Abstract The effects of adenosine 5′-triphosphate (ATP) on human and mouse skeletal muscle fibres in primary culture were investigated. ATP-evoked changes in intracellular calcium concentration ([Ca^{2+}]_{i}) were measured and compared with those induced by agonists of the nicotinic acetylcholine (Ach)- and P2X purinoreceptors. While ATP was effective on both myoblasts and multinucleated myotubes in the micromolar range, Ach failed to induce any change in [Ca^{2+}]_{i} at early stages of development. In contrast, myofibres with peripheral nuclei showed little response to ATP but responded to Ach with a large change in [Ca^{2+}]_{i}. The responsiveness of the myotubes to Ach paralleled that to potassium. The removal of external calcium abolished the response to ATP. P2X receptor agonists mimicked the response to ATP with the order of potency being ATP > 2′,3′-O-(4-benzoyl)-benzoyl-ATP > β,γ-methylene-ATP > α,β-methylene-ATP. Under voltage-clamp conditions ATP induced an inward current that showed little inactivation. These results are consistent with the existence of P2X receptor-mediated signal transduction pathway in cultured mammalian skeletal muscle cells.

Keywords Human · Skeletal muscle · Culture · ATP · Purinoreceptors · Development · Intracellular calcium

Introduction

Considerable evidence indicates that adenosine 5′-triphosphate (ATP) is released from nerve terminals and acts as a co-transmitter in many tissues [7]. Extracellular ATP exerts its diverse effects by binding to membrane proteins termed P2 purinoreceptors [14]. P2 receptors have been classified into two families: a P2X family consisting of ligand-gated channels (at least seven subunits have been cloned [30, 33]) and a P2Y family consisting of G protein-coupled receptors. Individual P2X subunits with unknown stoichiometry form an essentially non-selective cation channel with an approximately two- to fourfold higher permeability for Ca^{2+} than for Na^{+} [16].

ATP activates cation channels in the membranes of fusion-competent myoblasts and myotubes obtained from chicken embryos [25]. This action of ATP was first believed to be mediated through binding to the nicotinic acetylcholine (Ach) receptor (nAchR). A similar conclusion has been drawn from measurements on cultured Xenopus laevis myotomal muscle cells [22] and on Xenopus oocytes expressing the nAchR from either BC3H1 cells (a skeletal muscle-type receptor) or from the Torpedo californica electric organ [15]. Work on embryonic chick skeletal muscle cells has, however, demonstrated that the ATP-induced inward currents represent an influx pathway that is independent of the nAchR [20] and is, most likely, mediated through the activation of P2X purinoreceptors [28, 36].

Recently, a novel gene associated with a P2X receptor, specifically expressed in human skeletal muscle, has been identified [37]. A similar gene and the encoded receptor have been found in the mouse with the receptor showing 83% homology to the human counterpart [29]. Examination of altered expressions of the human receptor suggests that it might play a significant role in the proliferation and/or differentiation of skeletal muscle cells. These findings indicate that a P2X receptor-mediated signal transduction mechanism might operate in developing skeletal muscle fibres in mammals and that it could play an important role in the regulation of cell differentiation and/or maturation.

This and the accompanying paper [10] therefore test the hypothesis that ATP is involved in the regulation of intracellular calcium homeostasis in developing mamma-
lidan skeletal muscle via the activation of P2X purinoreceptors. We show, for the first time, that ATP induces an inward current and a transient rise in intracellular calcium concentration ([Ca$^{2+}$]) in cultured human and mouse skeletal muscle cells. The calcium transients were not mediated through the nAChR but were mimicked by P2X receptor agonists. These findings indicate the presence of a P2X receptor-mediated signalling pathway in developing mammalian skeletal muscle. This work has been presented to the Physiological Society [11].

