

## Ca<sup>2+</sup> Influx and Opening of Ca<sup>2+</sup>-Activated K<sup>+</sup> Channels in Muscle Fibers from Control and *mdx* Mice

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**ABSTRACT** Using the patch-clamp technique, we demonstrate that, in depolarized cell-attached patches from mouse skeletal muscle fibers, a short hyperpolarization to resting value is followed by a transient activation of Ca<sup>2+</sup>-activated K<sup>+</sup> channels (K<sub>Ca</sub>) upon return to depolarized levels. These results indicate that sparse sites of passive Ca<sup>2+</sup> influx at resting potentials are responsible for a subsarcolemmal Ca<sup>2+</sup> load high enough to induce K<sub>Ca</sub> channel activation upon muscle activation. We then investigate this phenomenon in *mdx* dystrophin-deficient muscle fibers, in which an elevated Ca<sup>2+</sup> influx and a subsequent subsarcolemmal Ca<sup>2+</sup> overload are suspected. The number of Ca<sup>2+</sup> entry sites detected with K<sub>Ca</sub> was found to be greater in *mdx* muscle. K<sub>Ca</sub> activity reflecting subsarcolemmal Ca<sup>2+</sup> load was also found to be independent of the activity of leak channels carrying inward currents at negative potentials in *mdx* muscle. These results indicate that the sites of passive Ca<sup>2+</sup> influx newly described in this study could represent the Ca<sup>2+</sup> influx pathways responsible for the subsarcolemmal Ca<sup>2+</sup> overload in *mdx* muscle fibers.

### INTRODUCTION

High-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (K<sub>Ca</sub>) channels belonging to the maxi-K class (or BK) are present in the sarcolemma of adult mammalian skeletal muscle (Blatz and Magleby, 1987; Latorre et al., 1989; McManus, 1991; Kaczorowski et al., 1996; Vergara et al., 1998). The basic characteristic of these channels is that their opening is induced by an increase in intracellular [Ca<sup>2+</sup>], as well as membrane depolarization. The K<sub>Ca</sub> channel protein comprises an S4 voltage-sensing element and a C-terminal region involved in Ca<sup>2+</sup>-dependent activation (Butler et al., 1993; Bian et al., 2001). K<sub>Ca</sub> channels have been extensively studied in excised conditions, but less is known about the role of the channel in its physiological environment. Yet, during activation, muscles undergo massive increase in intracellular [Ca<sup>2+</sup>] associated with membrane depolarization, both of which should favor K<sub>Ca</sub> channel opening. Indeed, we previously showed that the increase in intracellular [Ca<sup>2+</sup>], resulting either from Ca<sup>2+</sup> entry through end-plate acetylcholine receptors or from long-lasting voltage-activated sarcoplasmic reticulum (SR) Ca<sup>2+</sup> release, could induce K<sub>Ca</sub> channel opening (Allard et al., 1996; Jacquemond and Allard, 1998). Additionally, in resting conditions, a high driving force for Ca<sup>2+</sup> exists. Ca<sup>2+</sup> enters cells in a continuous way but remains at a concentration of a few tens of nanomolar in the cytosolic bulk because of the activity of calcium extrusion systems (Rasmussen and Barret, 1984). At the global level, the Ca<sup>2+</sup> changes induced by this passive influx concomitant of a negative membrane potential are likely too small to produce opening of K<sub>Ca</sub> channels.

However, at the level of sarcolemmal sites of Ca<sup>2+</sup> influx, the local increase in intracellular [Ca<sup>2+</sup>] might be high enough to induce K<sub>Ca</sub> channel activation upon membrane depolarization associated with muscle activation as already shown in neurones and smooth muscle cells (Ganitkevich and Isenberg, 1996; Marrion and Tavalin, 1998).

The pathway for resting Ca<sup>2+</sup> influx in skeletal muscle is not clearly defined but has prompted renewed interest with investigation of the Duchenne muscular dystrophy (DMD). In patients suffering from DMD, as well as in the *mdx* mouse, an animal model of the disease, skeletal muscles lack the protein dystrophin (Hoffman et al., 1987). Recently, an increase in Ca<sup>2+</sup> entry into *mdx* muscle fibers in resting conditions has been revealed by measuring the quenching of the fluorescence signal of Fura-2 induced by extracellular Mn<sup>2+</sup> influx (Tutdibi et al., 1999). Additionally, electrophysiological studies of this pathology have led to the identification of Ca<sup>2+</sup> channels open at rest in the muscle sarcolemma whose activity was demonstrated to be higher in the dystrophic muscle (Gillis, 1999). These channels have been shown either to be modulated by the degree of stretch of the plasma membrane, and hence labeled as mechanosensitive channels (Franco-Obregon and Lansman, 1994), or to behave as nonselective cation leak channels (Hopf et al., 1996). However, what is not known is the magnitude of the intracellular Ca<sup>2+</sup> load resulting in resting conditions from the activity of these Ca<sup>2+</sup> channels in *mdx* as well as in control muscles. Classic Ca<sup>2+</sup> fluorescence methods are thought not to offer the required resolution to estimate these [Ca<sup>2+</sup>] changes supposed to take place in the immediate vicinity of the membrane and possibly not to affect the bulk intracellular [Ca<sup>2+</sup>]. In contrast, the resulting [Ca<sup>2+</sup>] changes at the subsarcolemmal level can be estimated by using endogenous plasmalemmal Ca<sup>2+</sup> sensors. In this respect, the activity of K<sub>Ca</sub> channels in cell-attached patches has been recently used as an index of subsarcolemmal [Ca<sup>2+</sup>] in control and *mdx* skeletal muscles, demon-

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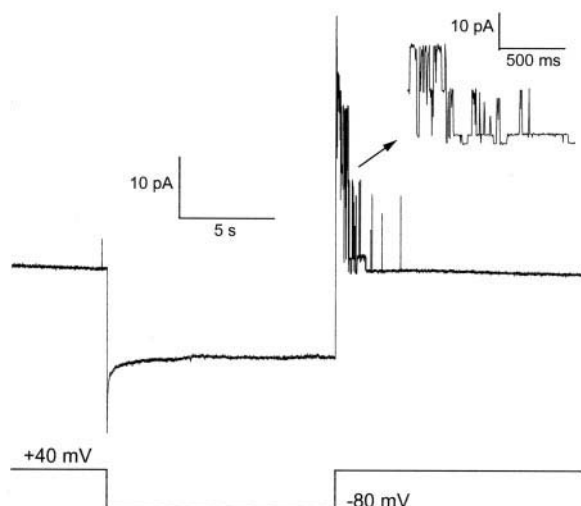


FIGURE 1 Activation of a high-conductance channel in a depolarized cell-attached patch after a transient hyperpolarization. The membrane potential of the patch was changed from +40 mV to -80 mV as shown by the voltage protocol presented below the current trace. The pipette contained Tyrode solution and the bath a  $K^+$ -rich solution with normal  $Ca^{2+}$ . The inset shows a short segment of the main trace on an expanded scale.

strating that subsarcolemmal  $[Ca^{2+}]$  was higher in *mdx* muscles (Mallouk et al., 2000).

