Prolonged contraction-relaxation cycle of fast-twitch muscles in parvalbumin knockout mice


1Institute of Histology and General Embryology and "Program in Neuroscience," University of Fribourg, CH-1705 Fribourg, Switzerland; 2Department of Anatomy, University College, London WC1E 6BT; 3Royal Veterinary College, London NW1 0TU, United Kingdom; 4Institute of Toxicology, Faculty of Biology, University of Constance, D-78457 Constance, Germany; and 5Roche Vitamins Division, Hoffmann-LaRoche, 4070 Basel, Switzerland

Schwaller, B., J. Dick, G. Dhoot, S. Carroll, G. Vrbova, P. Nicotera, D. Pette, A. Wyss, H. Bluethmann, W. Hunziker, and M. R. Celio. Prolonged contraction-relaxation cycle of fast-twitch muscles in parvalbumin knockout mice. Am. J. Physiol. 276 (Cell Physiol. 45): C395–C403, 1999.—The calcium-binding protein parvalbumin (PV) occurs at high concentrations in fast-contracting vertebrate muscle fibers. Its putative role in facilitating the rapid relaxation of mammalian fast-twitch muscle fibers by acting as a temporary buffer for Ca²⁺ is still controversial. We generated knockout mice for PV (PV⁻⁻) and compared the Ca²⁺ transients and the dynamics of contraction of their muscles with those from heterozygous (PV⁺⁻) and wild-type (WT) mice. In the muscles of PV-deficient mice, the decay of intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) after 20-ms stimulation was slower compared with WT mice and led to a prolongation of the time required to attain peak twitch tension and to an extension of the half-relaxation time. The integral [Ca²⁺]ᵢ in muscle fibers of PV⁻⁻ mice was higher and consequently the force generated during a single twitch was ~40% greater than in PV⁺⁻ and WT animals. Acceleration of the contraction-relaxation cycle of fast-twitch muscle fibers by PV may confer an advantage in the performance of rapid, phasic movements.

EF hand; calcium-binding protein; muscle relaxation; homologous recombination

Parvalbumin (PV) is a low-molecular-weight, high-affinity calcium-binding protein of the EF hand family found in a limited number of vertebrate tissues, the most important being skeletal muscle and specific nerve cells (8, 15). The highest concentrations of PV are found in the fast-contracting and -relaxing skeletal muscles, whereas in the slow-twitch skeletal muscles, cardiac or smooth muscle, there is little or no PV expressed (16). In lower vertebrates, up to five isoforms classified into α- and β-PVs have been detected, whereas in adult rodents a single isoform of PV is expressed (3). A second gene belonging to the β-PVs called oncomodulin (OM) is expressed in the cytotrophoblasts of the fetal placenta, but in adult mice OM expression is completely absent (3). The fact that PV is only found in the cytosol and is not found in intracellular organelles or associated with membranes makes it a strong candidate as a physiological Ca²⁺ buffer as opposed to Ca²⁺ sensors or Ca²⁺ modulators like the ubiquitous calmodulin. Suggestive evidence supporting a buffer role for PV comes from structural studies in which it was demonstrated that rat PV at 25°C does not undergo significant Ca²⁺-dependent conformational changes (31). PV contains two high-affinity Ca²⁺-binding sites (approximate affinity 10⁸ M⁻¹) that are occupied by Mg²⁺ under resting conditions [intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) < 100 nM] (14). On cell activation [Ca²⁺]ᵢ rises to micromolar levels, and the Mg²⁺ ions are displaced by these. The net rate of Ca²⁺ uptake by PV during contraction is determined by the rate of dissociation of Mg²⁺ from this protein. The Ca²⁺ association rate of PV is slower than the rate of Ca²⁺ binding to troponin C (28). Hence, Ca²⁺ binds preferentially to troponin C during muscle activation, and the PV-buffering activity is somewhat delayed. On the basis of these observations, PV was expected to promote the relaxation of fast-contracting skeletal muscles (5, 12). Since PV can deplete isolated myofibrils of Ca²⁺, and isolated sarcoplasmic reticulum can deplete PV of Ca²⁺ (12), the hierarchy of relative Ca²⁺ affinities is a prerequisite for this protein to act as a shuttle for Ca²⁺ from the contractile machinery to storage sites in the sarcoplasmic reticulum. Simulation studies indicate that the exchange of Ca²⁺ for Mg²⁺ on PV can occur with sufficient rapidity for it to contribute to the relaxation of frog skeletal muscles maintained at 0°C (13), a prediction that has been confirmed experimentally (19).

