Volume 212, number 2, 313–316 FEB 04361 February 1987

Increased chloride efflux in fibroblasts from X-linked muscular dystrophies and clones from Duchenne carriers

Michela Rugolo, Mariano Rocchi*, Giorgio Lenaz and Giovanni Romeo*

Dipartimento di Biologia, Istituto Botanico, Via Irnerio 42, 40126 Bologna and **Laboratorio di Genetica Molecolare, Istituto G. Gaslini, Via 5 Maggio 39, 16148 Genova, Italy*

Received 23 October 1986

Previous studies have suggested an increased chloride membrane permeability in Duchenne muscular dystrophy (DMD) fibroblasts. We report that an increased chloride efflux with respect to controls is present not only in fibroblasts from DMD, but also from two other X-linked muscular dystrophies, Becker and Emery-Dreifuss, as well as in clones from DMD carrier females. The latter observation suggests that, at least in DMD, the increased chloride efflux phenotype might be subject to lyonization.

Cl^- efflux; Fibroblast; X-linked muscular dystrophy; Duchenne muscular dystrophy; Becker muscular dystrophy; Emery-Dreifuss muscular dystrophy; (Human)

1. INTRODUCTION

Among the X-linked muscular dystrophies, DMD is the most serious and frequent, while BMD and EDMD are characterized by a milder course and survival into adulthood compatible with a normal reproductive function. The biochemical defects of these X-linked dystrophies are as yet unknown. Histopathological features typical of primitive muscular dystrophies are observed in skeletal muscle from these patients, while other abnormalities have been detected in heart [1], nervous system [2], red blood cells [3], lymphocytes [4] and skin fibroblasts [5–8] (review [9]). In particular cultured skin fibroblasts might represent a suitable model in the search for the basic defect(s) of X-linked muscular dystrophy, if it can be convincingly demonstrated that they express it.

Correspondence address: M. Rugolo, Istituto Botanico, Via Irnerio 42, 40126 Bologna, Italy

Abbreviations: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; EDMD, Emery-Dreifuss muscular dystrophy

Recently, Davis and co-workers [10] have described several changes involving the Cl^--dependent lysosomal proteolytic enzyme dipeptidyl aminopeptidase I in DMD fibroblasts. The most significant changes were pronounced decreases in both enzyme activity and latency. These observations led to suggest the presence of an underlying abnormality in chloride permeability or concentration [10]. A significant increase in membrane permeability of DMD fibroblasts to Cl^- has been subsequently observed by the same group [11].

Here, we report that an increased Cl^- efflux with respect to controls is present not only in fibroblasts from DMD, but also from BMD and EDMD patients, as well as in clones from DMD carrier females. The latter observation suggests that, at least in DMD, the increased chloride efflux phenotype might be subject to lyonization.

2. MATERIALS AND METHODS

Skin fibroblasts, derived from biopsies of the upper arm, were cultured in Dulbecco's modified Eagle's medium containing L-glutamine, an-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

tibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and 10% fetal calf serum, in a humidified atmosphere of 5% CO_2 in 95% air at 37°C.

Fibroblasts from controls, patients and obligate carrier females (with at least one brother and one son both affected) were developed in our laboratory. For the experiment, cells were seeded into multiwell tissue culture plates (2 cm^2, Nunc) at a density of 4×10^4 cells per well and allowed to grow to confluency in 1 ml of growth medium for 7 days. Cells were matched for passage number ± 5.

Cloned fibroblasts were obtained by seeding approx. 1 cell per well in multiwell plates with a feeder layer of male fibroblasts previously treated with mitomycin C. Clones from controls and DMD carriers were cultured in the same experimental set and utilized at complete confluency.

