Comparison of regulated passive membrane conductance in action potential–firing fast- and slow-twitch muscle

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In several pathological and experimental conditions, the passive membrane conductance of muscle fibers (G_m) and their excitability are inversely related. Despite this capacity of G_m to determine muscle excitability, its regulation in active muscle fibers is largely unexplored. In this issue, our previous study (Pedersen et al. 2009. J. Gen. Physiol. doi:10.1085/jgp.200910291) established a technique with which biphasic regulation of G_m in action potential (AP)-firing fast-twitch fibers of rat extensor digitorum longus muscles was identified and characterized with temporal resolution of seconds. This showed that AP firing initially reduced G_m via CIC-1 channel inhibition but after ~1,800 APs, G_m rose substantially, causing AP excitation failure. This late increase of G_m reflected activation of CIC-1 and K_ATP channels. The present study has explored regulation of G_m in AP-firing slow-twitch fibers of soleus muscle and compared it to G_m dynamics in fast-twitch fibers. It further explored aspects of the cellular signaling that conveyed regulation of G_m in AP-firing fibers. Thus, in both fiber types, AP firing first triggered protein kinase C (PKC)-dependent CIC-1 channel inhibition that reduced G_m by ~50%. Experiments with dantrolene showed that AP-triggered SR Ca^{2+} release activated this PKC-mediated CIC-1 channel inhibition that was associated with reduced rheobase current and improved function of depolarized muscles, indicating that the reduced G_m enhanced muscle fiber excitability. In fast-twitch fibers, the late rise in G_m was accelerated by glucose-free conditions, whereas it was postponed when intermittent resting periods were introduced during AP firing. Remarkably, elevation of G_m was never encountered in AP-firing slow-twitch fibers, even after 15,000 APs. These observations implicate metabolic depression in the elevation of G_m in AP-firing fast-twitch fibers. It is concluded that regulation of G_m is a general phenomenon in AP-firing muscle, and that differences in G_m regulation may contribute to the different phenotypes of fast- and slow-twitch muscle.

INTRODUCTION

In our companion paper (see Pedersen et al. in this issue), we presented a new technique that allows determination of G_m in action potential (AP)-firing muscle fibers with a temporal resolution of seconds. Using this approach, we identified biphasic regulation of G_m in AP-firing fast-twitch extensor digitorum longus (EDL) muscle fibers. In the first phase, Phase 1, the onset of AP firing led to a reduction in G_m that was caused by reduced resting membrane conductance for Cl^- (G_Cl) via underlying inhibition of CIC-1 channels. The second phase, Phase 2, was initiated after prolonged AP firing and was characterized by a dramatic rise in G_m that reflected greatly elevated resting membrane conductance for K^+ (G_K) in combination with elevated G_Cl. We further showed that the ion channels underlying the elevated G_K and G_Cl during Phase 2 were K_ATP and CIC-1 channels, respectively. Intriguingly, the elevations in G_K and G_Cl occurred synchronously during Phase 2, indicating that the K_ATP and CIC-1 channels responded to similar cellular signaling.

In this study, we further explore G_m dynamics in AP-firing muscle fibers by comparing G_m between fast- and slow-twitch muscle fibers. This comparative approach proved useful for two reasons. First, it allowed us to assess whether regulation of G_m is a general phenomenon in skeletal muscle or only restricted to fast-twitch muscle. Thus, it was possible to evaluate whether differences in G_m dynamics in active muscle fibers could contribute to phenotypic differences between fast- and slow-twitch muscle fibers. Second, because G_m dynamics in fast-twitch muscle fibers predominantly reflected underlying regulation of CIC-1 and K_ATP channels and these channels are expressed in both fiber types, the comparative approach could reveal how ion channel regulation depends on the cellular environment. In particular, because slow-twitch fibers have much larger oxidative capacity than fast-twitch fibers (Jackman and Willis, 1996;
Mogensen and Sahlin, 2005), the comparative approach allowed the same ion channels to be studied under different settings of cellular maintenance of metabolic status during repeated AP firing.

MATERIALS AND METHODS

Animal handling and muscle preparation
The aim of this study was to explore \( G_m \) dynamics during muscle activity in fast- and slow-twitch muscle fibers. Consequently, experiments were performed using either rat EDL muscle to represent typical fast-twitch fibers or rat soleus muscles that almost exclusively contain type I slow-twitch fibers (Edström et al., 1982). Experiments for \( G_m \) determinations used muscle from 12–14-wk-old female Wistar rats (\( \sim \)230 g), whereas for contraction experiments, muscles from male or female 4–5-wk-old animals (\( \sim \)70 g) were used. The handling and killing of animals followed Danish animal welfare regulations. The standard Krebs-Ringer bicarbonate solution and the solutions containing reduced or no Cl\(^-\) were prepared as described in our companion paper (Pedersen et al., 2009). Solutions with 10 rather than 4 mM K\(^+\) were made by substitution of KCl for NaCl. All experiments were conducted at 30°C. GF109203X, G6993, \( \alpha \)-naphyl-toluenium sulphonamide (BTS), dantrolene, phorbol 12,13-dibutyrate (PdBu), and blebbistatin were dissolved in DMSO. The maximal concentration of DMSO in experimental solutions was 0.15%, which did not affect resting conditions in muscles.

Determination of the passive membrane conductance in AP-firing fibers
To determine \( G_m \) in AP-firing fibers, the technique and methods of calculating \( G_m \) and \( G_k \) developed in our previous study (Pedersen et al., 2009) were used. In brief, direct measurements of the input conductance in between successive AP trains were used to extract \( G_m \) in AP-firing fibers. This required that the passive membrane conductance was assessed before AP firing (\( G_m \)) in a subset of muscle fibers under the appropriate experimental conditions. Values of \( G_m \) under the various experimental conditions are shown in Table I. The protocol for current injections, data sampling, filter settings, electrode resistance, and electrode solutions (2 M K-citrate) were all identical to those in our previous study.