In mammals, the role of PV is less clear. Indeed, it has been reported that this protein has little effect on relaxation rate (4) and Ca²⁺ sequestration in fast-twitch murine muscles (29). Nevertheless, the level of PV in various vertebrates skeletal muscles (17) and the dissociation rates of Ca²⁺ and Mg²⁺ from this protein in the temperature range 0–20°C in the frog (19) correlate fairly well with muscle relaxation speed. Decay of Ca²⁺ transients after electrical stimulation of fast- and slow-twitch skeletal muscle fibers in the rat (6) is proportional to the concentrations of PV in each. Furthermore, induced expression of PV by injection of its cDNA into the slow-twitch soleus muscle of rats leads to an increased relaxation rate (25).

To investigate the physiological role of PV in fast-twitch muscles of mammals, we have created PV-deficient mice by homologous recombination. These
mice develop and breed normally, and no significant alterations in their behavior or physical activity are observed under standard housing conditions. Isometric contraction in the tibialis anterior muscles and Ca\textsuperscript{2+} measurements in isolated fibers of fast-twitch muscles revealed significant differences in the PV–/– mice compared with wild-type (WT) animals. Surprisingly, both the half-relaxation and the rise time to the peak twitch tension were significantly increased, and concomitantly the twitch force was higher than in heterozygous or WT animals.

**MATERIALS AND METHODS**

**Targeting vector construction.** Two genomic clones for PV were isolated from a 129Sv library (Stratagene) using a full-length mouse cDNA probe. From the 5′ end of clone PV1.1, a 3.5-kb fragment (Sal I-Eco47 III), containing part of the promoter and terminating at the Eco47 III site in exon 2, was used as the 5′ fragment. For the 3′-flanking region we used a Hind III-Eco R I fragment from clone PV4.4, which lies entirely within intron 4 and has a size of 8.5 kb. Within the targeting vector pPVknock, the fragment from the Eco47 III site in exon 2 up to the Hind III one in intron 4 was replaced by a neocassette (1.8 kb) containing the neomycin resistance gene driven by the phosphoglycerate kinase (PGK) promoter and including the PGK polyadenylation signal. At the 5′ end of the targeting vector, we inserted the herpes simplex virus-thymidine kinase. A scheme of the targeting vector, pPVknock, is shown in Fig. 1A.

Generation of PV-deficient mice. Embryonic stem cells (E14/Ola), cultivated on feeder cells in the presence of the leukemia inhibitory factor, were electroporated with the targeting vector pPVknock and grown in the presence of G418 and gancyclovir. From resistant clones, genomic DNA was isolated from the ES cells and hybridized with two probes, P1 (1.4-kb Hind III fragment derived from a third genomic clone upstream from PV1.1) and P2 (2.3-kb Eco R I fragment derived from PV4.4), respectively, both located outside the targeting vector (Fig. 1A). The absence of heterologous integration in the recombinant ES clones was tested using a 0.7-kb Eco RI fragment of the neomycin resistance cassette as a probe (not shown). Of the five positive clones (occurring at a frequency of 1/15), two were injected. The highly chimeric mice from the 134 line were bred with C57/Bl6 WT ones, which crossing resulted in germ-line transmission. Genomic DNA was extracted from fresh mouse tail biopsies (2–3 mm in length) using a commercial kit. Ten micrograms of Sca I-digested genomic DNA were probed with the P1 fragment in a Southern blot.

**Immunohistochemistry.** Immunohistochemical analysis of muscle tissue was performed as described by Celio and colleagues.

![Image](attachment:image.png)

**Fig. 1. Disruption of the parvalbumin (PV) gene by homologous recombination.** A: the targeting vector, pPVknock, replaces the PV gene from the Eco47 III site in exon 2 to the Hind III one in intron 4 with the phosphoglycerate kinase (PGK) neocassette. The 2 probes, P1 and P2, were used to check for homologous recombination. B: Southern blot analysis of mouse genomic DNA digested with Sca I. The wild-type (WT) allele probed with P1 gives rise to a 13.7-kb fragment (top arrow), the targeted allele to a smaller one (11.4 kb; bottom arrow). Samples: DNA from heterozygous (+/–) (lane 1), WT (+/+) (lane 2), and homozygous (−/–) (lane 3) mice. C: Western blot analysis of 3 different muscles tested with the antiserum PV-28. No signal is apparent in the −/– mice, whereas a reduced intensity is visible in the heterozygous (+/–) one. HSV-TK, herpes simplex virus-thymidine kinase.
received by the avidin-biotin technique instead of the peroxidase-anti-peroxidase one.

