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Calcium-binding protein, parvalbumin, is reduced in mutant mammalian muscle with abnormal contractile properties

(mouse mutant/muscle contraction/two-dimensional electrophoresis/high-performance liquid chromatography/immunohistochemistry)

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ABSTRACT **To elucidate the biochemical basis of hereditary muscle diseases in an experimental mammal, we performed polypeptide analyses on skeletal muscles of neuromuscular mutants of the mouse. In one of these, "arrested development of righting response" (*adr*), the concentration of the soluble Ca^{2+}-binding protein parvalbumin was drastically reduced in comparison to wild type. This reduction was not an unspecific consequence of muscle disease, as it was not observed in two other neuromuscular mouse mutants, "wobbler" (*wr*) and "motor endplate disease" (*med* or *med*^{jo}). Isometric twitches of *adr* muscle had only slightly prolonged contraction and half-relaxation times, yet long-lasting after-contractions were observed upon repeated (20–100 Hz) direct stimulation. Thus, parvalbumin may be mainly involved in the relaxation after tetanic contraction of fast-twitch fibers.**

Currently under investigation are an increasing number of mutant mice that are affected in the development or maintenance of the neuromuscular system (1, 2). Apart from their possible significance as models for hereditary muscle diseases in man, neuromuscular mutants of the mouse may provide insights into the genetic control of the mammalian neuromuscular system since genetics, experimental embryology, and comparative biochemistry can be performed in parallel. We have started the analysis of polypeptide patterns of skeletal muscle from three such mutants (3). Here we report on a striking reduction of the soluble calcium-binding protein, parvalbumin, in the mutant "arrested development of righting response" (*adr*) (4, 5), a reduction that was not observed in two other mutants affected in the neuromuscular system. The parvalbumin content of skeletal muscles has been related to their fiber type composition (6): In the mouse, fast-twitch glycolytic [IIglyc, low succinate dehydrogenase (EC 1.3.99.1)] fibers contain high concentrations of parvalbumin (about 5 mg/g; fresh weight); whereas the parvalbumin content of fast-twitch oxidative (IIox, high succinate dehydrogenase) fibers is less than 1% of that value. Type I (slow) fibers are virtually lacking parvalbumin (ref. 7; for fiber types in the mouse see ref. 8). Since parvalbumin might be involved in fast fiber relaxation (7, 9, 10) or tetanic contraction (11–13), comparative contraction measurements were performed on mutant and wild-type muscles. In contrast to single twitches, upon repeated stimulation the behavior of *adr* muscle was found to be grossly abnormal. The correlation of abnormal muscle function and diminished parvalbumin content in the *adr* mutant should be useful to elucidate the physiological role of parvalbumin.

MATERIALS AND METHODS

A founder stock of mice carrying the recessive autosomal mutation *adr* on an A2G inbred background (4, 5) had been obtained in 1982 through the kindness of R. and D. Watts, Guy's Hospital, London. Mice affected by other neuromuscular mutations (1), motor endplate disease (*med*, from A. Searle, Harwell, England, and its mild allele *med*^{jo}, from F. Rieger, Institut National de la Santé et de la Recherche Medicale U 153, Paris, France) and wobbler (*wr*, from E. Trenkner, Harvard Medical School, Boston, MA) (14) were investigated in parallel.

Homozygous *adr/adr* animals were recognized in litters of strain A2G *adr*/+ matings about 2 weeks after birth. They were unable to right themselves immediately when turned on their backs and, in their attempts to do so, developed a characteristic stiffness of hindlimbs (4). Affected animals and sex-matched nonaffected (+/?) littermates or matched +/? animals of the same colony were sacrificed. Individual muscles were removed, frozen in isopentane/liquid nitrogen, and stored at −70°C for biochemical analysis. Some muscles were fixed in Bouin's solution at 4°C for immunohistochemistry of parvalbumin (6).

