Correction

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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.
Regulation of ClC-1 and \( K_{ATP} \) channels in action potential–firing fast-twitch muscle fibers

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Action potential (AP) excitation requires a transient dominance of depolarizing membrane currents over the repolarizing membrane currents that stabilize the resting membrane potential. Such stabilizing currents, in turn, depend on passive membrane conductance \( (G_m) \), which in skeletal muscle fibers covers membrane conductances for \( K^- \) \( (G_K) \) and \( Cl^- \) \( (G_{Cl}) \). Myotonic disorders and studies with metabolically poisoned muscle have revealed capacities of \( G_K \) and \( G_{Cl} \) to inversely interfere with muscle excitability. However, whether regulation of \( G_K \) and \( G_{Cl} \) occur in AP-firing muscle under normal physiological conditions is unknown. This study establishes a technique that allows the determination of \( G_{Cl} \) and \( G_K \) with a temporal resolution of seconds in AP-firing muscle fibers. With this approach, we have identified and quantified a biphasic regulation of \( G_m \) in active fast-twitch extensor digitorum longus fibers of the rat. Thus, at the onset of AP firing, a reduction in \( G_{Cl} \) of \( \sim 70\% \) caused \( G_m \) to decline by \( \sim 55\% \) in a manner that is well described by a single exponential function characterized by a time constant of \( \sim 200 \) APs (phase 1). When stimulation was continued beyond \( \sim 1,800 \) APs, synchronized elevations in \( G_K \) \( (\sim 14\text{-fold}) \) and \( G_{Cl} \) \( (\sim 3\text{-fold}) \) caused \( G_m \) to rise sigmoidally to \( \sim 400\% \) of its level before AP firing (phase 2). Phase 2 was often associated with a failure to excite APs. When AP firing was ceased during phase 2, \( G_m \) recovered to its level before AP firing in \( \sim 1 \) min. Experiments with glibenclamide \( (K_{ATP} \text{ channel inhibitor}) \) and 9-anthracene carboxylic acid \( (\text{ClC-1 Cl}^- \text{ channel inhibitor}) \) revealed that the decreased \( G_{Cl} \) during phase 1 reflected ClC-1 channel inhibition, whereas the massively elevated \( G_K \) during phase 2 reflected synchronized openings of ClC-1 and \( K_{ATP} \) channels. In conclusion, \( G_{Cl} \) and \( G_K \) are acutely regulated in AP-firing fast-twitch muscle fibers. Such regulation may contribute to the physiological control of excitability in active muscle.

**INTRODUCTION**

Excitation and propagation of action potentials (APs) are the hallmarks of excitable cells. Both of these cellular processes require that excitatory currents exceed the inhibitory currents that dampen cellular excitability via their stabilizing actions on the resting membrane potential. The magnitude of the inhibitory currents can be inferred from measurements of passive membrane conductance \( (G_m) \), which reflect the function of the ion channels that are open at the resting membrane potential. In resting skeletal muscle fibers, \( G_m \) represents the sum of conductances for \( K^- \) \( (G_K) \) and \( Cl^- \) \( (G_{Cl}) \), the latter accounting for \( 80\text{-}90\% \) of \( G_m \) (Hutter and Noble, 1960; Bryant and Morales-Aguilera, 1971; Pedersen et al., 2005; Pierno et al., 2007). The roles of \( G_K \) and \( G_{Cl} \) for skeletal muscle excitability can be illustrated by considering two extreme conditions: at one extreme, loss of function mutations of the muscle-specific \( Cl^- \) channel, the ClC-1 channel, is the cause of greatly reduced \( G_m \) and hyperexcitable muscles in patients suffering from myotonia congenita (McComas and Mrozek, 1968; Bryant and Morales-Aguilera, 1971; Koch et al., 1992; Pusch, 2002). At the other extreme, muscle excitability is completely lost when metabolic poisoning was used to reveal that \( G_{Cl} \) and \( G_K \) have the capacity to increase \( \sim 14\text{-fold} \) and \( \sim 110\text{-fold} \), respectively (Fink and Lütgau, 1976). Although these extreme conditions reflect pathological or unphysiological conditions, they do demonstrate that regulation of \( Cl^- \) and \( K^- \) channels can cause very large changes in \( G_m \) of skeletal muscle fibers and have dramatic consequences for muscle excitability. However, it is not known whether this potential of \( Cl^- \) and \( K^- \) channels to modulate muscle excitability via \( G_m \) is exploited in the normal physiological regulation of the excitability of active AP-firing muscle fibers. This lack of knowledge about regulation of \( G_m \) in active muscles is likely to be ascribable mainly to the experimental difficulties associated with measuring \( G_m \) while muscle fibers are firing APs.

In this study, we establish a new technique that allows the determination of \( G_{Cl} \) and \( G_K \) with a temporal resolution of seconds in AP-firing muscle fibers. This approach is used to demonstrate that \( G_{Cl} \) and \( G_K \) are both highly dynamic in fast-twitch extensor digitorum longus (EDL).
mature fibers firing APs and, furthermore, that these changes in \( G_m \) and \( G_k \) are caused by regulation of ClC-1 and \( k_{\text{ATP}} \) ion channels. This study is followed by a companion study (see Pedersen et al. in this issue) that compares the highly dynamic nature of \( G_m \) and \( G_k \) in fast-twitch muscle fibers with the dynamics of \( G_m \) and \( G_k \) in fatigue-resistant slow-twitch muscle fibers.

**MATERIALS AND METHODS**

**Animal handling and muscle preparation**

All experiments were performed on isolated EDL muscles from 12- to 14-wk-old female wistar rats (~290 g). Handling and killing of animals followed Danish animal welfare regulations. Most experiments were performed using standard Krebs-Ringer bicarbonate solution containing 122 mM NaCl, 25 mM NaHCO\(_3\), 2.8 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 1.3 mM CaCl\(_2\), and 5.0 mM \( \alpha \)-glucose. Total buffer \( [\text{Cl}^-] \) was 127.4 mM (127 mM). In Cl\(^-\)-free solutions, methylsulphate salts replaced NaCl and KCl. Ca(NO\(_3\))\(_2\) was used to replace CaCl\(_2\). Solutions with lower Cl\(^-\) concentration were prepared by mixing standard and Cl\(^-\)-free solutions. All solutions were maintained equilibrated with 5-95% of CO\(_2\)/O\(_2\), pH 7.2-7.4, throughout experiments. All experiments were performed at 30°C.

**Electrophysiological technique**

Electrophysiological investigations were performed on superficial fibers of intact muscles using a previously described setup (Macdonald et al., 2005; Pedersen et al., 2005). To measure resting membrane conductance before \( (G_m) \) and in between AP trains \( (G_m) \), two electrodes were inserted into the same fiber. One electrode was used to inject square current pulses, and the other electrode recorded the membrane potential. The protocols for injections of current pulses and the data sampling were controlled using Signal 2.09 software (Cambridge Electronic Design). Recordings were low-pass filtered at 10 kHz and sampled at 60 kHz. Electrodes were filled with 2 M K-citrate except for a few experiments in which 3 M KCl or 770 mM K-methanesulphonate was used to check for any influence on the results of the citrate ion. No effects of citrate except more stable resting membrane potentials were observed. Holding currents were not used in any of the experiments. Intracellular measurements of AP trains were achieved without interference from contractile activity by including 50 \( \mu \)M \( \alpha \)-benzyl-\( \beta \)-toluene sulphamidone (BTS) in the perfusion solution. BTS is a specific inhibitor of fast-twitch myosin II (Cheung et al., 2002) that does not markedly affect the excitability of the muscle fibers (Macdonald et al., 2005). Importantly, it has been shown that despite the reduction in contractile force, BTS only reduces muscle energy turnover by ~20% (Zhang et al., 2006). Fibers in which the resting membrane potential depolarized beyond -60 mV during electrophysiological assessments were disregarded.