Western blot detection of PV in various skeletal muscles. The panniculus carnosus, extensor digitorum longus (EDL), and abdominal muscle were removed from killed mice and homogenized, and the soluble proteins were separated by SDS-PAGE (12.5%) and transferred onto nylon membranes (Bio-Rad). After blocking [1% (wt/vol) bovine serum albumin and 10% (vol/vol) fetal calf serum], membranes were incubated with the PV-specific polyclonal antisemur PV-28 (1: 1,000; Swant, Bellinzona, Switzerland) and were then further processed by the avidin-biotin method, using 4-chloro-1-naphthol/hydrogen peroxide as a chromogen.

Analysis of proteins from extracts of tibialis anterior muscles. Muscle samples were analyzed by SDS-PAGE (10%) in Tris-glycine buffer without added calcium. Muscle extracts were prepared in 10 volumes of 0.0625 M Tris (pH 6.8), 2% SDS, 2% 2-mercaptoethanol, and 0.001% bromphenol blue. The presence of different isoforms of troponin I was detected using the monoclonal antibody 42/25 that detects fast, slow, and cardiac troponin I. The presence of fast troponin T was analyzed by staining of the Western blots with antibody F24 as described before (11).

[Ca²⁺]i measurements. Methods for enzymatic dissociation and agarose suspension of PV −/−, PV +/+ , and WT animal EDL fibers were essentially the same as described previously for flexor digitorum brevis (FDB) fibers (7). Before agarose suspension, fibers were loaded with the Ca²⁺ indicator dye indo 1-AM (cell permeant form; 5 µM) for 40 min at 37°C and pipetted onto a coverslip serving as the bottom of small culture dish placed on a Leica TCS 4D confocal microscope. Indo 1 was excited using an ultraviolet laser source set at 1,000; Swant, Bellinzona, Switzerland) and were then further processed by the avidin-biotin method, using 4-chloro-1-naphthol/hydrogen peroxide as a chromogen.

RESULTS

PV-deficient mice are not distinguishable from WT littermates under standard housing conditions. To eliminate the functional gene for PV, a targeting vector (pPVknock) was designed to replace the greater part of the coding sequence by a neocassette (Fig. 1A). By this strategy, ~85% of the coding sequence is replaced by the neocassette. Its purpose is to ensure that no truncated protein molecules, which could partially restore WT PV function, are produced. Of the 100 G418-resistant ES clones analyzed, 5 had undergone homologous recombination, as revealed by Southern blotting using two external probes, P1 and P2 (Fig. 1A). Injection of clone 134 gave rise to seven chimeric mice (~50% chimeric), which were mated with C57/B16 WT ones. Germ-line transmission could be demonstrated in several cases. Crossing of the heterozygous animals yielded homozygous mice at a frequency not statistically different from the expected Mendelian transmission of an autosomal gene. This suggests that elimination of the functional PV gene is neither lethal to embryos nor significantly affects embryonic development.

The three different genotypes were characterized by Southern blotting of genomic DNA (Fig. 1B). The WT allele gave rise to a fragment of ~13.7 kb, the targeted allele to one of only 11.4 kb. To demonstrate the absence of the PV protein, we performed either Western blotting or immunohistochemical analysis of various skeletal muscles. Western blot analysis of soluble proteins extracted from three different muscles revealed an intense signal in WT +/+ mice (Fig. 1C), one of intermediate intensity in the heterozygous (+/−) ones, and none whatsoever in PV −/− animals. For immunohistochemical analysis, we used sections from the fast-twitch tibialis anterior muscle. No signal was detected in the muscles of knockout animals, but the well-characterized checkerboard staining pattern was manifested in fast-twitch muscle fibers of WT and, less intensely, of heterozygous mice (Fig. 2).
Routine histological analysis of hematoxylin-eosin-stained sections of different organs revealed the microscopic anatomy of PV−/− animals to be normal. They grow and breed normally and have a normal lifespan (of >1 yr). At 4 and 8 wk of age, their body weight does not differ significantly from that of heterozygous and WT mice. The three genotypes were indistinguishable with respect to behavior and physical activity under standard housing conditions.