For polypeptide analysis, muscle samples were subjected to different extraction procedures: "total extracts" were prepared either with high salt/urea (15) or with hot sodium dodecyl sulfate ($NaDodSO_4$) solutions. Separation into "soluble" and "insoluble" fractions was achieved by glycerol treatment (16) or by extraction with water/EDTA (7). Polypeptides were separated by two-dimensional gel electrophoresis according to O'Farrell (17). LKB ampholines were used at concentrations of 1.8% pH range 4–6, 0.7% pH range 5–7, 0.5% pH range 3.5–10. The second dimension was $NaDodSO_4$ electrophoresis on exponential (8–20%, over 20 cm) polyacrylamide gels (18). Gels were fixed and stained according to Fairbanks *et al.* (19).

For radio-immunoblotting, proteins in $NaDodSO_4$ extracts of minced muscle were separated on a 10% polyacrylamide gel slab by one-dimensional electrophoresis and then transferred by blotting to a nitrocellulose sheet (20). Unspecific binding capacity of the sheet was saturated with 4% bovine serum albumin in phosphate-buffered saline. The sheet was then incubated with antibody to rat muscle parvalbumin (ref. 6; diluted 1:1000 in bovine serum albumin/phosphate-buffered saline) followed by the iodinated second antibody (sheep anti-rabbit IgG iodinated by the chloramine-T method, ref. 21, 40 μg/ml, specific radioactivity of 2×10^8 dpm/mg). Kodak X-Omat AR film with intensifying screens (DuPont) was used to localize the radioactive material. X-ray films as well as parallel stained gels were scanned on a Joyce–Loebl Chromoscan 3 to quantitate the bands in comparison to standard dilution series of purified parvalbumin.

Immunohistochemistry of parvalbumin (6) was done on Bouin-fixed 8-μm sections, which had been preabsorbed (to avoid unspecific binding) with 20% horse serum in calcium- and magnesium-free phosphate-buffered saline prior to the application of the first antibody, rabbit antiserum to rat par-

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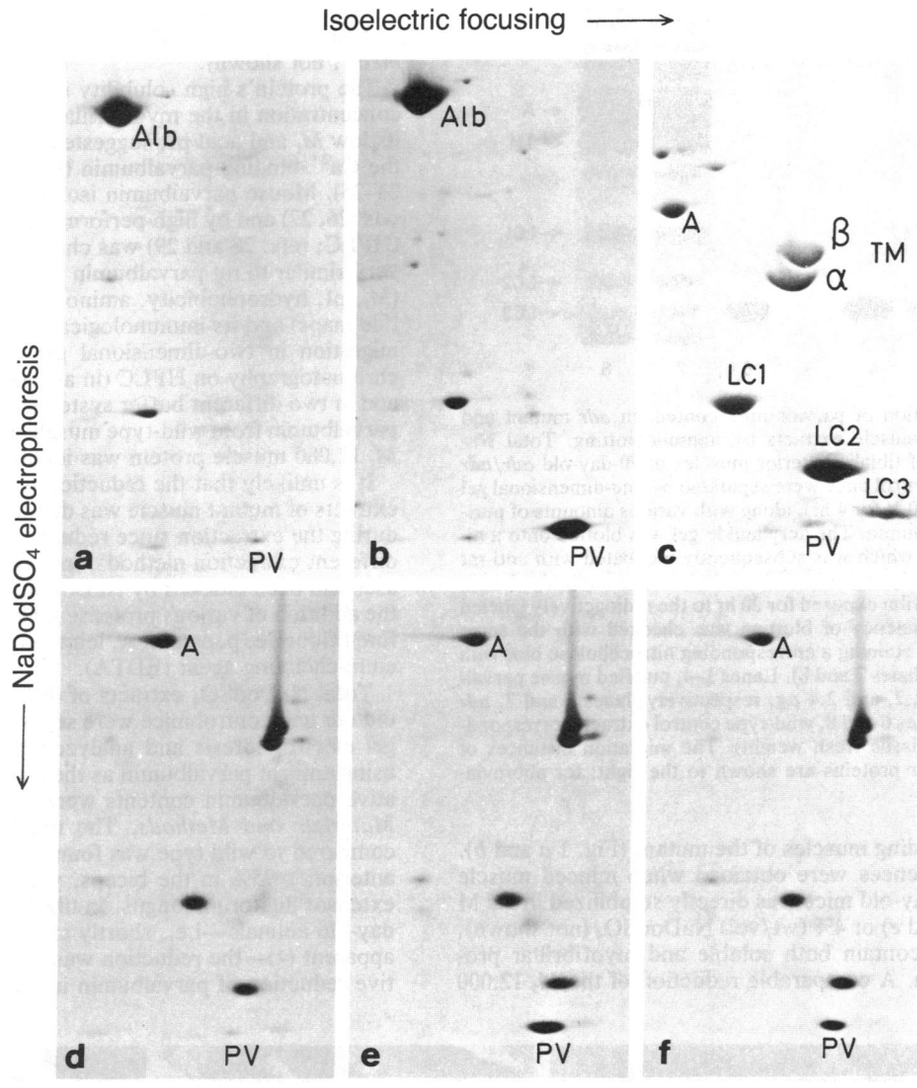