Blebbistatin was used to block contractile activity in soleus muscles
To measure the input conductance in between successive AP trains without encountering problems of microelectrode breakage due to contractile activity, the myosin II of the contractile proteins was inhibited using specific inhibitors. In most experiments with fast-twitch fibers, myosin II was inhibited using 50 \( \mu \)M BTS, which only reduces the energy consummation of active EDL muscles with fast-twitch fibers, myosin II was inhibited using 50 \( \mu \)M BTS, (Cheung et al., 2002; Macdonald et al., 2003), an alternate myosin II inhibitor, blebbistatin (25 \( \mu \)M) (Straight et al., 2003; Limouze et al., 2004), which inhibits contractions in both fast- and slow-twitch fibers (Fig. S1), was used in experiments with soleus muscles. To evaluate whether blebbistatin could be used as an experimental tool to block contractile activity during AP firing in both fast- and slow-twitch muscle fibers.

Intracellular pH (pH\(_i\)) measurements
Measurements of pH\(_i\), were performed using an epifluorescence system as described previously (de Paoli et al., 2007). In brief, BTS-treated EDL muscles were loaded with 20 \( \mu \)M 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) for \( \sim \)30 min and then washed. Whole muscles were stimulated to fire APs using field stimulation with protocols similar to those used in the single-electrode experiments. The emission ratio of BCECF-loaded muscle was calibrated to pH\(_i\), by the K+-nigericin technique (Thomas et al., 1979).

Contractions in whole muscle
Force production and M-waves were assessed in intact EDL and soleus muscles using an experimental setup that has been extensively described previously (Nielsen et al., 2001; Macdonald et al., 2005; Pedersen et al., 2005). Tetanic contractions were elicited via field stimulation at 30 V/cm for 2 (soleus) or 0.5 s (EDL) using 60-Hz trains of 0.02-ms pulses every 10 (soleus) or 20 min (EDL).

Quantification of \( G_m \) dynamics and statistics
In our companion paper (Pedersen et al., 2009), the biphasic dynamics of \( G_m \) in AP-firing fast-twitch fibers was well described by Eqs. 1 and 2. This approach for quantification was also used in the present study.

\[
G_{m,Phase1} = G_{m5} + A_1(1 - \exp(-t/\tau_1))
\]

\[
G_{m,Phase2} = G_{m5} + A_1 + \frac{A_2}{1 + \exp((-t/\beta))}
\]

Thus, Phase 1 was described by a single-exponential function containing information on the amplitude of the reduction in \( G_m \) during Phase 1, \( A_1 \), and how fast this reduction occurred, \( \tau_1 \), Phase 2 was described by a sigmoidal function that quantified the magnitude of the rise in \( G_m \) during Phase 2, \( A_2 \), when \( A_1 \) rose, \( \tau_2 \), and how fast \( G_m \) rose, \( \beta \), during Phase 2. The dynamics of \( G_m \) in soleus fibers was reminiscent of Phase 1 in EDL fibers, and it was well quantified by Eq. 1. Statistical analysis of data was performed as described in Pedersen et al. (2009).

Chemicals and isotope
All chemicals were of analytical grade. BTS and blebbistatin were from Toronto Research Chemicals Inc., and GF109203X (bisindolylmaleimide I), G6993, PdBu, and dantrolene were from Sigma-Aldrich. BCECF-AM was from Invitrogen. \(^{86}\)Rb\(^+\) (1,590 GBq mmol\(^{-1}\)) was from PerkinElmer.
RESULTS

Comparison of $G_{m}$ dynamics in AP-firing fast- and slow-twitch fibers

In the initial series of experiments, $G_{m}$ dynamics were determined in AP-firing muscle fibers from fast-twitch EDL or slow-twitch soleus muscles. Fig. 1 (B and C) shows representative recordings of the membrane potential from an EDL fiber (Fig. 1 B) and from a soleus fiber (Fig. 1 C) during experiments in which the fibers were activated to fire 3.5-s AP trains at 15 Hz repeatedly every 7 s. In the EDL fiber, the onset of AP firing was associated with an increased $\Delta V$, and when AP firing was continued beyond $\sim$1,800 APs, $\Delta V$ suddenly dropped to less than half of its value before AP firing. These observations in the EDL fiber reflect the biphasic dynamics of $G_{m}$ in AP-firing EDL fibers that we presented in the companion paper (Pedersen et al., 2009). In the soleus fiber, the onset of AP firing was similarly associated with increased $\Delta V$ but, in marked contrast to the observations in the EDL fiber, the drop in $\Delta V$ never appeared, even after $>15,000$ APs. Such experimental observations of $\Delta V$ in AP-firing fibers were next converted to $G_{m}$ using Eq. 3 from Pedersen et al. (2009). Fig. 1 D shows the dynamics of $G_{m}$ in AP-firing soleus fibers that were stimulated to fire AP trains of either 15 or 30 Hz, and Fig. 1 E shows the dynamics of $G_{m}$ in AP-firing soleus fibers that were stimulated to fire AP trains of either 15 or 30 Hz, and Fig. 1 E shows the average $G_{m}$ from EDL fibers that were stimulated to fire AP trains of 15 or 30 Hz, respectively. The magnitude of the $G_{m}$ reductions, $A_{1}$, reached 45–70% and 43–55% of $G_{m}$ at the start of the experiments in soleus and EDL fibers, respectively, whereas the time required for the development of the reductions, $\tau_{1}$, was substantially longer in the slow-twitch fibers ($60 \pm 9$ and $76 \pm 9$ s at 15 and 30 Hz, respectively; Table II) than in the fast-twitch fibers ($32 \pm 4$ and $17 \pm 1$ s at 15 and 30 Hz, respectively; Table II). However, the most striking difference between fast- and slow-twitch muscle fibers was the complete absence of a rise in $G_{m}$ with prolonged AP firing in slow-twitch fibers. Thus, comparing Fig. 1 (D and E) also shows that in clear contrast to the massive rise in $G_{m}$ after prolonged AP firing in EDL fibers (Phase 2), $G_{m}$ remained low throughout all experiments and at all frequencies in the soleus fibers, even if the AP firing was continued for much longer.

