Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 67-74

THE INFLUENCE OF DYSTROPHIN ON LATERAL DIFFUSION OF PROTEINS IN SARCOLEMMA OF L-185 AND C2 MYOBLASTS AND MATURE STRIATED MUSCLE CELLS OF RATS AND MICE, AS MEASURED BY FRAP TECHNIQUE

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Received August 24, 1995

The expression of dystrophin can be suppressed in cultured skeletal muscle cells (coded L-185 from rat, and C2 from mouse) after a proper genetic manipulation. The influence of presence or absence of dystrophin on the lateral diffusion constant of Con-A-receptors was studied in the cell membrane of such cells (and also of mature skeletal muscle fibres of rat and mouse) by means of the fluorescence recovery after photobleaching (FRAP) technique, applying a novel fluorescent label called Con-A-BODIPY-Fl conjugate. It has been established that the normal maturation of myoblasts into skeletal muscle fibres involves a significant decrease of the mobility of Con-A receptors in the sarcolemma. In the absence of dystrophin, this maturation process cannot take place; the membrane proteins display an increasing mobility during the culture time, which is of lethal effect for these cells.

Duchenne muscular dystrophy (DMD) is one of the most common lethal genetic diseases in man (1,2). The key problem of DMD is the deficiency of dystrophin (Dy) which is a recently identified protein of 427 kDa molecular mass (3,4). Dy is expressed in normal skeletal muscle fibres, but it is present also in smooth muscle cells and even in neurons (5-7). Immunohistochemical and immunoelectron microscopic studies have revealed that Dy is localized to the sarcolemma of the skeletal muscle cells (8-13). In DMD patients, Dy is practically not detectable by the same methods, whereas it displays a discontinuous occurrence in another human muscular disease called Becker muscular dystrophy (BMD) which is clinically less severe (14). It is known that the expression of Dy in normal cultured myoblasts is low, and increases with the differentiation of these cells (15,16), however, many details of the true role of Dy in muscle cell maturation and physiology are still missing.

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0006-291X/95 $12.00

A particular possibility of studying the molecular biology of Dy has emerged from experimental genetic manipulation of the cultured L-185 (from rat) and C2 (from mouse) skeletal muscle cell lines, as described in detail elsewhere (17). Essentially, a partial DNA sequence in antisense orientation of Dy together with a particular promoter gene have been introduced in these cells. This antisense, however, remains silent, unless its promoter gene is stimulated by dexamethason (DXM). Such a transfection results in (i) normal myoblast lines with perfectly expressed Dy genes (if no DXM is added to the cultures); and (ii) Dy(-) myoblast lines (if DXM is added), where Dy is not present at all. These cellular models represent convenient possibilites to study the effects of presence or absence of Dy on various parameters of the cells and their membranes.

In the present study, the lateral diffusion constant of the concanavalin A-receptors labeled with a Con-A-BODIPY-FL conjugate proteins in the cell plasma membrane was measured by the method of fluorescence recovery after photobleaching (FRAP) (18-22). This approach is entirely new, since, to best of our knowledge, no similar experiments have so far been described.

MATERIALS AND METHODS

The studied cells

L-185 and C2 cells were cultured on cover slips under optimalized conditions, at 37°C, in the presence of 5 % CO_2. Cell passages were performed by washing in phosphate buffered saline (PBS), and 0.25% trypsin treatment. Culturing medium was Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS) and 1% chicken embryo extract (CEE) for proliferation, as well as only 2% FBS for differentiation. Other details have been described elsewhere (17).

Cells attached to cover slips are easy to handle for microscopic purposes. The following cultured muscle cell stages have been studied on both types of myoblasts.

a. Normal myoblasts without any genetic manipulation, on the 6th day of culturing.

b. Normal myoblasts without any genetic manipulation, on the 14th day of culturing (fused cells).

c. Dy(-) myoblasts on the 6th day of culturing.

d. Dy(-) myoblasts on the 12th day of culturing.

e. Myoblasts transfected by the anticodon but without activation with DXM, on the 6th day of culturing.

Apart from the cultured cells, normal, mature skeletal muscle fibres were also studied in freshly made, strip-preparations. They were taken from the extensor digitorum longus (EDL), and quadriceps femoris (QF) muscles of 2-month-old, male Fischer 344 rats and from male white mice of the same age. The method used was very simple; immediately after decapitation of the rats, a piece of the muscles mentioned above were excised, and using a fine pincette, thin strips were pulled out from the muscle mass. Such strips contained bundles of several muscle fibres under microscopic control, which maintained their morphology, striation, etc.

