Human Myoblast Differentiation: Ca\textsuperscript{2+} Channels are Activated by K\textsuperscript{+} Channels

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In a paradigm of cellular differentiation, human myoblast fusion, we investigated how a Ca\textsuperscript{2+} influx, indispensable for fusion, is triggered. We show how newly expressed Kir2.1 K\textsuperscript{+} channels, via their hyperpolarizing effect on the membrane potential, generate a window Ca\textsuperscript{2+} current (mediated by \textalpha{}1H T-type Ca\textsuperscript{2+} channels), which causes intracellular Ca\textsuperscript{2+} to rise.

The generation of multinucleated muscle fibers during postnatal growth and the repair of skeletal muscle depend on the special ability of myoblasts to fuse. Muscle satellite cells, a kind of myogenic stem cell, are responsible for this growth and regeneration potential (13). Satellite cells are small quiescent mononucleated cells lying between the basal lamina and the sarcolemma of the muscle fiber (Fig. 1A). When they are activated, satellite cells proliferate as myoblasts. After a few days of proliferation, most myoblasts fuse either with preexisting muscle fibers or with other myoblasts. Fusion between myoblasts leads to multinucleated myotubes that will, with time, mature into new muscle fibers. A small proportion of proliferating myoblasts withdraws from the cell cycle and reconstitutes a new population of quiescent satellite cells (4).

Fusion between myoblasts, or between myoblasts and myotubes, has long been known to be a strictly Ca\textsuperscript{2+}-dependent process that requires an influx of Ca\textsuperscript{2+} (18). However, investigations in various preparations left several unsolved issues and suggested that different mechanisms may cause this influx of Ca\textsuperscript{2+} in different species or at different states of development (7, 19). We have attempted to characterize the molecular mechanisms involved in this process in clonal cultures of human myoblasts. Our current results suggest that the membrane resting potential and the biophysical properties of specific ionic channels are important actors in the fusion process in human myogenic cells.

A model linking ionic channels, membrane potential, and Ca\textsuperscript{2+} influx during myoblast fusion

Human myogenic cells can be studied as freshly isolated satellite cells, can be maintained in culture as undifferentiated proliferating myoblasts, or else can be triggered to differentiate and fuse, by a simple change of the culture medium (Fig. 1B). The last property allowed us to investigate events at the onset of fusion using fusion-competent myoblasts, that is, myoblasts cultured at a low density in the fusion-promoting medium. Under these conditions, myoblasts are induced to proceed through the differentiating steps preceding fusion, but because they cannot interact with neighboring cells, they remain mononucleated (11). By investigating membrane currents in these various preparations of myogenic cells, from satellite cells to fusion-competent myoblasts, we observed that there is a sequence in the expression of ionic channels as myoblasts differentiate. Native satellite cells and proliferating myoblasts express exclusively voltage-gated Na\textsuperscript{+} and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (10). Fusion-competent myoblasts, on the other hand, express additional ionic channels in succession: 1) nicotinic acetylcholine receptors (11); 2) ether-à-go-go (EAG) and delayed rectifier K\textsuperscript{+} channels (1, 3, 15); 3) inward rectifier K\textsuperscript{+} channels (Kir) channels (9, 12); 4) T-type Ca\textsuperscript{2+} channels (T channels; Ref. 2); and 5) L-type Ca\textsuperscript{2+} channels (L channels; Ref. 2). This review will concentrate mainly on Kir and T channels.

Our current understanding of the mechanisms controlling the Ca\textsuperscript{2+} influx is illustrated in Fig. 1C. Human myoblasts must hyperpolarize (i.e., their membrane potential becomes more negative inside the cell) before they can proceed through the fusion process. A similar observation has been made in chick embryonic myoblasts (20). For human cells, this hyperpolarization occurs through the sequential expression of two different K\textsuperscript{+} currents. An EAG current is expressed first and hyperpolarizes myoblasts to an intermediate resting potential of approximately −32 mV (1, 3). Then, slightly before fusion, the expression of a Kir current causes fusion-competent myoblasts to hyperpolarize to approximately −65 mV (12) (it should be recalled that under physiological conditions open K\textsuperscript{+} channels allow an efflux of K\textsuperscript{+} when the potential of the cell is more positive than the equilibrium potential for K\textsuperscript{+} ions (E\textsubscript{K}); this efflux of K\textsuperscript{+} leaves negative charges inside the cells). Inhibition of the Kir current depolarizes myogenic cells (12) and impedes fusion (9, 12), suggesting that hyperpolarization through functional Kir channels is required for fusion to proceed (9). We propose that the purpose of the hyperpolarization is to set the membrane resting potential of fusion-competent myoblasts in a voltage range at which the necessary inward Ca\textsuperscript{2+} flux can take place (2). In our model, this Ca\textsuperscript{2+} influx is generated by a “window current” through T channels that are expressed just before fusion takes place (2).