FIG. 1. Polypeptide analysis of *adr* mutant and wild-type muscle extracts by two-dimensional polyacrylamide gel electrophoresis. Isoelectric focusing was from left (basic) to right (acidic). $NaDodSO_4$ electrophoresis was from top to bottom. Fixed gel slabs were stained with Coomassie blue. Samples *a* and *b* in the second dimension were run together on a single gel slab, as were samples *d*, *e*, and *f*. Identified proteins are marked on the gels and characterized by their relative molecular masses and isoelectric points (M_r/pI). Only part of the gel area is shown in each case. (*a* and *b*) Soluble fraction of glycerol-extracted biceps of 80-day-old mice; Alb, albumin (67,000/6.3); PV, parvalbumin (12,000/5.0). (*a*) Mutant *adr/adr*, showing striking reduction in parvalbumin. (*b*) Wild-type +/? littermate. (*c*) Polypeptide pattern of wild-type myofibrils (insoluble fraction of glycerol extract), showing A, actin (43,000/5.8); TM, tropomyosins (36,000/4.8 and 34,000/4.9) and the three fast isoforms of myosin light chains (LC1, 25,000/5.3; LC2, 18,000/4.9; and LC3, 16,000/4.6). There is only little residual protein at the position of parvalbumin. (*d–f*) Total urea extracts of tibialis anterior muscles of 70-day-old mutant and wild-type mice. (*d*) *adr/adr*; (*e*) wild-type littermate, +/?; (*f*) wobbler mutant, *wr/wr*. Parvalbumin is strongly reduced in the *adr* but only slightly in the *wr* mutant.

valbumin (6), diluted 1:3000 in 20% horse serum, for 45 min at 37°C. The second antibody was horseradish peroxidase-conjugated swine antiserum to rabbit gamma globulin (Boehringer Ingelheim, FRG) (diluted 1:20). After 60-min incubation at 20°C enzyme activity was developed with 4-chloro-1-naphthol at 0.5 mg/ml (22) and 0.025% H_2O_2 at pH 7.35 for 60 min at 20°C.

For quantitative HPLC of parvalbumin (7), muscles were extracted in 10 μl of 4 mM EDTA, pH 7, containing protease inhibitors per mg of tissue. The heat-treated (30 min, 85°C) extracts from 15 mg of muscle, as well as purified rat parvalbumin (10 μg) were separated on an Aquapore RP-300 column (4.6 mm × 3 cm; Brownlee Labs, Santa Clara, CA). As a control only buffer was injected. The following buffer system was used: buffer A (0.1% H_3PO_4/10 mM $NaClO_4$, pH 2.2) and buffer B [buffer A with 60% (vol/vol) acetonitrile]. A gradient from 0% to 100% buffer B was applied to the column and the absorbance at 220 nm of the eluted material was used to quantitate the proteins.

