Ph. D. THESIS

REGULATION OF THE INTRACELLULAR CALCIUM HOMEOSTASIS IN SKELETAL MUSCLE CELLS

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Introduction

Skeletal muscle development

Skeletal muscle development can be subdivided into three distinct stages. During the first, so called embryonic stage or early differentiation, the undifferentiated mesodermal cells are transformed into myogenic cells. The myoblasts start the differentiation program that is the genes encoding muscle-specific proteins are activated and the cells start to fuse. In the second phase, after the fusion, the expression of muscle-specific proteins, as actin, myosin, troponin, tropomyosin, myokinase, kreatinphosphokinase, some receptors (e.g. acetilcholine receptor, AchR) and cell adhesion molecules increases. During this stage the cells grow, and adapt to their specific function. These two phases can be repeated *in vitro*, but due the lack of external influences, as innervation and specific hormones, the development unable to exceed this stage. The third phase, the terminal differentiation is characterized by the formation of different muscle types.

A subpopulation of myoblasts forms the embryonic muscle, while a separate cell line of myoblast gives rise to the satellite cell populations. Satellite cells are non proliferating cells, found between the sarcolemma and basal membrane of mature skeletal muscle. These cells are activated following cell damage and are the source for the regeneration of injured or destroyed muscles. During this process satellite cells are transformed into proliferating myoblasts and they undergo the same developmental and differentiation pathway as during the embryonic development.

Characteristic of striated muscle

Voltage-gated Na^+, delayed K^+, Cl^- and Ca^{2+} channels, as well as several ligand-gated receptors are found in the membrane of mature skeletal muscle. Myofilaments form myofibrils which are surrounded by an intracellular membrane system, the sarcoplasmatic reticulum (SR). This consists of longitudinal (L-) tubules and ends called the terminal cisternae. The latter are in close proximity of the transversal (T-) tubules, the folds of the sarcolemma and with them form the triads. The SR functions as the calcium store, and enable the simultaneous, rapid increase of calcium concentration in the intracellular space.

Excitation-contraction coupling (E-C coupling) is a series of events that span between the depolarisation of the surface membrane and contraction. Its first step is the propagation of the action potential along the T-tubules. Dihydropyrydine receptors (DHPR), non-functional L-type Ca^{2+} channels, serving as voltage sensors are located in the T-tubule membrane. While

ryanodine receptors (RyR), calcium release channels, are found in the membrane of the terminal cisternae.

According to our knowledge, the organisation and certain functions of striated muscles in amphibians differ from more in mammals. The explanation reside in different morphology of their sarcoplasmatic reticulum, and the different isoforms of their ryanodine receptors. In the frog two RYR isoforms are present, which are found in twin rows. One is closely associated with the voltage sensor DHPR-s, while the other isoforms are farther away and a small rise in the intracellular calcium contcentration opens them. This latter isoform is not expressed in mammalian skeletal muscle. There activation of RYR-s via the DHPR-s, causes calcium release. The increase in $[Ca^{2+}]_i$ then initiates contraction and other cellular processes.

The openings of one or some RYR-s, that underlie the global calcium relese and the global, transient intracellular calcium concentration ($[Ca^{2+}]_i$) increase, are termed elementary calcium release events (ECRE). They can be subdivided into sparks and embers. The spark is characterized by the relatively large amplitude, and short duration, while the ember is an event with long duration and small amplitude.

The purinergic signal transduction pathway

The ATP is a now clearly accepted neurotransmitter. It was firstly described in non-adrenerg, non-cholinerg nerves (NANC). The signal transduction pathway initiated by ATP is termed purinergic transmission. Its receptors are subdivided two groups, based on their effects and endogenous agonists. The adenosine or P1 purinoreceptors are activatable by adenosin, are connected to the adenilate-cyclase and are usually found on the presynaptic membrane of neurons. The other group is termed as P2 purinoreceptors, their agonists are ATP and ADP. There are found in several cell types, including neurons, where they are located the postsynaptic membrane and participate in the stimulation-transduction processes.

P2 receptors are further subdivided into two main groups according to their structure and signal transduction pathway:

1. P2X purinoreceptors are ligand-gated cationic channels, consisting of two transmembrane domains and an extracellular loop, the C- and N-terminal are intracellular.
2. P2Y purinoreceptors have 7 transmembrane domains, they are coupled to G-proteins, and activate different phospholipase C enzyme.

Effects of ATP in skeletal muscle

The sources of extracellular ATP at muscle cells are diverse. ATP is co-released with acetylcholine from nerve terminals at the neuromuscular junction and participates in the modulation of synaptic transmission. Furthermore, it can be released from the muscle cells during strenuous exercise or following cell damage. Recently, extracellular ATP has been suggested to participate in the regulation of muscle differentiation and to be involved in sarcoglycanopathies, since sarcoglycans function as ecto-ATP-ases.

Both ionotropic and metabotropic P2 receptors have been described on skeletal muscle cells. The expression of these receptors displays a differentiation-dependent pattern, certain subtypes that are expressed at early disappear at later stages of development. Fully differentiated mammalian fibres seem to lack P2X receptors, although denervation can induce their expression. P2Y receptors were described and characterised on C2C12 mouse myotubes, but embryonic cells also express them. The activation of both P2X and P2Y receptors were shown to lead to the increase in $[Ca^{2+}]_i$ but the relative contribution of the two pathways have not been determined.

Proteinkinase C enzymes in skeletal muscle cells

The activation of the phospho-inositide (PI) pathway via a P2Y receptor will not only increase $[Ca^{2+}]_i$ but will also lead to the activation of enzymes of the protein kinase C (PKC) family. PKC isozymes have been suggested to play a pivotal role in the proliferation and differentiation in a wide variety of tissues including skeletal muscle.

Protein kinase C comprises a family of serine/threonine kinases. Up to date, at least 11 different isoenzymes have been identified, which can be classified into the groups of calcium- and phorbol ester-dependent 'classical' (PKCα, βI, βII, and γ), the calcium-independent 'novel' (PKCδ, ε, η, and θ), the calcium- and phorbol ester-independent 'atypical' (PKCζ and λ/ι) isoforms, and the unique PKCμ. These isoforms possess a characteristic expression pattern in a given cell type, and isoenzyme-specifically regulate various cellular processes. Not only may some PKC isoforms be activated whereas others not for a given response, but different PKC isoenzymes may often have antagonistic effects on the same cellular events suggesting differential roles of specific PKC isoforms.