In the present paper, we first demonstrated the existence of sarcolemmal sites of passive  $Ca^{2+}$  influx that induce an increase in subsarcolemmal  $[Ca^{2+}]$  high enough to produce  $K_{Ca}$  channel opening upon depolarization. We then investigated this phenomenon in *mdx* muscles to compare the subsarcolemmal  $[Ca^{2+}]$  changes induced by the passive influx of  $Ca^{2+}$  occurring at resting potential in control and *mdx* muscle. Finally, we tried to determine in *mdx* muscle fibers whether  $K_{Ca}$  channel activity and hence subsarcolemmal  $[Ca^{2+}]$  could be correlated with the activity of channels carrying inward currents at resting membrane potential.

## MATERIALS AND METHODS

### Isolation of skeletal muscle fibers

All experiments were performed in accordance with the guidelines of the French Ministry of Agriculture (87/848) and of the European Community (86/609/EEC). For the first part of the study (Figs. 1–7), muscles were obtained from adult mice (swiss). For the second part of the paper (Fig. 8), muscles were removed from wild-type (C57BL/10ScSn) and *mdx* (C57BL/10 *mdx*) mice aged 3–5 weeks (period corresponding to the peak of degeneration in *mdx* muscle (DiMario et al., 1991)). Mice were killed by cervical dislocation. Isolated skeletal muscle cells were obtained from the flexor digitorum brevis and interosseal muscles by a classical enzymatic dissociation process; muscles were incubated for 1 h at 37°C in a Tyrode solution containing collagenase (2 mg/ml, Sigma type 1, Sigma, St. Louis, MO). After enzyme treatment, muscles were rinsed with Tyrode and stored in Tyrode at 4°C until use. Intact skeletal muscle fibers were separated from the muscle mass by gently triturating the muscle with a plastic Pasteur pipette. All experiments were carried out at room temperature (20° to 23°C).

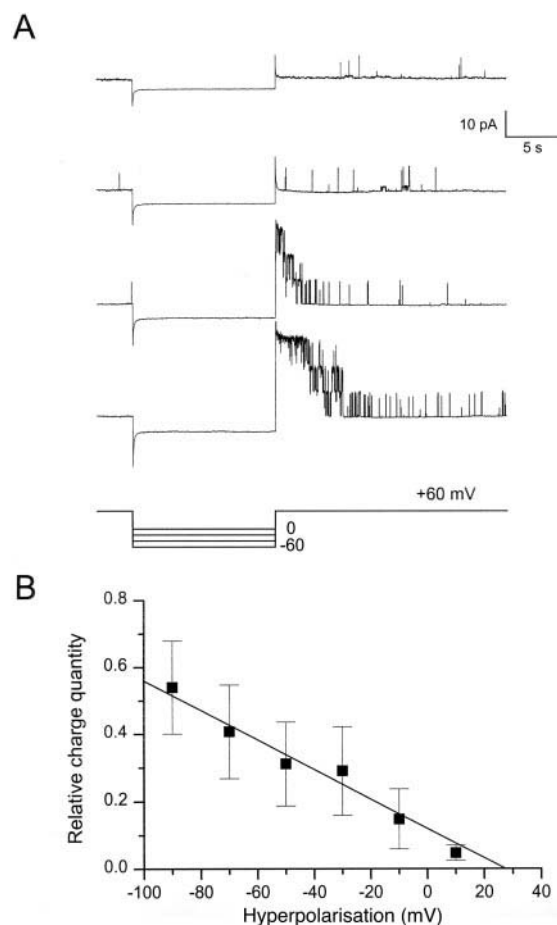


FIGURE 2 Activation of  $K_{Ca}$  channels in cell-attached patches after transient hyperpolarizations of fixed duration and increasing amplitudes. (A) Single-channel currents through  $K_{Ca}$  channels recorded in response to 8-s hyperpolarizations of increasing amplitudes by steps of 20 mV from a holding potential of +60 mV as indicated by the voltage protocol presented below the current traces. Pipettes contained Tyrode solution and the bath a  $K^+$ -rich solution with high  $Ca^{2+}$ . (B) Relationship between the relative charge quantity through  $K_{Ca}$  channels and the membrane potential reached during the hyperpolarization pulse (see text for details).

### Electrophysiology

Single-channel currents were recorded from cell-attached and inside-out membrane patches using a patch-clamp amplifier (model RK 400; Bio-Logic, Claix, France). Currents flowing into the pipette were considered to be positive. Command voltage pulse generation and acquisition were done using the Biopatch software (Bio-Logic) driving an A/D, D/A converter (Lab Master DMA board, Scientific Solutions, Solon, OH). Currents were analyzed using Biopatch software. Charges flowing through ion channels were quantified, after zeroing the current value corresponding to the closed state of the channels, by measuring the area under the current traces over 10 s recording periods for  $K_{Ca}$  channels and over 8-s recording periods for channels carrying inward currents during hyperpolarization. Relative charge quantity flowing through  $K_{Ca}$  channels was obtained in the following way:  $Q/(i \cdot N \cdot t)$  where  $Q$  is the measured charge quantity,  $i$  the unitary current amplitude,  $N$  the number of  $K_{Ca}$  channels in the patch, and  $t$  corresponds to the 10-s recording period. Channel open-state probability ( $P_o$ ) was determined from the average current ( $I$ ) as  $P_o = I/N \cdot i$ .  $I$  was measured after filtering at 300 Hz and sampling at 1 kHz over 30-s