Isometric contraction measurements were performed on tibialis anterior *in situ* or on isolated extensor digitorum longus muscles under mammalian Ringer solution at 34–35°C. Muscles were prestretched to their original length and stimulated directly with 0.2-ms rectangular pulses with amplitudes 25% above the voltage required for maximum twitch response. The force transducer was connected to Tectronix 5113 and Vuko VKS 22-60 digital storage oscilloscopes.

RESULTS

The most striking difference in polypeptide composition between *adr* and wild-type muscle was found in the soluble fraction. As revealed by two-dimensional gel electrophoresis, a heat-stable protein of M_r 12,000 and pI 5.0 was abundant in the fast-twitch muscles tibialis anterior, gastrocnemius, vastus, and biceps of the wild type but was much reduced

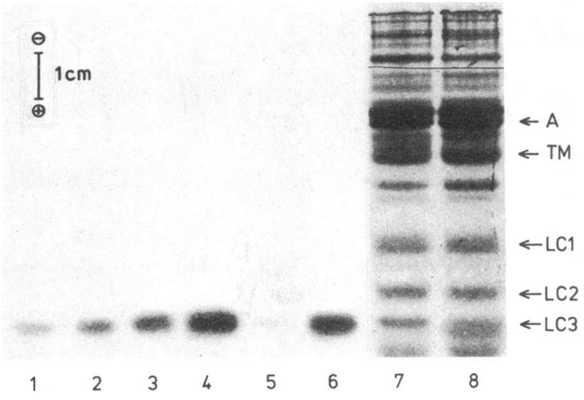

FIG. 2. Estimation of parvalbumin content in *adr* mutant and control wild-type muscle extracts by immunoblotting. Total $NaDodSO_4$ extracts of tibialis anterior muscles of 70-day-old *adr/adr* mutant and +/? control mice were separated by one-dimensional gel electrophoresis (100 V for 4 hr), along with various amounts of purified mouse parvalbumin. The acrylamide gel was blotted onto a nitrocellulose sheet, which was subsequently incubated with anti-rat parvalbumin antibody and then ^{125}I-labeled second antibody. Lanes 1–6 show an x-ray film exposed for 20 hr to the radioactively labeled nitrocellulose. Efficiency of blotting was checked with the same muscle extracts by staining a corresponding nitrocellulose blot with amido black 10 B (lanes 7 and 8). Lanes 1–4, purified mouse parvalbumin, 0.24, 0.6, 1.2, and 2.4 μg, respectively; lanes 5 and 7, *adr* mutant extract; lanes 6 and 8, wild-type control extract (corresponding to 2.5 mg of tissue fresh weight). The migration distances of known myofibrillar proteins are shown to the right; for abbreviations see Fig. 1.

in the corresponding muscles of the mutant (Fig. 1 *a* and *b*). The same differences were obtained when minced muscle from 26- to 80-day-old mice was directly solubilized in 9.5 M urea (Fig. 1 *d* and *e*) or 4% (wt/vol) $NaDodSO_4$ (not shown), extracts which contain both soluble and myofibrillar proteins in common. A comparable reduction of the M_r 12,000 protein was not found in the two other mutants investigated, wobbler (Fig. 1*f*) and motor endplate disease (*med* and med^{jo}, not shown).

The protein's high solubility in low salt extracts, and low concentration in the myofibrillar pellet (Fig. 1*c*), as well as its low M_r and acid pI, suggested that it may be identical to the Ca^{2+}-binding parvalbumin (for recent reviews see refs. 23–25). Mouse parvalbumin isolated by conventional methods (26, 27) and by high-performance liquid chromatography (HPLC; refs. 28 and 29) was characterized and shown to be very similar to rat parvalbumin in its biochemical properties (M_r, pI, hydrophobicity, amino acid composition, and peptide maps) and its immunological properties (cf. Fig. 2). Comigration in two-dimensional gel electrophoresis and cochromatography on HPLC (in absence or presence of Ca^{2+}, and in two different buffer systems) with the isolated mouse parvalbumin from wild-type muscle extracts showed that the M_r 12,000 muscle protein was identical to parvalbumin.

