SHORT COMMUNICATION

Analysis of Ca^{2+} Signaling Mechanisms – Our Experience on the Intercellular Communication in Muscle Remodeling

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Running Title: Evaluation of Ca^{2+} Signaling in Sketelal Muscle Remodeling

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Summary

The aim of this study was to evaluate cell diversity by considering how the activation of Ca^{2+} signaling has been adapted for the control of different skeletal muscle cell functions. We characterized single C2C12 myoblasts by determining intracellular Ca^{2+} signaling kinetics after the exposure to specific drugs and calcium blockers using fast fluorescence microspectrofluorimetry. The analysis of ATP effects confirmed that these cells expressed functional purinergic adenosine and P2 receptors. Furthermore, we found that the sensitivity of C2C12 cells to glutamate was mediated by ionotropic glutamate receptors. As expected, the majority of cells were responsive to cyclopiazonic acid, which inhibits the sarco-endoplasmic reticulum Ca^{2+}-ATPase pump. These results suggest that C2C12 cells possess functional L- and P/Q-type voltage-operated Ca^{2+} channels, ryanodine receptors and functional sarcoplasmic reticulum Ca^{2+} stores (typical for muscle cells), adenosine and P2 purinergic receptors, and ionotropic glutamate receptors. The evaluation of intracellular Ca^{2+} signaling is a promising approach towards a better understanding and control of the physiopathological properties of myogenic cells, which could be used as a predictive factor in the selection of optimal cells for the recellularization of scaffolds or tissue engineered constructs suitable for stem cell-based therapy.

Key words: Ca^{2+} signaling, homeostasis, skeletal muscle, muscle remodeling

The intracellular free Ca^{2+} measurement in single isolated cells can be an invaluable approach for identification of diverse cellular subtypes in heterogeneous cell population that occurs in the skeletal muscle. Excitation-contraction coupling in muscle cells occurs mainly through the activation by Ca^{2+} signaling cascades and the release of Ca^{2+} from intracellular stores. Since membrane depolarization is a key element for the activation of many muscle cells, much attention has been paid to the mechanisms responsible for depolarization of the cell membrane. To confront muscle cell diversity by considering how the activation mechanisms for generating Ca^{2+} signals have been adapted to control the different cell functions, we utilized the intracellular free Ca^{2+} measurement on single cells. For that purpose a fluorescence microspectrofluorimetry was adapted so that the control and test solutions could be applied using a temperature controlled multichannel polypropylene capillary perfusion system (Forostyak *et al.*

2013, 2016a and 2016b). A single outlet capillary tubing (100 μm inner diameter) with a flow rate of 250 μl/min was positioned close to the tested cell (<0.5 mm) and thus, the selected cell could be subjected to a constant flow of control buffer or test solutions. Each capillary was fed by a reservoir 45 cm above the bath and connected to a temperature control device. The temperature of all solutions was maintained at 37°C. In this approach, switching the flow from one capillary to the next will result in complete solution exchange within 1-3 seconds (Viero *et al.* 2010, Viero *et al.* 2014, Kortus *et al.* 2016). This approach could be used for characterization of diverse cell types present in tissues and organs, e.g. cells constituting the parenchyma vs. stromal cells. We tested this hypothesis on examination of myogenic (muscle) cells and non-myogenic cells. We characterized the C2C12 myoblasts by studying in detail the functional properties of voltage-operated Ca^{2+} channels (VOCC), glutamate and purinergic receptors, as well as intracellular sarcoplasmic Ca^{2+} stores and store-operated Ca^{2+} entry. To identify the specific involvement of the type(s) of active purinergic receptors, we tested the effect of the ATP (100 μM), ADP (100 μM) and adenosine (2 μM). ATP evoked $[Ca^{2+}]_i$ increase in 29 ± 5 % of cells (Fig. 1); ADP had no effect, whereas adenosine caused rise in $[Ca^{2+}]_i$ in 24 % of tested cells. ATP-induced $[Ca^{2+}]_i$ rise was partially inhibited by non-selective P2 receptor antagonist suramin. These data suggest that myogenic cells express functional purinergic adenosine and P2 receptors. Glutamate, applied at 50 μM concentration evoked Ca^{2+} increase in 68 ± 8.4 % of myogenic cells (Fig. 1). Furthermore, we tested the $[Ca^{2+}]_i$ responses to various concentrations of glutamate (1 μM – 1 mM). The amplitude of the glutamate-induced $[Ca^{2+}]_i$ responses at various concentrations ranged, respectively: at 1 μM = 224 ± 21 nM, n= 6; at 10 μM = 543 ± 36 nM, n = 6; at 50 μM = 611 ± 32 nM, n = 9; and at 100 μM = 726 ± 57 nM, n = 10. Glutamatergic signals in skeletal muscles are generally mediated by ionotropic (NMDA, AMPA, kainate) and metabotropic (mGlu) receptors. 25 % of tested cells were sensitive to the application of NMDA (100 μM), while only 1 out of 6 cells was sensitive to kainic acid (100 μM). In order to determine the contribution of metabotropic glutamate receptors, we applied glutamate in the absence of extracellular Ca^{2+}; this caused a $[Ca^{2+}]_i$ increase in only 15 % of tested cells suggesting that the sensitivity of myogenic cells to glutamate is mediated mainly *via* ionotropic glutamate receptors.