Expression of EAG K\textsuperscript{+} channels hyperpolarizes myoblasts to an intermediate potential

A hyperpolarization is observed as soon as human myoblasts differentiate. The resting potential of undifferentiated myoblasts is low (−8 ± 1 mV) (10), and that of newly formed myotubes (−65 ± 1 mV) (12) is similar to the resting potential...
measured in intact mammalian muscle fibers. We find that fusion-competent myoblasts can be subdivided into two distinct populations with respect to their resting potential. The majority of fusion-competent myoblasts (72% of the total population) has a mean resting potential of $-32 \pm 1$ mV, a potential that lies about halfway between the resting potential of undifferentiated myoblasts and that of newly formed myotubes. The second population (28%) has a mean resting potential of $-64 \pm 2$ mV, a potential that is statistically identical to that of newly formed myotubes (12). The hyperpolarization from $-8$ mV to $-32$ mV is linked to the expression of an EAG current (1, 3, 15). This current activates at voltages more depolarized than $-40$ mV and, unlike the delayed rectifier K$^+$ current, it does not inactivate at depolarized potentials, which explains why, when expressed, it can hyperpolarize myoblasts to $-32$ mV. EAG channels cannot, however, account for the hyperpolarization to $-65$ mV because, at this negative potential, EAG channels are not activated. We found that the second phase of the hyperpolarization (from $-32$ to $-65$ mV) is due to the expression of Kir channels.

Expression of Kir channels precedes fusion and is required for fusion to occur

We observed that the fusion-competent myoblasts that are hyperpolarized to $-65$ mV (28% of the total population) and all newly formed myotubes express a Kir current. An example of Kir current recorded in a hyperpolarized, fusion-competent myoblast is illustrated in Fig. 2A. The current-voltage relationship shows a marked change in slope (rectification) at potentials more negative than $E_K$, which means that the inward flux of K$^+$ (from the extracellular medium to the inside of the cells) is more efficient than the outward flux, hence the name “inward rectifier” K$^+$ current. It must be emphasized, however, that it is the small outward K$^+$ current that is responsible for driving the membrane resting potential from $-32$ mV (EAG channels alone) to $-65$ mV (at which Kir channels are activated).

Because Kir channels are sensitive to Cs$^+$ or Ba$^{2+}$ (12), we could examine the consequences of a block of these channels on the resting potential of fusion-competent myoblasts. Figure 2B shows that increasing concentrations of Cs$^+$ cause a progressive depolarization of fusion-competent myoblasts that express Kir channels from $-65$ mV to approximately $-40$ mV. EAG channels, which are insensitive to these concentrations of Cs$^+$ and which activate near $-40$ mV, prevent any further depolarization. A similar result was observed when Ba$^{2+}$ instead of Cs$^+$ was applied on fusion-competent myoblasts.

If a negative membrane potential or the activity of Kir channels were important for myoblast fusion, a block of Kir channels should affect fusion. We found that this was indeed the case. Figure 2C shows that fusion is drastically reduced in cultures treated with Cs$^+$. This cannot be explained by a toxic effect of Cs$^+$ on the cells because, immediately after removal of Cs$^+$, fusion proceeds normally. A similar inhibition of fusion can be obtained by blocking Kir channels with Ba$^{2+}$ (12). Although these results are consistent with the idea that Kir channel activity is required during the fusion process, it was important to characterize the molecular nature of the Kir channel involved to design more specific molecular tools that would definitely establish the involvement of Kir channels.

The whole cell characteristics of the native Kir current of myoblasts [steep inward rectification and insensitivity to intracellular ATP or nonhydrolyzable GTP analog GTP$\gamma$S (12)] suggested that the underlying channel was a member of the Kir subfamily (14), of which four members have been isolated so far. Using RNase protection assays, a method for detecting and distinguishing low levels of highly homologous transcripts, we established that Kir2.1 and Kir2.2 transcripts were present in fusion-competent myoblasts (9).

Concomitantly, we performed single channel recordings of fusion-competent myoblasts to characterize the endogenous Kir channels. We found that fusion-competent myoblasts express a unique type of Kir channel. We then compared the unitary conductance of this endogenous Kir channel to the unitary conductances of cloned Kir2.1 and Kir2.2 channels. Expression vectors coding for Kir2.1 and Kir2.2 channels were transfected in proliferating myoblasts (8), and single channel recordings were performed. We observed that the unitary conductance of the Kir2.1 channel (20.0 ± 0.9 pS) was nearly identical to that of the native Kir channel (21.5 ± 0.3 pS), whereas the unitary conductance of the Kir2.2 channel (29.4 ± 0.2 pS) was much larger and had, in addition, never been observed in native fusion-competent myoblasts (9). These results suggest that the Kir channel responsible for the hyperpolarization preceding fusion is formed by Kir2.1 subunits.