It is unlikely that the reduction in parvalbumin content in extracts of mutant muscle was due to the action of proteases during the extraction since reduced levels were obtained by different extraction methods, in which the activity of proteases had been blocked by $NaDodSO_4$ (cf. Fig. 2), heating, or the addition of various protease inhibitors (phenylmethylsulfonyl fluoride, pepstatin A, leupeptin hemisulfate) and a calcium-chelating agent (EDTA).

Total $NaDodSO_4$ extracts of different muscles of 70-day-old *adr* and control mice were separated by one-dimensional gel electrophoresis and analyzed by radio-immunoblotting using anti-rat parvalbumin as the first antibody (Fig. 2). Relative parvalbumin contents were estimated as described in *Materials and Methods*. The reduction in *adr* muscle as compared to wild type was found to be >97% in the tibialis anterior, >95% in the biceps, yet only around 80% in the extensor digitorum longus. In tibialis anterior muscles of 17-day-old animals—i.e., shortly after the disease had become apparent (4)—the reduction was about 85%. Thus, the relative reduction of parvalbumin in the *adr* mutant progresses

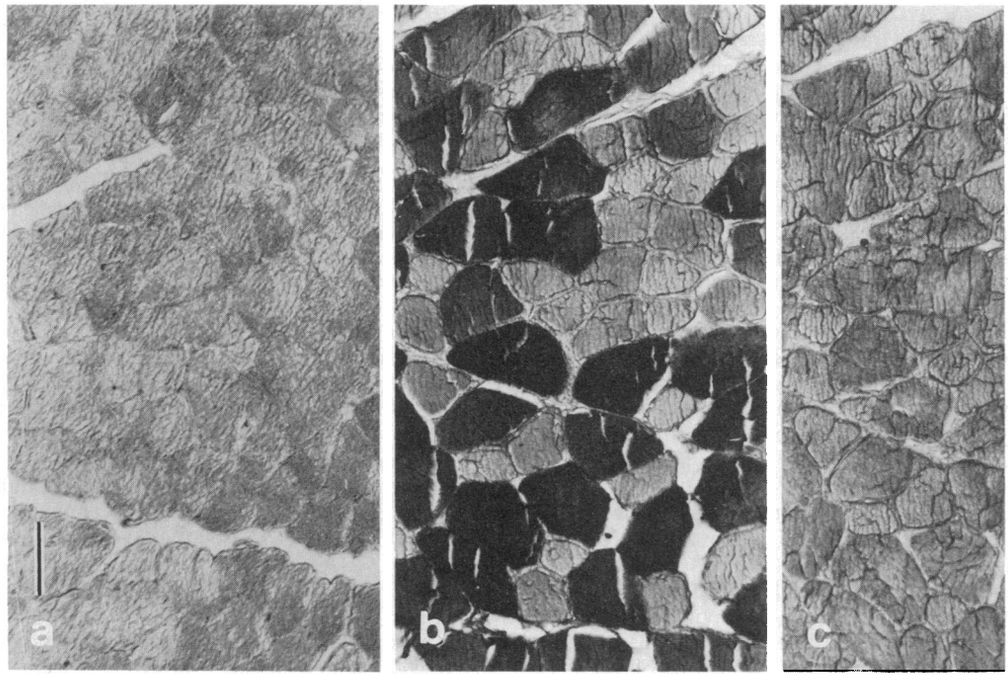

FIG. 3. Immunohistochemical demonstration of parvalbumin in *adr* mutant and wild-type muscle. Tibialis anterior muscles of a 70-day-old *adr/adr* mouse and its +/? littermate were fixed in Bouin's solution, processed for paraffin histology, sectioned (8 μm thick), and stained for parvalbumin by using a horseradish peroxidase-conjugated second antibody. (*a*) Mutant *adr/adr* and (*b*) wild type +/?, both stained with anti-parvalbumin. (*c*) Wild type +/?, stained with preimmune serum (the same result was obtained by omitting the first antibody). (Bar, 50 μm.)

during postnatal development.

