

P2Y₂ Receptor Activation Regulates the Expression of Acetylcholinesterase and Acetylcholine Receptor Genes at Vertebrate Neuromuscular Junctions

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Received May 27, 2004; accepted July 9, 2004

ABSTRACT

At the vertebrate neuromuscular junction (nmj), ATP is known to be coreleased with acetylcholine from the synaptic vesicles. We have previously shown that the P2Y₁ receptor is localized at the nmj. Here, we extend the findings to show that another nucleotide receptor, P2Y₂, is also localized there and with P2Y₁ jointly mediates trophic responses to ATP. The P2Y₂ receptor mRNA in rat muscle increased during development and peaked in adulthood. The P2Y₂ receptor protein was shown to become restricted to the nmjs during embryonic development, in chick and in rat. In both rat and chick myotubes, P2Y₁ and P2Y₂ are expressed, increasing with differentiation, but P2Y₄ is absent. The P2Y₂ agonist UTP stimulated there inositol trisphosphate production and phosphorylation of extracellular signal-regulated kinases, in a dose-dependent manner. These UTP-induced responses were insensitive to the P2Y₁-specific antagonist

MRS 2179 (2'-deoxy-N⁶-methyl adenosine 3',5'-diphosphate diammonium salt). In differentiated myotubes, P2Y₂ activation induced expression of acetylcholinesterase (AChE) protein (but not control α -tubulin). This was shown to arise from *AChE* promoter activation, mediated by activation of the transcription factor Elk-1. Two Elk-1-responsive elements, located in intron-1 of the *AChE* promoter, were found by mutation to act in this gene activation initiated at the P2Y₂ receptor and also in that initiated at the P2Y₁ receptor. Furthermore, the promoters of different acetylcholine receptor subunits were also stimulated by application of UTP to myotubes. These results indicate that ATP regulates postsynaptic gene expressions via a common pathway triggered by the activation of P2Y₁ and P2Y₂ receptors at the nmjs.

In the developing vertebrate neuromuscular junction (nmj), when a motor nerve terminal contacts a myotube, acetylcholine receptors (AChRs), acetylcholinesterase (AChE), and certain other proteins become localized and stabilized in a specialized postsynaptic apparatus. A few subsynaptic nuclei at each developing junction, it is now known, become transcrip-

tionally specialized to sustain the local synthesis of those proteins, including the postsynaptic AChR and AChE (Krejci et al., 1999; Sanes and Lichtman, 1999). In a parallel action, when the neural contacts are established, the evoked electrical activity selectively represses transcription of *AChR* genes in the non-synaptic nuclei (Schaeffer et al., 2001). Trophic factors from the nerve have been deduced to initiate and/or maintain the postsynaptic specialization, notably agrin and neuregulin (Sandrock et al., 1997; Sanes and Lichtman, 1999).

Among these nerve-derived factors, adenosine 5'-triphosphate (ATP) is an additional potential such trophic factor at the nmj. In the synaptic vesicles in vertebrate nmjs or the related electroplaques, ATP stabilizes acetylcholine and is coreleased quantally with it in a ratio of about 1 ATP to 5

This work was supported by grants from the Research Grants Council of Hong Kong (6098/02M, 6283/03M, and AoE/B-15/01 to K.W.K.T.) and from the Wellcome Trust (to E.A.B.). R.C.Y.C. was supported by the postdoctoral matching fund from Hong Kong University of Science and Technology. N.L.S. holds a Croucher Foundation Scholarship.

E.K.K.T. and R.C.Y.C. contributed equally to this study.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.104.003269.

ABBREVIATIONS: nmj, neuromuscular junction; AChR, acetylcholine receptor; AChE, acetylcholinesterase; PKC, protein kinase C; 2-MeSADP, 2-(methylthio)adenosine 5'-diphosphate; BSA, bovine serum albumin; CPK, creatine phosphokinase; CP, creatine phosphate; Luc, luciferase; kb, kilobase; bp, base pair; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; ECL, enhanced chemiluminescence; TMR-BuTX, tetramethyl-rhodamine-labeled α -bungarotoxin; IP₃, inositol trisphosphate; E, embryonic day; P, postnatal day; TPA, 12-O-tetradecanoylphorbol 13-acetate.

acetylcholine (Zimmermann, 1994; Silinsky and Redman, 1996). We have shown that the P2Y₁ receptor for ATP is localized at the nmj in chicken, rat (Choi et al., 2001), and amphibian (Cheng et al., 2003) muscles. Furthermore, evidence was obtained that ATP contributes to the induction and maintenance of expression of AChE and of AChR subunits in muscles (Choi et al., 2001, 2003). Some of the regulatory elements within promoter regions of the mammalian *AChE* gene have been identified in several laboratories (Ben Aziz-Aloya et al., 1993; Mutero et al., 1995; Chan et al., 1999). Likewise, for *AChR* subunit genes some promoter elements have also been identified (for review, see Schaeffer et al., 2001). For the aforementioned effect on chick or mouse myotubes heterologously expressing promoter-reporter constructs for genes of AChE or the AChR subunits, P2Y₁ receptor agonists were shown to stimulate the transcriptions of those genes (Choi et al., 2001, 2003). The pathway to activation of the *AChE* gene was shown to involve protein kinase C (PKC) and intracellular Ca²⁺ release. In turn, the activation of PKC triggered a mitogen-activated protein kinase signaling cascade and culminated in activation of the transcription factor Elk-1.

In cultured myotubes, the ATP-induced responses, including the gene activations, were always higher than those evoked by the P2Y₁ subtype-specific agonist 2-(methylthio)adenosine 5'-diphosphate (2-MeSADP). This suggests a possible existence of other subtype(s) of P2Y receptor in muscle that can also respond to ATP challenge. Although the P2Y₁ receptor has been known for more than a decade and occurs widely in tissues, its functional in situ relationship, if any, to others of the seven additional P2Y subtypes now known in mammals (Abbracchio et al., 2003) is in general very unclear. The one exception, which is instructive, is in the case of platelet aggregation induced by ADP, for which process the PKC-linked P2Y₁ receptor co-occurs with and acts cooperatively with the G_i-linked P2Y₁₂ receptor (Dorsam and Kunapuli, 2004). Here, we examine whether the two other PKC-linked P2Y receptors that are responsive to ATP, namely, P2Y₂ and P2Y₄, could be localized with P2Y₁ at the nmjs and could contribute to the signaling induced by ATP in muscle cells.

Materials and Methods

Materials. Materials not specified were as in Choi et al. (2001). MRS 2179 was purchased from Tocris Cookson Inc. (Bristol, UK). The antibodies, where not specified, and bovine serum albumin (BSA; fraction V) were purchased from Sigma-Aldrich (St. Louis, MO) or Cappel Laboratories (Durham, NC). All tissue culture reagents were from Invitrogen (Carlsbad, CA). Purity of all nucleotides used in this study was ensured by a hexokinase/glucose treatment for the diphosphates (extended to 90 min here for UDP) or a creatine phosphokinase (CPK)/creatine phosphate (CP) treatment for the triphosphates (extended to 3 h with 50 mM CP for UTP), all as described previously (Simon et al., 2001). In inositol phosphate assays and during the stimulation of AChE/AChR expression or promoter activity by triphosphates in myotubes, CPK at 2 U/ml, CP at 5 mM, and Mg²⁺ at 2.5 mM were also present throughout. When diphosphate agonists were used in these assays, hexokinase at 2 U/ml, glucose at 5 mM, and Mg²⁺ at 2.5 mM were also included. Those media, if used alone in the control incubations in each case, had no effect. For the longer incubations with an agonist, renewal of that agonist solution was also made at intervals, as stated.

Animals. Chicken, rat, and *Xenopus* muscles (or spinal cord) at the stated ages (from embryonic to adult) were collected and stored as described in Choi et al. (2001) or in Cheng et al. (2003). Rat embryos were from the Animal Care Facility at Hong Kong University of Science and Technology. All procedures conformed to the Guidelines of the Animal Research Panel of the university for the use and care of laboratory animals in research.

Cell Cultures. Eggs of New Hampshire chicks were purchased from a local farm and hatched in the University Animal Care Facility. Primary chick myotube cultures were prepared as in Choi et al. (2001). Mouse C2C12 muscle cells were cultured, differentiated into myotubes, and treated as described previously (Siow et al., 2002). The human 1321N1 astrocytoma cell line was originally from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). The astrocytoma cell lines stably expressing either rat P2Y₁ or P2Y₂ receptor were generated by electroporation of the respective plasmid constructs (rP2Y₁/pcDNA 3.1, rP2Y₂/pcDNA 3.1) into 1321N1 cells, followed by antibiotic selection with 600 µg/ml geneticin sulfate (G-418) and dilution cloning. After selection, the cell lines stably expressing either rat P2Y₁ or P2Y₂ receptors at high levels were maintained in Dulbecco's modified Eagle's medium/Nutrient Mix F-12 medium (1:1) containing Glutamax I, pyridoxine hydrochloride, 10% fetal bovine serum, and 600 µg/ml G-418 at 37°C in a humidified atmosphere (95% air, 5% CO₂) and passaged by trypsinization every 4 to 5 days (Sellers et al., 2001).