Knowing the molecular identity of the Kir channel expressed by fusion-competent myoblasts, it was now possible to use an antisense strategy to demonstrate its involvement in myoblast...
T-type Ca\(^{2+}\) channels allow an influx of Ca\(^{2+}\) at a hyperpolarized potential

In our model, we propose that the purpose of the Kir-associated hyperpolarization is to set the membrane resting potential of fusion-competent myoblasts in a voltage range that triggers a Ca\(^{2+}\) influx through T channels. Although both L channels and T channels are expressed in human myoblasts just before fusion, we could determine that only T channels are required for myoblasts to fuse (2). First, the inhibition of T channels by amiloride or Ni\(^{2+}\) prevents an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) that we normally observe at the onset of fusion (2). Specifically, we find that 25% of fusion-competent myoblasts have a higher [Ca\(^{2+}\)]\(_i\) and that this particular population disappears in the presence of T channel blockers. In contrast, the inhibition of L channels by nifedipine (10 μM) does not affect [Ca\(^{2+}\)]\(_i\). Second, Fig. 3 shows that myoblast fusion is drastically inhibited in the presence of T channel blockers (the figure also shows that the inhibition is not due to toxic effects of amiloride or Ni\(^{2+}\) on the cells, since removing the drugs from the culture medium restores normal fusion), whereas the inhibition of L channels by nifedipine (10 μM) does not affect fusion (2).

The next step was to understand how a hyperpolarization of the membrane potential mediated by Kir channels could lead to an activation of T channels, since this was somewhat counterintuitive. Indeed, it is well established that T channels activate rapidly from a closed to an open state upon depolarization and that they rapidly switch and stay in an inactivated state as depolarization persists. To move the T channels out of the inactivated state, the cell membrane has to hyperpolarize, which resets the T channels to the closed state. How then would T channels be activated by a hyperpolarization? A careful analysis of the T channel characteristics of myoblasts showed that a steady-state Ca\(^{2+}\) current through T channels exists in the voltage domain where the activation and inactivation current-voltage relationships overlap (Fig. 4A). In this domain, the equilibrium among the closed, open, and inactivated states is such that T channels cycle continuously among the three states and that a tiny fraction of the T channels is always open. This gives rise to a steady-state current that is referred to as a “window current.” As we shall see, a steady-state Ca\(^{2+}\) current through T channels, i.e., a T window current, is indeed generated in human myoblasts at potentials between −35 and −75 mV.

Figure 4A illustrates the biophysical characteristics of a T window current recorded in a fusion-competent myoblast. It can be seen that there is an overlap of the activation and inactivation curves centered on −55 mV. Figure 4B represents the T window current computed from the product of the two curves at various membrane resting potentials. From the voltage dependence of activation and inactivation measured in 11 fusion-competent myoblasts, we evaluated that a maximum T window current should be generated at a potential of −58 ± 2 mV and that its amplitude should amount to −0.37 ± 0.01 pA.

To confirm that the computed T window current actually participates in Ca\(^{2+}\) influx during fusion, we used diffuse-light microscopy to monitor fusion events. The experiments described in Fig. 2D show that the formation of multinucleated myotubes is impeded when myoblasts are transfected with a vector expressing Kir2.1 antisense, whereas the inhibition of Kir2.2 channel expression does not affect fusion. These results bring compelling evidence for the implication of functional Kir2.1 channels in human myoblast fusion and demonstrate, without having to rely on pharmacological agents such as Cs\(^{+}\) or Ba\(^{2+}\), that a hyperpolarization of the membrane potential of myoblasts is a prerequisite for fusion (9).
the T current by 78%. On the other hand, neither α1G, α1I, nor scrambled antisense oligonucleotides had any significant effect on the T current (2).

**T window current is large enough to increase [Ca\(^{2+}\)]\(_i\)**

The T window current observed in fusion-competent myoblasts is <1 pA, but it is sustained as long as the potential remains in the window domain. Because such a small current could theoretically generate a relatively large increase in [Ca\(^{2+}\)]\(_i\) over time, we designed an experiment to demonstrate that the T window current can indeed modify [Ca\(^{2+}\)]\(_i\) in human myogenic cells. For that purpose, the membrane potential of small myotubes was held for >80 s at three different membrane potentials and the steady-state [Ca\(^{2+}\)]\(_i\) was simultaneously evaluated with the Ca\(^{2+}\) indicator indo 1. The membrane potential had to be maintained constant for >80 s because we had found that this was the time required for [Ca\(^{2+}\)]\(_i\) to reach a steady state after an abrupt change in voltage triggering a large T-type Ca\(^{2+}\) current.