Different parvalbumin contents were shown *in situ* by immunohistochemistry. To avoid loss of the soluble antigen, small pieces of muscle were fixed in Bouin's solution and paraffin sections were stained with antibody against rat parvalbumin and horseradish peroxidase-labeled second antibody. While the known "checkerboard" pattern of staining typical of a fast-twitch muscle—i.e., most intense in the large fast glycolytic (type IIglyc) fibers (6)—was observed in wild-type sections, the reaction of mutant sections was hardly above background as defined by using preimmune serum as a first antibody (Fig. 3).

Parvalbumin was determined quantitatively by HPLC (7) previously applied for its isolation (28, 29). Tibialis anterior muscle of the wild type showed a high concentration of parvalbumin, averaging 4.8 mg/g of wet weight, in agreement with its fast contraction and relaxation (7, 30) and predominantly fast fiber-type composition (type I 1%, type II 99%) (7, 30). The parvalbumin concentration in the tibialis anterior of the *adr* mutant was decreased to 0.1 mg/g, which is in accordance with the estimate based on immunoblots.

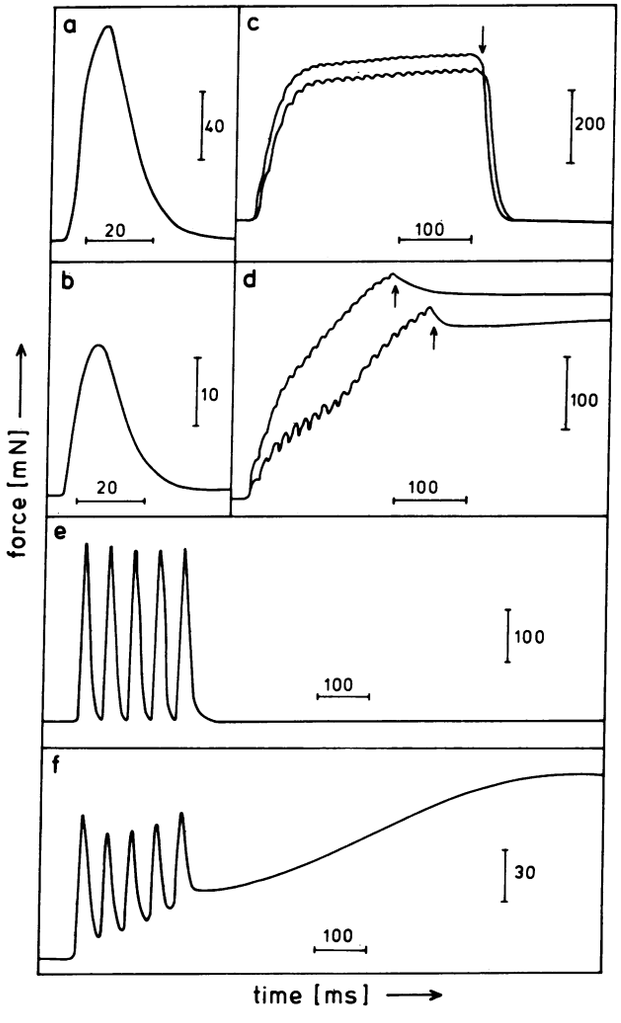

FIG. 4. Isometric contraction of *adr* mutant and wild-type muscles. Tibialis anterior muscles *in situ* were subjected to isometric contraction measurements using direct stimulation. (*a* and *b*) Single twitches. (*c* and *d*) Tetanic contractions, stimulation frequencies 80 Hz (lower traces) and 100 Hz (upper traces). Arrows, termination of stimulus. (*e* and *f*) Repeated stimulation (20 Hz, five stimuli) of curare-treated muscle (10 min after application of 20–50 μl of 10 mM *d*-tubocurarin to the muscle surface, a dose that abolished contraction after indirect stimulation). (*a*, *c*, and *e*) Wild-type controls (A2G +/?). (*b*, *d*, and *f*) Mutants (*adr*/*adr*). (*a*–*d*) Females, (*e* and *f*) males; ages of mutants 58–63 days.