Since muscle cell cultures can release considerable ATP into the medium, the cultures were pretreated with apyrase (2 U/ml, 1 h) or when loading with [³H]myo-inositol (24 h), to eliminate all such free nucleotides, followed by a gentle wash with apyrase-free medium before drug application.

Northern Blots. Total RNA was prepared by the LiCl method from the tissues or cells stated (Choi et al., 2001). A nominal loading of 30 µg of RNA per gel lane was used. The consistency of the RNA loading in every lane was confirmed by ethidium bromide staining of the ribosomal RNAs. The blots were hybridized at 42°C overnight with [³²P]dCTP-labeled probes against mouse AChE catalytic subunit cDNA, rat AChR α-subunit cDNA, or rat P2Y₁ receptor cDNA, all as detailed by Choi et al. (2001). For rat P2Y₂ receptor the ~2.0-kb probe of Chen et al. (1996) and for rat P2Y₄ receptor the ~1.2-kb probe of Webb et al. (1998) were prepared and used. Quantitation of the 28S ribosomal RNA bands and of the ³²P-labeled bands was made, using calibration curves constructed for the same gel (Choi et al., 2001).

Reporter Gene Constructs and cDNA Transfections. A 2.2-kb DNA fragment containing the human AChE promoter region (Ben Aziz-Aloya et al., 1993) was subcloned into pGL3 vector (Promega, Madison, WI) that contained the luciferase reporter gene downstream, to form pAChE-Luc (Choi et al., 2001). The chick AChR α, rat AChR δ, and rat AChR ε subunits and the mouse AChE promoters were tagged likewise with the luciferase reporter to form the pAChRα-Luc, pAChRδ-Luc, pAChRε-Luc, and pAChE_m-Luc constructs, respectively (Choi et al., 2003). The full-length rat cDNAs encoding P2Y₁ (Tokuyama et al., 1995) and P2Y₂ (Chen et al., 1996) receptor were subcloned into pcDNA 3.1 mammalian expression vector (Invitrogen) for use where stated. Two fragments from the human AChE promoter, previously identified by gel-shift analysis with Elk-1 binding and containing bp -1431 to -1412 or bp -1102 to -1083 (Elk-1[1] and Elk-1[3]; Choi et al., 2003), were subcloned into a pTA-Luc luciferase reporter vector (BD Biosciences Clontech, Palo Alto, CA). To enhance the promoter activity, three copies of each of those sequences were placed in tandem (without linkers) into the reporter vector, to form pElk-1[1]-Luc and pElk-1[3]-Luc, respectively. The aforementioned 2.2-kb human AChE promoter-containing fragment was directly mutated made within one or other of both of those two segments in their Elk-1 site, as defined under *Results*, and again used with the luciferase reporter to generate the pAChE_{ΔElk-1[1]}-Luc, pAChE_{ΔElk-1[3]}-Luc, and pAChE_{ΔElk-1[1,3]}-Luc constructs.

Myoblasts from 11-day chick embryos were cultured at 37°C for 2 days and transiently transfected with the plasmid constructs (2 μ g plasmid per 35-mm dish or 1 μ g per 12-well plate) using calcium phosphate, and then allowed to fuse to myotubes. The transfection efficiency in each case was determined from control cells, cotransfected with same vector containing the β -galactosidase gene; it was consistently ~30%. For mouse C2C12 myoblasts in 12-well plates, the transfection was by Lipofectamine Plus reagent (Invitrogen), and the transfected cultures were selected for stable expression in the presence of G-418 (400 μ g/ml). Simultaneous cotransfection was made with a vector containing the β -galactosidase gene under a cytomegalovirus promoter (BD Biosciences Clontech). In each case, the luciferase activity was measured in a lysate of the cells, and the values were normalized relative to the parallel β -galactosidase activity.

Immunoblotting and Phosphorylation Assay. Treated mouse C2C12 or chick myotubes cultured on 35-mm dishes were homogenized in lysis buffer (400 μ l) containing 10 mM HEPES (pH 7.5), 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mg/ml bacitracin, and 1 M NaCl and centrifuged (12,000g, 10 min) at 4°C. The cultured neurons were homogenized in 100 μ l of lysis buffer and similarly centrifuged. Protein samples were denatured at 100°C for 5 min in sample buffer containing 1% sodium dodecyl sulfate (SDS) and 1% dithiothreitol, separated by SDS polyacrylamide gel electrophoresis (20- μ g sample per lane) and immunoblotted with all procedures as described previously (Choi et al., 2001), probing with anti-rat P2Y₁ or anti-rat P2Y₂ antibodies (Alomone Labs, Jerusalem, Israel; 1:400–1:1000 dilutions). The selectivity of the P2Y₁ and the P2Y₂ antibodies in immunoblotting was verified by incubation of each (2 μ g) with 10 μ g of its peptide antigen for 1 h at 4°C before applying them to the blots. The following primary antibodies were also used on the same or parallel samples: the anti-mouse AChE antibody (BD Transduction Laboratories, San Diego, CA), at 1:10,000 dilution; the anti-chick AChE antibody (Tsim et al., 1997), at 1:5000; the anti- α -tubulin antibody (Sigma-Aldrich), at 1:50,000 dilution. The peroxidase-conjugated secondary antibodies used at 1:5000 dilutions in each case were from Zymed Laboratories (South San Francisco, CA).

In phosphorylation studies, myotube cultures (transfected or not) were serum-starved for 8 h and treated with drugs as stated in serum-free culture medium. They were then washed with 1 mM Na₃VO₄/phosphate-buffered saline (PBS), followed by addition of 400 μ l of lysis buffer containing 10 mM HEPES, pH 7.5, 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM Na₃VO₄, and 5 mM phenylmethylsulfonyl fluoride. Lysates were then collected for immunoblotting as described above, with probing by the anti-phospho-specific ERK 1/2 antibodies and reprobing of those blots with the phosphorylation state-independent ERK 1/2 antibodies (New England Biolabs, Beverly, MA) at 1:5000 dilution.

The immunocomplexes were visualized by the enhanced chemiluminescence (ECL) method (Amersham Biosciences Inc., Piscataway, NJ). The intensity of the protein bands in the control or agonist-stimulated samples run on the same gel was determined using an image analyzer under strictly standardized ECL conditions. A calibration curve in each case was constructed using serial dilutions of a maximally stimulated sample from that set and used in the linear, nonsaturating range of the ECL conditions (Choi et al., 2001).

Immunocytochemistry. Gastrocnemius muscle at different developmental stages from chicken and rat or from adult *Xenopus* was dissected and rapidly frozen in isopentane/liquid nitrogen. Sections (15 μ m) were cut, fixed in 2% paraformaldehyde/PBS (15 min at room temperature), and briefly washed with PBS, as detailed by Tsim et al. (1997). Astrocytoma cultures were cultured on coverslips until confluence (Sellers et al., 2001) and fixed similarly. All anti-P2Y receptor antibodies were applied for 24–48 h at 4°C at 1:500 dilution on the muscle sections (to further ensure specificity) and 1:200 on the astrocytoma cells. To prevent cross-reactivity with other P2Y subtypes, as shown under *Results*, the anti-P2Y₁ antibody was

applied in 6% normal serum (goat or human, without difference) plus 2% BSA. The anti-P2Y₂ receptor antibody was applied in 5% serum without BSA. All media contained 1% Triton X-100 for penetration. A blocking preincubation was given in the same medium alone for 30 min at room temperature. Where additional blocking by the relevant peptide antigen was to be tested, pretreatment of the antibody with it was as described above. Reaction with each antibody was followed by washes and application of a secondary antibody conjugated to fluorescein-5-isothiocyanate or (on astrocytoma cultures) to cyanine-3, by methods described previously for other antibodies (Tsim et al., 1997; Sellers et al., 2001). The muscle sections were double-labeled for P2Y₁ receptor and for AChR with 10 nM tetramethylrhodamine-labeled α -bungarotoxin (TMR-BuTX; Molecular Probes, Eugene, OR; Tsim et al., 1997). Staining of muscle was viewed under a 20 to 40 \times objective alternately with phase-contrast and fluorescence optics, the latter using excitation at 555 or 488 nm and emission at 580 or 515 nm for TMR or fluorescein-5-isothiocyanate, respectively. To measure the percentage colocalization in muscles, about 50 fields were selected at random and all sites there clearly labeled with either stain and not coinciding with the other stain were taken as not colocalized and compared with the total number of discrete sites seen for both stains.