Note also that the observations in the EDL fibers presented in Fig. 1 D were obtained using blebbistatin as a myosin II inhibitor. These very closely replicated the observations in our previous study, where BTS rather than blebbistatin was used to inhibit contractile activity during AP firing. Thus, none of the parameters used to quantify Phase 1 ($A_{1}$ and $\tau_{1}$) and Phase 2 ($A_{2}$, $\tau_{2}$, and $\beta$) were substantially different when comparing the effects of blebbistatin and BTS (Table II).

Table II shows the magnitudes of the $G_{m}$ reductions, $A_{1}$, reached 45–70% and 43–55% of $G_{m}$ at the start of the experiments in soleus and EDL fibers, respectively, whereas the time required for the development of the reductions, $\tau_{1}$, was substantially longer in the slow-twitch fibers ($60 \pm 9$ and $76 \pm 9$ s at 15 and 30 Hz, respectively; Table II) than in the fast-twitch fibers ($32 \pm 4$ and $17 \pm 1$ s at 15 and 30 Hz, respectively; Table II). However, the most striking difference between fast- and slow-twitch muscle fibers was the complete absence of a rise in $G_{m}$ with prolonged AP firing in slow-twitch fibers. Thus, comparing Fig. 1 (D and E) also shows that in clear contrast to the massive rise in $G_{m}$ after prolonged AP firing in EDL fibers (Phase 2), $G_{m}$ remained low throughout all experiments and at all frequencies in the soleus fibers, even if the AP firing was continued for much longer.

### Table I

| Muscle fiber type | Group       | Treatment           | $G_{mS}$ (µS/cm²) | $G_{mS,25}$ (µS/cm²) | $G_{KS}$ (µS/cm²) | $G_{KS,127}$ (µS/cm²) | $G_{KS,50}$ (µS/cm²) |
|------------------|-------------|---------------------|-------------------|----------------------|-----------------|----------------------|----------------------|
| EDL*             | 50 µM BTS   | —                   | 1,458 ± 70        | 361 ± 21             | 144 ± 16        | 1,314 ± 72           | 217 ± 26             |
|                 | 25 µM blebbistatin | —                 | 1,317 ± 71        | —                    | —               | —                    | —                    |
|                 | 50 µM BTS 25 µM dantrolene | 1,538 ± 80        | —                    | —                    | —               | —                    | —                    |
|                 | 50 µM BTS 1 µM GF109203X | 1,927 ± 115       | —                    | —                    | —               | —                    | —                    |
|                 | 50 µM BTS Glucose free | 1,622 ± 126       | —                    | —                    | —               | —                    | —                    |
| Soleus           | 25 µM blebbistatin | —                 | 970 ± 61           | 289 ± 29             | 191 ± 13        | 779 ± 62             | 98 ± 26              |
|                 | 25 µM blebbistatin 1 µM GF109203X | 1,306 ± 92       | —                    | —                    | —               | —                    | —                    |

Subscript “S” indicates values for $G_{m}$ before AP firing under the various experimental conditions as determined using the classical technique (Pedersen et al., 2009). $G_{mS,50}$ represents $G_{m}$ before AP firing at 50 mM Cl⁻. $G_{KS}$ and $G_{KS,127}$ represent component conductances of $G_{mS}$ for K⁺ and Cl⁻, respectively, whereas $G_{KS,50}$ represents the component conductance of $G_{mS,50}$ for Cl⁻. All data are presented as means ± SEM.

aIncluded from our companion paper (Pedersen et al., 2009).

Online supplemental material

The online supplemental material contains the outcome from experiments where the effects of blebbistatin on the excitability of EDL and soleus muscles were evaluated. It is available at http://www.jgp.org/cgi/content/full/jgp.200910291/DC1.
The onset of AP firing was associated with reduced $G_m$ in both fast- and slow-twitch fibers, whereas prolonged AP firing induced a massive elevation in $G_m$ only in fast-twitch fibers. For experiments, electrodes were inserted into a fiber at close proximity ($\approx 70 \mu m$). One electrode repeatedly injected a current protocol while the other electrode recorded the membrane potential. (A) Current injection protocol that consisted of low amplitude ($\pm 10–20$ nA) pulses, which were used for $G_m$ determination, and the train of AP trigger pulses (3 ms and 300 nA) that could be delivered at variable frequency, here as 15 Hz. This protocol was repeated without intermittent breaks until the fiber had been exposed to $\approx 5,000$ trigger AP pulses in EDL or up to 15,000 pulses in soleus fibers. (B) Representative recordings of AP trains in a fast-twitch EDL fiber during the first, the 23rd, and the 61st AP train. Note the dropout of APs at the end of the AP train on the right. (C) Representative recordings of AP trains in a slow-twitch soleus fiber during the first, the 23rd, and the 308th AP train. In B and C, the experimental durations and the total number of AP trigger pulses are shown above the AP trains. $G_m$ was calculated from the voltage deflections, $\Delta V$, resulting from the small current pulses between the successive AP trains. The underlined $\Delta V$s have been enlarged below the representative traces. (D) The biphasic dynamics of $G_m$ in AP-firing EDL fibers treated with blebbistatin.
were different between EDL fibers treated with the two types of myosin II inhibitors (Table II).