Lack of PV alters Ca\(^{2+}\) transients in single isolated EDL fibers. Temporal as well as spatial aspects of Ca\(^{2+}\) transients are modulated by the presence of Ca\(^{2+}\) buffers such as EF hand calcium-binding proteins (9). Depending on the intracellular concentration and the kinetic parameters of metal binding of a particular protein, different aspects of Ca\(^{2+}\) transients (e.g., amplitude, duration) can be modified. To investigate the effect of PV in fast-twitch muscles, Ca\(^{2+}\) measurements on isolated EDL fibers were carried out. A prolonged Ca\(^{2+}\) decay in the PV−/− mouse for the 20-ms stimulation period was observed, whereas no difference existed between WT and PV−/− fibers after 50-ms stimulation, when nearly all the PV Ca\(^{2+}\)-binding sites with a high Mg\(^{2+}\) off rate are expected to be saturated with Ca\(^{2+}\) (Fig. 3, A and B). The rate constant of Ca\(^{2+}\) decay was 33% lower in the PV−/− compared with the WT animals (Fig. 3C). Although no conspicuous differences existed between the peak [Ca\(^{2+}\)]\(_i\) values (Fig. 3D), there was a twofold elevation of the integral [Ca\(^{2+}\)]\(_i\) in the PV−/− compared with the WT mice (Fig. 3E).

Twitch tension force in PV−/− mice is higher than in WT mice, but maximal tetanic force is not affected. Isometric contractions were recorded in mice anesthetized with chloral hydrate. The time required to attain maximal twitch tension force was increased by 40% in PV-deficient mice (Table 1 and Fig. 4A). This variable reflects the duration of active state in the contractile machinery, which, in turn, depends on the length of time that [Ca\(^{2+}\)]\(_i\) is increased. The delay in attaining maximal isometric twitch tension was due to a larger amplitude of the Ca\(^{2+}\) transient in PV−/− mice but to the elevated [Ca\(^{2+}\)]\(_i\) integral (Fig. 3E). The half-relaxation time of tibialis anterior muscles in PV−/− mice was significantly longer than that in either WT or heterozygous animals (Table 1 and Fig. 4A). The contractile properties of muscles in heterozygous and WT mice were similar. This latter result is not a surprising one given that a pronounced effect on the half-relaxation time requires the PV level to drop below 50% (23).

The PV−/− mice also developed an ~40% greater force during a single twitch than did WT animals (Table 2). The value in heterozygous (+/−) mice was marginally higher than that in the control group (34 ± 2.9 vs. 31 ± 1.7 g), but the difference was not statistically significant. When the force was measured during maximal tetanic contraction (elicited at 80 Hz), no significant differences between the three groups of mice were observed (Table 2 and Fig. 4B). This suggests that under conditions in which all of the PV molecules would be expected to be in their Ca\(^{2+}\)-saturated form, the maximum generatable force is independent of this protein. The calculated ratio between tetanic and twitch force was smaller in the PV−/− than in either the WT or the heterozygous animals (Table 2).

When stimulated repetitively at 20 Hz, the tibialis anterior muscle of WT mice relaxed almost completely during the interval (~50 ms) between successive stimuli, the maximum force attained being only marginally higher than that achieved during a single twitch (Fig. 4B). In PV−/− mice, on the other hand, ~70% of the tetanic force (elicited at 80 Hz) was achieved after only three successive stimuli at 20 Hz. This is a consequence of the slower time course of the twitch in the PV−/− mice (Fig. 4A) and hence of their inability to relax during the intervals between successive stimuli. The characteristics of the traces recorded for heterozygous mice at 20 Hz were intermediate between those documented for WT and PV-deficient ones. This finding demonstrates that a reduced level of PV can thwart the tibialis anterior muscle’s capacity to relax under the conditions investigated. It is interesting to note that, although the measured parameters for single twitches (time required to attain peak force, half-relaxation time, maximum force) did not differ significantly between PV+/− and WT mice, tibialis anterior muscles of the former exhibited an obvious tendency to achieve higher forces, which were sustained for longer periods of time, than those of the latter (Fig. 4A). When a series of pulses were administered at 20 Hz, these small differences in twitch parameters between PV+/− and WT mice accumulated and were manifested in the building up of the force in PV+/− mice, which was not detected in the WT animals. To quantify these results, the ratios of the tetanic forces (Tet) elicited at 20 and 80 Hz (Tet\(_{20\text{Hz}}\)/Tet\(_{80\text{Hz}}\)) were calculated (Table 3). As expected, the Tet\(_{20\text{Hz}}\)-to-Tet\(_{80\text{Hz}}\) ratios for PV−/− and PV+/− mice were significantly different (P < 0.001 and P < 0.05, respectively) from that for WT ones.