Other Analyses. Other measurements, including those of protein content, of luciferase, of AChE protein and enzymatic activity, as well as statistical analysis, were as specified by Choi et al. (2001), as were inositol phosphate assays, except that [³H]inositol triphosphate ([³H]IP₃) was separated from other labeled inositol species by standard chromatographic procedures. The [³H]IP₃ accumulation was expressed as a percentage of the level present (basal) before nucleotide addition. Where gel blots or microscope images are shown they are representative of at least three complete replications.

Results

Expression of the P2Y₂ Receptor mRNA in Developing Rat Muscles and Spinal Cord. A single transcript (~3.2 kb) for the P2Y₂ receptor was found in Northern blots of RNA isolated from rat gastrocnemius muscles (Fig. 1A). Up to embryonic day 14 (E14) muscle, the P2Y₂ mRNA was at a low level; this increased significantly from E16 until birth. It was at a high and essentially constant level from 10 to 31 days postnatally (P31; Fig. 1A). The P2Y₂ transcript content further increased at birth, but its major increase was shown to occur around P4 (Fig. 1B). The expression profile of the P2Y₁ receptor mRNA (~4.2 kb) in the same muscles, from E14 to P31, was similar to that of the P2Y₂ receptor. The expression of the AChE catalytic subunit was also compared in the same muscles. As found previously (Choi et al., 2001), two transcripts are present, at ~2.4 and ~3.6 kb. The AChE developmental profile followed closely that of the P2Y₁ receptor (Fig. 1A). The P2Y₄ receptor transcript (at ~4.0 kb) was not detected in the same muscle specimens at any stage (Fig. 1A).

In rat spinal cord, there was a detectable but very low level of P2Y₂ mRNA in the late embryo; this increased greatly after birth, up to an essentially constant level at P20 to P31 (Fig. 1C). The postnatal expression profile was similar for the P2Y₁ and P2Y₂ receptors. The expression of P2Y₁ receptor, however, occurs noticeably earlier in the embryonic spinal cord than that of P2Y₂. When the blots were quantitated (taking the means from three replicate gels in each case) and normalized for the amount of RNA loaded onto the gel and the specific radioactivity of the probes, it was shown that the blots (specimens shown in Fig. 1) were quantitatively reproducible and that there was (at all postnatal stages) ~3-fold

higher expression (relative to total RNA content) of P2Y₂ receptor mRNA in muscle than in the spinal cord. In addition, the amount of the P2Y₂ receptor mRNA in adult (P31) rat muscle was higher (~5-fold) than that of P2Y₁ receptor.

Transcripts for the P2Y₂ but not for the P2Y₄ receptors were also found in mouse C2C12 myotubes. We had previously found (Choi et al., 2003) that those cells during differentiation strongly express P2Y₁ receptors. Here, they showed a 5-fold increase in P2Y₂ transcript level from day 1 to day 5 during differentiation (Fig. 1D). This profile of P2Y₂ expression is in line with the C2C12 expression of myogenin, a marker for the myotube formation, and of AChR and AChE (Siow et al., 2002). In rat brain, which served as a positive control, transcripts of ~3.2 kb for P2Y₂ and ~4.0 kb for P2Y₄ receptors were detected at high levels (Fig. 1D).

Immunoreactivity of P2Y Receptor Subtypes in Muscle Cells. To identify and localize the P2Y subtypes at rat nmjs, the commercially available (Alomone Labs) polyclonal antibodies to the P2Y₁ or P2Y₂ or P2Y₄ receptors were tested. The antibodies recognize both human and rat orthologs of these receptors. We used the astrocytoma (1321N1) human cell line to express those receptors, a host cell that we have found is totally unreactive to any of those antibodies (data not shown), in agreement with previous evidence on the

absence in it of their mRNAs (Moore et al., 2001). We isolated stable cell lines in that host expressing, singly, the rat P2Y₁ or P2Y₂ or P2Y₄ receptors. Each cell line was reacted in turn with the three antibodies. Under standard conditions (medium containing 3% normal serum and 1% BSA), the anti-P2Y₁ antibody recognized in immunocytochemistry the P2Y₁ receptor, but also the P2Y₂ receptor (Fig. 2, A and B). In the same conditions, the anti-P2Y₂ antibody was specific for the P2Y₂ receptor, neither reacting with the P2Y₁ receptor (Fig. 2, A and B) nor (not shown) staining P2Y₄-expressing cells. Pretreatment with the P2Y₁ or the P2Y₂ peptide antigen blocked the corresponding antibody reaction, as shown in Fig. 2.

Since nonspecific antibody reactions can often be prevented by competition with serum proteins, the serum content of the incubation medium was varied. When the medium contained 10% serum the reaction of the anti-P2Y₁ antibody became specific but the intensity of its reaction at the P2Y₁ receptors was greatly decreased (Fig. 2, C and E). In that medium the anti-P2Y₂ antibody remained specific, but again its reaction was greatly weakened (Fig. 2, C and E). These comparisons are significant for the present study since others have recently reported (Cheung et al., 2003) with the same commercial antibodies different findings to ours (as given

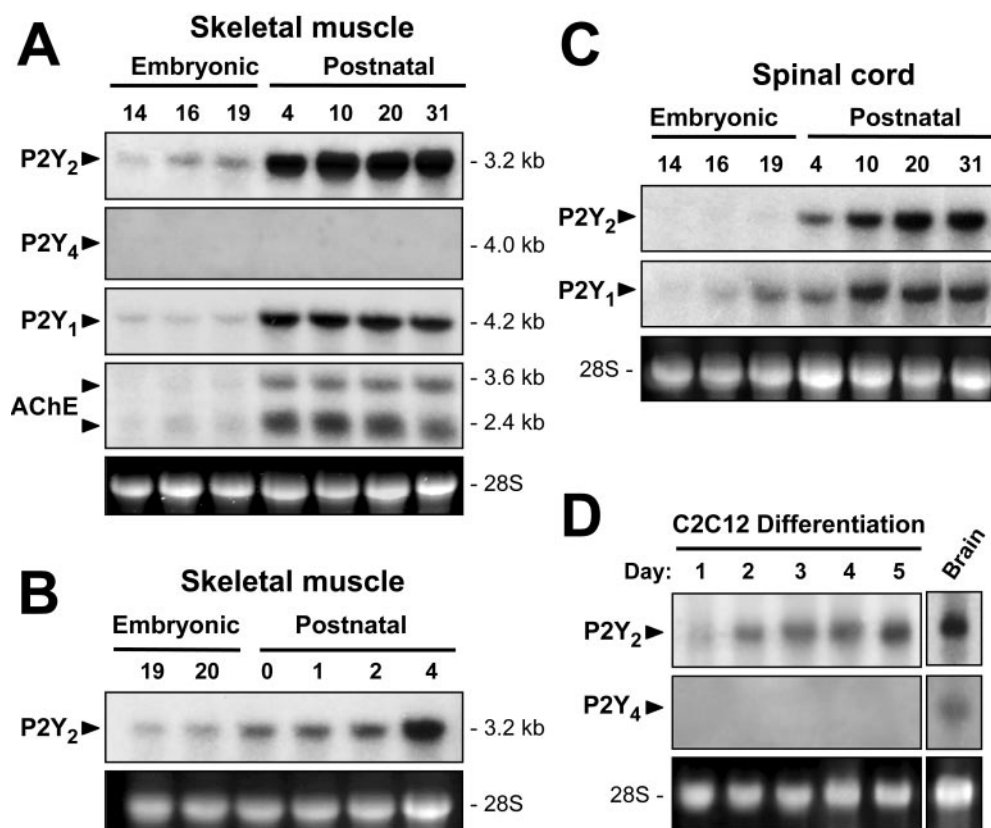


Fig. 1. Developmental changes of P2Y₂ receptor mRNA levels in rat or muscle and spinal cord. A, a single P2Y₂ receptor transcript at ~4.2 kb was detectable in rat gastrocnemius muscle, from E14 to P31. Transcripts of expected sizes encoding the P2Y₁ receptor (~3.2 kb) and AChE catalytic subunit (~2.4 and ~3.6 kb) were also detected, as shown. The P2Y₄ receptor transcript (expected size ~4.0 kb) was never detected. B, from E19 to P4 in the rat, the muscle exhibits a major change in its content of the P2Y₂ receptor transcript. C, in rat spinal cord P2Y₂ receptor mRNA increases similarly during development, and P2Y₁ receptor mRNA somewhat earlier. D, the mouse C2C12 myoblast cell line was transferred at the start of day 1 into differentiation medium and maintained thus. The formation of myotubes was almost complete by day 4, with a concomitant increase in the content of the P2Y₂ transcript. The mouse P2Y₄ transcript was absent throughout. Total RNA from mouse brain was used as a positive control (last lane) to show the transcript sizes of ~3.2 and ~4.0 kb corresponding to P2Y₂ and P2Y₄ receptors. It is known that the P2Y₄ receptor is expressed in rodent brain but at a much lower level than P2Y₁ or P2Y₂, being only expressed on supporting structures such as the vestibules. In all cases, A to D, 28S ribosomal RNA loading markers are shown.

below) on P2Y₁ and P2Y₂ immunoreactivity in rat muscle, but there the medium included 10% serum throughout the blocking and staining procedure (see *Discussion*).