Ion channels involved in \(G_m\) dynamics in AP-firing slow-twitch fibers

To explore which ion channels that were involved in the reduction in \(G_m\) in AP-firing soleus fibers, \(G_K\) and \(G_{Cl}\) were determined using the approach described in our previous study (Pedersen et al., 2009). This required experimental observation of \(G_m\) dynamics at two extracellular \(\text{Cl}^-\) concentrations (Eq. 4 in Pedersen et al., 2009). Thus, Fig. 2 A shows the dynamics of \(G_m\) at 50 and 127 mM \(\text{Cl}^-\) with an AP-firing frequency of 30 Hz. Two major effects of reduced \(\text{Cl}^-\) were apparent. First, the resting membrane conductance before AP firing was reduced substantially (Table I). Second, AP firing was associated with an initial increase in \(G_m\), followed by a slight reduction in \(G_m\), whereas at 127 mM \(\text{Cl}^-\), \(G_m\) became reduced from the onset of AP firing. This indicated that the reduction of \(G_m\) in AP-firing soleus fibers could largely be ascribed to reduced \(G_{Cl}\). This notion was confirmed by calculations of \(G_K\) and \(G_{Cl}\) (Fig. 2 B), which demonstrated that the reduced \(G_m\) in AP-firing soleus fibers reflected the net outcome of a pronounced reduction in \(G_{Cl}\) and a slight increase in \(G_K\). Similar changes in \(G_K\) and \(G_{Cl}\) were observed in soleus fibers stimulated with AP trains of 6 and 60 Hz (not depicted). Because CIC-1 channels are the major \(\text{Cl}^-\) channel in muscle (Koch et al., 1992; Lueck et al., 2007) this shows that, similar to the situation in EDL fibers (Pedersen et al., 2009), the onset of AP firing in soleus fibers leads to CIC-1 channel inhibition and a minor activation of \(K^+\) channels.

Cellular mechanism underlying CIC-1 inhibition at the onset of AP firing

The above experiments showed that inhibition of CIC-1 channels at the onset of AP firing was common to both fiber types. It has previously been demonstrated experimentally that the resting membrane conductance for \(\text{Cl}^-\) can be reduced in muscle fibers either by acidosis (Hutter and Warner, 1967; Pedersen et al., 2004, 2005) or by activation of PKC (Tricarico et al., 1991; Rosenbohm et al., 1999; Pierno et al., 2007; Dutka et al., 2008).

First, to explore for a role of PKC in the CIC-1 channel inhibition at the onset of AP firing, experiments were conducted in which the muscles were pretreated with a PKC inhibitor, GF109203X (Toullec et al., 1991). Under such conditions, the reduction in \(G_m\) at the onset of AP firing was largely abolished in both fiber types. Thus, Fig. 3 A shows observations from an EDL muscle fiber pretreated with the inhibitor during the first, the 22nd, and the 70th AP train. The enlarged recordings of \(\Delta V\) below the AP trains show that the increase in \(\Delta V\) that was usually observed at the onset of AP firing was completely abolished by the inhibitor, whereas the drop in \(\Delta V\) during prolonged AP firing was still present with PKC inhibition. Fig. 3 B shows that the increase in \(\Delta V\) at the onset of AP firing could also be abolished by the PKC inhibitor in a soleus fiber. Fig. 3 (C and D) presents the average \(G_m\) with the inhibitor in 11 EDL fibers (Fig. 3 C) and in 5 soleus fibers (Fig. 3 D). In the soleus fibers, the PKC inhibitor completely abolished the reduction in \(G_m\) at the onset of AP firing, whereas in the EDL fibers, a minor reduction of \(G_m\) that reached 81 ± 4% of the \(G_m\) at the start of the experiments in EDL could still be detected with the inhibitor. This reduction in EDL fibers was, however, much less than the reduction of \(G_m\) in control fibers, where it became reduced to 45 ± 4% of its value at the start of the experiment. Furthermore, the reduction in \(G_m\) in the EDL fibers with the PKC inhibitor appeared to occur later than the reduction

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to inhibit myosin II. Fibers were stimulated with AP trains of either 15 or 30 Hz. (E) The \(G_m\) dynamics in AP-firing soleus fibers. Fibers were stimulated with AP trains of 6, 15, 30, or 60 Hz. Average data are presented as mean ± SEM.

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in control fibers and, accordingly, \( G_m \) in the fibers treated with the PKC inhibitor could not be quantified by fitting to Eq. 1. In contrast, to the effect of PKC inhibition on the reduction of \( G_m \) at the onset of AP firing, the PKC-inhibited EDL fibers developed a pronounced increase in \( G_m \) with prolonged AP firing that was very similar to the increased \( G_m \) during Phase 2 in control fibers. In an additional series of experiments with four EDL fibers, which had been pretreated with an alternate PKC inhibitor, \( \text{Gö6976} \), \( G_m \) was only reduced to 79 ± 11% of the \( G_m \) at the start of the experiment during Phase 1, whereas a pronounced increase of \( G_m \) was also observed during Phase 2 in these fibers.

In our previous study (Pedersen et al., 2009), the ClC-1 channel inhibition during Phase 1 in EDL fibers was demonstrated to be independent of whether the APs were triggered using inserted electrodes or whether the APs were triggered via the more physiological approach of motor nerve stimulation. To ensure that PKC activation was also activated during such motor nerve stimulation, whole muscles were stimulated to fire 686 APs, after which the two electrodes were rapidly inserted (<1 min) to monitor the recovery of \( G_m \) after AP firing. Although muscle contractions were clearly visible during the motor nerve stimulation, Fig. 5 E shows that after the AP firing, \( G_m \) was still high and did not display any change throughout 7 min of recovery in the fibers treated with the PKC inhibitor. This contrasted the rise in \( G_m \) that was observed after identical motor nerve stimulation in muscles without the inhibitor (Fig. 3 E).

Second, despite the very clear involvement of PKC in the fast reduction of \( G_m \) at the onset of AP firing (Fig. 3), it was possible that acidosis played a role for activating PKC or was involved in the delayed reduction of \( G_m \) during Phase 1 in the EDL fibers treated with the PKC inhibitor. Thus, to evaluate the role of acidosis for the reduction in \( G_m \) at the onset of AP firing, recordings of \( \text{pHi} \) in whole, field-stimulated BTS-treated EDL muscle was compared with the observed \( G_m \) dynamics during Phase 1. Fig. 4 A shows that the development of \( \text{pHi} \) had similar magnitudes when muscles were stimulated using AP trains of 6, 15, or 30 Hz. Also, the changes in \( \text{pHi} \) appeared to depend on the number of APs fired rather than on the duration of the experiment (Fig. 4 A). Hence, during the first 500 APs, \( \text{pHi} \) either rose a little or remained constant, and then it began to drop. The lowest \( \text{pHi} \) occurred after ~1,500 APs. In Fig. 4 (B–D), the dynamics of \( \text{pHi} \) and \( G_m \) have been plotted against time for observations at 6 (Fig. 4 B), 15 (Fig. 4 C), and 30 Hz (Fig. 4 D). Comparing the dynamics of \( \text{pHi} \) and \( G_m \) shows that although \( \text{pHi} \) did not change markedly during the first 500 APs, this was actually the period when the largest decline in \( G_m \) occurred. This strongly indicated that the PKC-mediated ClC-1 channel inhibition at the onset of AP firing was not triggered by acidification. However, in the EDL fibers exposed to PKC inhibition,
a minor reduction in $G_m$ during the early stages of AP firing was still observed (Fig. 3 C). When the dynamics in pH during AP firing were compared with the dynamics of $G_m$ in the AP-firing fibers treated with the PKC inhibitor, a close temporal correlation was observed (Fig. 3 E). Thus, although the majority of the reduction in $G_m$ at the onset of AP firing was caused by PKC-mediated ClC-1 channel inhibition, the close temporal correlation between the dynamics of pH and $G_m$ in PKC-inhibited EDL fibers indicates that acidification may have imposed a minor inhibitory effect on the ClC-1 channels.