**Determination of the resting membrane conductance before AP firing, \( G_m \)**

To calculate \( G_m \) in AP-firing fibers, it was necessary to know its value before AP firing \( (G_{m0}) \). This constant was measured in resting fibers using the classical technique for \( G_m \) determination in skeletal muscle fibers (Boyd and Martin, 1959; Pedersen et al., 2005) that relies on measurements of the membrane potential deflections \( (\Delta V) \) in response to the injection of square current pulses at three to five locations along the muscle fibers (Fig. 1 A; Hodgkin and Rushton, 1946). After plotting the steady-state amplitudes of these deflections as a function of the interelectrode distance \( (x) \) (Fig. 1 B), the input conductance \( (G_m) \) and the length constant \( (\lambda) \) of the fibers can be extracted by fitting the data to a two-parameter exponentially decaying function \( (r^2 \geq 0.99; \text{Eq. 1}) \),

\[
\Delta V(x) = \Delta V_{i=0} \exp(-x\lambda^{-1}) \\
\text{and} \quad \Delta V(x) = I_{V_{i=0}} \exp(-x\lambda^{-1}),
\]

where \( \Delta V_{i=0} \) represents the steady-state membrane potential deflection at the point of current injection \( (x = 0) \) and \( I \) represents the amplitude of the square current. \( G_m \) could then be calculated by adopting an internal resistivity, \( R_i \), of 180 \( \Omega \)cm from Albuquerque and Thesleff (1968), which they had calculated \( (R_i = \sigma/\pi r) \) on the basis of an optimally measured muscle fiber diameter \( (2\pi) \) and a measured intracellular resistance \( (r) \) that was similar to our observations:

\[
G_m = \lambda^{-1.5} \frac{G_m}{\sqrt{8\pi R_i}} \quad \text{(2)}
\]

Table I provides the \( G_m \) values for the different experimental conditions used in this study.

**Determination of \( G_m \) between successive AP trains from measurements of \( G_m \)**

The aim of this study was to determine changes in \( G_m \) in muscle fibers that were being activated intermittently to fire trains of APs. Clearly, the aforementioned classical technique for the determination of \( G_m \) in muscle fibers is too slow to allow \( G_m \) measurements in AP-firing skeletal muscle fibers. Instead, the two intracellular microelectrodes were inserted at close proximity \( (~70 \mu \text{m}) \), whereby direct measurements of \( G_m \) became possible in between AP trains. The following equation was then used to relate \( G_m \) to \( G_m \) (Appendix 1 in supplemental text):

\[
G_m = \frac{G_m^2}{G_m^2 - G_{m0}^2} \quad \text{(3)}
\]

\( G_{m0} \) refers to the input conductance of the investigated fiber before AP firing, and \( G_m \) represents the input conductance measured in between the AP trains. \( G_{m0} \) represents the mean resting membrane conductance measured in a subset of fibers that were not exposed to AP firing but kept under the same conditions as the fibers in which \( G_m \) was determined between the AP trains (Table I). The \( G_{m0} \) values for the different experimental conditions were obtained using the aforementioned classical technique and are presented in Table I (also see Fig. 1). Thus, \( G_m \) is the calculated membrane conductance between the AP trains for the fiber in question based on measurements of its \( G_{m0} \) and measurements of \( G_{m0} \) in other fibers under similar conditions using the classical technique. Essentially, Eq. 3 provides a way to extract absolute values of \( G_m \) from \( G_{m0} \) obtained during muscle activity, whereas non-S subscripts indicate
mean conductances determined in AP-firing muscle fibers at either 50 or 127 mM Cl\(^-\). \(G_{\text{Cl}}\) was subsequently determined by subtracting \(G_K\) from \(G_m\) at 127 mM Cl\(^-\).

Chemicals
All chemicals were of analytical grade. BTS was obtained from Toronto Research Chemicals, whereas all other chemicals were purchased from Sigma-Aldrich. Glibenclamide, BTS, and 9-anthracene carboxylic acid (9-AC) were dissolved in DMSO. The maximal concentration of DMSO in experimental solutions was 0.15%, which did not affect resting conditions in muscles. Muscles were incubated for at least 15 min with drugs before experiments were conducted.

Statistics
All mean data are presented as means ± SEM. Statistical significance between two data groups was ascertained using a Student’s two-tailed \(t\) test for unpaired observations. Fisher’s exact test was used for two categorical data groups. One-way ANOVA was used for comparisons of more than two data groups. Tukey’s posthoc test was used to detect a significant difference between individual data groups. \(P < 0.05\) was considered significant.

Online supplemental material
Part I of the supplemental text contains detailed derivations of Eqs. 3 and 4. Part II investigates the influence of the possible volume increase of muscle fibers or changes in \(R_i\) during AP firing for \(G_m\) determinations. Part III presents results from experiments in which the dynamics of \(G_m\) were elicited in AP-firing muscle fibers without inserted electrodes. These are compared with observations of \(G_m\) dynamics with inserted electrodes. Fig. S1 shows the estimated effect of muscle fiber swelling on \(G_m\) during AP firing. Fig. S2 shows that the regulation of ion channels underlying phase 1 is independent of inserted electrodes. Fig. S3 shows that the cellular mechanisms that triggered phase 2 were activated by APs independently of inserted electrodes. Online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.200910291/DC1.

**Experiment data (Table 1)**

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>(G_{\text{Na}}) (\mu\text{S/cm}^2)</th>
<th>(G_{\text{Na50}}) (\mu\text{S/cm}^2)</th>
<th>(G_K) (\mu\text{S/cm}^2)</th>
<th>(G_{\text{Cl}}) (\mu\text{S/cm}^2)</th>
<th>(G_{\text{Cl50}}) (\mu\text{S/cm}^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µM BTS</td>
<td>1.458 ± 70 ((n = 26/7))</td>
<td>361 ± 21 ((n = 15/4))</td>
<td>144 ± 16 ((n = 13/6))</td>
<td>1.314 ± 72 (217 ± 6)</td>
<td></td>
</tr>
<tr>
<td>50 µM BTS + 10 µM glibenclamide</td>
<td>1.252 ± 42 ((n = 24/4))</td>
<td>524 ± 27 ((n = 11/2))</td>
<td>195 ± 32 ((n = 6/2))</td>
<td>1.057 ± 53 (329 ± 42)</td>
<td></td>
</tr>
<tr>
<td>50 µM BTS + 100 µM 5-HD</td>
<td>1.609 ± 82 ((n = 14/2))</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>50 µM BTS + 100 µM 9-AC + 0.01 µM TTX</td>
<td>143 ± 13 ((n = 9/2))</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

The subscript S indicates values for \(G_m\) before AP firing under the various experimental conditions as determined using the classical technique (Fig. 1 and Eq. 1). \(G_{\text{Na50}}\) represents \(G_m\) before AP firing at 50 mM Cl\(^-\). \(G_K\) and \(G_{\text{Cl}}\) represent component conductances of \(G_{\text{Na}}\) for \(K^+\) and Cl\(^-\), respectively, whereas \(G_{\text{Cl50}}\) represents the component conductance of \(G_{\text{Na50}}\) for Cl\(^-\). All data have been presented as means ± SEM. \(n\) represents fibers/muscles. NA, not applicable.
RESULTS

When $G_m$ in skeletal muscle fibers is measured using the classical technique, one intracellular microelectrode is used for injection of square current pulses while another electrode records the resulting membrane potential change at several locations along the fiber (Fig. 1 A). From the decay of the steady-state amplitude of such voltage changes along the fiber (Fig. 1 B), $G_m$ and $\lambda$ are extracted and used to calculate $G_m$ (Eq. 2). Although this classical technique is preferable in resting muscle fibers, it usually requires several minutes to extract the experimental observation required for each determination of $G_m$. This makes it highly unsuitable for determinations of fast changes in $G_m$ as might occur in AP-firing muscles. Here, we have overcome this problem by developing a method in which $G_m$ in AP-firing fibers is calculated from $G_{nm}$, which can be measured directly if the electrodes are inserted into the fiber at sufficiently close proximity. In the standard experimental protocol, the current electrode was used to trigger 3.5-s trains of APs repeatedly every 7 s (Fig. 2 A). Currents of small amplitude were injected in between the successive AP trains, and from the ratio of these injected currents and the resulting steady-state deflection in membrane potential ($\Delta V$), $G_m$ was monitored in the AP-firing fibers.