Testing several intermediate conditions, we found that the optimum effect of the anti-P2Y₁ antibody was obtainable in the presence of 6% normal goat serum and 2% BSA, where the intensity at P2Y₁ receptors is adequate (Fig. 2D), whereas the cross-reactions are negligible to P2Y₂ (Fig. 2F) and to P2Y₄ (data not shown). That medium was, therefore, used in the muscle P2Y₁ immunostainings. For the anti-P2Y₂ antibody, due to its lack of cross-reactivity throughout, serum contents in the 3 to 5% range with no additive effect gave the same satisfactory results, as shown in Fig. 2, A and B, and therefore were used for muscle P2Y₂ immunostaining.

For the anti-P2Y₄ antibody, no conditions were found in which it could show a specific reaction with the P2Y₄ recep-

tor. At the highest serum levels tested, it still reacted significantly with both P2Y₁ and P2Y₂ (Fig. 2, C and E) as well as with P2Y₄ receptors. Moreover, pretreatments with the epitope peptide antigens (in the conditions used in Fig. 2, A and B) could not be used to discriminate those subtypes. Thus, the P2Y₄ peptide fully blocked the reaction with the anti-P2Y₄ antibody on the P2Y₄ cell line (data not shown) but also greatly reduced its cross-reaction on the P2Y₁ or P2Y₂ cell lines, whereas failure of the peptide to block was seen for P2Y₄ peptide in the anti-P2Y₁ antibody cross-reaction on P2Y₄ and also for P2Y₁ peptide in the anti-P2Y₄ antibody cross-reaction on P2Y₁ (not shown). Inspection of the relevant sequences showed that there are three dipeptide matches between the C-terminal epitope used for P2Y₄ and other regions in P2Y₁ and likewise in P2Y₂. There is also one tripeptide match within the epitopes used for P2Y₁ and for

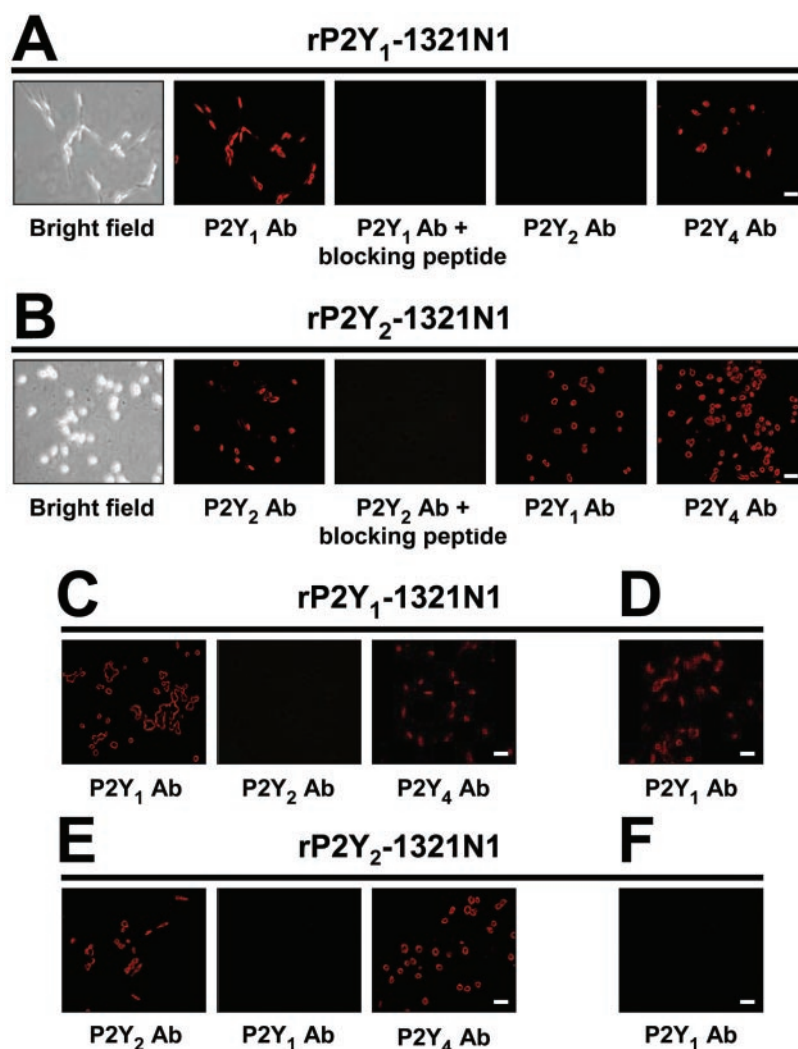


Fig. 2. Specificity of antibodies used in detecting P2Y receptor subtypes on muscle cells. In each case, 1321N1 cells transfected to stably express the single rat P2Y subtype indicated were reacted with each antibody raised against a given human P2Y subtype. The antibody incubation medium was, as stated, either low serum medium (3% goat serum/1% BSA) or high serum medium (10% horse serum) or intermediate serum medium (6% horse serum/2% BSA). Scale bar, 50 μm. A and B, reactions in low serum medium. The P2Y₁ receptor is recognized (A) by the anti-P2Y₁ antibody (reaction blocked by the presence of the P2Y₁ peptide antigen, 1 μg in 50 μl of incubation medium), but also by the anti-P2Y₄ antibody and *not* by the anti-P2Y₂ antibody. The P2Y₂ receptor is recognized (B) by the anti-P2Y₂ antibody (reaction blocked by the presence of the P2Y₂ peptide antigen, 1 μg in 50 μl), but also by the anti-P2Y₁ antibody and by the anti-P2Y₄ antibody. C and E, reactions in high serum medium. The P2Y₁ receptor is recognized (C) by the anti-P2Y₁ antibody though with very low intensity, but also by the anti-P2Y₄ antibody and *not* by the anti-P2Y₂ antibody. The P2Y₂ receptor is recognized (E) by the anti-P2Y₂ antibody, although with very low intensity, but also by the anti-P2Y₄ antibody and *not* by the anti-P2Y₁ antibody. D and F, reactions in intermediate serum medium. In this medium, the anti-P2Y₁ antibody stains the P2Y₁ receptor (D) but its cross-reactivity on the P2Y₂ receptor is again abolished (F).

P2Y₂ and another two between the P2Y₁ epitope and elsewhere on P2Y₄. The sum of those observations would be capable of explaining all of the findings here, since these membrane proteins are unlikely to be fully unfolded in the conditions of immunocytochemistry. We concluded that the presently available anti-P2Y₄ antibody could not be used in immunocytochemistry of P2Y receptors in muscle (although this does not exclude its use in Western blots). The optimum conditions as described above were then used in localizing the P2Y₁ or the P2Y₂ receptor proteins in muscle specimens.

Under conditions when both the P2Y₁ and P2Y₂ immunostaining are selective, the P2Y₂ immunoreactivity was colocalized with the AChR-specific α -bungarotoxin (TMR-BuTX) binding on muscle sections of adult chicken, rat, and *Xenopus*, indicating the restricted localization of P2Y₂ receptor at the nmjs (Fig. 3A). Furthermore, serial sections (10 μ m) were stained with anti-P2Y₁ and anti-P2Y₂ antibodies (1:500 dilution of each), and the colocalization of the two immunoreactivities was confirmed (Fig. 3B).

The extent of colocalization of P2Y₂ receptor with AChR at the nmjs was further analyzed in rat and chick muscles during development. In both chick and rat muscles, there was no significant colocalization of P2Y₂ receptors with AChRs up to E16. However, that colocalization was evident at E19 in both muscle samples; the extent of the colocalization steadily increased during later stages of development up to the adult (Fig. 4). A very weak staining by anti-P2Y₂ receptor antibody could still be observed in some extrajunctional areas.