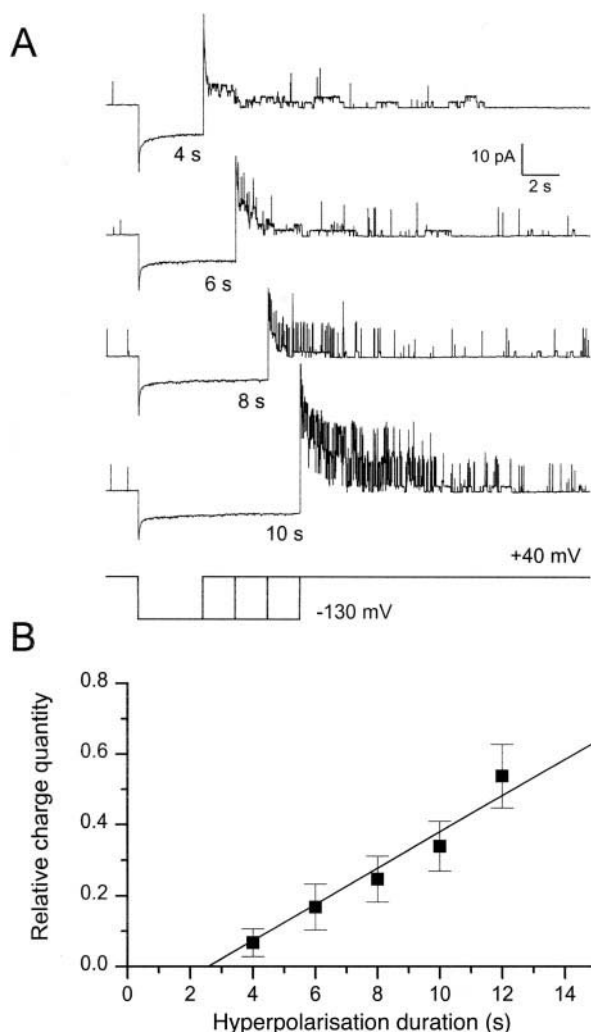


FIGURE 3 Activation of  $K_{Ca}$  channels in cell-attached patches after transient hyperpolarizations of fixed amplitude and increasing durations. (A) Single-channel currents through  $K_{Ca}$  channels recorded in response to hyperpolarizations to  $-130$  mV of increasing duration from a holding potential of  $+70$  mV as indicated by the voltage protocol presented below the current traces. Pipettes contained Tyrode solution and the bath a  $K^+$ -rich solution with high  $Ca^{2+}$ . (B) Relationship between the relative charge quantity through  $K_{Ca}$  channels and the duration of the hyperpolarization pulse (see text for details).

recording periods.  $N$  was determined in inside-out patches by exposing the cytoplasmic face to the  $Ca^{2+}$  containing external  $K^+$ -rich solution. Single-channel current amplitudes were determined using amplitude histograms.

### Pipette perfusion

A patch pipette perfusion system was adapted from the one described by Tang et al. (1990). The perfusion capillary was made from standard borosilicate glass capillaries pulled using a horizontal microelectrode puller so that an outside diameter of  $50 \mu m$  could be achieved. The capillary was connected to a plastic tubing that had been threaded through a second port in the pipette holder (two-port pipette holder (PE series), Phymep, France). The far end of the plastic tubing was connected to a syringe used to push the intracapillary solution to be perfused within the

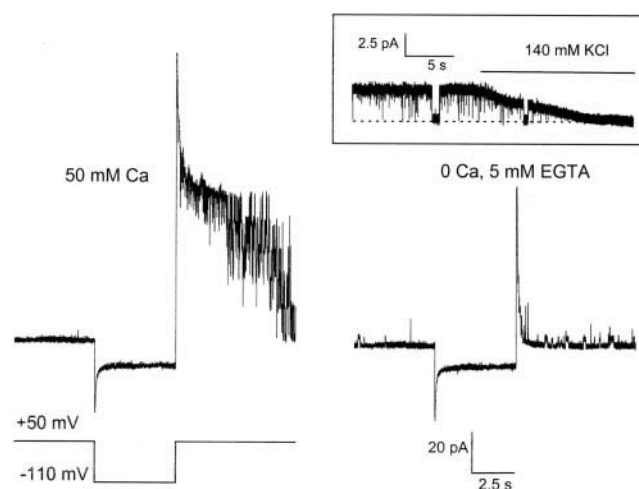


FIGURE 4 Inhibition of hyperpolarization-induced  $K_{Ca}$  channel activation in a cell-attached patch by removal of external  $Ca^{2+}$ . Inset shows the effect of a perfusion within the pipette of a  $K^+$ -rich external solution on the unitary current through  $K_{Ca}$  channel in an inside-out patch held at  $0$  mV in the presence of an internal  $K^+$ -rich solution. Elevated noise on the current trace was induced by the pipette perfusion system. In the main panel, the membrane potential was changed in a cell-attached patch as shown by the voltage protocol presented below the current trace. In the left panel, the pipette contained Tyrode solution plus  $50$  mM  $Ca^{2+}$ . The right panel shows the current trace recorded  $30$  s after the pipette had been perfused by a free- $Ca^{2+}$  Tyrode solution plus  $5$  mM EGTA. The bath contained a  $K^+$ -rich solution with high  $Ca^{2+}$ .

patch pipette. Bullet-shaped patch pipettes were used to allow positioning of the capillary between  $100$  and  $300 \mu m$  from the tip of the pipette. For each patch pipette used, the position of the capillary was adjusted under a microscope.

### Solutions and chemicals

In inside-out and cell-attached experiments, except for Figs. 4 and 7, pipettes were filled with Tyrode solution containing (in mM):  $140$  NaCl,  $5$

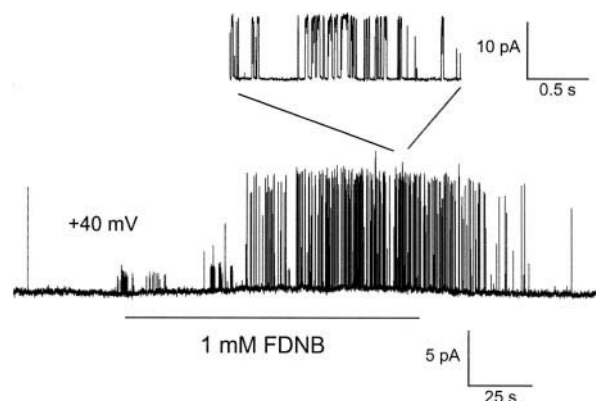


FIGURE 5 Activation of  $K_{Ca}$  channels in cell-attached patches in response to metabolic poisoning of the fiber. The patch membrane potential was  $+40$  mV. The pipette contained Tyrode solution and the bath a  $K^+$ -rich solution with normal  $Ca^{2+}$ . FDNB was added to the bathing solution during the period indicated by the bar. The inset shows a segment of the main current trace on an expanded scale.

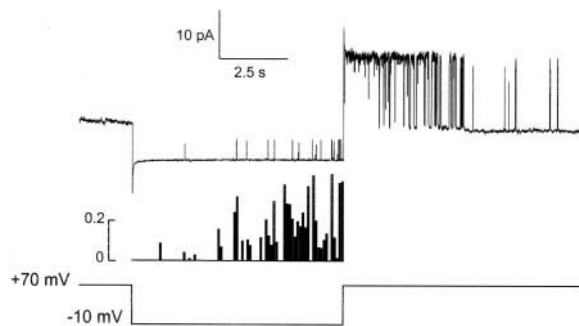


FIGURE 6  $K_{Ca}$  channel activation after transient hyperpolarization under inactivated conditions of SR  $Ca^{2+}$  release. The upper trace shows single-channel currents through  $K_{Ca}$  channels recorded in response to voltage changes as indicated by the voltage protocol (lower trace). The pipette contained Tyrode solution and the bath a  $K^+$ -rich solution with high  $Ca^{2+}$ . The middle trace illustrates the mean average current over successive 100-ms intervals in three cell-attached patches. Average currents were normalized to the maximal value obtained during hyperpolarization.