Proteins of the contractile complex of the tibialis anterior muscles of PV−/− mice are fast-twitch muscle isoforms. To exclude the possibility of the aforesaid changes in the contractile properties being secondary to alterations in other muscle-specific proteins, tibialis anterior proteins were separated by SDS-PAGE (Fig. 5). No significant differences in the staining intensity of
the proteins of the contractile complex (fast myosin heavy chain, fast troponin I, troponin C) were observed between the three genotypes. In addition, immunoblots for troponin T confirmed that only the isoforms specific for fast-twitch muscles were present and the amounts were similar in all three groups of mice (Fig. 5), the fastest migrating isoform being the most abundant.

With the monoclonal antibody 42/25 recognizing the different troponin I isoforms, only fast troponin I was detected irrespective of the genotype (not shown).

DISCUSSION

Since more than 20 years ago, PV has been predicted to promote relaxation in fast-contracting skeletal muscle (5, 12). A major problem in the debate on the role of PV
in mammalian muscle relaxation has been the purportedly slow rate of Mg\(^{2+}\) dissociation from this protein. It has been assumed that Ca\(^{2+}\) was transported into the sarcoplasmic reticulum via a Ca\(^{2+}\)-ATPase before PV had a chance to contribute to the buffering of this ion. But evidence has now accumulated that the dissociation rate of Mg\(^{2+}\) from PV at temperatures more physiological for mammals is significantly higher than that documented for the frog and fish in the 0–20°C range. In addition, the Mg\(^{2+}\) off rates are not identical for the two metal-binding sites, one being almost one order of magnitude faster than the other (20, 26).

Values between 11 and 25 s\(^{-1}\) have been estimated from fura 2 and mag-fura 2 recordings, respectively, in fast-twitch muscle of the rat at 26–28°C (6). Permiakov et al. (26) have reported the Mg\(^{2+}\) dissociation rate of PV to be as high as 33 s\(^{-1}\) at 30°C as against 4 s\(^{-1}\) at 10°C. Taken together, these results support the notion that the Mg\(^{2+}\) dissociation rate of PV at elevated temperatures higher than 30°C is sufficiently rapid for this protein to act as a Ca\(^{2+}\) acceptor during a single twitch.

With increasing knowledge about the different EF hand calcium-binding proteins, it becomes evident that not only binding constants but also the on/off rates for the different metal ions play a crucial role in understanding their functions. The spatial and temporal aspects of Ca\(^{2+}\) transients are regulated by the efficacy and geometry of the Ca\(^{2+}\)-release and Ca\(^{2+}\)-uptake/extrusion systems as well as by the concentration and kinetic parameters of soluble Ca\(^{2+}\) buffers such as EF hand calcium-binding proteins. It has been suggested that proteins with Ca\(^{2+}\)-specific sites and high on rates would strongly affect the amplitude of an intracellular rise in [Ca\(^{2+}\)], whereas “slow” Ca\(^{2+}\)-binding proteins would mainly increase the decay rate of [Ca\(^{2+}\)] without much affecting the amplitude. For the protein calbindin-D28k (CB), which contains four Ca\(^{2+}\)-specific sites, this was demonstrated in rat dorsal root ganglion neurons by Chard et al. (9). The addition of CB via patch clamp pipette lowered the amplitude of a Ca\(^{2+}\) transient induced by a brief depolarization stimulus and caused an eightfold decrease in the rate of rise in [Ca\(^{2+}\)]. Under the same conditions, the effect of PV was much less pronounced. It was postulated that because Mg\(^{2+}\) has to dissociate from PV before Ca\(^{2+}\) ions can be for deletion of the parvalbumin gene

