*Biochimica et Biophysica Acta*, 730 (1983) 71–75
Elsevier Biomedical Press



BBA 71605

# SELECTIVE LOSS OF A PLASMA MEMBRANE PROTEIN ASSOCIATED WITH CONTRACTION OF SKELETAL MUSCLE

HIROMICHI T. NARAHARA and JOHN D. GREEN

*Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201 (U.S.A.)*

(Received September 6th, 1982)

*Key words: Membrane protein; Plasma membrane; Contraction; $Ca^{2+}$; (Frog sartorius muscle)*

**Muscle proteins were labeled by incubating isolated frog sartorius muscles with [$^3$H]- or [$^{14}$C]phenylalanine. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of plasma membrane fractions revealed a major protein band with an apparent molecular weight of approx. 96000. Radioactivity in this band showed a clearly delineated decrease, relative to other bands, when previously labeled muscles were induced to contract either by electrical stimulation or by increasing the influx of $Ca^{2+}$ from the incubation medium. It is postulated that a $Ca^{2+}$-activated neutral protease may account for this decrease in labeled membrane protein.**

## Introduction

Isolated frog sartorius muscles that have been stimulated to contract in vitro exhibit a marked increase in permeability to 3-*O*-methyl-D-glucose (but not to mannitol) [1]. This change in permeability could be initiated by a small, transient increase in the concentration of $Ca^{2+}$ in the myoplasm; it is mimicked when contractures are caused by augmenting the entry of $Ca^{2+}$ into muscle cells during a brief exposure to $K^+$-Ringer's solution, in which $Na^+$ has been replaced by $K^+$ [2]. Under these conditions, raising the concentration of $Ca^{2+}$ in the medium from 0.2 to 10 mM increases its rate of influx and produces a graded increase in permeability to sugar.

The increase in permeability is remarkably persistent, lasting unabated for approx. 5 h at 19°C before it begins to subside [1]. Complete reversal takes approx. 20 to 24 h. Protein synthesis may be required for this slow recovery, since Garthwaite and Holloszy [3] have shown that cycloheximide can prevent the fall in permeability over a 24-h period. A persistent increase in permeability to sugar appears to be a general, physiological response of skeletal muscles to exercise; Ivy and Holloszy [4] have observed a prolonged increase in permeability to glucose in the hindlimbs of exercised rats.

Nothing is yet known about the manner in which events associated with muscle contraction interact with the plasma membrane to alter its permeability to sugar. In the present work we have examined the effects of stimulation of contraction on radioactively labeled proteins of the plasma membrane of frog muscle cells.

## Experimental procedures

*Chemicals.* [4-$^3$H]Phenylalanine, 23.5 Ci/mmol, and a mixture of [$^{14}$C]methylated protein molecular weight calibration standards (derivatives of myosin, $M_r$ 200000; phosphorylase *b*, 92500; bovine serum albumin, 69000; ovalbumin, 46000; carbonic anhydrase, 30000; and lysozyme, 14300) for gels were obtained from Amersham Corp. L-

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

0005-2736/83/0000–0000/$03.00 

[U-$^{14}$C]Phenylalanine, 0.5 Ci/mmol, was from New England Nuclear Corp. Crystalline bovine insulin was kindly donated by Dr. Walter N. Shaw of Lilly Research Laboratories. SDS from Matheson Coleman and Bell was recrystallized from ethanol.

*Radioactive labeling of muscle proteins.* Female *Rana pipiens*, approx. 8 cm long, from a dealer in Wisconsin were kept at room temperature and fed mealworms. Experiments were designed to allow for the slow rate of incorporation of amino acid into total protein of frog muscle. Then changes in labeled membrane proteins were examined after a brief period of muscle contraction. Rannels, McKee and Morgan [5] have recommended the use of labeled phenylalanine for measurements of protein metabolism in muscle because this amino acid is neither synthesized nor converted to other compounds in that tissue. In the present studies isolated sartorius muscles removed from pithed frogs were incubated in frog Ringer's bicarbonate solution [2] that contained 3 to 4 $\mu$Ci of carrier-free phenylalanine labeled with either $^{3}$H or $^{14}$C, plus (per ml) 0.24 $\mu$g (6000 $\mu$units) of insulin, 2 mg of bovine plasma albumin, 2 units of penicillin, and 0.1 mg of streptomycin. For each experiment 20 to 30 muscles were incubated at 19°C in a Dubnoff shaker [6,7] in 2 to 3 ml of medium. The muscles were transferred to fresh medium at 24-h intervals during 3 days of incubation. Normal permeability to sugar is well preserved under these conditions [7], and radioactive phenylalanine is taken up from the medium and incorporated into total muscle protein at a rate that remains essentially constant throughout the period of incubation.