Muscle activity is associated with biphasic $G_m$ dynamics

Fig. 2 (B–D) shows the effect of repeated AP trains on $\Delta V$ in a representative fast-twitch rat EDL muscle fiber, and Fig. 2 F shows enlargements of the underlined $\Delta V$ recordings in Fig. 2 (B–D). Remarkably, AP firing was associated with the pronounced biphasic development of $\Delta V$. Thus, at the onset of AP firing, $\Delta V$ was increased (Fig. 2, compare B with C), but as stimulation continued, a sudden drop in $\Delta V$ to less than half its value before AP firing was observed (Fig. 2, compare B with D). Fig. 2 E shows that when AP firing was stopped for 1 min after the fiber had fired 4,998 APs, $\Delta V$ fully recovered to its level before AP firing.

Next, $G_m$ during AP firing was evaluated from the ratio of injected current and the recorded $\Delta V$ values during the muscle activity. The mean $G_m$ during 18 experiments such as the one presented in Fig. 2 is shown in Fig. 3 A, and in Fig. 3 B the observations of $G_m$ have been used to extract $G_m$ in the AP-firing fibers (Eq. 3). Fig. 3 B shows that the onset of muscle activity led to a pronounced reduction in $G_m$ that was maintained during the initial $\sim$1,800 APs. This phase of $G_m$ regulation during muscle activity will be referred to as phase 1. When AP firing continued, $G_m$ suddenly rose to around 400% of its level before AP firing, marking the beginning of the second phase. This phase of $G_m$ regulation during muscle activity will be referred to as phase 2. If AP firing was stopped after 4,998 APs, at which point the second phase was fully developed, $G_m$ recovered within 1 min (Fig. 3 B). This fast recovery showed that the elevated $G_m$ during phase 2 was not caused by membrane damage. This was further supported by a repolarization from $-69 \pm 1$ to $-76 \pm 1$ mV during the development of phase 2 (18 fibers stimulated at 15 Hz), which would not occur under conditions of unspecific membrane damage (Clausen and Gissel, 2005). If AP firing was resumed after the 1 min of rest, $G_m$ rapidly rose. This indicates that the cellular signaling that triggered phase 2 had not completely recovered in the 1-min resting period (unpublished data).

To further ascertain that the dynamics of $G_m$ observed in the aforementioned experiments (Fig. 3 B) reflected the normal physiological behavior of active AP-firing muscle fibers, a series of experiments was conducted in which the APs were elicited either via motor nerve stimulation or via direct field stimulations. In these experiments, the electrodes were only inserted once the AP firing had ceased, and the recovery of $G_m$ (Fig. S2) or its behavior upon resumed AP firing in the impaled fiber (Fig. S3) was compared with the $G_m$ dynamics observed when all APs were elicited via the inserted electrodes (Fig. 3 A). These experiments confirmed that the $G_m$ dynamics observed when the APs were triggered by the current electrode could also be induced when the whole muscle was stimulated by more physiological stimulation without inserted electrodes (Part III in supplemental text). Also, the derivation of Eq. 3 assumed that the volume of the fiber did not change during the AP firing, which may not be absolutely valid because it is well known that muscles swell during activity (Usher-Smith et al., 2009). However, when the observation of $G_m$ in AP-firing fibers was corrected for volume changes of up to 30%, it was obvious that this could only marginally affect the $G_m$ dynamics (Fig. S1 and Part II of supplemental text).

To quantify and characterize the dynamics of $G_m$ in the AP-firing muscle fibers, the experimental observations were fitted to suitable functions. The dynamics of $G_m$ during phase 1 fit very accurately to a single exponential function,

$$G_{m,\text{Phase 1}} = G_{m3} + A_1(1 - \exp(-t/\tau_1)), \quad (5)$$

whereas the dynamics of $G_m$ during phase 2 were well described by a sigmoidal function,

$$G_{m,\text{Phase 2}} = G_{m3} + A_1 + \frac{A_2}{1 + \exp(-t/\beta)}, \quad (6)$$

Fig. 3 C shows experimental observations of $G_m$ dynamics in a representative fiber and illustrates what was described by the five parameters in the fits ($A_1$, $\tau_1$, $A_2$, $\tau_2$, and $\beta$): phase 1 was characterized by an amplitude, $A_1$, that describes the magnitude of the reduction of $G_m$.
Pedersen et al.

Figure 2. Representative recordings of successive AP trains in a fast-twitch EDL muscle fiber. Two electrodes were inserted in the fiber at close proximity (~70 µm): one electrode was used for injection of square current pulses, and the other electrode recorded the membrane potential. (A) The current injection protocol consisted of low amplitude (~10–20 nA) 75-ms square pulses, which were used for \( G_m \) determinations. These were followed by a train of AP trigger pulses (for 3 ms at 300 nA), here delivered at 15 Hz. The protocol was repeated until 4,998 trigger pulses had been injected. Recovery of \( G_m \) was assessed 1 min after the stimulation ceased. (B–D) Recordings of the membrane potential during the 1st, the 32nd, and the 85th current injection protocol. (E) An AP train 1 min of recovery after the fiber had fired 4,998 APs. (F) Enlargement of the resting membrane voltage deflections (ΔV) that are underlined in B–E. Because \( G_{in} \) and \( G_m \) are inversely related to ΔV, an increase in ΔV reflects reduced \( G_{in} \) and \( G_m \) and vice versa.

during this phase and a time constant, \( \tau_1 \), that quantifies the time or the number of fired APs that was required from the beginning of the experiment to reach 63% of \( A_1 \). Phase 2 was also characterized by an amplitude, \( A_2 \), that describes the magnitude of the rise in \( G_m \) from the steady level during phase 1 to the steady level during phase 2 and by a time constant, \( \tau_2 \), that quantifies the time or the number of fired APs that was required to reach half of \( A_2 \). In addition, phase 2 was described by \( \beta \), which quantifies how rapidly \( G_m \) rose during the development of phase 2. The mean amplitudes and time constants from the 18 fibers summarized in Fig. 3 B are shown in Fig. 3 D. Note that \( A_1 \) is negative because \( G_m \) drops during phase 1, whereas \( A_2 \) is positive because \( G_m \) rises during phase 2. On average, \( G_m \) was reduced by 55 ± 3% during phase 1, whereas during phase 2, \( G_m \) rose by 372 ± 34% of \( G_m \) at the start of the experiment. In Fig. 3 B, the mean of the five parameters obtained by the fits was used to calculate \( G_m \) in AP-firing muscle fibers during the firing of 5,000 APs (solid red line).
Regulation of $G_m$ depends most closely on the total number of APs fired