KCl, 2.5  $CaCl_2$ , 1  $MgCl_2$ , 10 Hepes, adjusted to pH 7.4 with NaOH. For Figs. 4 and 7, the pipette solution contained a Tyrode solution plus 50 mM  $CaCl_2$ . The intrapipette solution used in Fig. 4 for pipette perfusion corresponded to a free- $Ca^{2+}$  Tyrode solution plus 5 mM EGTA. In cell-attached experiments, fibers were bathed in an external  $K^+$ -rich solution containing (mM): 140 KCl, 50  $CaCl_2$ , 1  $MgCl_2$ , 10 Hepes, adjusted to pH 7.4 with KOH, except for Figs. 1 and 5 where the solution contained 2.5 mM  $CaCl_2$ . Despite the high  $[Ca^{2+}]$  and osmolarity of the 50 mM  $Ca^{2+}$  bathing medium, cells could stay for several hours in this solution without displaying any detectable signs of deterioration. In inside-out experiments, the internal solution corresponded to a 2.5 mM  $Ca^{2+}$  containing external  $K^+$ -rich solution. Glibenclamide (Sigma) and nitrendipine were dissolved in dimethyl sulfoxide at a concentration of 100 mM and 5 mM, respectively, and dinitrophenol (Merck, West point, PA) and 2,4-fluorodinitrobenzene (FDNB; Aldrich, Milwaukee, WI) at 1 M. All drugs were diluted to the required concentrations in the solutions. Cells were exposed to

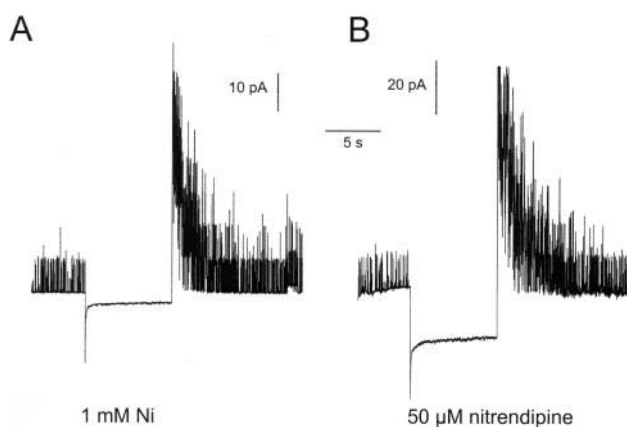


FIGURE 7 Effects of  $Ca^{2+}$  channel blockers on  $K_{Ca}$  channel activation after transient hyperpolarization. (A) 1 mM  $NiCl_2$  was present in the pipette and hyperpolarization was given to  $-80$  mV from a holding potential of  $+80$  mV. (B) 50  $\mu$ M nitrendipine was present in the pipette and an 8-s duration hyperpolarization was given to  $-80$  mV from a holding potential of  $+30$  mV. In (A) and (B), the bath  $K^+$ -rich solution and the pipettes' Tyrode solution both contained high  $Ca^{2+}$ .

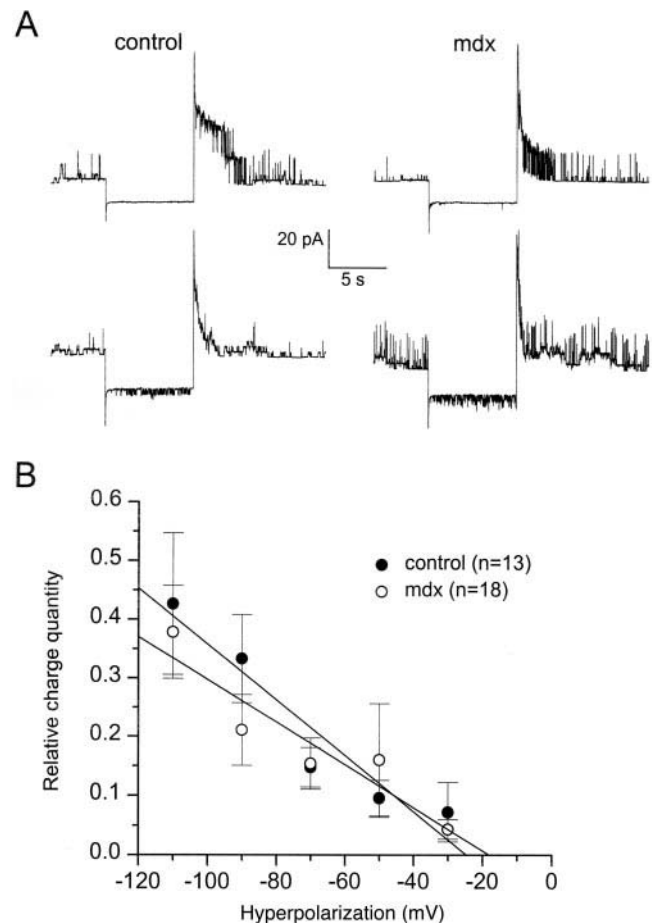


FIGURE 8 Comparison in control and *mdx* cell-attached patches of the degree of  $K_{Ca}$  channel activation after transient hyperpolarization. (A) Single-channel currents through  $K_{Ca}$  channels recorded in response to 8-s duration hyperpolarization to  $-110$  mV from a holding potential of  $+70$  mV in control (left panel) and in an *mdx* cell-attached patch (right panel). Pipettes contained Tyrode solution and the bath a  $K^+$ -rich solution with high  $Ca^{2+}$ . (B) Relationships between the relative charge quantity through  $K_{Ca}$  channels and the membrane potential reached during the hyperpolarization pulses in control (●) and in *mdx* cell-attached patches (○).

different solutions by placing them in the mouth of a perfusion tube from which the rapidly exchanged solutions flowed by gravity.

## Statistics

Least-squares fits were performed using a Marquardt-Levenberg algorithm routine included in Microcal Origin (Microcal Software, Northampton, MA). Data values are presented as means  $\pm$  SE.