Aims

In our experiments we examined the intracellular calcium homeostasis and the elementary calcium release events that underlie the global calcium increase in skeletal muscle cells.

We asked the following questions:

1. How does the extracellular ATP affect cultured skeletal muscle cells?
2. Are purinergic receptors present on skeletal muscle cells in culture, and if yes which isoforms? What are the possible mechanisms for calcium entry into the intracellular space on these cells and how ATP modulates these processes?
3. Does the extracellular ATP have any effect on the proliferation and differentiation and if yes, through which signal transduction pathway?
4. What are the characteristic parameters of the elementary events of calcium release from the SR?
5. Is it possible to determine the number of channels, underlying these events, from their characteristic parameters? What is the difference between the two main forms of elementary calcium release events, the sparks and the embers? Can they be altered?

Methods

Skeletal muscle cells in culture

Muscle tissues were obtained either from healthy human subjects undergoing orthopaedic surgery or from young mice following the guidelines and an approved protocol of the Ethics Committee of the University of Debrecen. A simple procedure was followed to obtaine satellite cells from the samples and to grow myotubes from the satellite cells. The muscle biopsy was dissociated at 37°C using collagenase and trypsin. The satellite cells were re-suspended in Ham's F-12 growth medium supplemented with 5% fetal calf serum (FCS), 5% horse serum (HS) in the case of human, and 10% FCS and 10% HS in the case of mouse cells. The cells were kept in a 5% CO2 athmosphere at 37°C. After 3 days in culture the medium was exchanged to Dulbecco's Modified Eagle's Medium supplemented with 2% FCS and 2% HS for human cells, and 4% HS for mouse cells to facilitate myoblast fusion and differentiaition.

Measurement of $[Ca^{2+}]_i$

Experiments were carried out on 7-14 days old cultures for human and 4-10 days old cultures for mice, carefully registering the degree of fusion for each myotube by counting the number of nuclei in the given cell. The calcium sensitive probe Fura-2 was introduced into the myoplasmic space in its AM form. Changes in intracellular calcium concentration were assessed measuring the fluorescence at 510 nm with alternating excitation between 340 and 380 nm using a Deltascan and an on-line connected computer. The intracellular calcium concetration ($[Ca^{2+}]_i$) levels were calculated from the fluorescence ratio.

Calculation of the calcium flux

To determine the calcium flux (Fl) entering the myoplasmic space, the calcium binding to intracellular binding sites and the removal of calcium from the intracellular solution was calculated. To this end calcium binding to the pump, to troponin C, to parvalbumin and to the dye, and the removal by the calcium pump was considered. The computer routine determined, as a single best fit parameter, the maximal transport rate of the pump (PV_{max}) from the declining phase of the calcium transient following the stimulation. All other parameters were held constant at values taken from the literature. A single fit was carried out for each myotube, and then used for all calcium transients on the given cell. Usually transients evoked by K^+-depolarisation were used for the fitting, starting at least 3 s after the peak of the

calcium transient. It should be noted that, unlike the so called “calcium release” Fl reflects all fluxes of calcium that enter the myoplasmic space. Fl thus includes calcium entering through the surface membrane and the calcium released from the sarcoplasmic reticulum (SR).

Voltage-clamp studies

Cells were voltage clamped using an Axopatch 200A amplifier. Patch-pipettes were fabricated of borosilicate glass capillaries, and had resistances of 3-4 MΩ when filled with an artificial internal solution. Passive electrical parameters of the cells were determined using 40 ms long pulses of 5 mV. The holding potential was set to –80 mV. The linear capacitance of the cells was between 100 and 600 pF.

The effect of externally applied ATP was followed either in voltage- or current-clamp mode. In case of the former, the holding current (I_h), necessary to maintain the -80 mV holding potential, was continuously recorded and the ATP-evoked current (I_{ATP}) was defined as the change in I_h in the presence of ATP. For the latter, the membrane potential was continuously monitored before, during and after the addition of ATP, and the magnitude of the depolarisation, caused by ATP, was determined.

Cell proliferation and fusion

To describe the effects of extracellular ATP on proliferation and myotube formation we followed the method previously described. The cells were cultured in 24-well plates and the culture medium was exchanged every second day. ATP, where appropriate, was present in the culture medium from day 2 till day 4. The number of nuclei was counted in 4 visual fields using an inverted microscope (200x), and changes in proliferation was expressed as proliferation rate, the relative increase in the number of myogenic nuclei. The degree of differentiation was assessed by calculating the fusion index (FI), the ratio of all nuclei in myotubes (cells with at least two nuclei) over the total number of nuclei in myogenic cells. Measurements were carried out in triplicates and averaged from 4 independent experiments. The time course of FI was fitted with a growth curve.

Immunofluoroscence studies

The myotubes at days 2 and 5 of culture were washed with PBS, fixed in acetone, air dried and treated with a 0.6% Triton X-100 and 5% BSA containing solution at room temperature for 30 min. An incubation with the rabbit anti-PKC antibody and a mouse anti-desmin antibody for an hour and a half was followed by a 1 h incubation with goat anti-rabbit IgG

labelled with fluorescein and an additional 1 h incubation with horse anti-mouse IgG labelled with Texas Red. Labelling of P2 receptors followed the same procedure using rabbit anti-P2 receptor antibodies instead of the anti-PKC antibodies. Fluorescence was measured using an inverted fluorescence microscope