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Twitch Tension, g</th>
<th>Tetanic Tension, g</th>
<th>Tetanic-to-Twitch Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+ (n = 10)</td>
<td>31 ± 1.7</td>
<td>90 ± 6.5</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>+/- (n = 8)</td>
<td>34 ± 2.9</td>
<td>87 ± 10.0</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>+/- (n = 8)</td>
<td>44 ± 1.5*</td>
<td>101 ± 7.7</td>
<td>2.3 ± 0.2†</td>
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Values are means ± SE. *Statistically significant vs. +/+ (P < 0.0003). †Statistically significant vs. +/- (P < 0.043).
An interesting finding in the PV-deficient mice is the observation that all the proteins of the contractile complex are isoforms found in fast-twitch muscles. It has been demonstrated before that fast-twitch muscles can be transformed into slower-contracting ones by chronic low-frequency stimulation (CLFS) (27). Whereas in rabbits the conversion of myosin heavy chain fast isoform MHCII(x) to slow isoform MHCIIb is almost complete after 60 days, rats exhibit a restricted capacity for fast-to-slow conversion (21). Because the disappearance of PV during CLFS precedes the conversion of MHC isoforms, it has been hypothesized that the downregulation of PV might in part be linked to the switch from fast- to slow-twitch isoforms of contractile proteins. Our results strongly disfavor this theory, because all the parameters investigated suggest that besides the lack of PV the muscle constituents are the ones found in fast-twitch muscles.

The only existing in vivo study that has addressed the function of PV in the rodent model is that reported by Münzener et al. (25). These authors injected the cDNA for PV into the soleus muscle of rats and observed an increase in its rate of relaxation. In this previous study, the contraction time was not significantly affected, although a decrease in this parameter from 43.5 ± 2.6 to 39.1 ± 7.1 ms was documented for transfected muscles that contained the highest levels of mRNA for PV. At first sight, these findings appear to be inconsistent with our own data pertaining to PV-deficient mice, in which not only the half-relaxation time but also that required to attain peak twitch in the tibialis anterior muscle were significantly longer than in the WT ones. During the early phase of contraction, it is principally the kinetics of Ca²⁺ release from the sarcoplasmic reticulum, the binding of the released ions to the proteins of the contractile complex (troponin C), and the subtype of myosin that determine those governing the rise in tension force. In the soleus muscle, the isoforms of troponin C and myosin heavy chain as well as those of other proteins are of the slow type, and, seemingly, overexpression of PV does not overtly affect the time to attain peak twitch force (although it might be a trend toward shorter times), but only the half-relaxation time. Furthermore, the levels of PV and the number of PV-expressing fibers in the transfected rat soleus muscle are considerably lower than in the murine tibialis anterior one. In the latter, >95% of the fibers express this Ca²⁺-binding protein (17), the concentration of which has been estimated to be higher than 0.4 mM (4.8 g PV/kg wet wt). In PV-deficient mice, all components of the tibialis anterior muscle thus far investigated (troponin C, troponin T, and myosin heavy chain) are of the fast type and identical to those in WT mice. That the myosin heavy chain isoforms remain unchanged in PV-deficient mice also argues against an alteration in the rate of rise in force during a single twitch. It indicates rather that the greater length of time required to attain peak force in PV −/− mice is due to an increased duration of the active state of the tibialis anterior muscle fibers. The results of Münzener et al. (25) pertaining to the rat slow-twitch soleus

bound, the effect in reducing d[Ca²⁺]/dt during the rapid rising phase would be significantly smaller for PV than for CB. A similar finding for CB was observed in CB-deficient mice, where the amplitude of Ca²⁺ transients in Purkinje cells evoked by extracellular stimulation of the afferent climbing fiber was increased by >80% in mice lacking CB (1).

In our calcium measurements in the EDL muscle fibers, the increase in [Ca²⁺] after 20- or 50-ms stimulation was not significantly different in the PV-deficient mice compared with the WT animals, whereas the rate constant of [Ca²⁺] decay was significantly smaller after a 20-ms stimulation in PV −/− mice. As a result, the integral [Ca²⁺] was increased during both 20- and 50-ms stimulations. Hence, the contraction-relaxation cycle in WT muscle fibers containing PV is shorter, owing to the increased rate of decay of [Ca²⁺] (6, 24), and maximal twitch force, although of lower amplitude, is more rapidly attained than in PV-deficient ones. It should be noted that our rate constants of calcium decay for the WT fibers are consistent with values reported previously for mouse fast-twitch fibers (18, 24). However, the amplitude of the calcium transient is significantly lower than the value reported by Hollingworth et al. (18) for mouse EDL. This difference is most likely to be due to differences in calcium indicators and Kd values chosen. We found that even after increasing the K1 by 10-fold (by decreasing the on rate for indo 1 by 10-fold), which increased the amplitude of the calcium transient by 10-fold, the rate constants of calcium decay were unaffected, and the observed differences between the WT and PV −/− mice fibers were still valid.