## RESULTS

A set of experiments was carried out on adult, control, skeletal muscle fibers. Pipettes filled with Tyrode solution were sealed on muscle fibers bathed with a  $K^+$ -rich solution containing 2.5 mM  $Ca^{2+}$  to clamp the cells near 0 mV. Fig. 1 shows that a very low channel activity was detected in a



patch held at +40 mV under these experimental conditions. However, after hyperpolarization to -80 mV during 12 s, returning the patch potential to +40 mV gave rise to a transient activation of high- and small-conductance channels carrying an outward current of 11.3 and 2.4 pA amplitude, respectively. The small-conductance channels correspond to the delayed rectifier, which is reactivated by hyperpolarization and inactivates during sustained depolarizations (Jacquemon and Allard, 1998; Hocherman and Bezanilla, 1996). The high-conductance channels shut after 3.5 s. Upon excision, the two high-conductance channels spontaneously opened in the presence of the bath solution containing 2.5 mM  $\text{Ca}^{2+}$  at the cytoplasmic face and their activity was completely inhibited by an intracellular solution containing 1 mM EGTA and no added  $\text{Ca}^{2+}$  (not shown). On the basis of these observations, the high-conductance channels activated in the cell-attached configuration were unambiguously identified as  $\text{K}_{\text{Ca}}$  channels as described in previous studies (Allard et al., 1996; Jacquemon and Allard, 1998).

Transient activation of  $\text{K}_{\text{Ca}}$  channels after hyperpolarization was observed in 3 of 17 patches containing  $\text{K}_{\text{Ca}}$  channels and tested under the precedent experimental conditions. Thus, to explore the mechanisms involved in  $\text{K}_{\text{Ca}}$  channel activation, the subsequent experiments were performed at highly depolarized membrane patch potentials (up to +70 mV) and in the presence of an external  $\text{K}^{+}$ -rich solution containing 50 mM  $\text{Ca}^{2+}$ . The increase  $[\text{Ca}^{2+}]$  at the subsarcolemmal level induced by the high  $[\text{Ca}^{2+}]$  in the bath (Turner et al., 1991) and the high depolarized membrane potentials were expected to both favor opening of  $\text{K}_{\text{Ca}}$  channels (Mallouk and Allard, 2000). Indeed, of 107 patches containing  $\text{K}_{\text{Ca}}$  channels and tested under these experimental conditions, 27 patches exhibited bursts of activation of  $\text{K}_{\text{Ca}}$  channels after hyperpolarization, much more sustained than under the preceding conditions. Any contribution of an influx of  $\text{Ca}^{2+}$  coming from extra-patch membrane under these high external  $\text{Ca}^{2+}$  conditions was also ruled out, since, on a same patch, removal of  $\text{Ca}^{2+}$  ions from the external medium did not alter the hyperpolarization-induced  $\text{K}_{\text{Ca}}$  channel activation (not shown). Therefore, in the next part of the study, experiments were all performed at highly depolarized patch membrane potential and in the presence of 50 mM  $\text{Ca}^{2+}$  in the bath.

Fig. 2 shows that the more negative the hyperpolarization, the higher the activation of  $\text{K}_{\text{Ca}}$  channels upon returning to depolarized membrane potential. In this patch, the potential was held at +60 mV, and 8-s duration hyperpolarizations of increasing amplitude were applied every 30 s. It can be seen that after hyperpolarizations to 0 mV and -20 mV no substantial  $\text{K}_{\text{Ca}}$  channel opening was observed, whereas after hyperpolarizations to -40 mV and -60 mV, relative charge quantity carried by  $\text{K}_{\text{Ca}}$  channels was gradually increased to 0.1 and 0.28, respectively. Fig. 2 B shows the relationship between the relative charge quantity

through  $\text{K}_{\text{Ca}}$  channels and the amplitude of the hyperpolarization obtained in seven patches. During these experiments the starting membrane potential was +70 mV, and hyperpolarizations were given during 8 s by steps of 20 mV. The relationship was fitted by a linear regression and the best fit to the mean data indicated that, for an increase of 20 mV of the hyperpolarization, relative  $\text{K}_{\text{Ca}}$  channel opening augmented 9%.

Fig. 3 shows that the longer the hyperpolarization, the higher the activation of  $\text{K}_{\text{Ca}}$  channels upon returning to depolarized membrane potential. The patch was held at +40 mV and hyperpolarization pulses to -130 mV of increasing duration were given every minute. After 4-s duration hyperpolarization, relative charge quantity through  $\text{K}_{\text{Ca}}$  channels was not changed and channel activity was dominated by opening of the delayed rectifier  $\text{K}^{+}$  channels. After 6-, 8-, and 10-s hyperpolarization duration, relative charge quantity through  $\text{K}_{\text{Ca}}$  channels gradually augmented and reached 0.06 and 0.45 for 8 and 10 s, respectively. Fig. 3 B shows the relationship between the relative charge quantity through  $\text{K}_{\text{Ca}}$  channels and the duration of the hyperpolarization in the seven patches in which this protocol was applied. The best fit to the mean data indicated that, for a lengthening of 2 s of the hyperpolarization duration, relative  $\text{K}_{\text{Ca}}$  channel opening augmented 10%.

In all the experiments reported above, activation of  $\text{K}_{\text{Ca}}$  channels after hyperpolarization was transient. Yet, in skeletal muscle,  $\text{K}_{\text{Ca}}$  channels are known not to exhibit inactivation; in the presence of a fixed voltage and cytoplasmic  $[\text{Ca}^{2+}]$ ,  $\text{K}_{\text{Ca}}$  channels usually display sustained activation during several tens of minutes. Activation of  $\text{K}_{\text{Ca}}$  channels after hyperpolarization observed in the above experiments likely suggests that the hyperpolarization step induced an increase in the intracellular  $[\text{Ca}^{2+}]$ . Likewise, the inactivation-like phenomenon observed within a few seconds after activation likely reflected a progressive decrease in the intracellular  $[\text{Ca}^{2+}]$ .

The following part of the study was then devoted to elucidation of the mechanisms by which intracellular  $[\text{Ca}^{2+}]$  increases during the hyperpolarization pulses. We first tried to determine whether the source of  $\text{Ca}^{2+}$  responsible for  $\text{K}_{\text{Ca}}$  channel activation was external. For this purpose, we used a pipette perfusion system which allowed us to demonstrate that removal of  $\text{Ca}^{2+}$  from the intrapipette external solution bathing the external face of the patch membrane led to a total suppression of the hyperpolarization-induced  $\text{K}_{\text{Ca}}$  channel activation. Inset of Fig. 4 first illustrates the quality of the pipette perfusion system.  $\text{K}_{\text{Ca}}$  channel activity was recorded at 0 mV in an inside-out patch in the presence of a Tyrode solution (5 mM  $\text{K}^{+}$ ) in the pipette and a  $\text{K}^{+}$ -rich solution (140 mM  $\text{K}^{+}$ ) containing 2.5 mM  $\text{Ca}^{2+}$  bathing the intracellular face of the membrane. As expected under these voltage and ionic conditions, current through  $\text{K}_{\text{Ca}}$  channel was outward. A high  $\text{K}^{+}$  extracellular solution, i.e., intrapipette, was then perfused so that