Chloride efflux studies were performed on confluent cultures in multiwell plates, at 37°C and in air without added CO_2, exactly as in [12]. Briefly, cells were loaded with the medium containing $^{36}Cl^-$ (7 μCi/ml) for 60–90 min, washed with ice-cold 0.25 M sucrose/5 mM Hepes (pH 7.4), and then incubated with the incubation medium without $^{36}Cl^-$. At defined times, the medium was removed and the cells were dissolved in 0.2 M NaOH; aliquots were removed for determination of radioactivity and protein content. The first-order rate constant for $^{36}Cl^-$ efflux from the plasma membrane of fibroblasts was determined as described [12]. Efflux parameters for each fibroblast line were determined from the best fit of the experimental points determined by a computer program based on standard non-linear least-squares minimization procedure [13]. Statistical evaluation was based on Student's t-test.

3. RESULTS

Cl^- efflux under steady-state conditions in normal fibroblasts has been shown to be biphasic, due to the presence of two intracellular compartments, both following first-order exponential decay [11,12]. According to this model, the sizes of the two compartments are very similar, while the rate constants of efflux are quite different. In the present experiments, performed in 12 control fibroblast lines (no significant difference being observed between male and female lines), the average values for the sizes of the two compartments were 109 ± 26 and 128 ± 50 nmol/mg protein, respectively. The average values for the rate constants of efflux were 0.081 ± 0.020 and 1.000 ± 0.113 min^{-1}, respectively. We have previously suggested that the efflux characterized by higher value of the rate constant is associated with transport across the plasma membrane of fibroblasts [12]. As a consequence, the fast-rate compartment would correspond to the cytoplasm of fibroblasts, while the identity of the slow-rate compartment remains unknown.

In fig.1, the rate constants of Cl^- efflux from the fast compartment (k) of fibroblast lines derived from male individuals normal and affected by X-linked muscular dystrophies have been reported. In comparison to controls, the value for the rate constant was increased on average by 55, 50 and 66% in DMD, BMD and EDMD fibroblasts, respectively. It is noteworthy that in all the experiments the value of the k from each cell strain of X-linked muscular dystrophy was significantly

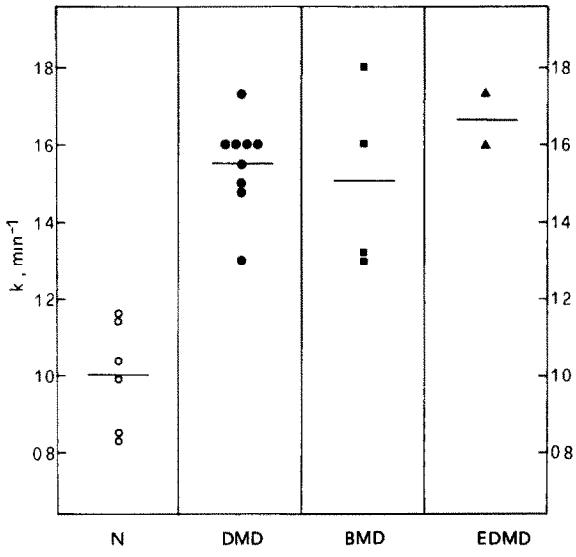

Fig.1. Rate constants of $^{36}Cl^-$ efflux from lines of fibroblasts derived from male individuals: (○) N, normal; (●) DMD; (■) BMD; (▲) EDMD. The values shown represent the average for each line determined in at least two experiments. The differences between normal and DMD, BMD and EDMD lines were statistically significant ($P < 0.001$; $P < 0.01$; $P < 0.001$, respectively). The horizontal bars are the means of the values.