To examine the importance of the AP firing intensity for the regulation of $G_m$ in AP-firing fibers, the development of $G_m$ was compared in fibers in which the AP firing frequency in the pulse trains was either 6 (Fig. 4 A), 15 (Fig. 4 B), or 30 Hz (Fig. 4 C). At all frequencies, the increase of $\Delta V$ that characterized phase 1 was observed at the onset of AP firing, and with prolonged AP firing, $\Delta V$ suddenly dropped, marking the beginning of phase 2. In Fig. 4 D and E, the measurements of $\Delta V$ have been converted to $G_m$, and the mean $G_m$ for each of the three frequencies has been plotted against either the experimental time (Fig. 4 D) or the number of APs fired (Fig. 4 E). Interestingly, a comparison of Fig. 4 D with Fig. 4 E suggests that $G_m$ dynamics in AP-firing fibers were more closely related to the number of APs elicited than to the experimental duration. To quantify this notion, the experimental observations at all frequencies were fitted to Eqs. 5 and 6, whereby the time constants for phase 1 ($\tau_1$) and phase 2 ($\tau_2$) were obtained both as functions of the number of APs fired and as functions of the experimental duration (Table II). Such quantification revealed that the 400% increase in AP firing frequency (6 to 30 Hz) was associated with 266% and 524% variations in $\tau_1$ and $\tau_2$, respectively, when these parameters were assessed as functions of experimental duration. In contrast, $\tau_1$ and $\tau_2$ only varied by 37 and 25% when assessed as functions of the number of APs fired. This confirmed that the $G_m$ dynamics during phase 1 and phase 2 were most closely related to the number of APs fired and further suggested that the dynamics of $G_m$ were elicited by mechanisms that, with the present experimental protocol, could sense and integrate the discrete
Figure 4. The dynamics of G_m in AP-firing fibers were more closely related to the number of APs fired than to the experimental duration. Experiments were conducted similar to that presented in Fig. 2 except that the frequency of the AP trains was varied. (A–C) Recordings from representative fibers in which the APs were triggered using trains of 6 Hz (A), the usual 15 Hz (B), or 30 Hz (C). Enlargements of the underlined parts in the recordings of the membrane potential have been included below. (D) G_m at the three frequencies have been plotted against the experimental time. At all frequencies, ~5,000 APs were fired, and the fibers were allowed to rest for 1 min before recovery of G_m was assessed. Arrows indicate G_m 1 min after cessation of AP firing. (E) G_m at the three frequencies have been plotted against the number of APs fired. (D and E) Mean data from eight fibers at 6 Hz, 18 fibers at 15 Hz, and 13 fibers at 30 Hz. For clarity of mean data, only every fifth error bar (SEM) has been included.
Experimental observations of $G_m$ in AP-firing fibers were fitted to Eq. 5 and Eq. 6 to obtain parameters that describe phase 1 and phase 2, respectively. $A_1$ describes the reduction in $G_m$ during phase 1, and $\tau_1$ describes either the time required or the number of APs fired before 63% of $A_1$ was observed. $A_2$ describes the magnitude of the increase in $G_m$ during phase 2, and $\tau_2$ describes the time or the number of APs that was fired before $G_m$ had risen to half of $A_2$. $\beta$ reflects the steepness of the rise of $G_m$ during phase 2. All data has been presented as means ± SEM.

All frequencies resulted in significantly different values.

*Significantly different from observations at 15 and 30 Hz.

### Regulation of $G_K$ and $G_{Cl}$ during AP firing

As a first approach in resolving which ion channels were involved in the regulation of $G_m$ in AP-firing fibers, the composite conductances of $G_m$ for K$^+$ and Cl$^-$ ($G_K$ and $G_{Cl}$, respectively) were calculated. Such calculations required experimental determinations of composite conductances before AP firing ($G_{K15, Cl}$, $G_{K5}$, and $G_{Na}$; Table I) and the dynamics of $G_m$ at two different extracellular Cl$^-$ concentrations (Eq. 4 and Appendix 2 in supplemental text). Thus, Fig. 5 shows AP trains and $\Delta V$ responses from representative fibers at 127 (Fig. 5 A) and 50 mM Cl$^-$ (Fig. 5 B). Comparison of the middle traces in Fig. 5 (A and B) shows that the increase in $\Delta V$, which was usually observed during phase I in fibers at 127 mM Cl$^-$, was almost absent in the fiber at 50 mM Cl$^-$. This indicated that increased $\Delta V$ during phase I was related to Cl$^-$ channel inhibition. In contrast, after prolonged AP firing leading to phase 2 (Fig. 5. A and B, right), the $\Delta V$ responses were similarly reduced in both fibers, indicating an involvement of both K$^+$ and Cl$^-$ channels in phase 2. The mean $G_m$ from fibers at 127 and 50 mM Cl$^-$ showed that the reduction of $G_m$ during phase 1 largely disappeared when extracellular Cl$^-$ was reduced to 50 mM and that the rise in $G_m$ during phase 2 was substantially reduced by the partial removal of Cl$^-$ (Fig. 5 D). Indeed, quantification by fitting of the observations at 50 mM Cl$^-$ to Eqs. 5 and 6 showed that the $G_m$ dynamics during phase 1 no longer achieved reliable fits to Eq. 5, whereas $G_m$ during phase 2 was still accurately described by Eq. 6. Analysis of phase 2 in the fibers at 50 mM Cl$^-$ showed that neither the steepness of the rise in $G_m$ during phase 2 ($\beta$) nor the number of APs that had to be fired before phase 2 appeared ($\tau_2$) was affected by removal of Cl$^-$ (compare Table II with Table III). However, the magnitude of the rise in $G_m$ during phase 2 ($A_2$) was substantially reduced by partial removal of Cl$^-$ (P < 0.05; Tables II and III). This indicated that Cl$^-$ channels were involved in both phase 1 and phase 2. Accordingly, calculations of $G_K$ and $G_{Cl}$ (Eq. 4) showed that phase 1 was caused by a small activation of K$^+$ channels and a more substantial inhibition of Cl$^-$ channels with $G_{Cl}$ decreasing by $\sim70\%$, whereas the large increase in $G_m$ during phase 2 was caused by a synchronized activation of both Cl$^-$ and K$^+$ channels as indicated by an $\sim3$-fold and $\sim14$-fold increase in $G_{Cl}$ and $G_K$, respectively.

To verify the calculation of $G_m$ and $G_K$ in AP-firing muscle fibers, additional experiments were conducted in Cl$^-$-free solution under which conditions $G_K$ could be calculated directly from development in $\Delta V$ during the experiments. In these experiments, a small concentration of the Na$^+$ channel inhibitor, tetrodotoxin (TTX; 10 nM), had to be included in the experimental solutions to avoid myotonic AP firing (Fig. 5 C). As shown in Fig. 5 E, there was a close agreement when $G_K$ was measured directly in the Cl$^-$-free solution and when $G_K$ was calculated from the experimental observations at 127 and 50 mM Cl$^-$. Indeed, quantification of the observations at Cl$^-$-free conditions (Table III) showed that $G_m$ reached a level during phase 2 (1,793 ± 197 μS/cm$^2$).
Figure 5. AP-firing fast-twitch fibers display a biphasic $G_m$ as a result of alterations in $G_{Cl}$ and $G_K$. Experiments similar to that in Fig. 2 were conducted in muscle fibers incubated in solutions with reduced or no extracellular Cl$. (A–C) Recordings from a control fiber at 127 mM Cl$^-$ (A) that was included for comparison with the representative fiber at 50 mM Cl$^-$ (B) and the fiber in the Cl$^-$-free solution (C). Note that in Cl$^-$-free solutions, 10 nM TTX had to be included in the extracellular solution to prevent myotonic discharges when the electrodes were inserted. Enlargements of the underlined parts in the recordings of the membrane potential have been included below. (D) The mean $G_m$ in fibers in the control solution of 127 mM Cl$^-$ ($n = 18$) and in fibers at 50 mM Cl$^-$ ($n = 9$). (E) The mean $G_m$ in Cl$^-$-free solution ($n = 11$) and the composite conductances, $G_K$ and $G_{Cl}$, calculated from recordings in fibers at 127 and 50 mM Cl$^-$ using Eq. 4. Mean data are presented with every fifth error bar (SEM).
that differed <2% from the level reached by the calculated $G_m$ during phase 2 ($\approx 1,715 \mu S/cm^2$). Comparing the observations at 127 mM Cl$^-$, 50 mM Cl$^-$ and Cl$^-$ free conditions showed that although the magnitude of the rise of $G_m$ during phase 2 ($A_2$) was inversely related to the Cl$^-$ concentration ($P < 0.05$), the numbers of APs fired to reach half of these increases ($\tau_d$) were similar at all conditions ($P > 0.11$). Furthermore, under all conditions, the rise in $G_m$ during phase 2 displayed similar steepness ($\beta$; $P > 0.45$). Thus, even though the removal of Cl$^-$ eliminated the majority of the rise in $G_m$ during phase 2, the parameters that characterized the kinetics of the rise of $G_m$ during phase 2 ($\beta$ and $\tau_d$) were not affected. This suggested that the K$^+$ and Cl$^-$ ion channels that conveyed the rise in $G_m$ and $G_{Cl}$ during phase 2 were responding to a similar cellular signal.

