the plasma membrane was induced by substitution of 50 mM NaCl with equal amount of KCl. For sodium-free conditions NaCl was replaced by *N*-methyl-D-glucamine (NMDG)-Cl, Na_2HPO_4 by K_2HPO_4, and KCl was reduced to 4.2 mM to keep the potassium concentration constant. Studies on the effect of lactate and pH were performed by adding 10 mM lactate and changing the pH from 7.4 to 6.8, respectively. The uptake medium was prepared by adding 25 μM fatty acid-free BSA and 25–100 μM NBD-conjugated fatty acid to the appropriate uptake buffer (37 °C). The superfusion rate during uptake was 40 ml/min. Uptake was measured for 300 s. Confocal images were generated every 10 s using the 488-nm argon laser line of a laser scanning microscope LSM 310 (Carl Zeiss, Jena, Germany), using the frame mode of the system. The emitted light was recorded after passing a 515–565-nm band pass filter and stored on a personal computer for later analysis. The scanning time per image was varied between 0.5 and 2 s. In subsequent analysis, regions of interest in the cytosol and the area of the plasma membrane with the adjacent cytoplasm were compared with respect to accumulation of 12-NBD-stearate fluorescence intensity. For construction of the uptake curve, changes in fluorescence intensity over a region of interest in the area of the plasma membrane or the cytosol were recorded. From these data the slope of the linear and maximal part of the uptake curve (V_0) was calculated by linear regression analysis using the stored intensity values.

TLC. To examine the possible metabolism of 12-NBD-stearate in muscle cells, TLC of extracted lipids was performed after incubation of the muscle cells with the fluorescent dye. For TLC analysis, 2.5 g of short skeletal muscles from rat hind feet was collected. Single myocytes were isolated as described and incubated for 24 h in normal cell culture dishes. The suspension was centrifuged at 200*g*, washed with prewarmed standard uptake buffer and stored for 10 min in this buffer at 37 °C. Afterwards, the cells were incubated for 5 min at 37 °C in a shaking waterbath with 100 μM NBD stearic acid and 25 μM fatty acid-free BSA in uptake buffer. Cells were then centrifuged for 2 min at 200*g*, resuspended in ice-cold 0.5% BSA in uptake buffer and centrifuged for 2 min at 400*g*. After a final washing step with ice-cold uptake buffer, lipid extraction was performed by a modification of the method described by [7]. Briefly, the supernatant was carefully sucked off, and the cells were homogenized in 24 ml of a mixture of

chloroform/methanol (2:1 v/v) by passing them 20 times through a 21-gauge needle. Distilled water (0.2 vol) was added and the mixture was shaken for 10 min, then phase separation was allowed. The upper phase was carefully removed, and the lower phase was concentrated under a nitrogen stream to 1.5 ml. Aliquots of the concentrated extract and of an NBD stearic acid stock solution were applied to silica gel 60 TLC plates (Merck, Darmstadt, Germany). The solvent mixture used for chromatography was hexane/ether/acetic acid (40:60:1 v/v/v). Spots were visualized under ultraviolet light.

Statistics. All results are expressed as means $\pm$ SD. Means of two groups were compared with Student's t test. Multiple means were compared by analysis of variance (ANOVA) followed by the Newman-Keuls test [8]. Linear regression analysis was performed by the least-squares method. The kinetics of 12-NBD-stearate uptake rates as a function of the stearate concentration in the medium were determined after computerized fitting of the weighted data to a rectangular hyperbola. $P < 0.05$ was considered statistically significant.

Results

Time course of 12-NBD-stearate uptake in single myocytes. For all fluorescent fatty acid derivative concentrations used in the present study the time course of intracellular accumulation was determined. A representative incubation experiment with 50 μM 12-NBD-stearate in the presence of 25 μM BSA is illustrated in figure 1. The increase of fluorescence intensity in a cytosolic and plasma membrane region of interest was recorded simultaneously and compared with the fluorescence of the incubation medium, which remained relatively constant over the observation period. In all experiments, an initial short lag phase was followed by a maximal and linear increase in cellular fluorescence. The pictures generated using the frame mode of the confocal system showed an initial pronounced labelling of the area of the plasma membrane, with subsequent time-dependent staining of the cytoplasm (fig. 2). To verify that uptake of the fluorescent fatty acid derivative was indeed dependent on the fatty acid moiety, control experiments were performed where the NBD molecule was covalently bound to hexanoic acid. In these experiments intracellular increase in fluorescence intensity could not be detected (data not shown), which suggests that uptake is determined by the fatty acid moiety.