The three groups of PKC isoforms that have thus far been discovered are distinguished by the cellular signals that convey their activation (Steinberg, 2008). Supposedly, the PKC inhibitors GF109203X and Gö6976 have the highest affinity for conventional PKC isoforms (Martiny-Baron et al., 1993), which require the presence of diacylglycerol as well as a rise in the free cytosolic Ca$^{2+}$ for activation (Steinberg, 2008). To test whether the release of Ca$^{2+}$ from the SR could be involved in activating the PKC-mediated ClC-1 channel inhibition, at the onset of AP firing a series of experiments was conducted in EDL fibers in which the Ca$^{2+}$ release channels of the SR had been partially blocked by 25 µM dantrolene. In these fibers, the biphasic $G_m$ dynamics that were usually observed in AP-firing EDL fibers were still observed.
The effect of dantrolene was to slow the ClC-1 channel inhibition during Phase 1 and not to abolish it as occurred with the PKC inhibition.

Cellular mechanism underlying the activation of ClC-1 and KATP channels during Phase 2

An intriguing difference in the $G_m$ dynamics between EDL and soleus fibers was the complete absence of $G_m$ during Phase 1 (Fig. 4 F). However, with dantrolene, the reduction in $G_m$ during Phase 1 was slower and less pronounced. Accordingly, the fitting of the observation to Eqs. 1 and 2 showed that $\tau_1$ of Phase 1, which reflects how fast the PKC-mediated ClC-1 channel inhibition proceeded, was markedly increased with dantrolene, whereas $A_1$, which reflects the magnitude of the reduction of $G_m$, was not significantly affected (Table III). Hence, the primary effect of dantrolene was to slow the ClC-1 channel inhibition during Phase 1 and not to abolish it as occurred with the PKC inhibition.
Phase 2 in soleus fibers (Fig. 1). Our previous study (Pedersen et al., 2009) proposed that Phase 2 arose when the fibers reached a critical level of metabolic depression. This hypothesis is supported by the absence of Phase 2 in soleus fibers (Fig. 1 E) because these fibers have a higher content of mitochondria and a larger oxidative capacity than fast-twitch fibers (Jackman and Willis, 1996; Mogensen and Sahlin, 2005). The absence of elevated $G_m$ with prolonged AP firing does not exclude that $G_m$ can rise in these fibers because in experiments with carbonyl cyanide m-chlorophenylhydrazone, an uncoupler of mitochondria, the input conductance of resting soleus fibers increased immensely (not depicted). Still, to further evaluate a role of the metabolic state in the etiology of Phase 2, two more series of experiments were conducted in EDL fibers.

First, experiments were performed in which 6 min of rest were introduced after every 20th train of AP. The average observations from three such experiments are presented in Fig. 5. These observations show that by introducing resting periods of 6 min, $G_m$ completely recovered after every set of 20 AP trains, and upon resumed AP firing, Phase 1 reappeared. Moreover, when compared with experiments without the intermittent resting periods, the appearance of Phase 2 became substantially delayed.

Second, a series of experiments was conducted with EDL fibers in glucose-free extracellular solution. Fig. 6 A

### Table III

| Muscle fiber type | Group | Myosin II inhibitor | $A_1$ (µS/cm²) | $\tau_1$ (s) | $A_2$ (µS/cm²) | $\tau_2$ (s) | $\beta$ (µS/cm²) | $\beta$ (s) |
|------------------|-------|---------------------|----------------|-------------|----------------|-------------|----------------|-------------|
| EDL 15 Hz ($n=10$) | BTS 25 µM dantrolene | -647 ± 38 | 565 ± 67 | 11,178 ± 2,101 | -2,566 ± 75 | -254 ± 24 |
| EDL 15 Hz ($n=18$) | glucose free | -890 ± 26 | 170 ± 9 | 11,056 ± 1,143 | -2,127 ± 163 | -194 ± 33 |

Experimental observations of $G_m$ in AP-firing EDL fibers with dantrolene or glucose-free conditions. $A_1$, $\tau_1$, and $\beta$ are as in Table II. All data are presented as means ± SEM.

Figure 5. Phase 1 reappears and Phase 2 becomes delayed in EDL fibers when AP firing is interrupted by intermittent resting periods. Experiments were performed as described in Fig. 1, except that for every 20th train of AP, a resting period of 6 min was introduced. Five sets of 20 AP trains were fired in each fiber (I–V). Average $G_m$ in three EDL fibers firing 15-Hz AP trains with intermittent resting periods after every 20th AP train. Recordings of $G_m$ in similar fibers stimulated to fire APs without the intermittent resting periods have been included for comparison. Average data are presented as mean ± SEM.