Measurement of calcium sparks

Single skeletal muscle fibers, isolated enzymatically from the *extensor digitorum communis* of rats, or mechanically from the *musculus semitendinosus* of frogs. Cells were treated with a Relaxing solution containing 0.002% Saponin and permeabilization of the surface membrane was monitored. This solution was then exchanged to a K_2SO_4, or K-glutamate based Internal solution. Fibers were imaged using an LSM 510 laser scanning confocal microscope. Line scan images ([x,t]) were taken with a 40x water immersion objective parallel to the fiber axis. Fluo-3 was excited with an Argon ion laser (at 488 nm; 5% laser intensity), emitted light was collected through a band-path filter and digitized at 12 bit. Any bleaching of the dye was corrected for. To avoid photo-damage, the same position was not imaged twice. The bleaching-corrected images (F[x,t]) were then normalized to baseline fluorescence (F_0[x]). Elementary calcium release events were captured using an automatic computer detection method. The program identified elementary events as regions with fluorescence above a relative threshold, calculated from the noise in the images, and having amplitudes greater than 0.3 $\Delta F/F_0$ units. The program also determined the parameters of the identified events. Amplitude (as $\Delta F/F_0$), spatial half-width measured at the time of the peak (FWHM), duration and rise time for sparks and sparks with embers, while average amplitude, duration and FWHM for lone embers. FWHM was obtained from fitting a Gaussian function to the spatial distribution obtained by averaging 3 lines at the peak for sparks or by all lines except the first and last 10 ms of the event for lone embers.

Results

ATP induced changes in $[Ca^{2+}]_i$

When ATP was administered into the bathing medium of cultured human myotubes the cells responded with a transient rise in $[Ca^{2+}]_i$ that varied both in kinetics and size with the state of development. Two typical kinetic responses were observed for 180 μM ATP in myotubes with more than 5 centred nuclei, where the transients were the largest in amplitude. In most cases these transients displayed a monotonic rising phase with a relatively slow rate of rise. In other myotubes, especially in this stage of development, the transients had an early, fast component followed by a second, more gradual increase in $[Ca^{2+}]_i$. The early component was not always followed by a clear decrease in $[Ca^{2+}]_i$, rather, the rising phase was composed of two phases, a fast followed by a slow rise. The fast component of the calcium transients was eliminated by tetrodotoxin (TTX) and was neither present in myoblasts nor in myofibres (large myotubes with peripheral nuclei). The calcium transients were characterised by relatively long latencies and were resistant to TTX, as well as to blocking the nicotinic acetylcholine receptor (nAchR) or the voltage-gated, L-type calcium channels since the addition of 20 μM d-tubocurarine or 100 μM verapamil into the bathing medium did not prevent ATP from eliciting a calcium transient.

The removal of calcium from the external milieu completely abolished the response to ATP. The lack of response to the second ATP challenge was not due to a full desensitisation of the signalling pathway since upon the readministration of calcium into the external solution ATP was again capable of evoking a calcium transient. Nevertheless, the increase in $[Ca^{2+}]_i$ was somewhat smaller during a second, and all subsequent, ATP applications, in all cells tested, indicating either a slow desensitisation or a run-down of the signalling pathway.

Changes in the calcium signals during maturation

The size and kinetics of the ATP evoked responses depended on the stage of development of the myotubes rather than on the days spent in culture. The ATP-evoked calcium transients were relatively small both at early and at late stages of development, while myotubes with more than 5 central nuclei responded with large changes in $[Ca^{2+}]_i$. In contrast, the amplitude of both the acetilcoline (Ach) and the potassium evoked signals increased monotonically with cell maturation being the largest in large myofibres. The age-dependence of the ATP-evoked calcium transients was bell-shaped, while that of the Ach- or potassium-evoked signals increased monotonically, reaching a maximum for myotubes with more than

10 nuclei. The myoblasts failed to respond to any challenge with Ach or potassium, ATP did induce a small elevation in $[Ca^{2+}]_i$ in these cells. While the responsiveness to these various agonists displayed a clear dependence on cell maturation, the resting $[Ca^{2+}]_i$ remained fairly constant in the examined period. The actual values varied between 81±6 and 91±2 nM, demonstrating that the cells maintained a low $[Ca^{2+}]_i$ similar to that reported earlier for cultured or adult skeletal muscle fibres.

Dose response curve for ATP

The dose-dependence of the ATP-evoked calcium transients was assessed by applying the agonist in concentrations ranging for 1 to 180 μM. Myotubes having more than 5 central nuclei, thus displaying the largest response to ATP, were used. To avoid any interference from possible desensitisation only the very first ATP-induced elevation in $[Ca^{2+}]_i$ was included into the analysis for any given cell. The changes in $[Ca^{2+}]_i$ caused by a given concentration of ATP were then averaged and plotted as a function of the concentration. The data points were fitted with the Hill-equation. The half activating concentration was 16.1±0.5 μM and the Hill-coefficient was 1.0±0.1.

Effects of ATP-analogues on $[Ca^{2+}]_i$

To address the pharmacology of the ATP-induced influx pathway various analogues of ATP were tested for their ability to reproduce the effects of ATP. Both α,β-methylene-ATP and β,γ-methylene-ATP were much less effective than ATP itself. In a concentration of 180 μM α,β-methylene-ATP failed to induce any measurable change in $[Ca^{2+}]_i$ in 7 out of 12 trials. Although β,γ-methylene-ATP was more effective, 17 out of 20 cells responded to the challenge, the amplitude of the calcium transient was still much smaller than that evoked by the subsequent addition of ATP. Although 2'3'-O-(4-benzoyl)benzoyl-ATP (BzATP) was found to be the more potent than the other ATP analogues, it still did not prove to be as efficient as ATP. On average, the 180 μM BzATP-evoked signals had half the amplitude if compared to those induced by 180 μM ATP. These values were 21 and 5% for β,γ- and α,β-methylene-ATP, respectively.

Skeletal muscle cells from mice in culture

Further experiments were carried out on cultured skeletal muscle cells from mice. First it was showed, that both the ATP-, and the depolarization-evoked calcium transients had similar amplitudes as on human cells. Furthermore the amplitude of these transients again increased with cell maturation up to the point where multi-nucleated myotubes were formed, and then it decreased.