Activation of P2Y₂ Receptors Induces IP₃ Accumulation and ERK Phosphorylation. We have demonstrated previously (Choi et al., 2001, 2003) that activation by adenosine tri- or diphosphates of the P2Y₁ receptors present in cultured chick myotubes mobilizes intracellular Ca²⁺ and also leads to an increase in the expression of AChE. Here, we have found that not only ATP but also UTP can stimulate IP₃ accumulation in mouse C2C12 myotubes and chick myotubes (Fig. 5A). UTP is inactive on rodent and avian P2Y₁ receptors, and agonist activity of UTP to increase IP₃ is characteristic of only two of the P2Y subtypes in the rat or mouse, the P2Y₂ and the P2Y₄ receptors (Webb et al., 1998; Kennedy et al., 2000; Suarez-Huerta et al., 2001; White et al., 2003; Wildman et al., 2003); an avian P2Y receptor whose highest homology is to mammalian P2Y₄ behaves likewise. However, as shown above, P2Y₄ mRNA is absent, whereas P2Y₂ mRNA and protein are amply present in the cells studied here. The increase in IP₃ accumulation was dose-dependent and at saturating (100 μ M) UTP application it has risen in mouse myotubes to 6-fold over the basal level (Fig. 5A).

The activation of the endogenous P2Y₂ receptors led to the phosphorylation of the ERK 1 (~42 kDa) and ERK 2 (~44 kDa) kinases in mouse C2C12 myotubes but only of ERK 1 in cultured chick myotubes (Fig. 5B), where ERK 2 has been shown to be present only at an extremely low level (Choi et al., 2003). The phosphorylation of ERK 1 and ERK 2 in cultured C2C12 myotubes was induced in a dose-dependent manner by UTP and reached a plateau (at ~7-fold the basal level) at 100 μ M UTP concentration (Fig. 5B). The UTP-induced phosphorylation was markedly higher in ERK 2 than in ERK 1. In chick myotubes, the UTP-induced ERK 1 phosphorylation was also dose-dependent and again reached a plateau at ~100 μ M UTP concentration (Fig. 5B). Chick

myotubes gave a lower response to UTP than mouse in activating ERK 1 phosphorylation, in line with their lower levels of UTP-induced inositol trisphosphate.

Those ERK phosphorylations by ATP or UTP were transient, declining sharply after 60 min (Fig. 5, C and D); the ERK 1 and ERK 2 total protein content remained invariant (Fig. 5C). Plots of scanned data (Fig. 5D) from four independent experiments of the type shown in Fig. 5C showed the maximal transient activation to be about 5-fold the basal level, for both ATP and UTP. UTP also induced ERK 1 phosphorylation to similar levels in cultured chick myotubes

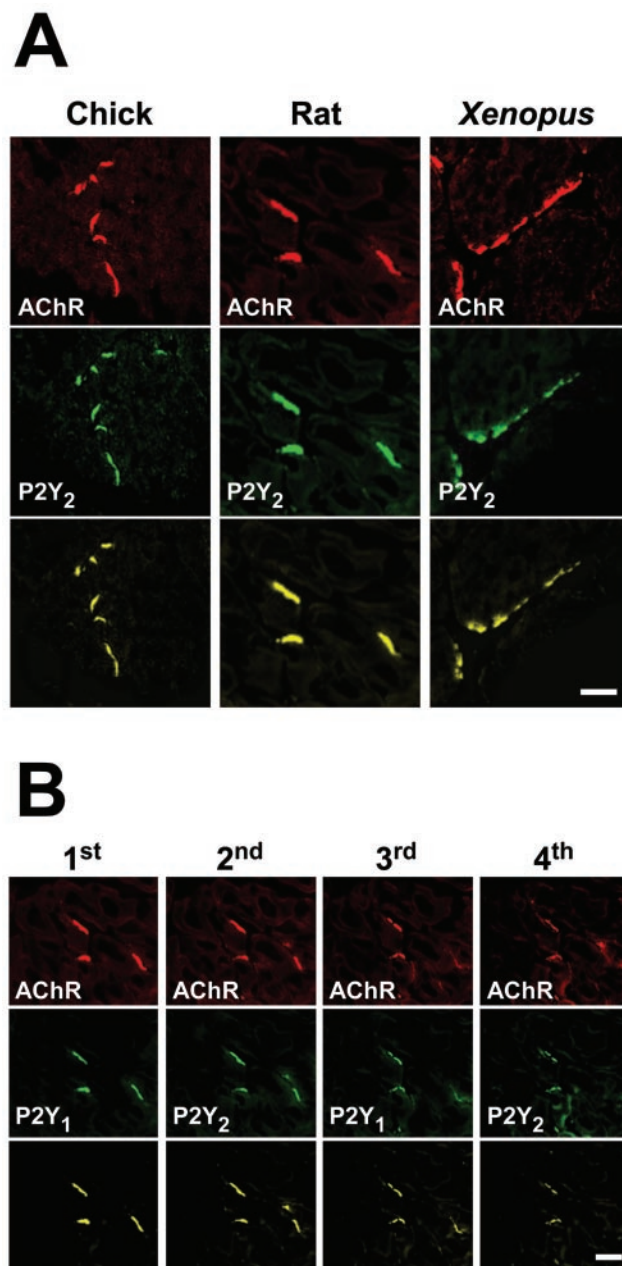


Fig. 3. Localization of P2Y₂ receptor in rat muscles. A, chicken, or rat, or *Xenopus* muscle sections (20 μ m) were used. For each, the same field is shown stained by the anti-P2Y₂-receptor antibody (green) or for AChR (red) by TMR-BuTX (10 nM) or superimposed (yellow). B, serial sections (10 μ m) were cut from rat muscles, and each of them was stained with anti-P2Y₁ and P2Y₂ antibodies alternatively or superimposed (yellow) on bottom panels. Scale bar, 20 μ m.

(data not shown). UDP, an agonist for the chick P2Y₃ (Webb et al., 1996) and for the similar rat P2Y₆ receptor (Chang et al., 1995), was however, unable to induce the phosphorylation of ERK 1 and ERK 2 (Fig. 5C). The PKC activator phorbol ester (TPA), serving as a positive control, induced a strong (7-fold basal) phosphorylation of ERK 1 and ERK 2, again transient (Fig. 5, C and D). When UTP was applied onto C2C12 myotubes transfected to overexpress P2Y₂ receptors, a much stronger activation of ERK 1 and ERK 2 was achieved, 15-fold basal (Fig. 5, C and D). This again reached a plateau at 10 min and again strongly declined after 60 min, serving to show that the transience of the ERK phosphorylation through the activated endogenous P2Y₂ receptor in myotubes is not due to limitation by low receptor density.

UTP-Induced Expression of AChE. Application of UTP onto cultured C2C12 myotubes increased the expression of the AChE catalytic subunit protein (~68 kDa) in a dose-dependent manner (Fig. 6A). UTP, likewise, induced the expression of the AChE catalytic subunit (~105 kDa) in cultured chick myotubes (Fig. 6A). Similar to the case in P2Y₁-induced AChE expression (Choi et al., 2001, 2003), the activation of P2Y₂ receptor in both mouse and chick myotubes did not show significant change of AChE enzymatic activity (Fig. 6A). The reason of this phenomenon could be due to the induction of intracellular inactive enzyme, which was discussed previously (Choi et al., 2001). The expression of α -tubulin (~55 kDa), as an internal control, remained unchanged in all treatments (Fig. 6A). Unstimulated myotubes secrete some of their AChE into the culture medium, but all of the UTP-mediated increase in AChE protein here was within the cells (both chick and mouse) the level of AChE protein in the medium being unaltered (data not shown). This increase in AChE expression by UTP is similar to that

mediated by the P2Y₁ receptor in chick myotubes as described previously (Choi et al., 2001).

To demonstrate that these stimulations arise directly from UTP-induced gene activation, the promoter of the human AChE catalytic subunit was inserted in a vector and tagged downstream with the *luciferase* reporter gene, giving the pAChE-Luc construct (Fig. 6B, top), which was transfected into the mouse C2C12 myotubes. Either ATP or UTP, applied onto those cells stably expressing the pAChE-Luc promoter, induced the promoter activity in a dose-dependent manner (Fig. 6B), with ATP (an agonist at both P2Y₁ and P2Y₂) producing a stronger effect than UTP. 2-MeSADP, a selective and more potent agonist for the P2Y₁ receptor (Filippov et al., 2000), also strongly induced this promoter activity; that action was completely blocked by a specific antagonist of the P2Y₁ receptor, MRS 2179, in both mouse and chick myotubes (Fig. 6C). The stimulation by UTP of the AChE promoter activity was, as predicted, insensitive to this antagonist. The activation by ATP was consistently greater than that by 2-MeSADP relative to that by UTP, interpreted as due to an additional effect of ATP occurring through the UTP-sensitive P2Y₂ receptor. All of these observations confirmed that not only P2Y₁ but also P2Y₂ receptor action can contribute to *AChE* gene activation here.