Figure 6. Glucose-free conditions accelerate the appearance of Phase 2 and enhance the magnitude of the rise in $G_m$ during Phase 2. (A) $G_m$ in 14 EDL fibers firing 15-Hz AP trains in the absence of extracellular glucose. Recordings of $G_m$ under control conditions of 5 mM glucose have been included for comparison. (B) $G_m$ before AP firing ($G_mS$, Table I) and $G_m$ 1 min after firing 5,000 APs in the presence and absence of glucose. Average data are presented as mean ± SEM.
shows that at the onset of AP firing, $G_m$ was reduced to a similar extent in the fibers in glucose-free and control conditions. Indeed, quantification showed that the magnitude of the reduction during Phase 1, $A_1$, was similar in the two situations, and that the time required for this reduction, $\tau_1$, was also similar in the control fibers and in the fibers under glucose-free conditions (Table III). However, with prolonged AP firing, the fibers under glucose-free conditions entered Phase 2 before the control fibers, and $G_m$ became substantially higher during Phase 2 in the fibers under glucose-free conditions than in the control fibers. Thus, quantification of Phase 2

Figure 7. PKC-mediated ClC-1 inhibition at the onset of AP firing reduces the rheobase current in the fibers. (A and B) Under control conditions, the recovery of $G_m$ after its initial reduction at the onset of AP firing lasted $\sim 5$ min in both EDL (A) and soleus fibers (B). Recordings of $G_m$ in AP-firing fibers with the PKC inhibitor showed that $G_m$ was not reduced at the onset of AP firing, and no change in $G_m$ was observed during the recovery period. This was exploited to examine the role of PKC-mediated ClC-1 channel inhibition for the excitability of the fibers as evaluated by their rheobase current. (C) Membrane potential recordings in a control EDL fiber in which the rheobase current was measured before and 1 min after firing 784 APs. (D) Membrane potential recordings before AP firing and 1 min after firing 784 APs in an EDL fiber treated with PKC inhibitor (1 µM GF109203X). (E) Average rheobase currents in the two fiber types before and 1 min after AP firing in the presence or absence of the PKC inhibitor. (F) Average resting membrane potential before and 1 min after AP firing in the EDL and soleus fibers. Average data are presented as mean ± SEM.
with Eq. 2 showed that under glucose-free conditions, Phase 2 arose faster ($\tau_2$ was $2,417 \pm 127$ APs and $2,127 \pm 163$ APs in control and in glucose-free conditions, respectively; $P < 0.05$) and reached a higher level than the in control fibers ($A_2$ was $5,426 \pm 494$ $\mu$S/cm$^2$ and $11,056 \pm 1,143$ $\mu$S/cm$^2$ in control and in glucose-free conditions, respectively; $P < 0.05$). Additionally, after 1 min of recovery from firing 4,998 APs, $G_m$ only recovered partly in the absence of glucose, whereas a more complete recovery of $G_m$ was observed in control conditions (Fig. 6 B).

**Effect of $G_m$ dynamics for the excitability of muscle**

The physiological importance of the PKC-mediated ClC-1 channel inhibition for muscle excitability was next considered using two different experimental approaches.

First, the effect of PKC inhibition on muscle excitability was evaluated. Fig. 7 (A and B) demonstrates that if AP firing was ceased during the initial reduction in $G_m$, it lasted ~5 min for full recovery of $G_m$ to be reached in both fiber types under control conditions. In contrast, neither EDL fibers nor soleus fibers showed any change in $G_m$ during AP firing or during the subsequent recovery period if the fibers were pretreated with the PKC inhibitor. This slow recovery from the PKC inhibition of ClC-1 channels during Phase 1 in the control fibers was exploited to evaluate the role of ClC-1 channel inhibition for the fiber excitability by comparing the rheobase currents before and 1 min after AP firing in control fibers and in fibers with the PKC inhibitor: Fig. 7 C shows experimental recordings from a representative EDL fiber under control conditions, whereas Fig. 7 D shows recordings from a representative EDL fiber exposed to the PKC inhibitor. These recordings illustrate that when the rheobase current was assessed 1 min after AP firing, a marked reduction, indicative of increased excitability, was observed under control conditions. In contrast, in the fiber with the PKC inhibitor, the rheobase was substantially elevated after AP firing. Fig. 7 E shows the average rheobase currents before and after AP firing under control conditions and with PKC inhibition in both EDL and soleus fibers. In both fiber types, the rheobase was reduced after the short duration of AP firing under control conditions, whereas increased rheobase current was observed if PKC was inhibited. Note that similar changes in the resting membrane potential were observed after AP firing in the absence and presence of the PKC inhibitor in the two muscle fiber types (Fig. 7 F).

Second, the effect of pharmacological PKC activation for muscle fiber excitability was assessed in muscles at elevated extracellular $K^+$, which repeatedly has been used to experimentally compromise muscle excitability and contractile force (Nielsen et al., 2001; Pedersen et al., 2005). Fig. 8 shows representative recordings of M-waves (Fig. 8 A) and tetanic force (Fig. 8 B) from an intact soleus muscle when stimulated every 10 min with a 2-s long 60-Hz train. When extracellular $K^+$ was elevated from 4 to 10 mM, a pronounced decline in both M-waves (Fig. 8 A) and force (Fig. 8 B) took place. After 1 h at elevated extracellular $K^+$, PKC was activated by the addition of 5 µM of PKC activator, Pbdu, which resulted in a pronounced recovery of both the excitability (M-waves) and force. In four similar experiments, the

![Figure 8](image-url)
addition of Pbdu to muscles at 10 mM K⁺ caused the force to increase from 43 ± 5 to 76 ± 12% of the control force at 4 mM K⁺. Similar results were obtained in two EDL muscles at 13 mM K⁺ (not depicted).

**DISCUSSION**

This study demonstrates that in active, AP-firing skeletal muscle, the ion channels that determine 
Gm undergo substantial regulation in both fast- and slow-twitch muscle fibers. We show that in both fiber types, ClC-1 channels are rapidly inhibited at the onset of AP firing via a PKC-dependent pathway that becomes activated via AP-mediated SR Ca²⁺ release. Such ClC-1 channel inhibition increases the excitability of the fibers. We further show that the synchronous openings of ClC-1 and KATP channels with prolonged AP firing in EDL fast-twitch fibers do not occur in slow-twitch soleus fibers. We propose that a significant reduction in the metabolic state of the fibers may induce such ion channel activations in fast-twitch fibers and thereby support recent suggestions that ClC-1 channels are sensitive to the metabolic state of muscle fibers (Bennetts et al., 2005; Tseng et al., 2007; Zhang et al., 2008).