Staining of cells

After a quick washing with F-10 medium of pH 7.2, the cells were stained with a Con-A-BODIPY-FL conjugate (Catalogue No.; C-2754, Molecular Probes, Inc. USA). The chemical structure of BODIPY-FL is N-(4,4-di-

fluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl); it is a novel green-emitting fluorophore (23) with particularly advantageous properties for cell biological purposes, such as relatively wide excitation, and very narrow emission spectra, high extinction coefficient and quantum yield, etc. (24). It conjugates with lectins, resulting in an intense, specific fluorescent label for the Con-A receptors.

A stock solution of 2 mg/ml was prepared from the Con-A-BODIPY-FL conjugate in the same buffer, which can be maintained at 4^{0}C for several months. The final dilution was prepared each time immediately before use, it contained 10 µg/ml of the label, in the same buffer.

Duration of staining of cells attached to coverslip was 10 min at 37^{0}C, followed by a quick washing with buffer, then putting on a fluorescence-free glass slide and wiping the excess liquid. At last, the edges of the cover slips were sealed with melted paraffin, in order to avoid drying of the cells.

The FRAP measurements were carried out by using the instrument described in detail elsewhere (25,26). The specimen was kept at 37^{0}C during the measurement by using a thermostated specimen holder. Excitation wavelength was the 476.5 nm line of the Argon-ion laser, cutting filter was of 495 nm. Using an Olympus FLPL dry objective (x40, NA = 0.75), the average half diameter of the measured spot size (at an intensity of $1e^{-2}$) was 1.5 µm. The computer program has been properly modified; the observation and recovery periods were 25 sec each, the fluorescence intensity was measured every second (Figure 1). The lateral diffusion constant of Con-A receptors (D) was calculated by two methods of computation, as described before (26), and the average of those results was considered for each cell as the definitive value of D. Since the distribution histograms per group were fairly close to normal distribution, group averages were compared by the two-tailed Student's t test.

RESULTS

Some typical FRAP computer plots are presented in Figure 1 demonstrating the recovery curves of the bleached fluorescence. The fluorescence intensity was safely measurable, and the myoblast cells proved to be convenient for this kind of FRAP studies. Although the muscle cells investigated sometimes showed some movements at microscopic scale under the measuring beam, causing some "jumps" in the fluroescence values, as one can see in the observation periods (Figure 1), the mechanical stability of the cells during the measurements can be regarded as acceptable. The strip-preparations of the EDL or QF muscles were somewhat more troublesome from this point of view, nevertheless, even in such preparations sufficient number of cells could safely be measured.

Table 1 summarizes the most relevant parameters obtained in all types of muscle cells studied. It should be noted that we present pooled results for each type of cells, taken from 2-5 different experiments per cell type. This pooling was justified by the fact that averages of single experiments did not differ from each other within the same cell group.

The mean values of F(0) (fluorescence intensity at zero postbleach time) were scattering between 27.6 and 43.1%, i.e., the amount of bleaching was optimal for the measurement (18). F(inf) means the maximum recovery values reached, it was in average between 56.4 and 78.3%, i.e., in agreement with other results on the mobility of Con-A receptors (27,28) the recovery re-