The ATP-evoked calcium flux displays a transient and a maintained component

To establish the general characteristics of the ATP-induced calcium flux, calcium transients were measured in response to stimulation with prolonged depolarisation and the application of ATP. Depolarisation was induced by replacing 120 mM NaCl with KCl in the external solution. Utilising the absence of calcium flux following the application of potassium a simple removal model was fitted to the declining phase of the calcium transients and the ATP-evoked calcium flux was calculated. After reaching an early peak, the flux declined to a quasi-maintained steady level and stopped only after removing the drug from the extracellular medium. The overall characteristics of the early, transient calcium flux were similar to those evoked by potassium. It had an amplitude of 125 μM/s, a time to peak of 5.8 s and a half relaxation time of 0.9 s. The steady flux was, on average, 11% of the early peak, thus representing a 13.7 μM/s flux of calcium into the myoplasm.

ATP-evoked fluxes have distinct characteristics at different stages of development

In myoblasts or in myotubes with only a few nuclei $[Ca^{2+}]_i$ rose rapidly, stayed elevated during the presence of the nucleotide and then declined to its resting value. The calculated Fl had a distinct peak followed by a relaxation to a maintained steady level. For slightly more mature myotubes (5-10 nuclei) the calcium transient had a distinct early peak. Although the general shape of Fl resembled that recorded for less differentiated cells, the early peak was narrower. This narrowing of the early peak of Fl with differentiation was found to be a general characteristic of the myotubes, and was described as a decresae in full width at half maximum. Not only did the early peak become narrower with maturation, it tended to completely separate itself from the following steady level with a clear notch. This notch, at first, only gave rise to a small secondary increase, but later, it became wide and deep enough to completely separate the steady level into an independent second component starting from the baseline. Finally, in large, multinucleated fibres (more than 10 nuclei) the ATP-evoked flux lost its early peak and only the slowly rising secondary component was present.

Repeated application partially suppresses the ATP-evoked flux

For pharmacological experiments it was essential to know if repeated applications of a drug gave similar calcium transients and fluxes. We were also concerned whether a K^+-depolarisation would reduce the content of the SR and thus alter the response to subsequent ATP stimuli.

The calcium transients elicited by repeated application of caffeine had similar amplitudes, the relative amplitude of the second and third transients were 0.87 and 0.81, respectively. Not only were the calcium transients close to identical but the calculated fluxes were also similar, with a slight decline in the early peak (relative amplitudes being 0.88±0.12 for the second transients, n=7) and with essentially the same maintained flux (1.09±11). The decline seen in the peak could be attributed to the slight depletion of calcium in the SR since depolarisation evoked calcium transients, through the influx of calcium from the external medium and re-loading of the SR, prevented this suppression.

In contrast, the amplitude of the ATP-evoked calcium transients clearly declined with repeated application. The effect was more pronounced at earlier (cells with less than 5 nuclei) than at later stages (cells with 5-10 nuclei) of development. Pooled data from the early stages of differentiation revealed that the second transient was suppressed by 26±8 while the third by 40±10% as compared to the first. The underlying calcium fluxes were also suppressed, but the suppression of the two kinetic components was not identical. The maintained component was hardly affected, whereas the early peak was greatly suppressed by subsequent applications of ATP. Expressing the peak and the steady component of the second and third transients as relative to those of the first revealed a 35±10 and 53±12% reduction in the former, while no significant change (4±11 and -12±9%) in the latter.

Cells with 5-10 nuclei showed a slightly different behaviour. The calcium transients were only slightly suppressed, due to the relatively constant early phase, while the calculated Fl showed a clear reduction in the steady component, too. Pooled data showed, that, in contrast to less differentiated cells, the early peak was less affected by repeated application than the steady level of Fl.

ATP induces a sustained inward current and a drop in membrane potential

The application of ATP for 20 s caused a sustained inward current, which did not desensitise since a subsequent addition of the drug evoked a current with similar amplitude. This observation was confirmed on 6 myotubes, with the relative amplitude of the second current trace being 97±12%. In myotubes with more than 10 nuclei I_{ATP} was found to be much smaller. On average, with a holding potential of -60 mV, the ATP-induced current was larger than 250 pA at the earlier, and smaller than 100 pA at the later stage of development. In parallel the resting membrane potential was more hyperpolarised for the more differentiated cells, and the size of the ATP-induced depolarisation was smaller, too.

To further characterise the P2X signalling pathway I_{ATP} was measured at different holding potentials (-80, -60, -40, -20 and 0 mV) and at different concentrations of ATP (from 1 to 300 μM). The ATP-evoked current was inward at the holding potentials tested indicating a non-specific cationic current. Furthermore, the channel showed an inward rectification characteristic to P2X receptors.

The kinetics of I_{ATP} did not depend significantly on the concentration of ATP used. Fitting the data points with the Hill-equation yielded a half-activating concentration of 68±5 μM and a Hill-coefficient of 1.66±0.14.

These findings establish that a P2X mediated pathway could play an important role in the measured ATP-evoked calcium flux. The observed reduction in the steady inward current, together with the smaller depolarisation also suggest that the down-regulation of the P2X pathway underlies the differentiation-related differences in $[Ca^{2+}]_i$ and Fl.

The transient phase of the ATP-evoked calcium flux critically depends on the depolarisation of the cell

Less differentiated cells, myoblasts and myotubes with less than 5 nuclei, usually did not respond with a calcium transient to depolarisation. ATP, on the other hand, was capable of inducing a calcium flux both in the absence and presence of KCl. Nevertheless, the ATP-evoked calcium transient was smaller and had a characteristically slower rising phase under depolarised conditions. The underlying flux lacked the early peak but kept its late component. Later in development, in cells with 5-10 nuclei, the depolarisation brought about a large increase in $[Ca^{2+}]_i$. This, again, did not prevent ATP from evoking a calcium transient and, furthermore, the pronounced effect of the depolarisation was to remove the early peak.

Since repeated application of ATP on its own was found to alter Fl, data from depolarised cells were normalised to the first ATP-evoked transient and expressed as relative

values. These were then compared to similarly calculated data from cells where the second ATP application was not preceded by a depolarisation. Pooled data from early and later in development show that the depolarisation significantly suppressed the calcium transient ($p<0.05$) and essentially eliminated the early peak component of Fl. On the other hand, the suppression of the steady flux by the depolarisation was not significantly different from the suppression caused by subsequently applied ATP alone ($p>0.1$).