Concentration dependency of 12-NBD-stearate influx. To further investigate the uptake kinetics of 12-NBD-stearate into single myocytes, the concentration dependency of 12-NBD-stearate influx at an albumin concentration of 25 μM was investigated. Initial uptake rates, calculated form the linear and maximal part of the uptake curve, were shown to be saturable as a function of the unbound FFA concentration (fig. 3) in the presence and absence of sodium. The concentration of unbound FFA concentration in the freshly prepared 12-NBD-stearate/albumin solutions was calculated by the stepwise equilibrium constant method of Wosilait and Nagy using the eight binding constants for stearic acid (but not including the dimerization-corrected constants) reported by Spector et al. [9]. After fitting the weighted data to a rectangle hyperbola, the Michaelis-Menten constant in the presence of sodium was 366 ± 118 nM and in the absence of sodium 679 ± 313 nM ($P < 0.05$). The maximal rate of reaction was similar in both experimental systems (V_{max}: 2.1 ± 0.3 vs. 2.2 ± 0.5 AU/s). This suggests that sodium enhances the affinity of the myocyte uptake system to 12-NBD-stearate.

Metabolism of 12-NBD-stearate by skeletal myocytes. Since cellular uptake of substrates can be regulated by intracellular metabolism, the cellular fate of the fatty acid derivative is of importance. Therefore, experiments were performed to analyse the metabolism of this fluorescent fatty acid. From previous studies it is known that 12-NBD-stearate is not metabolized in the liver [5]. In our experiments no significant metabolism of the dye

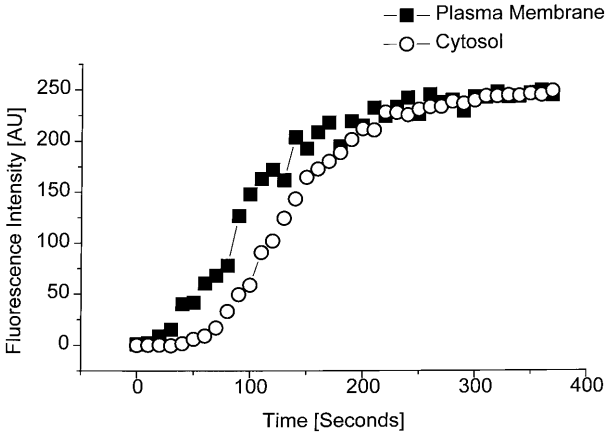

Figure 1. Time course of 12-NBD-stearate uptake into a single myocyte. In this representative experiment 100 μM 12-NBD-stearate in presence of 25 μM BSA was incubated. Fluorescence intensity values, measured in arbitrary units, were recorded in an intracytoplasmatic region of interest and for comparison in an area of the plasma membrane. It is shown that intracellular fluorescence intensity increases in a sigmoidal fashion. Maximal and linear uptake phase between 100 and 150 s represents unidirectional cellular influx of 12-NBD-stearate. Accumulation of fluorescence intensity in the area of the plasma membrane is faster than accumulation in the cytosol.