Next, we studied the influence of the external Ca^{2+} concentration and of Ca^{2+} channels at the plasma membrane on sarcoplasmic reticulum Ca^{2+} release. Functional intracellular Ca^{2+} stores were checked with 1 μM ryanodine (ryanodine receptor (RyR) activator) and 20 mM caffeine

(inhibitor of intracellular IP_3 receptors and a potent activator of RyRs). A brief application (10 s) of either caffeine or ryanodine induced a $[Ca^{2+}]_i$ rise in 72 % and 81 %, respectively (Fig. 1). Immunostaining for RyR1 and RyR3 was positive in all tested cells. The application of 10 µM cyclopiazonic acid (CPA), a potent, selective and reversible inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase pump (SERCA), caused an intracellular $[Ca^{2+}]_i$ increase in 92 % of cells tested with a mean amplitude of 604 ± 21 nM (Fig. 1). The removal of extracellular Ca^{2+} did not affect significantly the $[Ca^{2+}]_i$ responses induced by 20 mM caffeine on myogenic cells. However, a major reduction in the Ca^{2+} response to caffeine was observed in non-myogenic cells studied in the absence of extracellular Ca^{2+}. Because only few non-myogenic cells appeared to be sensitive to caffeine we focused on myogenic cells to dissect the functional interaction between RyR and Ca^{2+} channels of the plasma membrane. We subjected myogenic cells to successive exposures to caffeine, in the absence or presence of Ca^{2+} channel blockers. Only the L-type Ca^{2+} channel blocker was able to block the Ca^{2+} response induced by caffeine, suggesting that the response could not directly be mediated by the caffeine-sensitive channels. To check this, we monitored Ca^{2+} entry through various VOCC as $[Ca^{2+}]_i$ transients evoked by depolarization with 50 mM K^+. The application of high K^+ solution evoked a rapid increase in $[Ca^{2+}]_i$ in 62 % of the tested cells (Fig. 1) and this increase was significantly reduced by 91 ± 13 % (n =7) after the preincubation with Cd^{2+} and Ni^{2+}, indicating the involvement of voltage-operated Ca^{2+} channels in depolarization-induced Ca^{2+} entry. Application of selective L-type VOCC blocker nicardipine (10 µM) significantly reduced $[Ca^{2+}]_i$ responses ($p = 0.03$) in 82 % of myogenic cells suggesting the contribution of L-type Ca^{2+} channels. The application of 300 nM ω-conotoxin MVIIC, which is known to block the P/Q-type of VOCC, reduced the $[Ca^{2+}]_i$ responses by 67 ± 11 % ($p = 0.004$) in all cells. The specific N-type VOCC blocker, ω-conotoxin GVIA had no effect, suggesting the absence of functional N-type Ca^{2+} channels in myogenic cells. To confirm the above $[Ca^{2+}]_i$ measurement results, we performed a series of immunocytochemical analyses. No positive immunostaining for the α1B subunit of the N-type of VOCC was observed, but positive immunostaining for the α1C subunit of the L-type of VOCC and the α1A subunit of the P/Q-type of VOCC was observed, typical of myogenic cells, suggesting the presence of L- and P/Q-type Ca^{2+} channels in myoblasts.

In general, non-myogenic cells were much less responsive to various physiological stimuli. These cells did not show significant response to calcium channels blockers of T, L, N,

P/Q and R types, and failed to exhibit the $[Ca^{2+}]_i$-induced response to caffeine in a huge majority of cells (66 cells out of 71). A very small response was observed in only 5 out of 71 cells. In contrast to myogenic cells, a progressive desensitization of the $[Ca^{2+}]_i$ response was observed in non-myogenic cells exposed to repeated caffeine applications (3 min each). In addition, the peak amplitude of the responses induced by three successive shots of 20 mM caffeine was significantly lower ($p<0.01$) than in myogenic cells 56 %, 71 % and 93 % decrease, respectively (n=16).

Altogether our results suggest that C2C12 myoblasts possess functional L- and P/Q-types of voltage-operated Ca^{2+} channels, ryanodine receptors and functional sarcoplasmic reticulum Ca^{2+} stores typical for muscle cells, adenosine and P2 purinergic receptors as well as ionotropic glutamate receptors. Functional characterization of cells with fast fluorescence microspectrofluorimetry allows to distinguish not only diverse cell types but also their quality. The resulting data on cell heterogeneity have potential to be exploited for the reconstruction of the skeletal muscle as well as repair of other organs. The specificity of cellular action depends on the source, localization and mechanism of Ca^{2+} signal generation and can be used as a predictive factor for selection of optimal cells for recellularization of acellular muscle scaffolds or tissue engineered constructs or suitable cells for stem cell-based therapy.

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Conflict of Interest

The authors have indicated that they have no conflict of interest regarding the content of this article.

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Figure legend

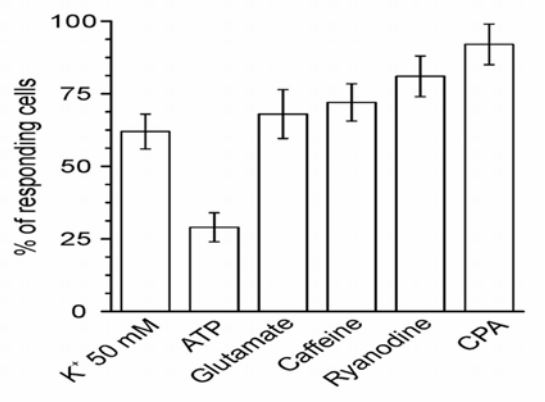

Fig. 1. Bar diagram showing the percent of myogenic cells C2C12 responding by a rise of intracellular Ca^{2+} to the application of various Ca^{2+} channels and receptors agonists, such as 50 mM K^+; 100 μM ATP; 50 μM glutamate; 20 mM caffeine; 1 μM ryanodine and 10 μM cyclopiazonic acid (CPA).