Activation of both P2X and P2Y receptors underlie the ATP-evoked calcium flux

The steady component of the ATP-evoked calcium flux was found to be resistant to depolarisation, therefore it must be, at least partially, independent of the activation of the P2X receptors. It was of interest to see if it corresponded to the activation of a specific purinergic pathway or to some unspecific action of ATP. Since suramin has been known to block the purinergic signal transduction, we tested if it was capable of interfering with the ability of ATP to evoke a sustained flux. To this end myotubes in the later stages of development were used where only the sustained component of Fl was present.

Subsequent applications of 10 and 300 μM suramin suppressed the ATP-evoked calcium transients and the underlying calcium flux. Following the removal of suramin a partial recovery was observed both in the calcium transients and in the calcium flux. Although neither $[Ca^{2+}]_i$ nor Fl returned to the value that was measured before the application of suramin, the obtained flux after recovery was comparable to the flux where subsequent applications of ATP were studied. 10 μM of the drug caused only a partial suppression (appr. 50%) both in the calcium transients and in the underlying flux. On the other hand, increasing the concentration of suramin to 300 μM almost completely abolished the ATP-evoked calcium flux and, consequently, the change in $[Ca^{2+}]_i$. Repeating the measurements on myotubes under voltage-clamp conditions and measuring the ATP-evoked current revealed, in line with the above, a partial suppression at 30 and an essentially complete abolishment at 300 μM suramin.

These observations suggested that the sustained component of Fl was, at least partially, due to the activation of a purinergic signalling pathway. Since this flux was present on depolarised cells, too, we suspected the presence of metabotropic P2 receptors on our cells. To identify the different purinergic receptors, cells were labelled with antibodies against several P2X and P2Y receptor. The desmin-positive cells were identified as myogenic in origin. A number of P2 receptors found to be on skeletal muscle cells in culture, namely, $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$ and $P2Y_4$. Cells at the stage of 5-10 nuclei were labelled with

anti-P2 and anti-desmin antibodies. Whereas the labelling was strong for $P2X_4$, $P2X_7$, $P2Y_1$ and $P2Y_4$, it was found to be relatively weak for $P2X_1$ and $P2Y_2$. We were unable to show any clear labelling for $P2X_2$. $P2Y_1$ had a distinct surface membrane localisation.

ATP inhibits the proliferation but increases the differentiation of muscle cells in primary culture

Previous studies have indicated that the addition of ATP to satellite cells from rat promotes their differentiation acting via $P2X_5$ receptors. To complement this result with observations from mouse myotubes we added 180 μM of the drug to our cultures from the first to the third day. Proliferation was visualised by following the proliferation rate, the relative change in the number of nuclei in myogenic cells. Under control conditions the proliferation rate had a clear peak, showing the largest rate at day 2. The addition of ATP did not alter the tendency for the proliferation rate to have a clear peak, however, the peak was suppressed and shifted to after the removal of the drug.

Together with suppressing proliferation ATP promoted differentiation as assessed by calculating the fusion index (Fi). The fusion index in control first rises slowly, indicating an increase in myogenic nuclei that are mostly in myoblasts. It's slope than increases rapidly as myoblasts fuse to form multinucleated myotubes. Finally, Fi(t) levels off at unity to show that all myoblasts have fused. The presence of ATP from day 1 to day 3 did not alter the overall shape of Fi(t) but it shifted the rapidly rising phase to earlier times. To quantify this difference the points were fitted to a simple growth curve. The fit gave 3.8±0.1 and 2.9±0.1 days for $t_{0.5}$ (the time where Fi is half its maximum) in control and in the presence of ATP, respectively, indicating that fusion starts earlier if ATP is present in the culture medium.

Characteristic changes in morphology paralleled the above described alterations in proliferation and fusion. Under control conditions, at day 2 in culture, myogenic cells were mostly myoblasts. They were partially scattered and partially grouped in an oriented way. In the presence of ATP, in a parallel experiment, most of the cells were found in the oriented groups, indicating that they were ready to fuse.

ATP alters the PKC isozyme pattern

To establish if the activation of the PI pathway and the alteration in proliferation/differentiation properties following ATP application involves the PKC family, PKC isoenzymes were immunostained. All isozymes, including the classical α, β and γ, the novel δ, ε, and η, and atypical θ, ζ, λ/ι and μ isoforms were labelled at days 2 and 5. The days

were selected to represent stages of intense proliferation and late differentiation, respectively. Although aPKC-s were labelled at both stages they were not explored further since, on the one hand, they require neither calcium nor diacylglicerol for their activation and, on the other hand, their labelling was unaltered during development.

Four isozymes of the cPKC and nPKC families, α, β, ε, and η showed clear labelling in control, while, for the remaining three, labelling was not detected neither in control nor after the treatment with ATP. All four isozymes were present at both the early and at the later stage of development. They were clearly present within the cytosol and no specific labelling of any subcellular organ was observed at day 2. While the cytosolic labelling was present at day 5 for all four isozyemes, PKCβ showed a dotted staining in the nucleus which was not present before.

The same four isozyemes of the cPKC and nPKC families were detected in myotubes kept in cultures supplemented with ATP from day 2 till day 4. Two characteristic differences, as compared to cells kept in normal culture medium were observed. The labelling of PKCα was considerably less intense at day 3 in cells treated with ATP then in control cells. In addition, dotted labelling of PKCβ was already present at day 3 in these ATP-treated cells.

Elementary calcium release events

We examined the elementary calcium release events (ECRE) that underlie the global calcium increase. Mammalian striated skeletal muscles differ from those of frogs in their triadic structure and RYR composition. Similarly elementary calcium release events in mammalian muscle fibers unlike those in amphibians exhibit a wide morphological variety. Calcium sparks, resembling those in frog fibers, were preceded and/or followed by embers. We established that the amplitude of sparks are greater in frog, while their full width at half maximum, full time at half maximum and duration are greater in mammals. These observation suggest that ECREs and RYR openings are small amplitude and long lasting in mammalian muscles. Comparing the signal mass of sparks and embers revealed that the proportion of channels involved was approximately 15. That is, if embers represent single channel events sparks are generated when some 20-30 channels open.