*Electrical stimulation of contraction.* Labeled sartorius muscles were laid on damp filter paper without stretching and were kept moistened with Ringer's solution while 10 5-s 20-V tetanic shocks were applied with platinum electrodes at 1-min intervals with a Simpson square-wave stimulator [1]. The muscles were then incubated in Ringer's solution at 19°C for 30 min; this interval had been shown previously to be required for permeability to attain its full increase after brief, intense stimulation [1].

*Chemical stimulation of contraction.* Chemical rather than electrical stimulation of contraction was used for other muscles. Labeled sartorius muscles were equilibrated for 20 min at 19°C with Ringer's solution that contained $CaCl_2$ at a concentration of 10 mM instead of the usual 1.3 mM; phosphate ions were omitted from these media of high $Ca^{2+}$ concentration. Contracture was produced by transferring the muscles for 6 min to $K^+$-Ringer's solution, in which $CaCl_2$ was 10 mM and all $Na^+$ was replaced by $K^+$ [2]. The muscles were then incubated for 30 min in regular frog Ringer's solution at 19°C to permit the full effect on membrane permeability to occur.

*Fractionation of muscles.* An important concern, especially for basal muscles, was to minimize the contraction that occurs during mincing. For this purpose the pooled sartorius muscles were equilibrated for 20 min with 5 ml of an ice-cold relaxing solution of 80 mM $MgCl_2$ [8] which also contained 1 mM EGTA, 2 mM phenylalanine, and 10 mM Tris-HCl, pH 8. Subsequent steps were carried out at 0 to 4°C. The muscles were chopped finely with a razor blade and radioactive, free phenylalanine was washed out by suspending the chopped tissue in 10 ml of a 0.19 M sucrose solution containing 0.1 mM $MgCl_2$, 0.2 mM EGTA, 2 mM phenylalanine, and 4 mM Tris-HCl. After centrifugation for 5 min at 800 $\times$ $g$ the pellet was resuspended in the same volume of fresh solution and centrifuged again.

The chopped sartorius muscles were combined with fresh nonradioactive frog thigh muscle that had been equilibrated with the $MgCl_2$ relaxing solution and chopped, to give a total muscle weight of 15 g. Fractionation of membranes was performed by a method reported previously [9], except that the modified isotonic sucrose solution described above was used for the first two Polytron homogenization steps, and $CaCl_2$ was not added to any of the fractionation media. Though the homogenizations were at very low speed, they were conducted in a fume hood because of the radioactivity.

*SDS-polyacrylamide gel electrophoresis.* Electrophoresis was carried out on 20-cm cylindrical gels, 0.6 cm in diameter. 5.3% gels made according to Fairbanks et al. [10] were used for most of the experiments, but 7.5% gels made by the method of Laemmli [11] were also employed in order to test the results in another system. Membrane fractions of two different types, labeled with different iso-

topes, were combined for each electrophoretic analysis. Approximately equal amounts of each isotope, in the range of 2.5 to 5.0 nCi, were incorporated into each sample. The samples contained 0.2 to 0.45 mg of protein, determined by the method of Lowry et al. [12].

The samples were heated at 100°C for 4 min in the presence of 1% SDS and 2.5% 2-mercaptoethanol to solubilize and disaggregate proteins, and were then diluted further in 0.25 M sucrose and appropriate gel buffer that contained 4 $\mu$g of Bromophenol blue tracking dye. Electrophoresis was performed at room temperature for 18 h at 1.9 mA in the presence of 0.1% SDS. Gels were sliced and crushed at 1- or 2-mm intervals on an automated device made by Gilson Medical Electronics, Inc. Slices were solubilized by the method of Kiehn and Holland [13] and counted on a 2-channel liquid scintillation spectrometer. Background was 12 to 16 cpm, and counting was continued until approx. 2000 to 3000 counts above background had been accumulated for gel slices from protein peaks of major interest. The counting characteristics of $^{14}C$ and $^{3}H$ were determined by preparing and slicing reference gels that contained membranes labeled with only a single isotope. The relative mobility of each protein band in the experimental gels, using the tracking dye as reference, was compared with that of [$^{14}C$]methylated protein standards that were run on separate gels in the presence of $^{3}H$-labeled plasma membrane fractions. Nonradioactive membrane samples and protein calibration standards were also run on 5.3% gels made by the method of Fairbanks et al. [10] and stained with Coomassie brilliant blue.