Our previous studies have shown that two binding site sequences (Table 1, cases 1 and 2) for the transcription factor Elk-1 (of the Ets family) are located in the human *AChE* gene upstream of the ATG start site and that these are jointly sufficient to drive P2Y₁ receptor-dependent *AChE* gene expression (Choi et al., 2003). Association of these Elk-1 binding sites on the *AChE* gene promoter with P2Y₂ receptor activation also was therefore considered here. Those two responsive elements, Elk-1[1] and Elk-1[3], which were

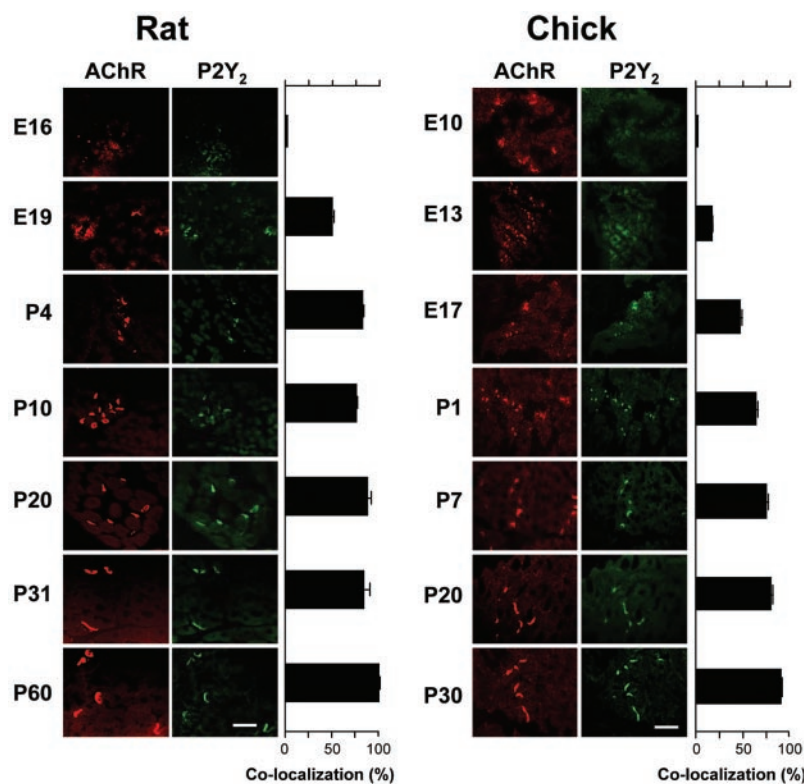


Fig. 4. Colocalization of P2Y₂ receptors and AChRs in muscle during development. Sections are double-stained as in Fig. 3. Rat gastrocnemius and chick pectoral muscles were used from E16 in rat or E10 in chick to adult (P30). The mean percentage of colocalization between P2Y₂ receptors and AChRs was determined across ~50 fields (see *Materials and Methods*) after revealing AChR staining first, and then shifting the red fluorescence filter to green for the detection of P2Y₂ receptor immunostaining. Data are mean \pm S.E.M. values, from counts over five or six fields from each of 10 sections from four animals. Scale bar, 10 μ m.

confirmed previously in gel mobility shift studies (Choi et al., 2003), were isolated, and each was inserted (in three adjacent copies) into the luciferase reporter vector. The resulting constructs, termed 3xElk-1[1]-Luc and 3xElk-1[3]-Luc, were stably transfected into myotubes. Application of ATP, or 2-MeSADP, or UTP, each produced significant promoter activation, in both mouse and chick myotube cultures (Fig. 7A). The PKC activator

TPA produced an even higher level (over 200% increase) of promoter activation in the same system, evidence for PKC involvement (Fig. 7A).

For confirmation, we sought to inactivate the Ets-type consensus sequences lying within Elk-1[1] and Elk-1[3]. The 2.2-kb genomic fragment containing all of the upstream untranslated DNA sequence from the human *AChE* gene (Ben

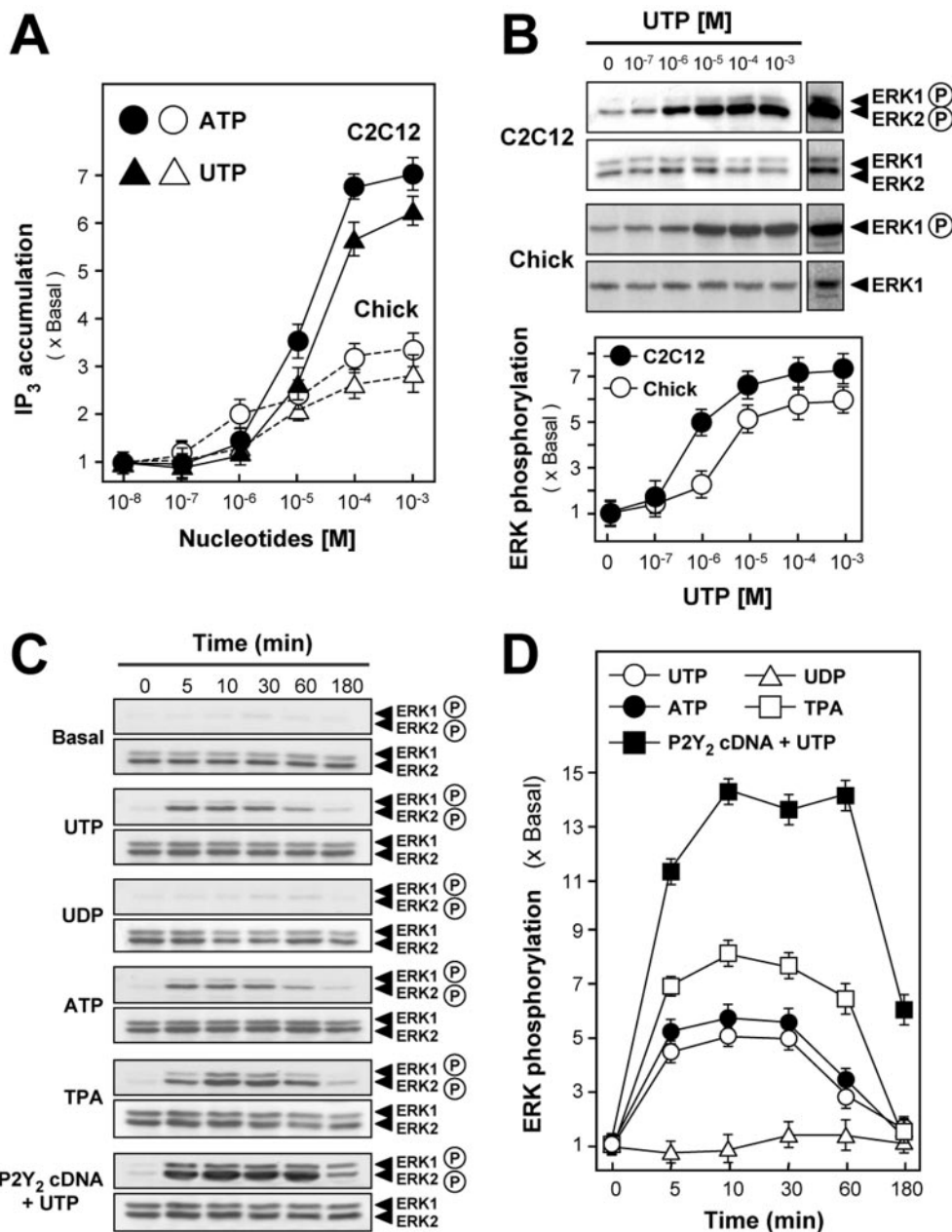


Fig. 5. Activation of P2Y₂ receptor induces total IP₃ formation and ERK phosphorylation in cultured myotubes. In each of the plots here, the ordinate shows the ratio of the stimulated over the basal level (no drug treatment). A, stimulation of IP₃ production in chick, or mouse myotubes, by application of P2Y₂ agonists. Myotubes were cultured for 5 days, and then treated with apyrase, washed, and incubated for 15 min with the indicated concentrations of ATP, or UTP, or with control medium. B, activation of P2Y₂ receptors in skeletal muscle induces phosphorylation of ERK. Myotubes (either mouse or chick) were exposed to UTP as in A. Phosphorylation changes were demonstrated with an antibody to ERK 1 and ERK 2, which recognizes the dually phosphorylated (hence active) forms of them (ERK-P). The nonphosphorylated forms of ERK 1 and ERK 2 are not increased, as seen using phosphorylation state-independent antibodies (with identification of all bands made using 10⁻³ M UTP and a long film exposure, shown in the separated end lanes). Essentially, only ERK 1 is expressed in chick myotubes, in contrast to mouse C2C12 myotubes where ERK 2 is the major and ERK 1 is the minor component. Quantitation from a set of such blots by calibrated densitometry is also shown (bottom). Values are expressed as the ratio of the stimulated over the basal level (no drug treatment). C, UTP and ATP induce transient phosphorylation of ERK 1 and ERK 2. Mouse myotubes were treated with ATP, UTP, or UDP at 50 μ M each, or with the PKC activator TPA at 50 nM. Myotubes were also pretransfected with rat P2Y₂ cDNA before the application of UTP (last panel). D, quantitation from a set of blots developed from C by calibrated densitometry. In all cases, the data shown are mean \pm S.E.M. values from four independent experiments, each with triplicate samples, and likewise in Figs. 6 and 8.