Elementary calcium release events are altered by thymol

Although excitation-contraction coupling (E-C coupling) in skeletal muscle critically depends on the concerted action of two proteins, the dihydropyrydine receptors and ryanodine receptors, a number of external influences can alter the function of the SR calcium channel.

Mammalian calcium release events not only differ in their appearance from those in frogs, they seem to be less affected by classic drugs, e.g. caffeine, that influence E-C coupling and calcium sparks in amphibians.

We examined, whether thymol show to alter RYR function is capable of influencing the parameters of ECRE. Under control conditions, calcium release events occurred with an appreciable frequency ($0.0587\pm0.0020\ s^{-1}sarcomere^{-1}$). Most abundant were lone sparks (63%), but lone embers (22%) or sparks with trailing embers were also readily observable. Sparks with leading embers were rare (1.1% of all events). In the presence of 30 μM thymol the frequency increased to $0.0654\pm0.0016\ s^{-1}sarcomere^{-1}$. More noticeable was the change in the overall characteristics of the events, since longer and complex events dominated the images with lone sparks decreasing in relative proportion (41%). Lone embers (35%) and sparks with trailing embers were still readily distinguishable, with sparks with leading embers increasing in proportion to more than 4% of all events. The drug decreased the amplitude or average amplitude of both sparks and embers by 20-24% leaving the rise time and FWHM essentially constant. In parallel, the duration of the calcium release events increased considerably. The drug, while reducing the relative proportion of shorter events, induced events that had durations of several hundred milliseconds.

Sparks were hardly ever observed in the presence of 150 μM thymol. Rather, long events resembling lone embers were detected. Their duration, however, often exceeded 500 ms, sometimes lasting longer than 1.5 s, the duration of the image. These events had characteristic amplitudes and full widths similar to the embers measured in the presence of low thymol concentrations.

These data clearly indicate that the long calcium release events correspond to the long-lasting open events seen for the thymol-modified isolated RyR. Since the long calcium release events were neither preceded nor followed by a spark, they should be considered as the opening of a single release channel or the concerted opening of a small group of channels.

Discussion

Human skeletal muscle cells in culture

Our results demonstrated the ability of ATP to induce an elevation of $[Ca^{2+}]_i$ in human skeletal muscle cells in culture. The amplitude of the calcium transients increased with cell maturation up to the point where multi-nucleated myotubes were formed, than it decreased. The transients displayed a variety of kinetics from slow monotonic rise to biphasic behaviour where the first, fast component was followed by a more gradual secondary increase. These effects of ATP were not affected by the nAChR antagonist d-tubocurarine, the voltage-dependent Na^+ channel antagonist TTX, or verapamil, a well known blocker of L-type Ca^{2+} channel. The removal of external calcium abolished the response to ATP. These results establish the presence of an ATP-induced calcium signalling pathway in cultured human skeletal muscle cells that critically depends on the presence of calcium in the external solution. In contrast the acetilcholine, caffeine or depolarisation evoked signals increased monotonically, reaching a maximum for myotubes with more than 10 nuclei.

Cultured skeletal muscle cells fom mice

The application of extracellular ATP induces a calcium flux that displays an early peak and then declines to a maintained steady level on cultured skeletal muscle cells from mice, similarly to human cells. The time course of the ATP-evoked flux changes with differentiation, the transient and steady components become clearly separated with the former completely disappearing on fully differentiated cells. We demonstrated the presence of both P2X and P2Y purinoreceptors on these cells and provided evidence that the activation of both underlie the observed calcium flux. The transient component depends on the activation of P2X receptors and the consequent depolarisation, while P2Y receptors are responsible for most of the maintained component.

Source of calcium ions underlying the observed flux

The effect of extracellular ATP on mammalian skeletal muscle cells in culture is the activation of P2X receptors. This activation, as well as the subsequent depolarisation, if large enough, would induce a series of events that could explain the observed calcium transient. Calcium might enter through the surface membrane via the P2X receptor itself or via voltage-gated calcium channels, or might be released from its internal store, the SR, via voltage- and/or calcium-induced calcium release.

Here we indeed show that the addition of ATP to the external medium of myotubes with 5-10 nuclei resulted in a large inward current and a consequent depolarisation of the cells. The depolarisation was large enough (20 mV) to initiate an action potential on these cells and, consequently, to activate the voltage gated T- and L-type calcium currents. These processes readily explain the large flux following the addition of ATP. On the other hand, the T-type calcium current inactivates rapidly and even the L-type current would completely inactivate in a few seconds. Since the inactivation of the voltage sensor is also complete within 10 s (for adult cells) all possible voltage-activated pathways are inactivated by approximately 10 s leaving the steady component of the ATP-evoked flux unexplained. In addition, mature myotubes had more negative membrane potentials and, furthermore, the ATP-evoked depolarisation was considerably smaller, therefore, the activation of voltage-gated channels or pathways can be excluded under these conditions.

One must, therefore, assume that the steady component of the ATP-evoked flux is either the consequence of a continuous calcium influx through permanently open P2X receptors, or a P2X receptor independent mechanism is responsible. I_{ATP} was found to be essentially constant during the application of the drug resembling currents through P2X7 receptors a subtype which was indeed found on these cells. Taking a permeability ratio of P_{Ca}/P_{Na} of 2, with the composition of our solutions gives that approximately 4% of the current is carried by calcium ions.

These data are thus consistent with a P2X receptor independent flux on myotubes in culture. The presence of P2Y receptors together with the suramin sensitivity of the calcium flux clearly argue in favour of the hypothesis that approximately 80% of the sustained component of Fl is due to the activation of P2Y receptors and the consequent release of calcium from the SR.

Time course of the ATP-evoked calcium flux

The effect of extracellular ATP on the P2X receptors is usually short-lived. There are at least two independent mechanisms that underlie this phenomenon. Some receptors, as $P2X_1$ and $P2X_3$ rapidly, while $P2X_2$ and $P2X_4$ slowly desensitise. In addition, extracellular ATP is degraded by ecto-nucleotidases. Although muscle tissue is rich in ecto-ATPases, their physiological roles have not been clearly established. Neither of the above mechanisms can, however, explain the observed rapid decline in Fl following the early peak.