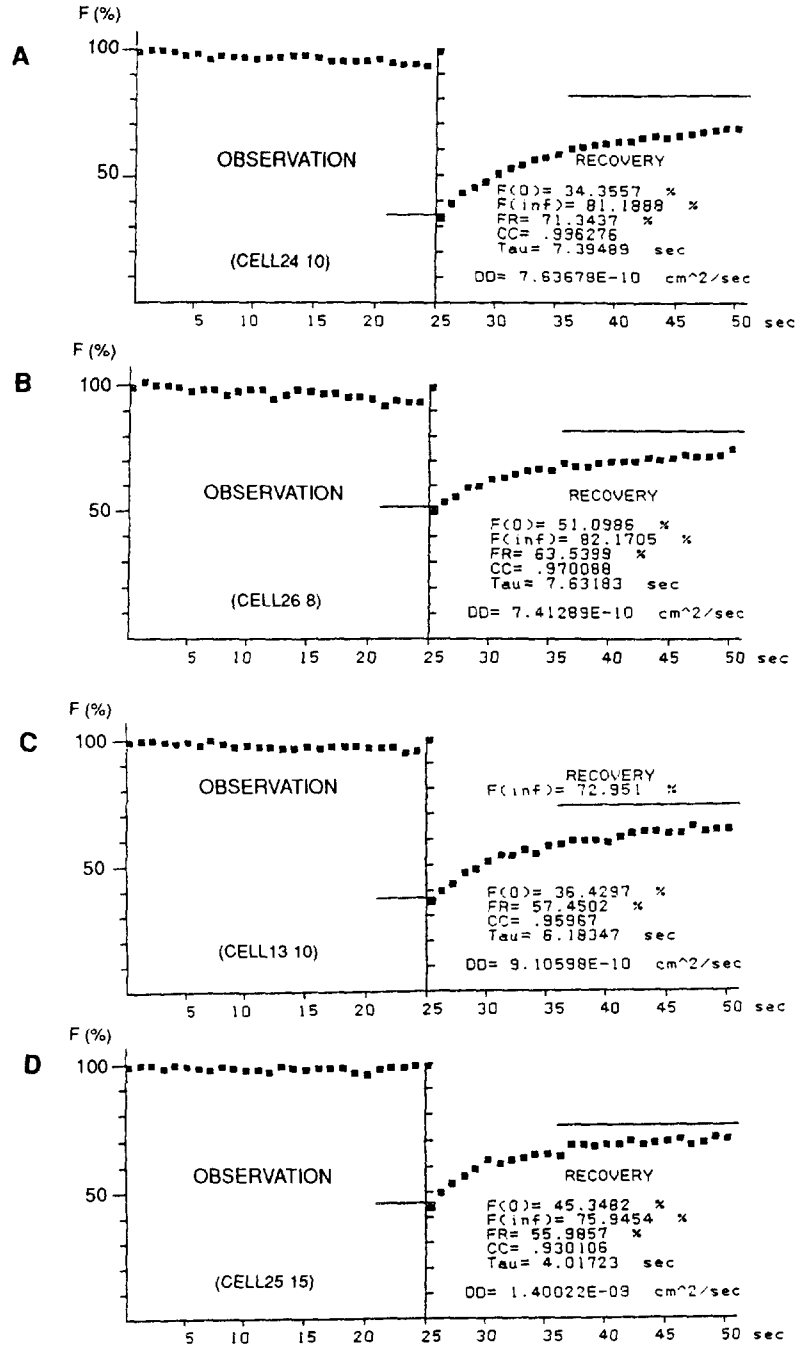

Figure 1. Some typical FRAP records of L-185 cells. A - normal myoblast of 6 days culturing; B - normal myoblast of 14 days culturing; C - Dy(-) myoblast of 6 days culturing; D - Dy(-) myoblast of 12 days culturing; Abbreviations not explained in the text; CC = correlation coefficient for the fitting of measured data to the reciprocal equation of Yguerabide et al. (22); Tau = the characteristic diffusion time; DD indicates the average diffusion constant from 2 different methods of computation.

Table 1

Group averages of the most relevant parameters from the FRAP experiments in L-185 and C2 cells and mature skeletal muscle cells of rats (R) and mice (M)

Cell types	Cell number	F(0)	F(inf)	FR (%)	D cm^2/sec	± S.E.M.	FRxD cm^2/sec	± S.E.M.
a. Normal myoblasts on the 6th day of culturing								
L-185	109	35.7	70.8	55.1	7.65E-10	2.42E-11	4.11E-10	1.46E-11
C2	96	43.1	77.6	61.4	7.61E-10	3.44E-11	4.57E-10	2.17E-11
b. Normal myoblasts on the 14th day of culturing (fused cells)								
L-185	36	43.1	77.3	60.0	7.69E-10	3.38E-11	4.45E-10	1.83E-11
C2	88	33.8	73.6	60.2	7.70E-10	3.93E-11	4.49E-10	2.23E-11
c. Dy(-) myoblasts on the 6th day of culturing								
L-185	105	32.3	67.6	52.3	9.20E-10	2.64E-11	4.81E-10	1.78E-11
C2	65	35.5	70.9	55.4	9.48E-10	6.56E-11	5.36E-10	4.64E-11
d. Dy(-) myoblasts on the 12th day of culturing								
L-185	55	39.0	78.3	64.1	1.38E-09	6.25E-11	8.83E-10	4.74E-11
C2	51	34.8	56.4	33.4	1.37E-09	1.31E-11	4.52E-10	4.60E-11
e. Myoblasts transfected but without DXM on the 6th day								
L-185	41	34.4	70.6	55.6	7.50E-10	4.22E-11	4.15E-10	2.90E-11
C2	90	38.9	65.1	42.9	7.47E-10	4.07E-11	3.08E-10	1.63E-11
f. Normal skeletal muscle fibres								
R EDL	70	36.8	63.1	42.1	5.28E-10	2.36E-11	2.14E-10	1.09E-11
R QF	60	40.1	68.4	47.9	5.42E-10	3.07E-11	2.41E-10	1.28E-11
M EDL	50	36.6	67.4	50.6	5.97E-10	2.64E-11	2.95E-10	1.91E-11
M QF	50	27.6	59.2	44.4	5.80E-10	2.78E-11	2.48E-10	1.60E-11