Aziz-Aloya et al., 1993) was mutated at those sequences in the Elk-1[1] and the Elk-1[3] elements, singly or together, to change six consecutive nucleotides in each, as shown in Table 1, case 6. The resultant mutant promoters were then again tagged downstream with *luciferase* as described before, to give the constructs: pAChE_{ΔElk-1[1]}-Luc, pAChE_{ΔElk-1[3]}-Luc and pAChE_{ΔElk-1[1,3]}-Luc. These were in turn stably transfected into cultured myotubes. When any of the three mutants were transfected into myotubes, they did not alter the responsiveness of the cells in P2Y₂ receptor-mediated ERK phosphorylation (data not shown). However, when the cells were treated with ATP or UTP, significantly less promoter

activity was seen with the mutant constructs than with the wild-type promoter when the cells were treated with ATP or UTP (Fig. 7B). For both mouse and chick myotubes, the double mutant pAChE_{ΔElk-1[1,3]}-Luc showed the least activity, suggesting that the two separate Elk-1 sites can operate additively.

UTP-Induced Expression of AChR. There is evidence that the expression and clustering of muscle AChE and AChR proteins have some but not all of their control mechanisms in common (Sanes and Lichtman, 1999). The regulation by ATP has been found to act on the expression of both (Choi et al., 2001, 2003). However, there is no information on

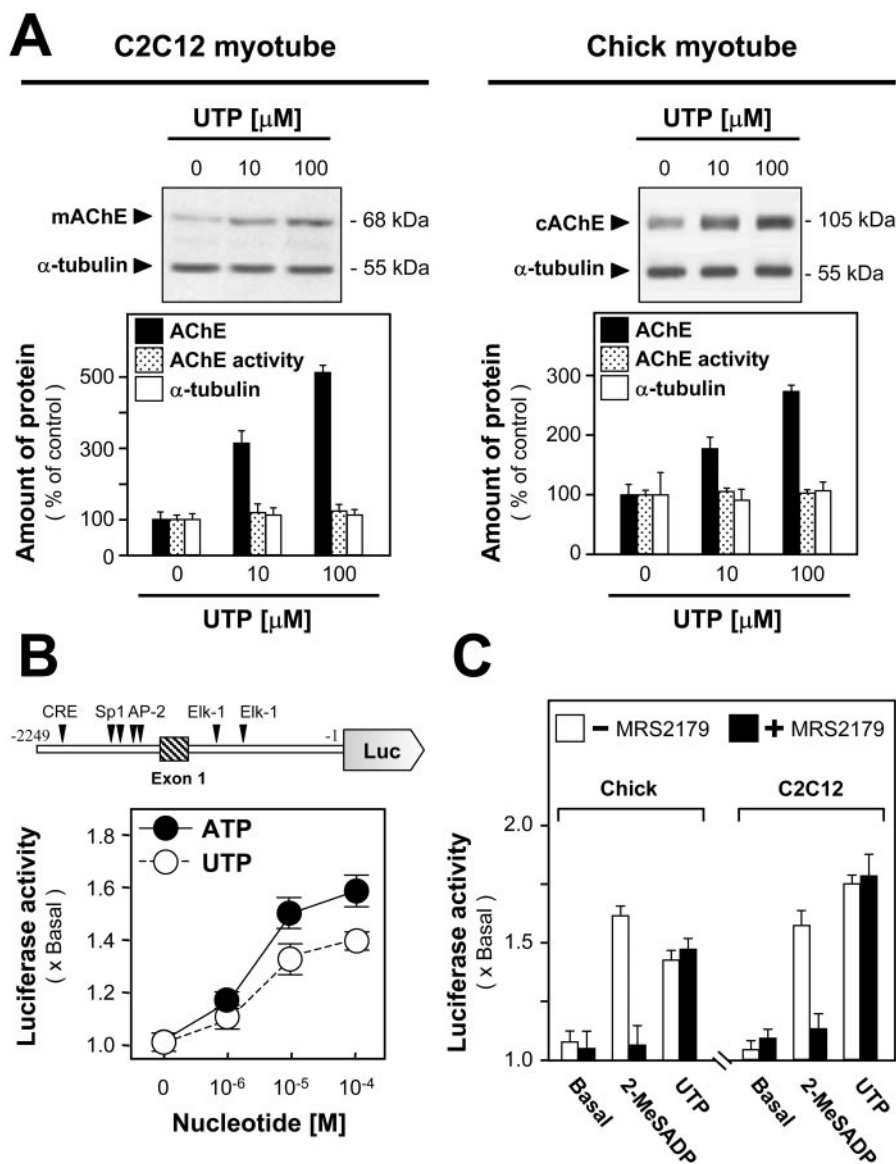


Fig. 6. P2Y₂ receptor agonists stimulate the expression of the AChE catalytic subunit. A, UTP at increasing concentrations (0–100 μ M) was applied onto cultured rat or chick myotubes for 24 h (with three changes of medium and the appropriate ligand-regenerating enzyme system present, during the incubation at 37°C). Analysis by immunoblotting of the AChE catalytic subunit (~68 kDa in rat; ~105 kDa in chick) is shown in the top panel. Quantitation (bottom) was made by calibrated densitometry. Values are expressed as the percentage of the control, which is measured in samples from myotubes incubated in parallel with buffer without UTP and run in an adjacent lane. AChE enzymatic activity was determined, and which was not affected by the agonist in both myotubes. As a control α -tubulin (~55 kDa) was detected by its antibody in the same gel lane in each case and quantitated similarly. Its amount was approximately the same in all samples. B, to mouse myotubes stably transfected with a human AChE promoter/luciferase reporter plasmid, pAChE-Luc, agonist (ATP or UTP) was applied as in A. The final luciferase activity is expressed as the ratio of the stimulated level to the basal level, the latter measured in samples from transfected myotubes incubated in parallel without ligand. C, a P2Y₁-specific antagonist (MRS 2179) does not block the UTP-induced gene activation. With procedures as in B, to pAChE-Luc transfected mouse, or chick, myotubes UTP (50 μ M) or the P2Y₁-selective 2-MeSADP (50 μ M), was applied, each with or without MRS 2179 (100 μ M).

UTP-mediated regulation of *AChR* expression, which would be a test of $P2Y_2$ receptor involvement therein (see above). Recently, we constructed AChR reporter constructs by inserting the *luciferase* reporter gene downstream of the promoter-containing regions of the chick α (930 bp), or rat δ (550 bp) or rat ϵ (2 kb) *AChR* subunit genes (Choi et al., 2003). Those constructs (designated as pAChR α -Luc, pAChR δ -Luc and pAChR ϵ -Luc) were here transfected singly into mouse and chick myotubes. Application of either ATP or UTP to these cultures stimulated that promoter activity in each case by ~50 to 100% (Fig. 8A). 2-MeSADP also induced the AChR promoter activities and those were blocked by a specific antagonist of the $P2Y_1$ receptor, MRS 2179, whereas the UTP-induced promoter activities were unaffected by this antagonist (exemplified in Fig. 8B).

For muscle *AChR* genes, it has been discovered (Schaeffer et al., 1998, 2001) that another Ets-family transcription factor, GABP, is used in their activation by a trophic factor, neuregulin (Sandrock et al., 1997), whose binding is at a responsive element containing a central 6-bp motif, the N-box. Similarly for muscle *AChE*, a GABP-binding N-box is present in an enhancer element required for normal expression of AChE in mouse C2C12 myotubes (as used here) and for its aggregation at the nmj (Chan et al., 1999). Since both *AChR* and *AChE* genes can also be activated via Elk-1 binding (Figs. 7 and 8) and since the N-box motif is also present in the Elk-1 sites of the *AChE* gene (Table 1), we considered what the relationship may be in this case between the gene sequences involved in binding Elk-1 and in binding GABP. The *AChE* genes from humans (Ben Aziz-Aloya et al., 1993)

TABLE 1

Elk-1 binding sites and N-box sequences in intron 1 of *AChE* genes

Two positions, which vary between species, are underlined. Bold type shows the core consensus. Line 6 shows the six nucleotides that were mutated here in the Elk-1[1] or Elk-1[3] site when in the intact 2.2-kb promoter-containing sequence for expression studies.