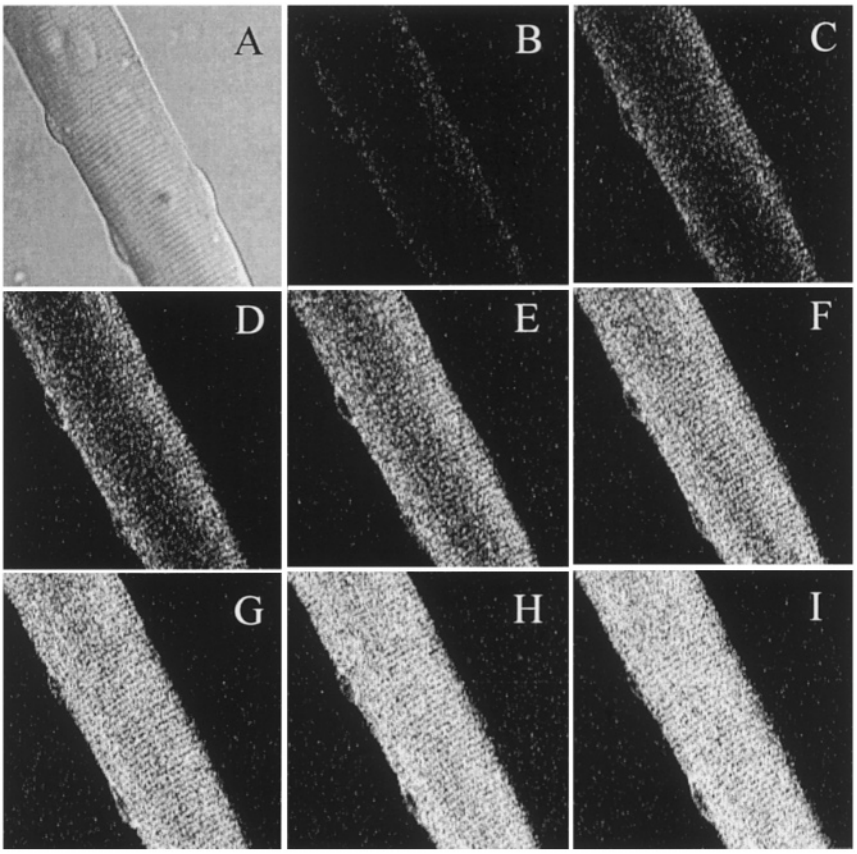

Figure 2. Confocal visualization of 12-NBD-stearate uptake into a single myocyte. Two-day cultured myocytes were superfused with the incubation medium containing 100 μM 12-NBD-stearate and 25 μM BSA. Panel *A* shows a phase contrast picture of a single myocyte. Panels *B–I* are pictures obtained at different time points during incubation using the frame mode of the confocal laser scanning microscope. Initial uptake into myocytes showed intense staining of the area of the plasma membrane.

was detectable after 5 min of incubation with skeletal muscles. The extracted fluorescent dye behaved like the natural compound (fig. 4), which demonstrates that the fluorescent moiety is still bound to the fatty acid tail and that the fatty acid is not metabolized.

Effect of pH, lactate and membrane voltage on the uptake rates of 12-NBD-stearate. In order to investigate possible driving forces for myocyte fatty acid uptake, the effect of buffer pH on the initial uptake rates at 50 μM 12-NBD-stearate concentration was analysed in the area of the plasma membrane and in the cytosol. During buffer pH of 6.8, membrane uptake rates increased by about 40% (fig. 5). The same was true for the uptake into the cytosolic compartment (data not shown). Lactate (10 mM) did not influence initial uptake rates (measured in the area of the plasma membrane and in the cytosol), neither at buffer pH 7.4 nor at buffer pH 6.8 (fig. 5).

During valinomycin preincubation to achieve membrane hyperpolarization, initial uptake rates were reduced by 65%. Membrane depolarization by replacing 50 mM NaCl with 50 mM KCl decreased the initial uptake only slightly. This reduction was more pronounced in the cytosolic compartment (fig. 6).

Discussion

This study utilizes confocal laser scanning microscopy to visualize and characterize the uptake of 12-NBD-stearate, a fluorescent fatty acid derivative, into isolated myocytes. In the liver this fluorescent fatty acid is neither metabolized nor secreted into bile, which is essential for kinetic analysis [5]. In our study we have demonstrated that no metabolism of 12-NBD-stearate occurred during the initial 5 min of incubation with isolated muscles cells. Thus, this compound is ideal to study fatty acid uptake into isolated myocytes. The uptake of this compound, as assessed by the confocal system, represents a saturable process with increasing concentrations of unbound fatty acids in the medium.