Regulation of CIC-1 and $K_{ATP}$ channels underlies the biphasic dynamics of $G_m$

In the last part of this study, we investigated which ion channels underlie the dynamics of $G_{Cl}$ and $G_K$ during AP firing. Because CIC-1 is the dominant Cl$^-$ channel in skeletal muscle fibers (Steinmeyer et al., 1991; Koch et al., 1992; Lueck et al., 2007), experiments were initially performed in the presence of a high concentration of the CIC-1 channel inhibitor 9-AC (100 µM; Palade and Barchi, 1977; Lueck et al., 2007). As expected, 9-AC reduced $G_{Cl}$ by $\sim$90% (Table I). Fig. 6 A shows the development of $G_m$ in AP-firing fibers at 127 mM Cl$^-$ in the presence of 9-AC and 10 nM TTX (added to avoid myotonic AP discharges). The decrease in $G_m$ usually observed with the onset of AP firing was now replaced by a small increase. This was consistent with the observations in Cl$^-$ free solutions (Fig. 5 E) and shows that the inhibition of CIC-1 channels underlies the reduction of $G_m$ during phase 1. As AP firing continued, $G_m$ suddenly increased substantially, reflecting the onset of phase 2, but the rise only represented $\sim29\%$ of the rise in $G_m$ during phase 2 in the absence of CIC-1 inhibition ($1,558 \pm 157$ vs. $5,426 \pm 494 \mu S/cm^2$; Tables II and III). Interestingly, in the presence of 9-AC, $G_m$ reached a level during phase 2 that was almost identical to the level reached at Cl$^-$ free conditions ($1,688 \pm 158$ vs. $1,730 \pm 197 \mu S/cm^2$) and to the level reached by the calculated $G_K$ ($\sim 1,715 \mu S/cm^2$; Fig. 5 E). Together, this provides three independent ways of showing that both K$^+$ and Cl$^-$ channels contribute to phase 2 and further demonstrates that the dynamics of $G_{Cl}$ in AP-firing fibers are being mediated entirely by CIC-1 channels.

To test for an involvement of $K_{ATP}$ channels in the regulation of $G_m$ in AP-firing fibers, experiments were performed in the presence of 10 µM of the $K_{ATP}$ channel inhibitor glibenclamide (Cifelli et al., 2008). Fig. 6 B shows mean $G_m$ dynamics in glibenclamide-treated fibers, 13 of which were at 127 mM Cl$^-$ and 16 of which were at 50 mM Cl$^-$. At 127 mM Cl$^-$, phase 1 appeared to be largely unaffected by the inhibitor ($A_1 = -579 \pm 27 \mu S/cm^2$; $\tau_1 = 299 \pm 27$ APs), whereas phase 2 was substantially reduced and delayed. In 5 out of the 13 fibers at 127 mM Cl$^-$, a clear phase 2 was never observed, even after firing $\approx$5,000 APs, and in the remainder of the glibenclamide-treated fibers, phase 2 was not accurately described by Eq. 6. Thus, the observations from the experiments with glibenclamide were not quantified by fitting. However, comparison of the maximum $G_m$ during phase 2 in the absence and presence of glibenclamide suggested that glibenclamide blocked phase 2 more than would be expected if this inhibitor only blocked the rise in $G_K$ during phase 2. Consequently, experiments with glibenclamide were also performed at 50 mM Cl$^-$ where extraction of $G_m$ and $G_{Cl}$ in AP-firing fibers treated with glibenclamide became possible (Eq. 4). Fig. 6 C shows that elevations in both $G_m$ and $G_{Cl}$ during phase 2 were markedly reduced and delayed by glibenclamide. This demonstrates that in addition to preventing the opening of $K_{ATP}$ channels during phase 2, the drug also prevented the reopening of CIC-1 channels during phase 2. In addition, phase 2 was usually associated with repolarization of the resting membrane potential in the absence of glibenclamide, whereas with glibenclamide, this repolarization was either not observed or was delayed in onset.

Because the involvement of $K_{ATP}$ channels in phase 2 was assessed with glibenclamide, which is known to target the $K_{ATP}$ channels in both the surface membrane of muscle fibers as well as in their mitochondria, a series of experiments was conducted using the inhibitor 5-hydroxydecanoate (5-HD), which predominantly targets the mitochondrial $K_{ATP}$ channel (Moses et al., 2005). In experiments with six fibers, both phase 1 ($A_1 = -992 \pm 51 \mu S/cm^2$; $\tau_1 = 125 \pm 12$ APs) and phase 2 ($A_2 = 7,686 \pm 1,464 \mu S/cm^2$; $\tau_2 = 2,440 \pm 275$ APs) were fully developed (Fig. 6 D). This indicated that the effect of glibenclamide on $G_K$ during phase 2 reflected direct interference with the surface membrane.
Pedersen et al.

An alternate method was developed in this study that extracts $G_m$ during muscle activity from on-line measurements of $G_m$. However, this approach only provides a relative measure of $G_m$, and to obtain absolute values for $G_m$, the value of the resting membrane conductance before AP firing, $G_{mS}$, had to be determined in a separate group of fibers under similar experimental conditions using the classical technique. Thus, a full description of $G_m$ in AP-firing fibers relies on observations obtained with both techniques. The massive benefit of combining the two techniques is witnessed by the possibility of separating $G_m$ dynamics into its $G_K$ and $G_{Cl}$ components from experiments conducted at two different $Cl^-$ concentrations. In this way, the involvement of $K^+$ and $Cl^-$ channels for $G_m$ dynamics may be assessed. In AP-firing EDL muscle fibers, we observed that upon the onset of AP firing, a substantial reduction in $G_{Cl}$ and a minor increase in $G_K$ summed to give the substantial reduction in $G_m$ during phase 1. With prolonged AP firing leading to phase 2, $G_{Cl}$ and $G_K$ rose to stable levels corresponding to ~3-fold and ~14-fold of their initial values, respectively.

**Figure 6.** The dynamics of $G_m$ during AP firing in rat EDL muscle fibers reflect regulation of ClC-1 and K<sub>ATP</sub> ion channels. (A) Mean $G_m$ from 13 fibers in the presence of 100 µM 9-AC and 10 nM TTX. (B) Mean $G_m$ from experiments at 127 ($n = 13$) or 50 mM Cl<sup>−</sup> ($n = 16$) in the presence of 10 µM of the K<sub>ATP</sub> channel inhibitor glibenclamide. (C) From the recordings in B, $G_K$ and $G_{Cl}$ in the presence of glibenclamide were calculated using Eq. 4. (D) Mean $G_m$ from six experiments in which the mitochondrial K<sub>ATP</sub> channels were inhibited using 5-HD. Under all conditions, $G_m$ recovered fully within 1 min after cessation of AP firing (not depicted). Mean data are presented with every fifth error bar (mean ± SEM).

**DISCUSSION**

This study demonstrates for the first time that $G_m$ is acutely regulated in AP-firing muscle fibers. We report that modulation of the functions of ClC-1 and K<sub>ATP</sub> ion channels underlies biphasic dynamics of $G_m$ in AP-firing rat EDL muscle fibers: at the onset of AP firing, inhibition of ClC-1 channels conveys a reduction in $G_m$, whereas with continued AP firing, reopening of ClC-1 channels and activation of K<sub>ATP</sub> channels massively elevate $G_m$.