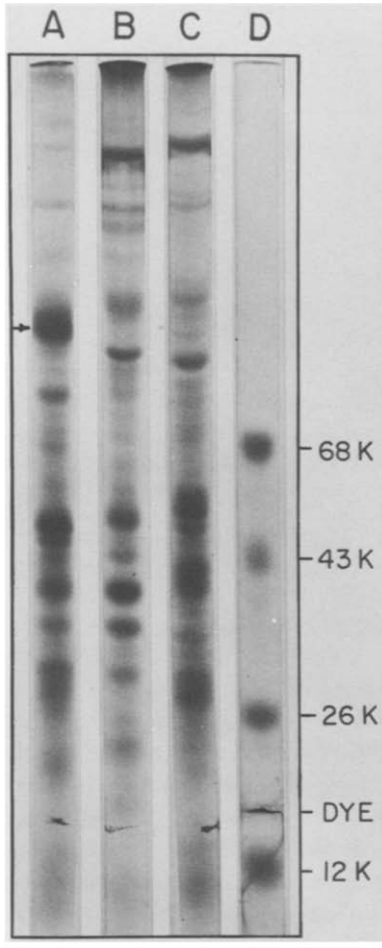


Fig. 1. Coomassie blue-stained Fairbanks-type gels of nonradioactive basal muscle membrane fractions: (A) Plasma membrane, (B) sarcoplasmic reticulum, and (C) mitochondrial fraction. Protein standards in gel D were (top to bottom) bovine serum albumin, ovalbumin, chymotrypsinogen A, and cytochrome *c*. An arrow indicates the position of a major protein band in (A).

## Results

Gel electrophoresis of plasma membrane fractions of nonradioactive muscles (Fig. 1) as well as radioactively labeled ones (Figs. 2–5) revealed a major protein band with an apparent molecular weight of approx. 96 000 ± 3000 (mean and S.E. of eight determinations). The band at this position was more prominent in the plasma membrane fraction than in the sarcoplasmic reticulum and mitochondrial fractions of basal, nonradioactive muscles (Fig. 1). In agreement with this observation, when a $^{3}H$-labeled plasma membrane fraction was electrophoresed together with a $^{14}C$-labeled sarcoplasmic reticulum fraction, plasma membrane made the predominat contribution to the total radioactivity of slices in the region of this band (Fig. 2). A similar result was obtained when differentially labeled plasma membrane and mitochondrial fractions were compared (not shown). Therefore it is possible to examine changes in the large plasma membrane peak without significant interference from cross contamination by the other membrane fractions.

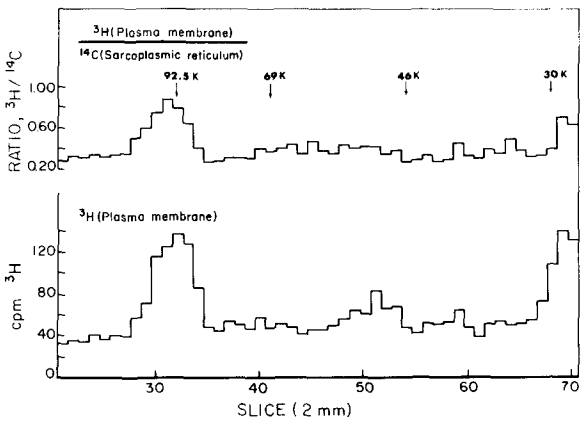


Fig. 2. Comparison of the plasma membrane fraction from $^3$H-labeled muscles with the sarcoplasmic reticulum fraction from $^{14}$C-labeled muscles on a Fairbanks-type gel. The lower panel shows a major peak of radioactivity in the plasma membrane in the region of slice 32. The upper panel shows the relative contribution of plasma membrane to total radioactivity of slices in this region.