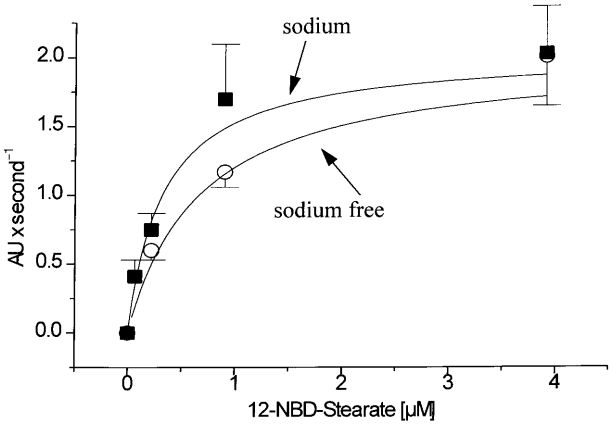

Figure 3. Concentration dependency of 12-NBD-stearate uptake into single myocytes. Uptake experiments were performed with increasing concentrations of 12-NBD-stearate at a constant BSA concentration in the presence and absence of sodium. Free 12-NBD-stearate concentrations were plotted against uptake velocity. Kinetic parameters were determined after computerized fitting of weighted data to a rectangular hyperbola (Michaelis constant in the presence of sodium 366 ± 118 nM, in sodium-free medium 679 ± 313 nM, $P < 0.05$, $n = 3–5$ different preparations).

This suggests the presence of a carrier protein in the plasma membrane of myocytes. Several putative carrier proteins are isolated, and three complementary DNAs (cDNAs) have been cloned [10–12]. However, it is unclear which carrier protein is expressed in myocytes. A recent study has suggested the presence of a membrane protein (FAT) homologous to CD 36 in the plasma membrane in both rat heart and skeletal muscle [2]. In human skeletal muscle membranes, a 40-kD membrane fatty acid binding protein-liver (mFABP-L) was detected by Western blotting. Its functional role, however, remains to be established [3].

This is the first study in which isolated skeletal muscles were used to investigate fatty acid uptake. The flexor digitorum brevis muscle (which was used in the study) is a small muscle composed of short myocytes. It allows experimental handling without damage. The problem faced in this confocal laser scanning microscopy study was to establish conditions for the myocytes to be firmly fixed at the dish and to remain motionless during superfusion with the buffer. We found that after 48-h incubation on Matrigel, most myocytes were attached to the gel along their length. The myocytes were viable, as judged by trypan blue exclusion, and had distinct striation. The myocytes accumulated 12-NBD-stearate with saturation kinetics. This is in accordance with previous studies, which demonstrated criteria of a carrier-mediated transport process in isolated perfused rat hindquarters [13] and several other organs [14, 15]. The stepwise equilibrium constant method of Wosilait and Nagy using the eight binding constants for stearic acid (but not including the dimerization-corrected constants) reported by Spector et al. [9] was used to calculate the FFA concentration. This method has recently been questioned, and different models to calculate the FFA concentration were proposed from different groups with differing absolute values [16–18]. They all share the notion that the FFA concentration is dependent on the molar ratio of fatty acid to albumin. The higher the ratio, the higher the FFA concentration. Thus we feel justified in using the method reported here for calculation of the FFA concentration until a final solution of this problem has been settled.

Studies in isolated perfused liver and in isolated hepatocytes have shown that fatty acid uptake is sodium- and

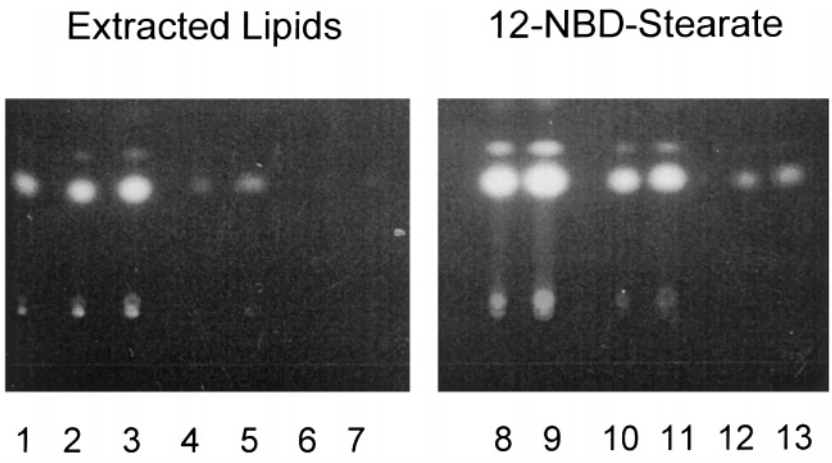

Figure 4. TLC and visualization of fluorescence of extracted lipids from muscle tissue after incubation with 50 μM 12-NBD-stearate for 5 min. The extracted lipids (lane 1 undiluted, lanes 2 and 3 1:10, lanes 4 and 5 1:100, and lanes 6 and 7 1:1000 diluted with ethanol) behaved like the natural compound (lanes 8 and 9 1:10, lanes 10 and 11 1:100, and lanes 12 and 13 1:1000 diluted with ethanol). These results demonstrate that during the incubation period no metabolism of the dye occurs.