$G_K$ and $G_{Cl}$ can be determined on a time scale of seconds in AP-firing muscle fibers

With the classical technique of measuring $G_m$, muscle fibers must be penetrated multiple times, making it an unsuitable approach for determinations of rapid regulation of $G_m$ in AP-firing fibers. To determine $G_m$ with a temporal resolution of seconds in AP-firing fibers, an alternate method was developed in this study that extracts $G_m$ during muscle activity from on-line measurements of $G_m$. However, this approach only provides a relative measure of $G_m$, and to obtain absolute values for $G_m$, the value of the resting membrane conductance before AP firing, $G_{mS}$, had to be determined in a separate group of fibers under similar experimental conditions using the classical technique. Thus, a full description of $G_m$ in AP-firing fibers relies on observations obtained with both techniques. The massive benefit of combining the two techniques is witnessed by the possibility of separating $G_m$ dynamics into its $G_K$ and $G_{Cl}$ components from experiments conducted at two different $Cl^-$ concentrations. In this way, the involvement of $K^+$ and $Cl^-$ channels for $G_m$ dynamics may be assessed. In AP-firing EDL muscle fibers, we observed that upon the onset of AP firing, a substantial reduction in $G_{Cl}$ and a minor increase in $G_K$ summed to give the substantial reduction in $G_m$ during phase 1. With prolonged AP firing leading to phase 2, $G_{Cl}$ and $G_K$ rose to stable levels corresponding to ~3-fold and ~14-fold of their initial values, respectively.
Reduced $G_m$ during phase 1 reflects inhibition of ClC-1 channels

When experiments were performed in Cl$^-$-free conditions or the ClC-1 channels were blocked by 9-AC, $G_{m5}$ was reduced by almost 90%, and the normal reduction in $G_m$ during phase 1 in AP-firing fibers was absent. This suggests that during early stages of physical activity, AP firing in muscle initiates a cellular signaling pathway that leads to inhibition of ClC-1 channels and, consequently, to a reduction in $G_m$. Interestingly, $G_m$ during phase 1 was well described by a single exponential function, which suggests that inhibition of the ClC-1 channels during phase 1 proceeds via a cellular signaling system that has a single rate-limiting step.

The physiological role of reduced $G_{m1}$ for the excitability and contractile properties of working muscle have been investigated previously using intact muscle and mechanically skinned muscle fibers. Because it is well documented that exercise is associated with marked elevation in the extracellular K$^+$ concentration (for review see Sejersted and Søgaard, 2000), several of these studies have focused on the role of Cl$^-$ channels for the effect of elevated K$^+$ on muscle function. These studies show that under conditions of elevated K$^+$, muscle excitability becomes substantially depressed, but if ClC-1 channels are inhibited via intracellular acidification, 9-AC, or by removal of Cl$^-$ from the extracellular solution, the muscle fibers substantially regain excitability and function (Nielsen et al., 2001; Pedersen et al., 2004, 2005; van Emst et al., 2004; de Paoli et al., 2007). This could indicate that loss of excitability in working muscles can be counteracted if ClC-1 channels are inhibited. This study demonstrates for the first time that the onset of muscle activity is indeed associated with an inhibition of ClC-1 channels, and, consequently, it can be speculated that this reduction in $G_m$ would assist in the maintenance of muscle excitability in working muscle.

ClC-1 and K$_{ATP}$ channels open synchronously during phase 2

A remarkable characteristic of phase 2 was its consistent appearance after firing roughly ~1,800 APs irrespective of whether the APs were fired in trains of 6, 15, or 30 Hz. This suggests that the recovery of the pathway that signals phase 2 was slow compared with the difference in experimental time with the different stimulation frequencies. Experiments showed that after the fibers had fired ~5,000 APs, $G_m$ achieved full recovery if the fibers rested for 1 min. However, upon resumed AP firing, phase 2 rapidly reoccurred. From the full recovery of $G_m$ during the 1-min resting period and the fast reappearance of phase 2 upon resumed AP firing, it can be envisaged that phase 2 relies on a cellular signaling system that has a distinct threshold such that once this threshold is crossed, it leads to the opening of Cl$^-$ and K$^+$ channels. If this cellular signal behind phase 2 recovers below the threshold in the 1 min of rest, $G_m$ should fully recover. However, upon resumed AP firing, the cellular signal starts to increase again, but this time from a much higher level. Therefore, the threshold is crossed much faster in the second round of AP firing. Indeed, such a system should be well represented by a sigmoidal function, which typically describes systems that can alternate between two states.

Experiments with reduced extracellular Cl$^-$, Cl$^-$-free solutions, and 9-AC (a) showed that reopening of ClC-1 channels caused the rise in $G_m$ during phase 2 and (b) demonstrated that the elevations in $G_K$ and $G_{Cl}$ during phase 2 appeared after the same number of APs and rose with similar steepness. This indicated that the reopening of ClC-1 channels and the rise in $G_K$ were temporally very closely synchronized and suggested that the ClC-1 and K$^+$ channels that mediated the elevations in $G_{Cl}$ and $G_K$ during phase 2 responded to similar cellular signaling. To this end, three lines of evidence suggest that this common signal may be a substantial reduction in the cellular energetic state of the muscle fibers: (1) Fink and Lüttgau (1976) showed that metabolic poisoning of frog skeletal muscle causes enormous elevations in both $G_K$ (110-fold) and $G_{Cl}$ (14-fold). Importantly, these elevations in $G_K$ and $G_{Cl}$ occurred almost simultaneously, as occurs during phase 2. Furthermore, such elevations occurred without alterations in the resting membrane potential, which indicates that the cellular integrity of the poisoned muscle fibers was conserved. Such observations with metabolic poisoning have been linked to opening of K$_{ATP}$ channels in mammalian muscle (Allard et al., 1995). Collectively, the experiments with metabolic poisoning demonstrate that skeletal muscle fibers are equipped with Cl$^-$ and K$^+$ ion channels that are sensitive to the metabolic state of the cell and that these channels appear to open at the same level of metabolic depression. However, the present study provides the first direct evidence that similar regulation of K$^+$ and Cl$^-$ channels may be involved in physiological regulation of ion channels in active skeletal muscle fibers. (2) Experiments performed in this study demonstrate that the dramatic elevation of $G_K$ in phase 2 is significantly reduced and delayed by the K$_{ATP}$ channel inhibitor glibenclamide. These channels are generally considered to constitute a link between the metabolic state and excitability of cells because they open when the energy level becomes low (Nichols, 2006). Thus, the experiments with glibenclamide indicate that the rise in $G_K$ during phase 2 was initiated by the depressed metabolic state of the fibers that caused K$_{ATP}$ channels to open. (3) The synchronicity of ClC-1- and K$_{ATP}$-channel opening in phase 2 suggests that ClC-1 channels should also be sensitive to the metabolic state of the cells.

Bennetts et al. (2005) have recently demonstrated in expression systems that conditions of low cellular energy levels increase the opening probability of ClC-1 channels. This important contribution to the understanding
of ClC-1 channels has now been further developed to show that the sensitivity of ClC-1 channel to the metabolic state of cells is inversely related to the intracellular proton activity (Bennetts et al., 2007; Tseng et al., 2007) and is completely lost upon cell oxidation (Zhang et al., 2008). However, one study has raised doubt that ClC-1 channels are sensitive to the metabolic state of the cells (Zifarelli and Pusch, 2008). All of these observations on the regulation of ClC-1 channels were obtained using various expression systems, and whether native ClC-1 channels are sensitive to the metabolic state of muscle fibers has not been explored. In the present study, we demonstrate that ClC-1 channels open after prolonged AP firing, and the synchronicity of opening of ClC-1 and K\textsubscript{ATP} channels strongly suggests that ClC-1 channels do respond to depressed metabolic conditions in native skeletal muscle fibers. Because the metabolic state can be severely depressed in fast-twitch fibers during physical exertions (Esbjörnsson-Liljedahl et al., 1999), it is possible that such openings of ClC-1 and K\textsubscript{ATP} channels occur in fatigued muscles in vivo.