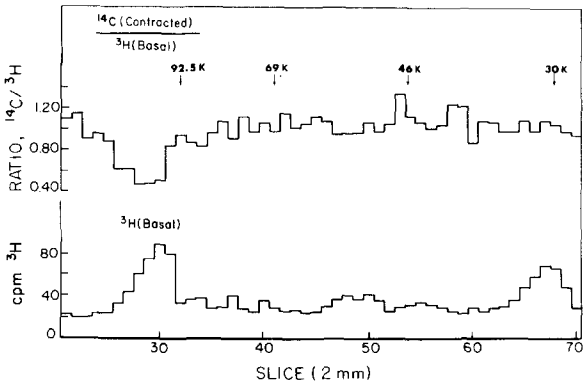


Fig. 4. Radioactivity of gel slices containing plasma membrane fractions from $^3$H-labeled basal muscles and $^{14}$C-labeled, chemically stimulated muscles on a Fairbanks-type gel. Contraction had been induced by increasing the influx of $Ca^{2+}$ from the medium.

Plasma membranes of muscles that had been stimulated electrically showed a significant decrease of radioactive label in the region of the protein band of molecular weight approx. 96 000 (Fig. 3). When the influx of $Ca^{2+}$ into muscle cells was augmented, a similar decrease was seen (Fig. 4). These analyses were done on gels made according to the procedure of Fairbanks et al. A Laemmli-type gel, which allowed compressing of a larger sample into thinner slices, clearly confirmed the change in protein pattern caused by an influx of $Ca^{2+}$ (Fig. 5).

Muscle contraction did not appear to cause extensive aggregation of plasma membrane proteins; no increase of radioactivity was found at the top of gels to complement the decrease in the region of the major peak. Minor increases of radioactivity were seen in several bands, including those corresponding to molecular weights of 88 000, 61 000, and 47 000 (Figs. 3–5), but definitive evaluation of these small changes must await further verification and analysis.

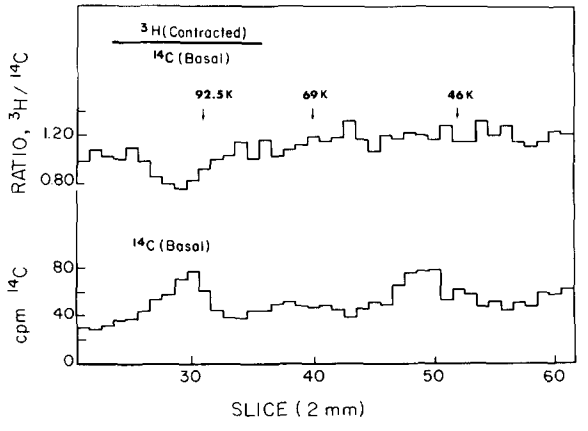


Fig. 3. Gel pattern for a mixture of plasma membrane from $^{14}$C-labeled basal muscles plus plasma membrane from $^3$H-labeled, electrically stimulated muscles on a Fairbanks-type gel. The upper panel shows that there is a decreased contribution of stimulated muscle to the total radioactivity of the major peak in the region of slice 30.

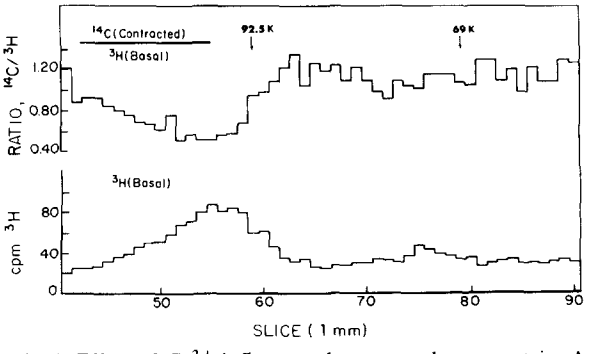


Fig. 5. Effect of $Ca^{2+}$ influx on plasma membrane protein. A membrane sample similar to that used for Fig. 4 but of larger volume was run on a Laemmli-type gel.

## Discussion

The selective loss of labeled protein from the plasma membrane of muscles that have been induced to contract could reflect an increased rate of degradation. Dissociation of loosely bound protein seems less likely, but has not been ruled out. The effect appears to be mediated by a transient increase in the concentration of $Ca^{2+}$ in the myoplasm since it can be reproduced under conditions [2] that increase the influx of $Ca^{2+}$ into the cells. The present experiments were not designed to detect short-term changes in rates of protein synthesis, but Lewis et al. [14] have reported that an increase in the intracellular concentration of $Ca^{2+}$ in rat skeletal muscle in the presence of ionophore A23187 inhibits the overall rate of protein synthesis in addition to enhancing protein breakdown.