eventually the concentration of  $K^+$  are the same on both sides of the patch membrane. Attesting the effectiveness of our perfusion system, in  $\sim 15$  s the current declined to zero as the  $[K^+]$  at the external face of the membrane reached its final value of 140 mM. The main panel of Fig. 4 illustrates the effect of perfusion of a free-calcium external solution on the hyperpolarization-induced  $K_{Ca}$  channel activation in a cell-attached patch. In this experiment, 50 mM  $Ca^{2+}$  was present in the bath as well as in the pipette. The patch was first held at +50 mV and no  $K_{Ca}$  channel was active. After a hyperpolarization to  $-110$  mV lasting 5 s, five  $K_{Ca}$  channels transiently activated revealing a  $Ca^{2+}$  increase at the inner face of the membrane. A free- $Ca^{2+}$  external Tyrode solution containing 5 mM EGTA was then perfused within the pipette, and 30 s later the same voltage protocol was applied. It can be observed that removal of external  $Ca^{2+}$  in the pipette caused a complete inhibition of the hyperpolarization-induced  $K_{Ca}$  channel activation. Furthermore, of 17 cell-attached patches containing  $K_{Ca}$  channels tested in the absence of  $Ca^{2+}$  in the pipette, activation of  $K_{Ca}$  channels after hyperpolarization steps up to  $-110$  mV was never observed (data not shown). These experiments demonstrate that the  $Ca^{2+}$  responsible for  $K_{Ca}$  channel activation came from outside.

Measurement of  $K_{Ca}$  channel activity in cell-attached patches from metabolically poisoned cells also allowed us to demonstrate that  $Ca^{2+}$  enters the muscle cells in a continuous manner. Single-channel activity was recorded in a cell-attached patch while the cell was exposed to the metabolic poison fluorodinitrobenzene (FDNB), an inhibitor of creatine kinase (Fig. 5). Our aim was to block the plasma membrane  $Ca^{2+}$ -ATPases, which are known to represent the most effective mechanism in charge of  $Ca^{2+}$  removal from the submembranous compartment (Guerini et al., 1998; Penniston and Enyedi, 1998). The bath contained a  $K^+$ -rich solution with 2.5 mM  $Ca^{2+}$ , the patch potential was brought to +40 mV and 10  $\mu$ M glibenclamide was present in the pipette to avoid activation of ATP-dependent  $K^+$  channels. No channel opening was observed in control. Upon superfusion of the cell with 1 mM FDNB, after a delay of 1 min, channels that could be identified as  $K_{Ca}$  channels on the basis of their conductance (90 pS at +40 mV; Fig. 5, *inset*) gradually opened and  $P_o$  reached a maximum of 0.1. This effect was reversible on removal of FDNB from the external solution. Similar results were obtained in three other fibers with FDNB and in two other fibers with the other metabolic poison dinitrophenol. It has to be noticed that, in these experiments, the cells never contracted during metabolic poisoning, indicating that the intracellular  $[Ca^{2+}]$  rise was certainly restricted to the submembranous domain. These latter experiments can be interpreted in terms of a continuous  $Ca^{2+}$  influx through the sarcolemma taking place outside of the pipette, progressively loading the submembranous compartment, and re-

vealed because of the inhibition of the sarcolemmal  $Ca^{2+}$  pump.

The precedent results strongly suggest that the origin of the  $Ca^{2+}$  activating  $K_{Ca}$  channels in cell-attached experiments is external and that  $Ca^{2+}$  may enter the cell in a continuous manner.

However, sites of peripheral coupling between the surface membrane and the SR have been reported in skeletal muscle (Spray et al., 1974). Hence, the transient activation of  $K_{Ca}$  channels may be caused, at least partially, by  $Ca^{2+}$  release from the SR as a result of the removal of inactivation of the voltage sensor by the hyperpolarizing pulse (Rios and Pizarro, 1991). The results obtained in the cell-attached patch in Fig. 6 rule out such a hypothesis. From a starting potential of +70 mV, the patch was weakly hyperpolarized to  $-10$  mV during 8 s. It can be observed that  $K_{Ca}$  channels gradually opened during the hyperpolarizing pulse, indicative of a growing of submembranous  $Ca^{2+}$ . Returning to +70 mV led to a transient and strong activation of  $K_{Ca}$  channels revealing the  $Ca^{2+}$  accumulated at the inner face of the membrane. Similar results were obtained in two other patches. The middle trace represents the evolution of the average current through  $K_{Ca}$  channels in the three patches where  $K_{Ca}$  channel opened during hyperpolarization; it clearly shows that  $K_{Ca}$  channel activity, hence submembranous  $Ca^{2+}$ , gradually increased during the hyperpolarization step. As  $-10$  mV is known to be unable to reactivate the voltage sensor controlling the gating of the SR  $Ca^{2+}$  release channel (Melzer et al., 1995), any contribution of internal stores in the hyperpolarization-induced  $K_{Ca}$  channel activation can be excluded.

We then tested pharmacological compounds susceptible to affect sarcolemmal  $Ca^{2+}$  entry. In these experiments, 50 mM  $Ca^{2+}$  was present in the bath as well as in the pipette. A set of experiments was first carried out in the presence of 1 mM  $Ni^{2+}$  in the pipette, an inhibitor of the  $Na^+/Ca^{2+}$  exchange and voltage-dependent  $Ca^{2+}$  channels (Gonzalez-Serratos et al., 1996; McDonald et al., 1994). In Fig. 7 A, the patch was first held at +30 mV. Channel activity was high certainly because of the presence of high  $[Ca^{2+}]$  in the bath and in the pipette. After a hyperpolarization to  $-80$  mV lasting 8 s, a transient activation of  $K_{Ca}$  channels occurred and relative charge quantity through  $K_{Ca}$  channels changed from 0.017 before hyperpolarization to 0.17 after the hyperpolarizing step. Similar results were obtained in two other patches. In Fig. 7 B, 50  $\mu$ M nitrendipine, an inhibitor of the L-type voltage-dependent  $Ca^{2+}$  channel, was present in the pipette. Membrane potential was held at +80 mV. After a hyperpolarization to  $-80$  mV lasting 8 s,  $K_{Ca}$  channels transiently activated and relative charge quantity through  $K_{Ca}$  channels changed from 0.016 before hyperpolarization to 0.2 after the hyperpolarizing step. A similar response was obtained in three other patches. Taken together, these results suggest that neither voltage-dependent  $Ca^{2+}$  channel (L- or T-type), nor  $Na^+/Ca^{2+}$  exchange is

implicated in the intracellular  $[Ca^{2+}]$  increase during hyperpolarization pulses. Additionally, in the major part of this series of experiments and those presented above, acute analysis of the current traces during hyperpolarization pulses failed to reveal any discernible activity of channels carrying inward currents.