Until now, our understanding of the regulation of G\textsubscript{m} in working muscles has been limited to extrapolations from knowledge obtained in resting muscles. Using a new technical approach, however, we here identify significant activity-induced regulation of G\textsubscript{m} in fast-twitch skeletal muscle fibers that provides new perspectives on the roles of ClC-1 and K\textsubscript{ATP} channels for muscle function. We demonstrate that at the onset of muscle activity, ClC-1 channels are rapidly inhibited. Such inhibition of ClC-1 channels may assist working muscle in their maintenance of excitability. The data further implicate ClC-1 channels in a mechanistic alliance with K\textsubscript{ATP} channels that at times of low cellular energy levels, opening of these channels generates a large G\textsubscript{m}. Because such an increase in G\textsubscript{m} tends to lower fiber excitability, we suggest that elevation of G\textsubscript{m} in response to the reduced metabolic state of the muscle fibers may switch off further muscle fiber activation and thus prevent complete depletion of the energetic state of the fibers that otherwise might result in loss of cellular integrity. This scenario is supported by the loss of AP excitation when G\textsubscript{m} became elevated during phase 2 and by observations of severe muscle damage in K\textsubscript{ATP} channel knockout mice after treadmill running (Thabet et al., 2005; Cifelli et al., 2007).

We thank T.L. Andersen, V. Uhre, and M. Stürup-Johansen for technical assistance. This work was supported by the Danish Research Medical Council (grants to T.H. Pedersen and O.B. Nielsen) and the Faculty of Health Science at Århus University (grants to T.H. Pedersen and F.V. de Paoli). Christopher Miller served as editor.

Submitted: 6 July 2009
Accepted: 11 September 2009

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RESTING MEMBRANE CONDUCTANCE IN AP-FIRING MUSCLES


Part I: Conversion of $G_m$ measurements into $G_m$

The aim of this study was to determine changes in $G_m$ in muscle fibers that were being activated intermittently to fire trains of APs. To do this, it was necessary to develop a technique that allowed measurements of $G_m$ in the 3.5-s periods between the trains of APs. Clearly, the classical technique of measuring resting $G_m$ in muscle fibers, which hinges on measuring the steady-state deflection in membrane voltage in response to a square current pulse at three or more locations along the muscle fiber, was not possible during muscle activity (Boyd and Martin, 1959; Pedersen et al., 2005). Instead, two intracellular microelectrodes were placed in the fiber at very close proximity (~70 µm), and $G_m$ could then be measured directly throughout AP firing. Eq. 3, which is derived below from Eq. S1, was subsequently used to relate $G_m$ to $G_m$. This provided us with a one-point measuring technique for $G_m$ determination.

Appendix 1: Expression used to calculate $G_m$ from measurements of $G_m$ in AP-firing muscle fibers. The following equations express some basic relationships that are often used in linear cable theory (Jack et al., 1975):

$$r_m = 2\lambda/G_m,$$

$$r_a = \frac{2}{G_m\lambda},$$

$$r_a = \frac{4R_m}{\pi D^2},$$

and

$$G_m = \frac{1}{\pi D r_m},$$

where $r_m$ is the membrane resistance (in ohm centimeters), $\lambda$ is the length constant of the fiber (in centimeters), $r_a$ is the resistance of the intracellular space (ohm centimeter$^{-1}$), $R_m$ is the resistivity of the intracellular space (ohm centimeters), $G_m$ is the input conductance (in microsiemens), and $D$ is fiber diameter (in centimeters). These expressions can be used to relate $G_m$ and $G_m$:

$$G_m = \frac{G_m^2 R_m}{\pi^2 D^2}.$$

Thus, if the resting membrane conductance had been determined before AP firing ($G_{m0}$) using the classical method and $G_m$ was monitored between trains of APs, $G_m$ between the AP trains will be given by

$$G_m = \frac{R G_m^2 D^2}{R_{m0} G_{m0} D^2} G_{m0},$$

Note that the subscript zero indicates membrane parameters before AP firing in the given fiber, and $G_{m0}$ represents the mean membrane conductance measured in a subset of resting fibers under the same conditions using the previously described classical three-point technique (Table I). Now, assuming that the diameter of the fibers and the internal resistivity remain unchanged during the experiment (see Part II), Eq. S2 can be reduced to

$$G_m = \frac{G_{m0}^2}{G_{m0}} G_{m0}.$$

Again, $G_{m0}$ refers to the input conductance of the investigated fiber before AP firing, and $G_m$ represents the measured input conductance in between the AP trains. Essentially, the equation gives a way to extract absolute values for $G_m$ from the changes in $G_m$ during muscle activity, provided $G_{m0}$ and $G_{m00}$ are known (Table I).

Appendix 2: Expression used to extract $G_K$ and $G_{CI}$ during AP firing. After establishing that muscle activity was associated with large alterations in $G_m$, the focus of the study turned toward clarifying the roles of the $K^+$ and $CI^-$ channels for the overall dynamics of $G_m$ during muscle activity. As a first approach, $G_K$ and $G_{CI}$ had to be determined. To develop an approach of extracting $G_K$ from experimental data, the following three assumptions were made.

1) $G_K$ was assumed to be unaffected by a change in extracellular $Cl^-$ concentration. Thus,

$$G_K = G_{K,X} = G_{K,X1}.$$

$G_{K,X}$ and $G_{K,X1}$ represent potassium conductances at two different $Cl^-$ concentrations.

2) The relative change that will be observed in $G_{CI}$ in resting fibers before AP firing when extracellular $Cl^-$ is changed will also reflect the relative change in $G_{CI}$ that is observed in AP-firing fibers when $Cl^-$ is changed. This means that the ratio between the $Cl^-$ conductances at $X$ and $X_1$ mM $Cl^-$ is the same regardless of whether the fibers are resting or firing APs. Thus,

$$\frac{G_{CI,X0}}{G_{CI,X1}} = \frac{G_{CI,X}}{G_{CI,X1}},$$

where $G_{CI,X}$ and $G_{CI,X1}$ are the chloride conductances at $X$ and $X_1$ mM $Cl^-$ during muscle activity, respectively, and $G_{CI,X0}$ and $G_{CI,X1}$ are the chloride conductances at $X$ and $X_1$ mM $Cl^-$ before muscle activation.

3) The membrane conductance at rest is composed only of $G_K$ and $G_{CI}$. Consequently,

$$G_{m,X} = G_K + G_{CI,X}.$$
and \( G_{m,X1} = G_K + G_{Cl,X1} \) \(^\text{(S6)}\)

where \( G_{m,X} \) and \( G_{m,X1} \) are the total membrane conductances at \( X \) and \( X1 \), respectively.

Based on Eqs. S3–6, an expression of \( G_K \) during muscle activity was derived:

\[
G_K = \frac{G_{m,X} G_{Cl,X1,0} - G_{Cl,X,0} G_{m,X1}}{G_{Cl,X1,0} - G_{Cl,X,0}}.
\]

From this, \( G_{Cl} \) was found using Eq. S5.

The second assumption implies that the anion concentration dependence of the anion channels, mostly CIC-1 channels, did not change during muscle activity. To check this, it was important to compare the outcome of the calculations of \( G_K \) and \( G_{Cl} \) during muscle activity using the aforementioned approach against other experimental assessments of \( G_K \). Thus, experiments were also conducted in \( Cl^- \)-free solutions and in the normal solution with 100 µM 9-AC. Similar estimates of \( G_K \) were seen with all three approaches (Figs. 5 E and 6 A). This was taken as validation for the aforementioned approach for calculating \( G_K \) and \( G_{Cl} \) and further suggests that modification by various signaling mechanisms of anion concentration dependence of CIC-1 channels has minor importance in AP-firing fibers.