Materials and methods

Skeletal muscle cells in culture

Muscle tissues were obtained either from healthy human subjects undergoing orthopaedic surgery or from young mice in accordance with the guidelines and an approved protocol of the Ethics Committee of the University of Debrecen. The procedure for obtaining satellite cells from the samples and growing myotubes from the satellite cells is described elsewhere [5, 32]. In brief, the muscle biopsy was dissociated at 37 °C using collagenase (Type II, Sigma, St. Louis, Mo., USA) and trypsin (Difco, Detroit, Mich., USA) in a calcium/magnesium-free phosphate buffer. After filtration and centrifugation, the pellet was resuspended in Ham’s F-12 growth medium (Sigma) supplemented with 5% FCS, 5% horse serum (HS), 2.5 mg/ml glucose, 0.5 mg/ml glutamate, 1.2 mg/ml NaHCO$_3$, 50 U/ml penicillin, 50 µg/ml streptomycin and 1.25 µg/ml fungizone (Biogal, Debrecen, Hungary). The cells were seeded onto sterile cover-slips (32 mm diameter, 0.07 mm thick; Biophysical Technologies, Sparks, Md., USA) and kept in a 5% CO$_2$ atmosphere at 37 °C. After 3 days in culture the medium was changed to DMEM (Sigma) supplemented with 2% FCS and 2% HS to facilitate myoblast fusion and differentiation.

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

Experiments were carried out on 7- to 14-day-old cultures for human and 5- to 8-day-old cultures for mice, carefully registering the degree of fusion for each myobute by counting the number of nuclei in the given cell. To introduce the calcium-sensitive probe into the myoplasmic space cells were incubated with 150 nM MitoTracker Deep Red FM (Molecular Probes, Eugene, Ore., USA) in the presence of 150 nM neotestimine (Pharmamagist, Budapest, Hungary) for 1 h at 37 °C. Before each measurement the myotubes were kept at room temperature (22–24 °C) in normal Tyrode’s solution (in mM): 110 K-aspartate, 2 KCl, 2 MgCl$_2$, 5 EGTA, 5 HEPES and 2 MgATP (pH 7.3 using KOH). The passive electrical parameters of the cells were determined with 40-ms, 5-mV pulses. The holding potential was ~80 mV. The linear capacitance of the cells was 150–200 pF. The effect of externally applied ATP was followed by continuously recording the holding current necessary to maintain the ~80 mV holding potential.

Experimental procedure and chemicals

Agonists were applied through a fast perfusion system that allowed rapid (delay approximately 2.5 s) and local application of the compound onto the cell investigated [3]. All experiments for measuring [Ca$^{2+}$]$_i$ were performed in either normal Tyrode’s solution or in a calcium-free Tyrode’s solution of the same composition as the normal Tyrode’s except for 5 mM EGTA and no added CaCl$_2$, ATP and other chemicals were from Sigma.

Statistical analysis

Data are presented as means±SEM or as best fit value±SEM in non-linear curve fits. Where appropriate, the significance of differences between experimental groups was assessed using Student’s t-test. $P<0.05$ was regarded as significant. The traces shown here-in are results from representative single experiments.

Results

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

When ATP was added to the bathing medium of cultured human myotubes the cells responded with a transient rise in [Ca$^{2+}$], that varied both in kinetics and amplitude with the state of development. Figure 1A and B illustrates the two typical kinetic responses observed for a saturating (180 µM; Fig. 4) concentration of ATP in myotubes with more than five centred nuclei, in which the transients were the largest in amplitude. In most cases these transients displayed a monotonic rising phase with a relatively slow rate of rise (Fig. 1A). 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$^{2+}$], (Fig. 1B). The early component was not always followed by a clear decrease in [Ca$^{2+}$], as in Fig. 1B; rather, the rising phase comprised two phases, a fast followed by a slow rise. The fast component of the calcium transients was eliminated by tetrodotoxin (TTX, as shown in Fig 1D) and was present neither in myoblasts nor myofibres (large myotubes with peripheral nuclei). Since the observations indicated that this component was due to depolarisation, most likely a propagating action potential induced by the addition of ATP, the following will focus on the detailed characterisation of the slow component.