Contraction measurements were performed on mutant and wild-type muscles. Isometric twitches of the *adr* tibialis anterior were like those of fast muscles, with time to peak and relaxation times equal to or slightly longer (up to 1.3-fold in 80-day-old individuals) than those of wild-type control muscles (Fig. 4 *a* and *b*). However, when stimulated repeatedly at frequencies of 20–100 Hz, *adr* muscle developed long-lasting after-contractions (Fig. 4*d*), which were not found in the wild-type controls (Fig. 4*c*).

These after-contractions were observed after acute nerve transection and in isolated extensor digitorum longus muscles (not shown) and were therefore not due to altered feedback control in the spinal cord. Furthermore, denervation 3.5 days prior to contraction measurement (not shown) or a complete block of nerve transmission by curare (Fig. 4 *e* and *f*) did not abolish the after-contraction, which must thus reflect a property of mutant muscle itself. Possible artifacts due to direct stimulation were excluded in experiments in which after-contractions were generated by indirect stimulations *in situ*. No abnormalities upon repeated stimulation were observed in the muscle of another mutant, *wr*, with no drastic reduction of parvalbumin in muscle.

DISCUSSION

We have described a drastic reduction in the calcium-binding protein parvalbumin caused by the *adr* mutation in the mouse. Could this observation be related to altered fiber type populations in the mutant muscle? In the wild-type mouse, all the muscles used in this study are composed nearly exclusively of fast fibers (7, 30, 31). Therefore, the wild-type staining patterns for succinate dehydrogenase reflect the proportions of fast-twitch glycolytic (IIglyc) and fast-twitch oxidative (IIox) fibers. The succinate dehydrogenase pattern regresses as the symptoms develop in the *adr* mouse (5), all fibers gradually acquiring a high-succinate-dehydrogenase phenotype. In the wild type, most of the parvalbumin has been shown to occur in type IIglyc fibers (6, 7), so that parvalbumin reduction could reflect a transformation of IIglyc to IIox fibers. However, fiber type pattern as revealed by ATPase staining (corresponding to the IIA and IIB fast fiber types; cf. ref. 8) persists in the *adr* mutant (ref. 5; E. M. Füchtbauer, personal communication).

Contraction measurements, in agreement with ATPase histochemistry, showed typical fast twitches in *adr* muscle, with time-to-peak and half-relaxation times hardly affected by the mutation. The fast twitching of *adr* muscles at stages at which the parvalbumin content is already strongly reduced would argue against a role of parvalbumin in single contraction relaxation cycles. However, the lack of parvalbumin could be the cause of the abnormal after-contractions that we observed in response to repeated stimulation. If this were supported by further experiments it would suggest a physiological role of parvalbumin in tetanic contraction of fast muscle. Alternatively, the after-contractions may be related to some membrane defect that could be another aspect of the *adr* syndrome.

Our results, taken together with the known dependence of parvalbumin content on innervation (7, 32), suggest that the *adr* mutation affects either the neural signal responsible for normal fast fiber development or the muscles' response to it. We did not find a drastic reduction of parvalbumin content in mutants *wr* and *med*, for which histological and electron microscopical evidence favor a primary defect in the motor nerves (1, 2, 4, 33). Definite proof as to which tissue—nerve, muscle, or other (2)—is primarily affected in *adr* mice may be obtained by transplanting mutant muscle to a wild-type host and vice versa (34).

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