**Increase in Gm after prolonged AP firing only occurs in fast-twitch fibers**

In slow-twitch fibers, the onset of AP firing was associated with a reduction in Gm that, despite developing at a slower rate, was reminiscent of the ClC-1 channel inhibition that caused a reduction in Gm during Phase 1 in the fast-twitch fibers (Pedersen et al., 2009). Further support for this notion was provided by the observation that in both fiber types, the reduction in Gm at the onset of AP firing primarily reflected a pronounced reduction in GCl. Because the ClC-1 channel is generally considered to be the main Cl⁻ channel in muscle (Koch et al., 1992), and because GCl was reduced by as much as 70–80% at the onset of AP firing, reduced GCl must have also reflected ClC-1 channel inhibition in soleus fibers. Collectively, these observations show that the onset of muscle activity triggers a pronounced reduction in GCl that reflects underlying inhibition of ClC-1 channels in both fast- and slow-twitch muscle fibers.

In marked contrast to the similar Gm dynamics in the two fiber types during early stages of muscle activity, the two fiber types displayed strikingly different Gm dynamics after prolonged AP firing. Thus, in contrast to the development of a very large Gm after firing more than ~1,800 APs in EDL fibers, Gm in slow-twitch fibers remained low even after firing as many as 15,000 APs. If Phase 2 reflected a reduced metabolic state of the EDL fibers, as it will be argued below, it can be speculated that Phase 2 did not appear in slow-twitch fibers because these fibers have a larger oxidative capacity and hence would have a better preservation of their energetic state during repeated AP firing. Indeed, when the cellular metabolic state is evaluated after intensive contractions in muscles containing both fast- and slow-twitch fibers, the reduction in ATP and elevation in IMP are much more pronounced in the fast-twitch fibers as compared with slow-twitch fibers (Esbjörnsson-Liljedahl et al., 1999).

**SR Ca²⁺ release triggers PKC-mediated ClC-1 channel inhibition at the onset of AP firing**

Previous studies have demonstrated that ClC-1 channels can be inhibited by acidification and via intracellular pathways involving PKC (Hutter and Warner, 1967; Tricarico et al., 1991; Rosenbohm et al., 1999; Pedersen et al., 2004, 2005; Pietro et al., 2007; Dutka et al., 2008). Hence, to explore for a role of pH in ClC-1 channel inhibition at the onset of AP firing, the dynamics of pH in whole EDL muscle were measured during field stimulation and compared with the corresponding Gm dynamics. This showed that the majority of the ClC-1 channel inhibition occurred while the fibers were in an alkaline state, presumably caused by breakdown of creatine phosphate. Hence, the rapid ClC-1 channel inhibition at the onset of AP firing was not caused by acidification. In contrast, in both muscle fibers, the initial inhibition of ClC-1 channels during AP firing was largely abolished by PKC inhibition. The ClC-1 channel inhibition could also be partly prevented by blockage of SR Ca²⁺ channels with dantrolene. This indicates that Ca²⁺ release from SR during AP firing was important for activation of PKC. Such interactions between increased cytosolic Ca²⁺ and PKC for ClC-1 channel inhibition implicate the conventional PKC isofrom in the inhibition, and this is supported by a recent study, which suggests that statin treatment of rats causes a chronic elevation in cytosolic Ca²⁺ that leads to PKC-mediated ClC-1 channel inhibition and hyperexcitability (Pietro et al., 2009). Because the reduction in Gm at the early stages of AP firing was well described by a single-exponential function in both muscles, it is likely that there was one rate-limiting step in the ClC-1 channel inhibition. Because dantrolene slowed down the process of ClC-1 inhibition at the onset of AP firing, it can be speculated that the rise in cytosolic Ca²⁺ represents this rate-limiting step in PKC-mediated ClC-1 inhibition in active muscle. Our observations demonstrate that the modulation of ClC-1 channels by statins (Pietro et al., 2009) that act via an elevated cytosolic Ca²⁺ reflects a physiological mechanism for inhibition of ClC-1 channels that occurs in active muscles. Thus, as summarized in Fig. 9, our findings demonstrate that during muscle activity, SR Ca²⁺ release triggered by AP firing activates PKC, which then inhibits the ClC-1 channels causing Gm to drop.
were incubated under glucose-free conditions. These fibers were likely to have a moderately reduced metabolic state even before the AP firing and consequently would reach the level of metabolic depression required for the induction of Phase 2 faster than control fibers. Third, when resting periods of 6 min were introduced after every 20th train of AP, the appearance of Phase 2 was substantially postponed. This suggests that by introducing resting periods, the fibers were recovering their metabolic state in the resting periods sufficiently to delay the onset of Phase 2. Collectively, Phase 2 was accelerated if the muscles were exposed to either glucose-free conditions or prestimulated with field stimulation (Fig. S3 in Pedersen et al., 2009), it was delayed when AP trains had the lowest frequency (6 Hz) and when intermittent resting periods were introduced during the AP firing, and, finally, it was completely absent when explored in soleus fibers that have a larger oxidative capacity and show a better maintenance of the energetic state during activity (Esbjörnsson-Liljedahl et al., 1999). These findings can all be explained by the metabolic state of the fibers being a key determinant for the induction of Phase 2.

Functional significance of $G_m$ dynamics for muscle function

In both fiber types, full recovery from ClC-1 channel inhibition was observed after $\sim$5 min. It is interesting that this was substantially longer than the time required for recovery of $G_m$ after Phase 2 in the EDL fibers. This indicated that in fast-twitch fibers, the PKC-mediated inhibition of ClC-1 channels during Phase 1 disappeared once Phase 2 was initiated. The role of PKC for ClC-1 channel inhibition also implies that some proteins, possibly the ClC-1 channels, became phosphorylated. Thus, as it will be argued below, if Phase 2 reflected a substantial reduction in the metabolic state of the fibers, it is possible that dephosphorylation becomes predominant rather than PKC-mediated phosphorylation, when the ATP levels have declined substantially.