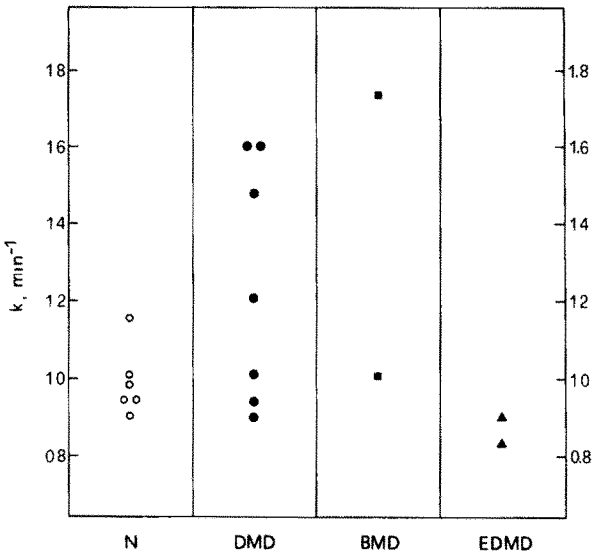

Fig.2. Rate constants of $^{36}Cl^-$ efflux from lines of fibroblasts derived from female individuals: (○) N, normal; (●) DMD; (■) BMD; (▲) EDMD. The values shown represent the average for each line determined in at least two experiments.

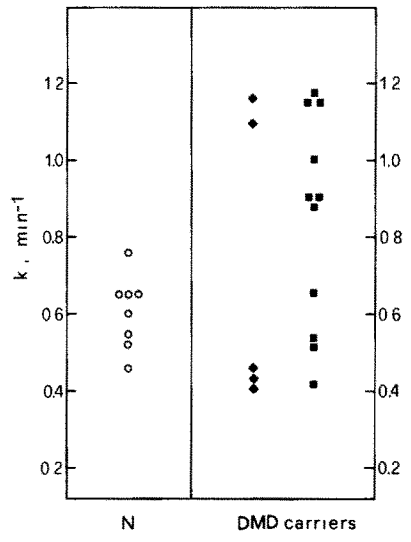

Fig.3. Rate constants of $^{36}Cl^-$ efflux from clones of fibroblasts derived from a normal female individual (○) and two DMD carrier females (■, ◆). If the highest value of rate constant observed among normal clones is taken as threshold, the clones from the two DMD carriers fall into two different groups with significantly different rate constant ($P < 0.001$).

higher than that of controls. No significant differences were observed on the rate constants from the slow compartment or on the amplitudes, in nmol/mg protein, of the two compartments.

Fig.2 shows the rate constant of Cl^- efflux from female individuals. It can be observed that the values of the rate constant for DMD and BMD carriers were very heterogeneous, ranging between those of controls and those of affected male individuals. The values of the rate constant of efflux in EDMD carriers resulted to be identical to those obtained in controls.

Fig.3 shows the values of the rate constant obtained in different clones derived from a control female and from two DMD carriers. A population of clones with rate constant values significantly higher than those found in clones from the control female occurs in both DMD carriers, who also present a second population of clones with rate constant values not significantly different from the control clones.

4. DISCUSSION

Our data demonstrate that in fibroblasts derived from patients with X-linked muscular dystrophies the kinetics of Cl^- efflux is altered, with a significant increase of the rate constant of efflux through the plasma membrane. The alteration described in this investigation in DMD fibroblasts is in good agreement with previous results [11]. In the latter studies, however, the increase in the average value for the rate constant of efflux was less pronounced (+30%). The alteration described here in BMD and EDMD fibroblasts, to the best of our knowledge, has not been previously reported. The increased Cl^- efflux observed in BMD is not very surprising since the most recent molecular genetic evidence [14–16] makes it likely that mutations causing DMD or BMD affect the same gene or a family of closely related genes. In contrast, the altered Cl^- efflux observed in EDMD is more difficult to explain, because the locus responsible for the latter disorder is located in a region of the long arm of the X chromosome which is clearly different from that of the DMD-BMD locus(i) [17,18]. The alteration in Cl^- efflux shared by the three X-linked muscular dystrophies might therefore be explained as a secondary phenomenon or as a common cellular phenotype due to muta-

tions in two different subunits of the same protein. In addition, the heterogeneous results obtained in uncloned fibroblasts from carrier females might reflect the percentage of somatic cells carrying the X-linked mutation on the genetically active X chromosome, according to Lyon's theory [19]. If the hypothesis of lyonization for this phenotype expressed in fibroblasts is correct, one would also expect to observe an increased Cl^- efflux in one of the two clonal populations which should be present in somatic cells of carriers. The data reported in fig.3 indicate that two types of clones can be distinguished in fibroblasts of DMD carriers. It should be noted, however, that the values of the rate constant observed in control clones, as well as in one of the two clonal populations from DMD carriers (fig.3), are significantly lower than those observed in control fibroblasts (figs 1,2).