Notes; All normal myoblasts (groups a, b and e) show statistically identical values of D; Dy(-) myoblasts (groups c and d) display significantly higher values of D ($p < 0.001$) than the normal myoblasts; D of mature skeletal muscle fibres (group f) is significantly lower ($p < 0.001$) than that of normal myoblasts; comparison of the respective values FRxD results in similar statistical trends.

mains consequently below 100%. As a matter of fact, the fractional recovery (FR) was in average between 0.334 and 0.641 for the myoblasts, and essentially identical in the mature skeletal muscle cells (Table 1 reports FR values in %).

The stability of the values of D during the time of measurement was also checked by analyzing the time-dependence of the values obtained. It was proven by these analyses that the 0-hypothesis for the time-correlation was valid throughout our measuring periods (up to 120 - 140 min from the beginning of the staining).

The results in Table 1 show some very conspicuous differences in the values of D between various cell types. These tendencies are the same for the myoblasts of both rat and murine origin. All normal myoblasts (i.e., groups a, b and e) gave identical values for D, indicating that these cell types have a common characteristics; D is not changed even when the myoblasts become fused on the 14th day of culturing. The presence of the silent anticodon has apparently no effect on the value of D (group e). On the other hand, the range of D found in normal myoblasts changes significantly in both the Dy(-) cells and the mature skeletal muscle fibres. While in

the Dy(-) cells D increases considerably by the 6th day, and even more so by the 12th day of culturing, mature skeletal muscle sarcolemma displays significantly lower values of D in both EDL and QF muscles, as compared to the normal myoblasts (Table 1).

Due to the fact that the values of FR did not differ considerably between the various cell types, the trends of changes described above for D remain valid also for the parameter FRxD. This letter parameter indicates the real value of the lateral mobility of the proteins in the whole sarcolemma, since it considers the immobile fraction of the membrane proteins (which are not recovering after photobleaching).

DISCUSSION

There is no doubt on the validity of the FRAP method in the revelation of membrane physicochemical properties such as lateral mobility of the components. The early available data were obtained by using so much different methods that no direct comparison is possible (27,28). However, more recent experiments (29) on cultured rat heart myocytes resulted in an average D value for the Con-A receptors in the range of $(4.5 \pm 0.6) \times 10^{-10}$ cm^2/sec, using tetramethyl-rhodamine-succinyl-Con-A as a label, which is quite close to our D values obtained in the mature EDL or QF muscle fibres in the present experiments (Table 1), especially if considering that our results were obtained at $37^{o}C$, whereas those of the cited authors at $22^{o}C$.

The significance of our data can be summarized as follows:

(i) The maturation of myoblasts into skeletal muscle fibres involves a considerable decrease of the mobility of Con-A receptors in the sarcolemma.

(ii) Dy is apparently a necessary factor in this maturation process demonstrated by the facts that (a) D is much higher in the normal myoblasts than in the mature skeletal muscle cells, and biochemical measurements have proven the scarcity of Dy in the normal myoblasts (15,16); (b) in the Dy(-) myoblasts where Dy is virtually absent, D increased even further. As a matter of fact, the value of D found in the Dy(-) cells of 12 days of culturing is practically identical with the lateral diffusion of membrane lipids, indicating a complete absence of the membrane maturation phenomena, accompanied by the inability of the cells not only for differentiation, but even for confluence. The sarcolemmal localization of Dy is in perfect harmony with the assumption that this compound may play a real role in the formation of the quaternary structure of the cell membrane which is an essential process for cell maturation.

It is tempting to speculate on the fact that Dy is present in the subsynaptic region of normal neurons as well (5,6), and the absence of it in muscle fibres causes an imperfect muscle function and death in the DMD patients. This suggests that Dy is most probably a necessary factor also in the

processes of elaboration of interneural and neuromuscular signal transduction. This possibility underlines further the significance of the measurement of the lateral diffusion of membrane components, especially of proteins, as it can be realized by the FRAP technique.

ACKNOWLEDGMENTS

Prof. I. Zs.-Nagy expresses his grateful thanks for the Tokyo University for the Visiting Professorship, and especially to Professor K. Kitani for being the host of him, and to Professor Y. Nonomura for the excellent cooperation in this work.

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