Fig. 5. A: proteins in muscle extracts of tibialis anterior from WT (+/+), heterozygous (+/−), and homozygous (−/−) mice were separated by SDS-PAGE (10%) and stained with Coomassie blue. No differences were observed for myosin heavy chain (Mhc), troponin I (TnI), and troponin C (TnC). For PV, strong bands are seen in the +/+ mice, reduced bands in the +/− mice, and no bands in the protein samples isolated from −/− animals. B: electrochemiluminescence-Western blot stained with antibody F24 specific for fast troponin T (TnT) (11).
muscle overexpressing relatively low levels of PV cannot therefore be directly compared with our results on the murine fast-twitch tibialis anterior muscle. It is evident, however, that an investigation of the physiological effects of PV during contraction is better addressed using the tibialis anterior muscle, within which this Ca\(^{2+}\)-binding protein is normally expressed, than the soleus one, in which it is absent.

In the tension experiments, tibialis anterior muscles of the three genotypes were stimulated at different frequencies (20, 40, and 80 Hz). At 80 Hz, no significant differences between the three genotypes were observed, the effect of PV deficiency being evident only when pulses were delivered at 20 Hz. In WT animals, the muscles stimulated at 20 Hz relaxed almost completely between successive stimuli, whereas in PV-deficient ones, this process was significantly compromised. These results qualitatively resemble those reported by Jiang et al. (22). These authors investigated the role of PV in the relaxation of frog skeletal muscle fibers at 10°C in which activity of the sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase was inhibited by 2,5-di-tert-butyl-1,4-benzoquinone. After induction of a series of twitches (at a frequency that resulted in complete relaxation of control fibers) the rate of twitch relaxation decreased progressively and approached zero after the seventh pulse. As an explanation for this finding, the authors suggested that a soluble relaxation factor was lost in a time-dependent manner. After conducting additional experiments, they came to the conclusion that loss of the capacity to relax paralleled the saturation of PV with Ca\(^{2+}\) and that Mg\(^{2+}\)-bound PV could induce relaxation at a rate that is defined by the Mg\(^{2+}\) off rate from this protein.

From the observation that PV is enriched in the I band region (2), and from the present finding that the integral [Ca\(^{2+}\)] is about twofold elevated in the PV−/− phenotype, an additional role of PV may be suggested: Ca\(^{2+}\) not associated with PV would be more homogeneously distributed in the sarcoplasm and therefore less accessible to the SR Ca\(^{2+}\)-ATPase. The evidence that PV-bound Ca\(^{2+}\) is a substrate of the SR Ca\(^{2+}\)-ATPase has been demonstrated long ago (12). PV-bound Ca\(^{2+}\) would thus be enriched in the I band and the role of PV as a “vehicle” of Ca\(^{2+}\)-transport between the contractile apparatus and the SR Ca\(^{2+}\)-ATPase would be similar to that of other calcium-binding proteins that selectively shuttle calcium signals to nearby sites of elevated Ca\(^{2+}\) (10).

It has been postulated that PV would have its greatest effect on the relaxation time in skeletal muscles of poikilotherms at low temperatures, when the activity of the Ca\(^{2+}\) pump of the sarcoplasmic reticulum is depressed (13). In view of our results, the very high concentrations of PV found in fish and amphibians could be considered as a mobile “emergency Ca\(^{2+}\) store” that would facilitate rapid contraction and relaxation at low temperatures. Because the body temperature in mammals is closely regulated and Ca\(^{2+}\) uptake into the sarcoplasmic reticulum via its Ca\(^{2+}\)-ATPase is very efficient, we postulate that the advantage conferred by PV lies in the gain in speed to be achieved at the expense of force in a single twitch. The absence of PV under “wildlife” conditions may be a selective disadvantage for knockout mice.

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Address for reprint requests: B. Schwaller, Institute of Histology and General Embryology, University of Fribourg, CH-1705 Fribourg, Switzerland.

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