This difference among control cells might be explained by aging of cloned fibroblasts. Although the passage number for clones from a control female and from the two DMD carriers was approximately the same, it would still be possible that the differences observed between the two clonal populations present in DMD carriers reflect some sort of heterogeneity in aging rather than a true lyonization phenomenon. A similar explanation has been recently invoked to justify the apparent lyonization of the limited proliferative potential of cultured myoblasts from DMD carriers [20]. A critical test to discriminate whether the increased Cl^- efflux is subject to lyonization is represented by experiments, now under way, with clones of fibroblasts from DMD carriers who are also heterozygotes for another X-linked phenotype, like a glucose-6-phosphate dehydrogenase deficiency variant.

ACKNOWLEDGEMENTS

This work was supported by grants from the Progetto Finalizzato 'Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie', CNR, Rome, and from a grant from the Muscular Dystrophy Association.

REFERENCES

[1] Hunter, S. (1980) Br. Med. Bull. 36, 133–134.
[2] Emery, A.E.H. and Gosden, C. (1974) J. Med. Genet. 11, 76–79.
[3] Pilshker, G.A. and Appel, S.H. (1980) Muscle Nerve 3, 70–81.
[4] Horenstein, A.L. and Emergy, A.E.H. (1980) Neurology 30, 1330–1332.
[5] Rounds, P.S., Jepson, A.B., McAllister, D.J. and Howland, J.L. (1980) Biochem. Biophys. Res. Commun. 97, 1384–1390.
[6] Fingerman, E., Campisi, J. and Pardee, A.B. (1984) Proc. Natl. Acad. Sci. USA 81, 7617–7621.
[7] Rodemann, H.P. and Bayreuther, K. (1984) Proc. Natl. Acad. Sci. USA 81, 5130–5134.
[8] Rodemann, H.P. and Bayreuther, K. (1986) Proc. Natl. Acad. Sci. USA 83, 2086–2090.
[9] Rowland, L.P. (1984) Ital. J. Neurol. Sci. 3, 13–28.
[10] Davis, M.H., Gelman, B.B. and Gruenstein, E. (1982) Neurology 32, 486–491.
[11] Pato, C.N., Davis, M.H., Doughty, M.J., Bryant, S.H. and Gruenstein, E. (1983) Proc. Natl. Acad. Sci. USA 80, 4732–4736.
[12] Rugolo, M., Romeo, G. and Lenaz, G. (1986) Biochem. Biophys. Res. Commun. 134, 233–239.
[13] Bevington, P.R. (1969) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York.
[14] Kunkel, L.M. and co-authors (1986) Nature 322, 73–77.
[15] Monaco, A.P., Neve, R.L., Colletti-Feener, C., Bertelson, C., Kurnit, D.M. and Kunkel, L.M. (1986) Nature, 323, 646–650.
[16] Ferlini, A., Roncuzzi, L., Nobile, C. and Romeo, G. (1986) Am. J. Hum. Genet., submitted.
[17] Boswinkel, E., Walker, A., Hodgson, S., Benham, F., Bobrow, M., Davies, K., Dubmitr, V. and Granata, C. (1985) Cytogenet. Cell Genet. HGM8, 40, 586.
[18] Romeo, G., Sangiorgi, S., Mochi, M., Tessarolo, D., Liguori, M., Marcelli, M. and Rocchi, M. (1986) Hum. Genet., submitted.
[19] Lyon, M.F. (1961) Nature 190, 372–373.
[20] Webster, C., Filippi, G., Rinaldi, A., Mastropaolo, C., Tondi, M., Siniscalco, M. and Blau, H.M. (1986) Hum. Genet. 74, 74–80.