In the following part of the paper, we tested whether the elevated  $Ca^{2+}$  influx which has been postulated in *mdx* muscle could give rise to an overactivation of  $K_{Ca}$  channels after hyperpolarization in *mdx* muscle. In control as well as in *mdx* muscle fibers, from a holding potential of +70 mV, patches were hyperpolarized by an 8-s duration voltage step to -110 mV. The upper trace in control shows that after hyperpolarization, a transient activation of  $K_{Ca}$  channel opening occurred and relative charge quantity carried by  $K_{Ca}$  channels was 0.36. Similar results could also be obtained in *mdx* patches; the upper current trace in the right panel of Fig. 8 A shows that after hyperpolarization,  $K_{Ca}$  channel transiently activated and relative charge quantity carried by  $K_{Ca}$  channels was 0.29. In average, the number of patches exhibiting bursts of activation of  $K_{Ca}$  channels was 26 of 107 tested in control patches containing  $K_{Ca}$  channels (24%) compared with 48 of 110 tested in *mdx* patches containing  $K_{Ca}$  channels (44%). The respective 95% confidence intervals, 16 to 32% in control and 34.5 to 53.5% in *mdx* muscle, did not overlap, demonstrating that the percentage was significantly higher in *mdx* muscle and did not result from a sampling artifact. A series of experiments carried out in the presence of 2.5 mM  $Ca^{2+}$  in the pipette and in the bath indicated that 2 of 25 control patches containing  $K_{Ca}$  channels compared with 8 of 28 *mdx* patches containing  $K_{Ca}$  channels displayed transient activation of  $K_{Ca}$  channels after hyperpolarization steps.

Using the same voltage protocol as the one used in Fig. 2 B, from a holding potential of +70 mV, patches were then hyperpolarized by an 8-s duration voltage step of increasing amplitude. Fig. 8 B presents the relationships between the relative charge quantity through  $K_{Ca}$  channels and the amplitude of the hyperpolarization in *mdx* patches and in control patches superimposed. In *mdx* patches, the best fit to the mean data indicated that for an increase of 20 mV of the hyperpolarization pulse, relative  $K_{Ca}$  channel opening augmented 7% although it augmented 9.5% in control.

In this set of experiments performed on young mice, a striking observation was that, during hyperpolarization steps, some patches exhibited activity of channels carrying inward currents in control as well as in *mdx* fibers (*lower traces* of Fig. 8 A). However, as illustrated in the two patches of Fig. 8 A, the sustained activity of channels carrying inward current during hyperpolarization was not necessarily associated to a potentiation of  $K_{Ca}$  channel activity upon returning to depolarized levels in the control and in the *mdx* patch (see Discussion). The conductance properties of this channel were characterized in control and in *mdx* patches. The best fit to the current-voltage relation-

ships indicated a conductance of 20 and of 18 pS and a reversal potential of +6 and +5.7 mV in control and in *mdx* patches, respectively. Using the experimental procedure of Fig. 8 B, we then tried to determine whether a correlation exists between the degree of activation of  $K_{Ca}$  channels and the quantity of charge carried by the channels open during the hyperpolarization steps in *mdx* patches. The degree of  $K_{Ca}$  channel activity was plotted as a function of the quantity of charge carried by the channel open during hyperpolarization. In some patches  $K_{Ca}$  channel activation was high although the quantity of inwardly carried charges during hyperpolarization was low or zero as illustrated by the upper current traces in Fig. 8 A, and reciprocally, in other patches,  $K_{Ca}$  channel activation was low although the quantity of inwardly carried charges during hyperpolarization was high (*lower current traces* in Fig. 8 A). On the whole, no clear correlation was found between the two parameters.

## DISCUSSION

In this study, we showed that, at a depolarized membrane potential and in the presence of physiological  $[Ca^{2+}]$ ,  $K_{Ca}$  channels open transiently after a hyperpolarization to resting values of a few seconds duration. Because  $K_{Ca}$  channels are known not to exhibit inactivation in skeletal muscle, this behavior was interpreted in terms of an increase in submembranous  $[Ca^{2+}]$  occurring during the hyperpolarization followed by a progressive decrease in submembranous  $[Ca^{2+}]$  upon returning to depolarized potentials. The fact that the phenomenon was not observed in the absence of external  $Ca^{2+}$  and inhibited by the perfusion of a free- $Ca^{2+}$  external solution within the pipette demonstrated that the  $Ca^{2+}$  giving rise to  $K_{Ca}$  channel opening had an external origin. Moreover, we observed that submembranous  $Ca^{2+}$  increased during the hyperpolarization pulse, at a membrane potential for which the voltage sensor of excitation-contraction coupling was inactivated; together with the fact that the phenomenon was not inhibited in the presence of nitrendipine, which acts as a blocker of the voltage sensor of the excitation-contraction coupling (Rios and Brum, 1987), it is most likely that the internal stores do not contribute to the hyperpolarization-induced  $K_{Ca}$  channel activation. The more negative the membrane potential during the hyperpolarization step, the higher the activity of  $K_{Ca}$  channels upon returning to depolarized membrane potentials. This observation is compatible with a leak  $Ca^{2+}$  entry during hyperpolarization proportional to the driving force for  $Ca^{2+}$ . Upon returning to the initial depolarized potential,  $K_{Ca}$  channel activity likely reflected  $Ca^{2+}$  accumulated underneath the membrane which rapidly dropped to prehyperpolarization levels because of the sudden reduction in the driving force for  $Ca^{2+}$  influx and the constant activity of surface membrane  $Ca^{2+}$  extrusion systems. Likewise, when hyperpolarization was made longer,  $K_{Ca}$  channel activity was also potentiated as expected if  $Ca^{2+}$  entered the cell and



loaded the submembranous space as hyperpolarization continued. The finding that metabolic poisoning of the muscle cell led to a reversible  $K_{Ca}$  channel activation confirms that  $Ca^{2+}$  enters the muscle cell in a continuous manner. Under these conditions, surface membrane  $Ca^{2+}$ -ATPases were thought to be inhibited, thus allowing accumulation of  $Ca^{2+}$  underneath the membrane and eventually  $K_{Ca}$  channel activation. These results also indicate that a diffusion barrier is likely to exist between the subsarcolemmal compartment and the intracellular bulk permitting  $Ca^{2+}$  to accumulate underneath the membrane.