$Ca^{2+}$-activated neutral proteases have been implicated in the digestion of high-molecular-weight proteins of human erythrocyte membranes [15] and rat brain synaptic membranes [16]. Huston and Krebs [17] found a neutral protease in rabbit skeletal muscle that acts on phosphorylase kinase when activated by millimolar concentrations of $Ca^{2+}$. More recently one form of this protease from muscle has been shown to be activated by physiologic (micromolar) concentrations of $Ca^{2+}$ [18–20], that is, concentrations that are attained during muscle contraction. Thus, it would be of interest to see whether or not a $Ca^{2+}$-activated neutral protease is responsible for the changes in plasma membrane protein seen after muscle contraction.

Additional studies will be needed in order to ascertain what relationship, if any, there is between the changes in labeled membrane protein observed in the present study and the enhancement of permeability to sugar that was found previously after muscle contraction [1,2]. The possibility of a causal relationship is worth considering because limited tryptic digestion of proteins on the outer surface of muscles [21–23] or adipose tissue cells [24] can enhance permeability to sugar. Moreover, the findings of Garthwaite and Holloszy [3] suggest that protein synthesis is required in order to reverse the changes in permeability to sugar that occur after contraction of frog sartorius muscles.

## Acknowledgments

We thank Drs. Nando Chatterjee, Robert MacColl, and Lawrence F. Sturman for helpful advice on gel electrophoretic techniques.

## References

1 Holloszy, J.O. and Narahara, H.T. (1965) J. Biol. Chem. 240, 3493–3500
2 Holloszy, J.O. and Narahara, H.T. (1967) J. Gen. Physiol. 50, 551–562
3 Garthwaite, S.M. and Holloszy, J.O. (1982) J. Biol. Chem. 257, 5008–5012
4 Ivy, J.L. and Holloszy, J.O. (1981) Am. J. Physiol. 241, C200–C203
5 Rannels, D.E., McKee, E.E. and Morgan, H.E. (1977) in Biochemical Actions of Hormones (Litwack, G., ed.), pp. 135–195, Academic Press, New York
6 Narahara, H.T. and Ozand, P. (1963) J. Biol. Chem. 238, 40–49
7 Narahara, H.T. and Holloszy, J.O. (1974) J. Biol. Chem. 249, 5435–5443
8 Ashkenaz, E.W. (1938) J. Cell. Comp. Phsyiol. 11, 163–174
9 Narahara, H.T., Vogrin, V.G., Green, J.D., Kent, R.A. and Gould, M.K. (1979) Biochim. Biophys. Acta 552, 247–261
10 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
11 Laemmli, U.K. (1970) Nature (London) 227, 680–685
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
13 Kiehn, E.D. and Holland, J.J. (1970) J. Virol. 5, 358–367
14 Lewis, E.M., Anderson, P. and Goldspink, D.F. (1982) Biochem. J. 204, 257–264
15 Triplett, R.B., Wingate, J.M. and Carraway, K. L. (1972) Biochem. Biophys. Res. Commun. 49, 1014–1020
16 Baudry, M., Bundman, M.C., Smith, E.K. and Lynch, G.S. (1981) Science 212, 937–938
17 Huston, R.V. and Krebs, E.G. (1968) Biochemistry 7, 2116–2122
18 Mellgren, R.L. (1980) FEBS Lett. 109, 129–133
19 Dayton, W.R., Schollmeyer, J.V., Lepley, R.A. and Cortés, L.R. (1981) Biochim. Biophys. Acta 659, 48–61
20 Kubota, S., Suzuki, K. and Imahori, K. (1981) Biochem. Biophys. Res. Commun. 100, 1189–1194
21 Rieser, P. and Rieser, C.H. (1964) Proc. Soc. Exp. Biol. Med. 116, 669–671
22 Weis, L.S. and Narahara, H.T. (1969) J. Biol. Chem. 244, 3084–3091
23 Kendrick, E.G., Takano, T. and Narahara, H.T. (1972) Fed. Proc. 31, 873
24 Kono, T. (1969) J. Biol. Chem. 244, 5777–5784