The first holding potential was set below the activation range for T channels (−75 mV), and hence neither a Ca\(^{2+}\) current nor a change in [Ca\(^{2+}\)]\(_i\) should be observed. The second holding potential was set at a voltage value at which T channel activation and inactivation currents overlap (~55 mV), and thus a sustained Ca\(^{2+}\) current should be generated with a sustained change in [Ca\(^{2+}\)]\(_i\). The third holding potential was set at a voltage at which a large fraction of T channels first activate and

**T channels expressed in human myogenic cells are mainly formed by α1H subunits**

We examined whether the T channels expressed in fusion-competent myoblasts were encoded by one of the three known α1 subunit genes (17). Antisense oligonucleotides were designed to specifically hybridize to nonconserved 5′-regions of the α1G, α1H, or α1I transcripts. Oligonucleotides were injected with a micropipette into newly formed myotubes that, unlike fusion-competent myoblasts, always express a T current. Again, this choice is justified by the fact that the biophysical properties of the T current in fusion-competent myoblasts are identical to those in myotubes. We found that antisense oligonucleotides directed against the α1H transcript reduce
then fully inactivate with time (~35 mV). This step potential should only generate a transient Ca\(^{2+}\) current, and thus no sustained [Ca\(^{2+}\)] change should be observed at 80 s.

The results presented in Fig. 4C confirm that the small T window current is indeed able to modify [Ca\(^{2+}\)]. At a steady holding potential of ~55 mV, a potential at which the T window current is elicited, we did not observe a sustained increase in [Ca\(^{2+}\)]. This increase was not seen when the myotubes were maintained at potentials 20 mV above or below ~55 mV. In addition, we observed [Ca\(^{2+}\)] increase seen at ~55 mV did not occur when a potent T channel blocker, mibefradil, was added to the superfusion solution. From these results we conclude that at defined hyperpolarized potentials T channels can generate a window current that is large enough to modify [Ca\(^{2+}\)].

**Issues raised by the present model and conclusions**

It is immediately apparent that the simple model illustrated in Fig. 1C will generate a persistent Ca\(^{2+}\) flux that will load the cell with Ca\(^{2+}\) if no compensatory mechanisms are activated. A possible mechanism that might prevent Ca\(^{2+}\) overload is an upregulation of Ca\(^{2+}\)-ATPase pumps at the time of fusion. Indeed, a muscle-specific isoform of Ca\(^{2+}\)-ATPase is expressed at the time of myotube formation in mouse C2 myoblasts (6). Another possible way of preventing Ca\(^{2+}\) overload is to act on the inward Ca\(^{2+}\) flux. This could be done by activating other K\(^+\) channels such as Ca\(^{2+}\)-activated or ATP-dependent K\(^+\) channels, because these would drive the membrane potential toward E\(_{K}\) i.e., away from the window domain. This mechanism would reduce the Ca\(^{2+}\) influx and allow Ca\(^{2+}\) extrusion mechanisms to restore basal [Ca\(^{2+}\)]. Incidentally, the interplay between Ca\(^{2+}\) influx and K\(^+\) channels may lead to oscillations in membrane potential and/or [Ca\(^{2+}\)]. Either oscillating signal could be used by the cell to orient its differentiation.

Another issue that deserves additional investigation is the role of EAG channels. Indeed, because specific pharmacological blockers of EAG channels are not available yet, the precise role of these K\(^+\) channels in fusion-competent myoblasts could not be established. Also, because EAG channels have been implicated in cell-cycle regulation (16), a possible role in the transition between proliferating and fusion-competent myoblasts deserves consideration as well.

Due to our past experience with the study of ionic channels in various differentiating preparations, our approach to myoblast fusion, a subject of intense investigation, has been somewhat unusual. We have explored the possibility that myoblast fusion might be controlled in part by the functional expression of ionic channels. We reviewed here the available evidence in human myogenic cells suggesting that one type of influence exerted by ionic channels on cell differentiation is mediated by their action on membrane potential. Other domains to be explored are the bidirectional interactions between ionic channels and the intracellular signaling network, which comprise kinases, phosphatases, and expression or activation of transcription factors that control specific genes involved in myogenic differentiation (5). Because all cells express ionic channels, it will be interesting to see whether some of the conclusions reached in differentiating human myogenic cells also apply to other cell types.

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**References**