Further evidence of reduced metabolic state being the trigger for Phase 2

In our companion paper (Pedersen et al., 2009), it was demonstrated that the elevated $G_m$ during Phase 2 in fast-twitch fibers reflected the opening of ClC-1 and $K_{ATP}$ channels. In that study, it was hypothesized that the opening of these channels during Phase 2 reflected a substantial reduction in the metabolic state of the fibers. Three lines of evidence in favor of this hypothesis have been shown in the present study. First, the absence of Phase 2 in slow-twitch fibers supports the hypothesis that Phase 2 occurred in fast-twitch fibers when their metabolic state reached a critical level of depression. This support comes from the observations that slow-twitch fibers show less reduction in their cellular energetic state during prolonged contractile activity when compared with fast-twitch fibers (Esbjörnsson-Liljedahl et al., 1999). Second, a role of the metabolic state of the fibers for the induction of Phase 2 was implied by the early appearance and larger magnitude of Phase 2 in EDL fibers that were incubated under glucose-free conditions. These fibers were likely to have a moderately reduced metabolic state even before the AP firing and consequently would reach the level of metabolic depression required for the induction of Phase 2 faster than control fibers. Third, when resting periods of 6 min were introduced after every 20th train of AP, the appearance of Phase 2 was substantially postponed. This suggests that by introducing resting periods, the fibers were recovering their metabolic state in the resting periods sufficiently to delay the onset of Phase 2. Collectively, Phase 2 was accelerated if the muscles were exposed to either glucose-free conditions or prestimulated with field stimulation (Fig. S3 in Pedersen et al., 2009), it was delayed when AP trains had the lowest frequency (6 Hz) and when intermittent resting periods were introduced during the AP firing, and, finally, it was completely absent when explored in soleus fibers that have a larger oxidative capacity and show a better maintenance of the energetic state during activity (Esbjörnsson-Liljedahl et al., 1999). These findings can all be explained by the metabolic state of the fibers being a key determinant for the induction of Phase 2.

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Functional significance of $G_m$ dynamics for muscle function

Generally, the resting membrane conductance of muscle fibers and their excitability appear to be inversely related. Accordingly, the PKC-mediated ClC-1 channel inhibition at the onset of AP firing was associated with enhanced muscle fiber excitability, as indicated by a reduction in rheobase current after short-duration AP firing. Note that this effect could not be related to changes in the resting membrane potential because this was not affected by the PKC inhibitor before or after AP firing. Also, in experiments where the muscle excitability
was initially depressed by exposing whole muscles to elevated extracellular K⁺. PKC activation with a phorbol ester markedly recovered the excitability. Using a similar experimental approach of elevated extracellular K⁺, we have previously shown that ClC-1 channel inhibition either via acidification or directly by the addition of 9-AC can recover muscle excitability in both fast- and slow-twitch muscles (Nielsen et al., 2001; Pedersen et al., 2004, 2005). Thus, the addition of the phorbol ester, which is known to reduce the resting G_Cl in rat muscle (Tricario et al., 1991), is likely to have mediated the recovery of excitability and force in K⁺ depressed via inhibition of ClC-1 channels, although other mechanisms may contribute.

Collectively, these observations suggest a scenario in which at the onset of muscle activity, AP-mediated SR Ca²⁺ release leads to an increased PKC activity, which in turn inhibits the ClC-1 channels and thereby enhances the excitability of the working muscle (Fig. 9). Conversely, the openings of ClC-1 and K_ATP channels during Phase 2, which was exclusive for fast-twitch fibers, resulted in increased G_m and dropout of APs (Fig. 9). It can be speculated that such a mechanism may contribute to the larger fatigability of fast-twitch fibers, and could serve as a protective mechanism that, by hindering prolonged muscle activation, limits metabolic depletion and excessive fiber damage during exercise. This notion is supported by observations of severe muscle damage in K_ATP channel knockout mice after treadmill running (Thabet et al., 2005; Cifelli et al., 2007).

**Conclusion**

In combination with our previous study (Pedersen et al., 2009), our findings demonstrate for the first time that acute regulation of G_m is a general phenomenon in active muscle. Our studies also show that this G_m regulation results from changes in the functions of ClC-1 and K_ATP channels and, thereby, reveal new physiological aspects on ClC-1 and K_ATP channels in skeletal muscles. In so doing, our observations complement several previous studies that have focused on specific pathways for ion channel regulation. Our approach allows for the determination of whether and when G_m is regulated in AP-firing fibers and which cellular signaling pathways that are involved. Because the observed changes in G_m had significant effects on muscle fiber excitability, our observations indicate that the excitability of skeletal muscles is far more dynamically regulated during muscle activity than previously expected.

Furthermore, the regulation of G_m differs significantly between fast- and slow-twitch fibers, with fast-twitch fibers being much more prone to a regulated reduction in excitability via massively elevated G_m. This fits with slow-twitch fibers often being involved in low intensity but prolonged contractile activity in the intact organism, whereas fast-twitch fibers usually are involved in intensive contractions of short duration. As such, the observed regulation of G_m may contribute to these well-established differences in the phenotypes of fast- and slow-twitch fibers.

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Comparison of regulated passive membrane conductance in action potential–firing fast- and slow-twitch muscle

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Please note that Frank Vincenzo de Paoli’s name appeared incorrectly in the author line. The html and pdf versions have been corrected.
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Please note that in the original Fig. 2 B, the circles were mislabeled. The data for $G_K$ are indicated by filled circles, whereas the data for $G_{Cl}$ are indicated by open circles. The correct figure appears below.

**Figure 2.** Component conductances, $G_K$ and $G_{Cl}$, in slow-twitch soleus fibers firing APs. From experiments conducted at two concentrations of extracellular $Cl^-$, it was possible to extract $G_K$ and $G_{Cl}$ using Eq. 4 from Pedersen et al. (2009). (A) $G_K$ dynamics at 50 and 127 mM $Cl^-$ obtained using AP trains of 30 Hz. (B) Calculated $G_K$ and $G_{Cl}$. Average data are presented as mean ± SEM.