I_{ATP} was found to have essentially constant amplitude, therefore, supplying a nearly constant calcium flux, thus rendering P2X receptor desensitisation an unlikely cause of Fl

decline. On the other hand, we have clearly shown that the peak component of Fl was suppressed by chronic depolarisation clearly arguing in favour of being carried by calcium currents through the surface membrane and calcium released from the SR. These fluxes are likely to be initiated by an action potential triggered by the depolarisation caused by the opening of the P2X receptors. The termination of the action potential will then terminate these fluxes. In addition, due to the prolonged depolarisation caused by the permanently open P2X receptors, as discussed above, both T- and L-type currents would inactivate. Furthermore, $[Ca^{2+}]_i$ stayed elevated throughout the application of ATP suggesting the involvement of a calcium-dependent inactivation of SR calcium release channels in the decline of Fl. In this framework the peak component of Fl should reflect, to some extent, the time course of the action potential on these cells. Myotubes are known to display slow, calcium-current based action potentials at early, while fast sodium-current based action potentials at later stages of development. In line with the above, the early peak became narrower with differentiation.

It should be mentioned here that the absence of a clear peak on large myotubes is most likely due to two interconnected phenomena. With maturation the depolarisation caused by the activation of P2X receptors becomes smaller. In addition, the cells have more hyperpolarised resting membrane potentials. The addition of ATP thus fails to initiate an action potential, leaving only the sustained flux through P2X receptors and the calcium released from internal stores by IP_3 due to the activation of P2Y receptors.

Purinoreceptor subtypes on cultured skeletal muscle cells

Several P2X receptors are known to be expressed in skeletal muscle. These include $P2X_1$, $P2X_2$, $P2X_5$, $P2X_6$. Recent experiments have pointed out that certain subtypes have a precisely regulated expression-pattern with $P2X_5$ appearing first, followed by $P2X_6$ and $P2X_2$ during pre-natal development in rat. From these $P2X_5$ was shown to be involved in the regulation of muscle differentiation. Our results, on the other hand, showed the presence of three - $P2X_1$, $P2X_4$ and $P2X_7$ - subtypes on primary cultures of mouse mytubes at the stage of 5-10 nuclei. The absence of the $P2X_2$ receptor on our cells could most likely be explained by its expression pattern, namely, this receptor subtype was reported to be present at later stages of differentiation

The demonstration of the $P2X_4$ and $P2X_7$ subtypes on skeletal muscle, on the other hand, is a novel finding. These receptors were shown to be expressed in smooth but not in skeletal muscle cells. Our finding could thus be explained by species differences, differences between cells in primary culture and in situ, or, finally, by cross-reactivity.

Furthermore, the kinetics of I_{ATP} observed in the present study, large sustained current for several 10 s, are characteristic to $P2X_7$ receptors. In addition, the Hill-coefficient for binding was found to be close to two (n=1.66), suggesting the presence of two binding sites for ATP in accordance with earlier studies.

In mice, the presence of both $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors were demonstrated during embryonic development. The expression levels were, however, dynamically changing, with all three receptors down-regulated postnatally. Here we show the presence of all three receptors in cultured mouse myotubes, although $P2Y_2$ receptor expression was low. AchR-s are known to be homogenously distributed on the surface membrane of cultured muscle cells due to the absence of definite neuromuscular junctions. It is intriguing to speculate that the homogenous distribution of the $P2Y_1$ receptor found in this work is also related to the absence of innervation.

PKC isoforms in proliferating and differentiating myotubes

Previous reports have demonstrated that a number of isozymes of the PKC family are present in muscle cells. Studies on cultured human and rat skeletal muscle also revealed that the isoform expression pattern is different in different species. We showed evidence for the presence of PKCα, β, ε, and η. We also found intense labelling for PKCζ and very weak labelling for PKCλ/ι and μ. In comparison with other species both similarities and differences can be pinpointed. The former includes the presence of PKCα, β, ε, and η. From the later the absence of PKCθ, the isoform originally assumed to be skeletal muscle specific is the most remarkable. However, reports from cultured muscle cells indicate that under these conditions PKCθ is not expressed.

PKC isoforms, and especially the specific pattern of expression has been connected with cell proliferation and differentiation in a number of tissues including skeletal muscle. Previous reports suggested that classical isoforms, most importantly PKCα is directly involved in the regulation of proliferation of muscle cells. Here we found that myotubes in culture, while continuously proliferating, display a distinct peak in the rate of proliferation under control conditions which is clearly suppressed if ATP is present in the culture medium. In parallel, PKCα was expressed at both early and late stages during differentiation under control conditions. The addition of ATP, on the other hand, suppressed the amount of PKCα at the time where proliferation was suppressed. In addition, a dotted labelling of PKCβ in the nucleus was observed in ATP treated cultures, as early as two days in culture, which was characteristic of late differentiation under control conditions. These observations suggest that

ATP, acting via P2Y receptors and the PI pathway interferes with the proliferation/differentiation program of skeletal muscle cells in culture.

Calcium release events in permeabilized fibers

Elementary calcium release events in mammalian skeletal muscle preparations have recently been reported from a number of laboratories. These events differ from those measured in amphibians mainly in their morphological variability, namely, that sparks and embers coexist, and in their relative frequency, events are more numerous in frogs.

Calcium release events in the presence of thymol

Thymol, in a concentration of 30 μM, has slightly increased the frequency of the elementary calcium release events, from 0.0587 to 0.0654 s^{-1}sarcomere^{-1}, which is in agreement with its effects on the calcium release measured on cut fibers and on the open probability of isolated RyR-s. The increase in event frequency was, however, not as marked as the increase in global calcium release. Nevertheless, the relative proportion of embers, which are more likely to be the elementary events in mammals, were increased more than the actual frequency.

In higher concentrations the drug resulted, in most cases, in a massive release of calcium rendering the detection of individual events impossible. In the few cases where the fiber survived the addition of 150 μM thymol the frequency of the events was reduced and the relative proportion of embers was further increased.