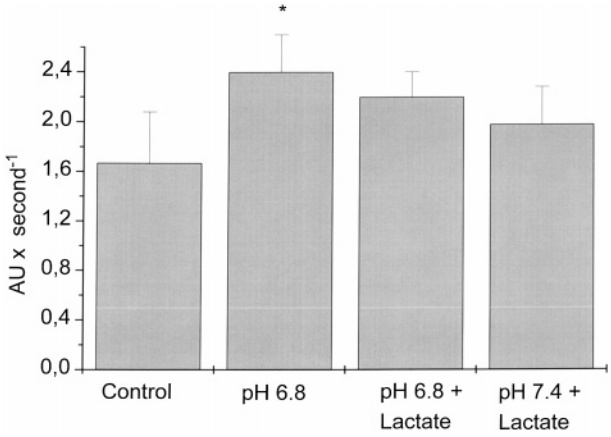

Figure 5. Effect of pH and lactate on initial uptake velocity. During buffer pH of 6.8, uptake velocity increased ($P < 0.01$) ($n = 3–5$ preparations), while lactate (10 mM) had no influence on uptake of 12-NBD-stearate.

potential-dependent [15, 19]. Other studies have questioned this observation [20, 21]. In the present study, omission of sodium in the perfusion buffer resulted in a significant reduction of the affinity of the myocyte transport system to fatty acids. This suggests that sodium is necessary for optimal transport and supports the hypothesis of a sodium-dependent recognition system of the fatty acid molecule by the transport system [22].

In contrast to other studies [5, 19] hyperpolarization, achieved by valinomycin pretreatment, resulted in a decrease of initial uptake rates of 12-NBD-stearate. The discrepancy may be due to different experimental procedures. Furthermore, in the present study, the change in membrane potential could not be measured. It is possible that pretreatment of myocytes with valinomycin does not significantly change the resting potential. The resting potential in myocytes is about the equilibrium for potassium (90 mV) [23]. Thus, the potassium ionophore valinomycin can effect only minor changes in this potential.

In vitro, a reduction in pH of the incubation medium was shown to increase FFA uptake into different cell types. This is due to the existence of a transmembrane proton gradient which supports fatty acid uptake [24]. In addition, low pH enhances the FFA concentration [25]. Our results obtained in isolated myocytes confirm these hypotheses.

Of interest is the observation that lactate had no effect on fatty acid uptake. Increased production of lactate is accompanied by increased participation in anaerobic metabolism for energy production in skeletal muscles [26, 27]. Our study, in which acidosis and lactate did not inhibit initial fatty acid uptake, therefore demonstrates that the uptake of fatty acids into myocytes is regulated independent of metabolic pathways.

In conclusion, our study is the first to show the existence of a carrier-mediated uptake process for fatty acids in isolated muscle cells. This uptake process is dependent on sodium and can be stimulated by an inwardly directed proton gradient.

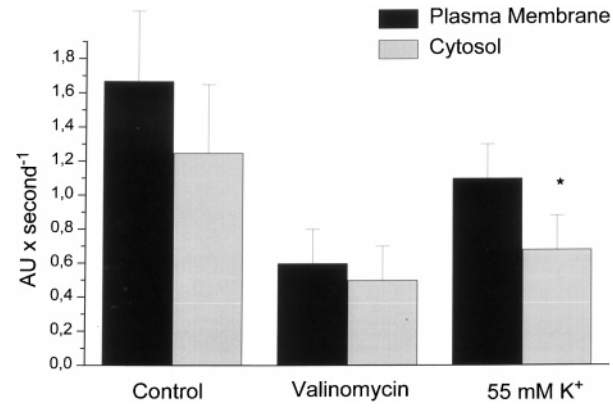

Figure 6. Effect of membrane potential on initial uptake velocity in the area of the plasma membrane and the cytosol. Hyperpolarization was achieved by incubation with valinomycin (10 μM) and membrane depolarization by replacing 50 mM NaCl with 50 mM KCl in the incubation buffer. Both manoeuvres decreased initial uptake velocity, with differing effects of depolarization in the plasma membrane and cytosol. * $P < 0.05$, $n = 3–5$ different preparations.

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