Voltage-operated  $Ca^{2+}$  channels do not seem to be involved because  $Ca^{2+}$  influx should have decreased with increasing hyperpolarization. Moreover,  $K_{Ca}$  channel activation after hyperpolarization was revealed in the presence of the  $Ca^{2+}$  channel blockers nitrendipine and  $Ni^{2+}$  at the external face of the membrane. These latter data also discard the possible contribution of the  $Na^+/Ca^{2+}$  exchange. In addition, opening of channels carrying inward currents was very rarely detected during hyperpolarization and, in no case could  $K_{Ca}$  channel activation be correlated to such an activity. We thus conclude that, under our experimental conditions,  $Ca^{2+}$  flows across the sarcolemma via channels whose conductance is too low for the unitary currents to be resolved, or transports or leak which are not gated and electrically silent. Along this line, one possible candidate could be the store-operated channel that is known to display very low unitary conductance in the presence of external  $Ca^{2+}$  (Parekh and Penner, 1997); however, there is no reason for the SR to be depleted under our experimental conditions so activation of store-operated channels should not occur.

Comparable mechanism of activation of  $K_{Ca}$  channels has been described in endothelial cells. In this preparation, membrane hyperpolarization was shown to raise intracellular  $[Ca^{2+}]$  by increasing the driving force for  $Ca^{2+}$  across the surface membrane (Cannell and Sage, 1989; Carter and Ogden, 1997). The intracellular  $[Ca^{2+}]$  reached at the end of the hyperpolarization was also found to be proportional to the amplitude as well as to the duration of the hyperpolarization. The route for  $Ca^{2+}$  was not characterized at the unitary level in this cell type although an inward macroscopic membrane current recorded on whole cells was found to be associated with the elevation of intracellular  $[Ca^{2+}]$  (Carter and Ogden, 1997). Interestingly, in frog skeletal muscle, it was described that hyperpolarization pulses from a holding potential of 0 mV gave rise to an inward macroscopic current which disappeared in  $Ca^{2+}$ -free solution indicating that this current could be carried by  $Ca^{2+}$  through noninactivating  $Ca^{2+}$  channels (Brum and Rios, 1987). In rat skeletal muscle, an inward macroscopic current also develops in response to similar voltage protocols but was found to be dominated by a  $Cl^-$  current (Fahlke and Rudel, 1995).

Our results demonstrate that a passive  $Ca^{2+}$  influx at negative membrane potentials close to resting value ( $-80$  mV) is high enough to load the subsarcolemmal domain with  $Ca^{2+}$ , to lower the threshold for  $K_{Ca}$  channel activation, and eventually to induce  $K_{Ca}$  channel activation upon depolarization to values reached by the spike of an action potential ( $+40$  mV). The fact that the phenomenon was only observed in 25% of the patches containing  $K_{Ca}$  channels (in the presence of high bath  $[Ca^{2+}]$ ) suggests that sites of  $Ca^{2+}$  influx are distributed at a low density along the muscle sarcolemma. At the level of these sparse  $Ca^{2+}$  microdomains, we can hypothesize that  $K_{Ca}$  channel opening may slow down or possibly stop the spread of action potential especially during sustained muscle activity or in exhausted fibers that have been shown to display an increased resting intracellular  $[Ca^{2+}]$  and decreased action potential amplitude (Lännergren and Westerblad, 1987; Westerblad and Allen, 1991).

Sites of passive  $Ca^{2+}$  influx were also detected with  $K_{Ca}$  channels in *mdx* muscle fibers from young mice. We found that bursts of opening of  $K_{Ca}$  channels after hyperpolarization pulses occurred  $\sim 1.8$  times more frequently in *mdx* patches than in control patches containing  $K_{Ca}$  channels (in the presence of high bath  $[Ca^{2+}]$ ). Because  $K_{Ca}$  channels have been found to display the same properties in control and *mdx* muscle fibers (Mallouk et al., 2000), these data suggest that there is a greater number of sites of passive  $Ca^{2+}$  influx in *mdx* than in control muscle fibers. One can not totally exclude that this result might be attributable, at least partially, to a higher density of  $K_{Ca}$  channel in *mdx* muscle. However, for such an hypothesis to be valid, it would be required not only to determine the density of  $K_{Ca}$  channels in control and *mdx*, but also their spatial distribution relative to the one of site of  $Ca^{2+}$  influx, which under the present conditions would prove hard to be achieved. The fact that a difference in the occurrence of the phenomenon was also observed between control and *mdx* muscle in the presence of normal external  $[Ca^{2+}]$  rules out the possibility that our observations would have been distorted by the high  $[Ca^{2+}]$  present in the bath during the course of experiments. We found that, in patches exhibiting  $K_{Ca}$  channel activation subsequent to hyperpolarization, the degree of  $K_{Ca}$  channel activation with hyperpolarization was found similar in *mdx* and control fibers, indicating that, at the level of sites of  $Ca^{2+}$  influx, the leak of  $Ca^{2+}$  is apparently of the same magnitude in the two muscle types. During the course of experiments, we also detected activity of channels carrying inward currents spontaneously open at negative membrane potentials. The conductance properties of these channels indicated that these channels likely correspond to the channels open at rest described by Hopf et al. (1996) and Haws and Lansman (1991) in control and in *mdx* fibers (20 pS with 150 mM external NaCl (Haws and Lansman, 1991)). We did not attempt to compare activity of these channels in control and in *mdx* fibers but found that the degree of  $K_{Ca}$



channel activation, which is thought to reflect the extent of  $\text{Ca}^{2+}$  accumulation underneath the membrane, was apparently not related to this channel activity. Along this line, the fact that the reversal potential of the current carried by these channels was found close to zero is consistent with a weak ion selectivity of the channel and could explain the apparent small  $\text{Ca}^{2+}$  influx supported by these channels. Taken together, our results suggest that sarcolemmal sites of  $\text{Ca}^{2+}$  influx are more efficient in loading the submembranous domain with  $\text{Ca}^{2+}$  than the 20-pS conductance channels open at rest. It can then be speculated that the elevated subsarcolemmal  $\text{Ca}^{2+}$  recently detected in dystrophin-deficient muscle fibers with  $\text{K}_{\text{Ca}}$  channels (Mallouk et al., 2000) might be the consequence of a greater number of sarcolemmal sites of  $\text{Ca}^{2+}$  influx rather than the higher activity of  $\text{Ca}^{2+}$  channels open at rest. As previously suggested by others (Menke and Jokusch, 1991), one can postulate that, in the absence of dystrophin, the local destabilization of the membrane could be reinforced, producing focal lesions, at the level of which the sarcolemma becomes leaky for  $\text{Ca}^{2+}$ .

## CONCLUSION

We have described the existence of scattered sites of passive  $\text{Ca}^{2+}$  influx along the sarcolemma at resting membrane potentials that locally elevate the subsarcolemmal  $[\text{Ca}^{2+}]$  and induce activation of  $\text{K}_{\text{Ca}}$  channels upon depolarization. The number of these sites of  $\text{Ca}^{2+}$  entry was found to be greater in *mdx* as compared with control muscle fibers and they could represent the  $\text{Ca}^{2+}$  influx pathway responsible for the subsarcolemmal  $\text{Ca}^{2+}$  overload in *mdx* muscle fibers.

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