Comparing the average amplitudes of sparks or embers before (0.93 and 0.21) and after (0.70 and 0.17) the addition of thymol reveals a 20-24% decrease in both cases. This suppression of event amplitude can only be explained as a drop in SR content due to an increased leak of calcium from the SR. Together with the decrease in amplitude the rise time and the duration of events were increased, from 9.4 to 9.6 and from 56 to 79 ms, respectively. The increase in rise time and duration is, on the one hand, likely to be due to the effect of thymol on the open time of the release channel. On the other hand, the decrease in the amplitude of sparks reflects a lower concentration of calcium at the release site and, therefore, a possibly less prominent negative feed-back of the released calcium.

Sparks were hardly ever observed in the presence of 150 μM thymol. Rather, long events resembling lone embers were detected. Their duration, however, often exceeded 500 ms, sometimes lasting longer than 1.5 s, the duration of the image. These events had characteristic amplitudes and full widths similar to the embers measured in the presence of

low thymol concentrations. The fact that the amplitude of the fluorescence was steady over the entire event suggests that the channel, or channels involved were continuously open. These data clearly indicate that the long calcium release events correspond to the long-lasting open events seen for the thymol-modified isolated RyR. Since the long calcium release events were neither preceded nor followed by a spark, they should be considered as the opening of a single release channel or the concerted opening of a small group of channels. It should be stressed, however, that were the latter the actual arrangement, the channels opening synchronously must always open together, constituting, therefore, the elementary release unit. These findings thus strongly argue in favor of the lone ember being the elementary building block of calcium release in mammalian skeletal muscle.

If embers represent single channel events sparks are generated when some 20-30 channels open. On the other hand, if embers are generated by a number of synchronously opening channels, the number of channels opened during a spark could easily reach a 100, that is the entire couplon.

Summary

We examined the intracellular calcium homeostasis in skeletal muscle cells and the elementary calcium release events (ECRE) that underlie the global calcium increase.

Our results demonstrated the ability of ATP to induce an elevation of $[Ca^{2+}]_i$ in human or mouse skeletal muscle cells in culture. ATP-evoked transients displayed a variety of kinetics from a slow monotonic rise to biphasic behaviour where the first, fast component was followed by a more gradual secondary increase. The amplitude of these transients increased with cell maturation up to the point where multi-nucleated myotubes were formed, and then it decreased. On the other hand acetilcholine, caffeine or depolarisation evoked signals increased monotonically, reaching a maximum for myotubes with more than 10 nuclei. These could be attributed to the changes in the voltage-dependent pathways and in the resting membrane potential, as well as to the development of intracellular stores following cell differentiation. In contrast the expression of purinoreceptors decreases during muscle differentiation. ATP evoked a non-desensitising inward current with a rise time around 300 ms. These effects of ATP were not affected by the nicotinic Ach receptor antagonist d-tubocurarine verifying the idea that ATP did not act via the nAChR, rather through an ionotropic purinoreceptor. A depolarisation, the blocking of voltage-dependent channels or the removal of external calcium partially suppressed the ATP-evoked calcium transients and eliminated the early peak but not the sustained component of the calcium flux. Suramin, a well known blocker of the purinerg signal transduction, suppressed the ATP-evoked signal. In parallel, P2X and P2Y purinoreceptors were detected in myotubes with immunocytochemical labeling. ATP inhibited the proliferation but promoted the differentiation of muscle cells in primary culture. These observations suggest that ATP acting via P2Y receptors and the PI pathway interferes with the proliferation/differentiation program of skeletal muscle cells in culture.

We optimized the conditions for determining the characteristic parameters of ECRE in mammalian muscle. Thymol altered these parameters, slightly increasing the frequency of events, but decreasing their amplitude and full width half maximum. In parallel the duration of ECRE increased considerably. In low thymol concentrations the relative proportion of events were also changed, the appearance of lone embers and complex events were increased. Higher concentrations favored long events, resembling embers in control, with duration often exceeding 500 ms. These experiments should contribute to the understanding of the calcium homeostasis in skeletal muscle cells.

***In extenso* articles that provide the basis of the thesis:**

1. J. Cseri, **H. Szappanos**, G. P. Szigeti, Z. Csernátony, L. Kovács, L. Csernoch (2002): A purinergic signal transduction pathway in mammalian skeletal muscle cells in culture. *Pflügers Archiv.* **443**, 731-738. **IF: 1,695**

2. P.Szentesi, **H. Szappanos**, Cs. Szegedi, M. Gönczi, I. Jóna, J, Cseri, L. Kovács, L. Csernoch (2004): Enhanced sarcoplasmic calcium release and altered elementary calcium release events in the presence of thymol in mammalian skeletal muscle. *Biophysical Journal* **86**, 1436-1453 **IF: 4,643**

3. **H. Szappanos**, J. Cseri, T. Deli, L. Kovács and L. Csernoch (2004): Determination of depolarisation- and agonist-evoked calcium fluxes on skeletal muscle cells in primary culture. *Journal of Biochemical and Biophysical Methods* **59**, 89-101 **IF: 1,383**

4. **H. Szappanos**, G. P. Szigeti, J. Cseri, L. Kovács, L. Csernoch (2004): Calcium fluxes and altered proliferation of cultured skeletal muscle cells from mice in the presence of extracellular ATP. *American Journal of Physiology* (közlésre beküldve)

Abstracts in the subject area of the thesis:

1. **H. Szappanos**, P. Szentesi, L. Csernoch, L. Kovács, J. Cseri (2002): Calcium transients and purinergic activation on mouse skeletal muscle cells in primary culture. *Acta Physiologica Hungarica* **89**, 35

2. **H. Szappanos**, M. Gönczi, J. Cseri, L. Kovács, L. Csernoch (2003): Elementary calcium release events (ECRE) in the presence of thymol on mammalian skeletal muscle. *Journal of Muscle Research and Cell Motility.* **24**, 359.

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3. L. Csernoch, **H. Szappanos**, J. Cseri, M. Gönczi, J. Sabatier, X. Altafaj, M. DeWaard, M. Ronjat (2004): Elementary calcium release events (ECRE) in the presence of the scorpion toxin maurocalcine. *Biophysical Journal.*

The cumulative impact factor of these articles: 9.69.