Part II: The role of muscle fiber swelling and \( R_i \) for the estimated \( G_m \) in AP-firing fibers

It is well established that muscle fiber volume increases during physical activity (for review see Sejersted and Sjøgaard, 2000; Usher-Smith et al., 2006, 2009). To be able to evaluate the error in the calculation of \( G_m \) from \( G_{m,rest} \) that was introduced by assuming a constant fiber diameter during AP firing, Eq. S2 was expressed with fiber volume (\( V_{fs} \)) rather than \( D \). Here, it was assumed that the muscle fiber could be considered to be infinitely long under all conditions, at least when compared with the length constant:

\[
G_m = \frac{R G_{m,0}^2 V_{f,b,0}^{1.5}}{R_i G_{m,0}^2 V_{f,b}^{1.5} + G_{m,0}} \times G_{m,0}. \quad \text{(S7)}
\]

Fig. S1 shows the recordings of \( G_m \) during AP firing when corrected for 10, 20, and 30% increases in fiber volume. The change in fiber volume is assumed to occur linearly throughout. This assumption is based on the findings by Usher-Smith et al. (2006), who showed a gradual and almost linear increase in fiber volume during muscle fiber stimulation. Clearly, the assumption of a constant muscle fiber volume during muscle activity, which we used in the calculations of \( G_m \), only leads to a minor underestimate of the inhibition of CIC-1 channels during phase 1 and an overestimate of the large \( G_m \) during phase 2. However, the errors introduced in the determination of \( G_m \) by this assumption of constant fiber volume are moderate compared with the large alterations that occur in \( G_m \) in active muscles, and they do not affect the conclusions from our study in any significant way.

Eq. S7 shows that changes in \( R_i \) could also have affected \( G_m \) in the AP-firing fibers. \( R_i \) depends on ion concentrations, which were unlikely to have changed markedly, as the osmolarity of the intracellular space should largely remain constant in the AP-firing fibers. \( R_i \) also depends on the electrical mobility of the ions in the intracellular space. Although such mobilities could have changed during repetitive AP firing, it appears unlikely that ion substitutions (methylsulfate for \( Cl^- \)) and use of different pharmacological tools (9-AC and glibenclamide) should affect these ion mobilities in similar ways.

Part III: \( G_m \) dynamics in AP-firing fibers reflect the normal physiological regulation of ion channels

To evaluate whether the dynamics of \( G_m \) in AP-firing fibers (Fig. 3 B) reflected physiological regulation of ion channels that could also be induced when the whole muscle was stimulated to fire APs without inserted electrodes, we investigated the development in \( \Delta V \) and \( G_{m,rest} \) in AP-firing whole muscles that were stimulated via their connected motor nerve (Overgaard et al., 1999) or with field stimulation pulses (Pedersen et al., 2005).

The approach for assessing whether phase 1 occurred when whole muscles were stimulated without inserted electrodes was to compare the recovery of \( G_{m,rest} \) after a period of stimulation known to induce phase 1 in (a) fibers stimulated with inserted electrodes (Fig. S2 A) and (b) in fibers in which the APs had been induced via stimulation of the motor nerve and in which the electrodes were inserted after cessation of this stimulation (Fig. S2 B). With inserted electrodes and with motor nerve stimulation, fibers were activated to fire APs every 7 s with 3.5-s trains of pulses at 15 Hz until a total of 686 APs had been fired. This number of APs corresponded to around three times the time constant for the development of phase 1 (Table II). Accordingly, as shown in Fig. S2 C, in the experiments with fibers stimulated via inserted electrodes, the mean \( G_{m,rest} \) in 17 fibers decreased by around 27% (from 3.7 ± 0.1 to 2.7 ± 0.1 pS). During the subsequent 7 min of rest, \( G_{m,rest} \) showed no recovery within the first min, recovered partly after 3 min, and reached a level of full recovery after 5–7 min. In experiments in which muscles were stimulated to fire AP via the motor nerve and the electrodes were rapidly inserted into fibers after the cessation of AP firing, the absolute levels of \( G_{m,rest} \) and the pattern of its recovery were very similar to the fibers stimulated via an intracellular electrode (Fig. S2 D). Note that in the experiments with nerve stimulation, neither BTS nor vehicle was added. These experiments demonstrate that the reduction in \( G_{m,rest} \) during phase 1 was not simply caused by insertion of the electrodes but rather reflected normal physiological regulation of ion channels in AP-firing fibers.

Because the recovery of \( G_{m,rest} \) from phase 2 was very fast (Fig. 3 B), a slightly modified experiment was used to test
for the ability of whole muscle stimulation to induce the rise in $G_m$ during this phase. First, whole muscles were excited via field stimulation every 7 s with 3.5-s-long 15-Hz trains of pulses until a total of 1,960 APs were fired, corresponding to the beginning of phase 2. Then two electrodes were inserted, and stimulation was resumed but via one of the intracellular electrodes and, therefore, only in the impaled fiber. Fig. S3 A shows recordings of membrane potential in the 1st and 14th train of APs after the electrodes were inserted in a fiber in a prestimulated muscle. After <637 APs, $\Delta V$ had dropped to a similar extent as during phase 2 in the fibers in which all APs were triggered by inserted electrodes (Fig. 2 D). Fig. S3 B shows the mean $G_m$ observed in eight fibers from prestimulated muscles and the observation from the muscle fibers that did not have a history of field stimulation. In the majority of prestimulated fibers (eight out of nine), phase 2 developed when only $\sim$400 APs were elicited via intracellular electrodes. This corresponded to <23% of the APs required to induce phase 2 via inserted electrodes in fibers in which no prestimulation was done (Fig. S3 C). In the ninth prestimulated fiber, phase 2 never developed during the stimulation with intracellular electrodes, although $\geq$3,900 APs were elicited. Despite this, these findings indicate that the cellular processes leading to the development of phase

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**Figure S1.** Estimated effect of muscle fiber swelling on $G_m$ during AP firing. The determinations of $G_m$ in AP firing in fast-twitch muscle fibers (15 Hz) have been corrected for a theoretical linear increase in fiber volume of 10, 20, and 30%.

**Figure S2.** The regulation of ion channels underlying phase 1 is independent of inserted electrodes. (A) Increased $\Delta V$ in a fiber after 686 APs and its recovery of $\Delta V$ during the subsequent 7 min of rest. Electrodes were inserted throughout the experiment. (B) Recovery of $\Delta V$ in a fiber in which 686 APs had been initiated via nerve stimulation and the electrodes were inserted <1 min after cessation of AP firing. (C) Mean values of $G_m$ as extracted from $\Delta V$ in AP-firing fibers (black bars) and during 7 min of subsequent recovery (white bars; $n = 17$) in experiments in which electrodes were inserted throughout. (D) Recovery of $G_m$ in eight fibers after 686 APs had been triggered via motor nerve stimulation in the absence of inserted electrodes and without addition of BTS or DMSO. Mean data are presented as means ± SEM.
2 were initiated already during the prestimulation with field pulses in which no insertion of electrodes had been done.

Because unspecific membrane damage can cause large increases in membrane permeability to ions, it could be speculated that damage imposed by the microelectrodes contributed to the large increase in $G_m$ in phase 2 after prolonged AP firing. However, four lines of evidence argue against such a notion. First, phase 2 occurred after firing roughly 1,800 APs irrespective of whether the AP was fired in trains of 6, 15, or 30 Hz, and when AP firing was stopped, $G_m$ recovered fully within 1 min (Fig. 4 D). Second, phase 2 was associated with repolarization of the resting membrane potential (Fig. 2 D), which is incompatible with the unspecific loss of fiber integrity. Third, the changes in $G_m$ in AP-firing fibers were sensitive to specific inhibitors of Cl$^-$ and K$^+$ channels. Fourthly, stimulation of fibers via the nerve or via field stimulation could be demonstrated to induce changes in $G_m$ that were similar to the dynamics of $G_m$ observed in fibers that were stimulated via inserted electrodes. Together, these observations speak strongly against an involvement of unspecific membrane damage and thereby argue that the dynamics of $G_m$ reflected normal physiological regulation of specific ion channels.

**REFERENCES**