Case	Sequence	Site	Species	Position ^a
1	GC ACT CGT CCG GAA CTC TTC CC	Elk-1[1]	Human	-1431
2	GA GGC TCG GCG GAA GCC CCG AG	Elk-1[3]	Human	-1102
3	CTG GAG <u>AAG</u> CCG GAA CTA CAG CAG	N-box ^b	Rat	-913
4	GA GGC TCA GCG GAA GCC CCG A	Elk-1[3]	Mouse	-1091
5	CTG GAG <u>ACG</u> CCG GAA CTA CAG CAG	N-box ^b	Mouse	-931
6	GAA TTC	(bases mutated in Elk-1 mutants)		

^a The position of the first nucleotide shown, numbering upstream from the ATG start site (which lies in the second exon).

^b Written in the reverse orientation, as operates here.

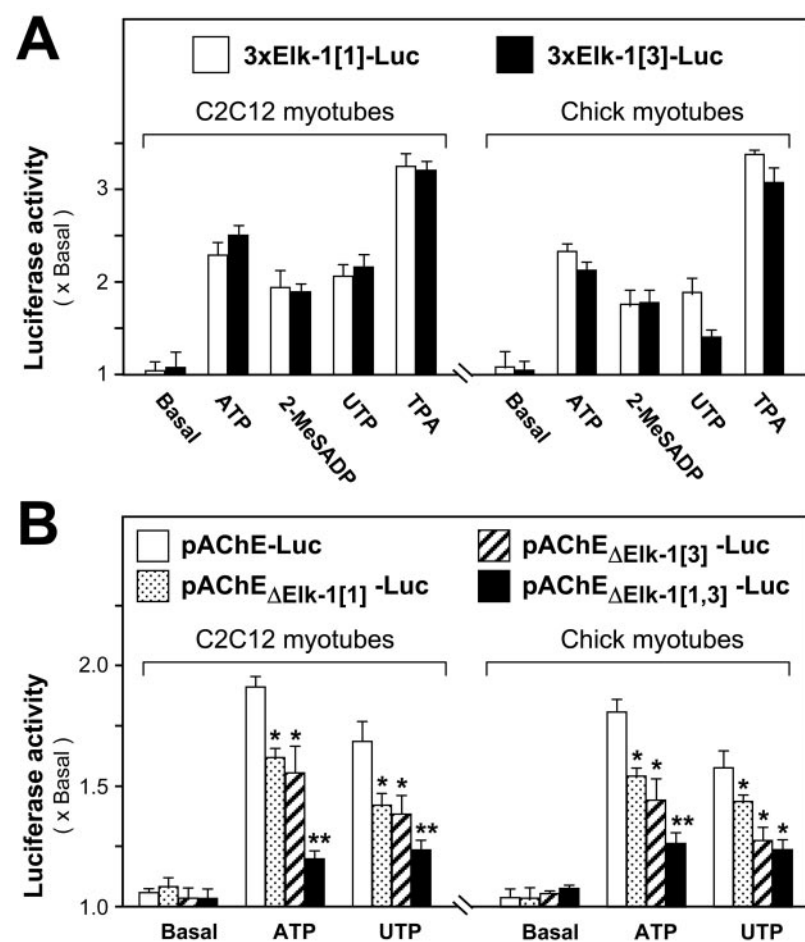


Fig. 7. Two identified binding sites for Elk-1 in the human AChE promoter region exert promoter activity and respond to $P2Y_2$ receptor activation. A, reporter gene constructs of Elk-1[1] or Elk-1[3], each carrying one of the identified Elk-1 binding sites from the AChE promoter, were transfected into mouse or chick myotubes as for Fig. 6. These cultures were exposed (16 h; 37°C) to the agents shown (50 μ M for ATP, 2-MeSADP, and UTP, with regeneration; 10 nM for TPA). B, mutation of the Elk-1 binding sites on the AChE promoter blocks its response to $P2Y_2$ receptor activation. Mouse or chick myotubes transfected with the mutated promoter constructs pAChE Δ Elk-1[1]-Luc, pAChE Δ Elk-1[3]-Luc and pAChE Δ Elk-1[1,3]-Luc, were used likewise, here the incubation being with 50 μ M ATP or UTP. In A and B, final luciferase values are expressed as in Fig. 6. Differences from the activity with nonmutated pAChE-Luc are significant at $p < 0.01$ (*) or at $p < 0.001$ (**).

and from mouse or rat (Mutero et al., 1995; Chan et al., 1999) possess known promoter-containing regions in their upstream noncoding sequence and in intron 1. In fact, a GABP-binding N-box located in the first intron regulates muscle AChE expression (whereas a second one there and two others in the upstream region are not involved; Chan et al., 1999), and we found that the above-described Elk-1[1] and Elk-1[3] sites also lie in that intron. The 6-bp canonical sequence CCG GAA of the N-box (as oriented in this case) is the same in the Elk-1[1] site, but not in Elk-1[3] (Table 1). However in the flanking sequence to that motif the Elk-1[1] and Elk-1[3] sites diverge greatly from the N-box (Table 1). We examined the genomic sequences and found that this GABP-binding N-box is, in fact, distant from either Elk-1 site, by 329 or 518 nucleotides (Table 1).

Discussion

Coexistence of P2Y₁ and P2Y₂ Receptors at Postsynaptic Membranes. The current findings extend our previous results on the nmjs (Choi et al., 2001, 2003), to show that ATP acts through both P2Y₁ and P2Y₂ receptors on the postsynaptic membrane to induce the gene expressions of

muscle AChE and AChR. Several lines of evidence support the coexistence and coactivity of P2Y₁ and P2Y₂ receptors at the nmjs. First, immunocytochemistry on rat muscle with anti-P2Y₁ and anti-P2Y₂ antibodies clearly demonstrated the abundance and colocalization of the two receptors at the junction (Fig. 3). This association arises at an early stage in postembryonic development and is maintained thereafter. Second, the colocalization of these two P2Y receptors at the nmj has now been observed in the four diverse species so far examined, i.e., mouse, rat, a bird, and an amphibian (Fig. 3; for P2Y₁, see Choi et al., 2001), suggesting that it has a distinct significance in muscle function. Third, the effect of ATP on various downstream signaling responses in myotubes has consistently been found to be higher than that of 2-MeSADP (at saturating concentrations of both), as in Figs. 6 and 7 and also in Choi et al. (2001, 2003), indicative of an additional presence of P2Y subtype(s) other than P2Y₁ in skeletal muscle. Fourth, UTP, an agonist at the P2Y₂ but not the P2Y₁ receptor, induced the downstream mitogen-activated protein kinase signaling cascade and led to activation of *AChE* and *AChR* gene expression. The UTP-induced responses were all insensitive to the P2Y₁-specific antagonist MRS 2179. Finally, the UTP-induced ERK phosphorylation was strongly potentiated by overexpression of P2Y₂ receptors in the myotubes (Fig. 5).

A cooperative action of the P2Y₁ and P2Y₂ receptors is a surprising finding, since the evidence on them at other locations (albeit limited) has shown apparently independent actions. However, their common characteristic of activation by ATP could obviously lead to a cooperative role for this pair at synapses such as the nmj where ATP is constantly released from presynaptic nerve terminals. A rationale for that dual role could be postsynaptic regulation at the levels of transcription and translation operated through two P2Y genes rather than one, to provide a greater range of control in, e.g., programs of differentiation or synaptic maintenance.

ATP is known to be generally coreleased with another transmitter at central and peripheral cholinergic and bioaminergic synapses (Zimmermann, 1994) and even some GABAergic neuronal synapses (Jo and Role, 2002). Besides neurons, glial cells have also been shown to release ATP to modulate activity in neighboring neurons (Newman, 2003, and references therein). The phenomena found here at the nmj may be a model for P2Y responses at some central nervous system neurons: preliminary studies on some central nervous system neurons have shown colocalizations of P2Y₁ and P2Y₂ receptors with postsynaptic density proteins (N. L. Siow et al., unpublished data).

Confinement of Postsynaptic Nucleotide Trophic Actions to the P2Y₁ and P2Y₂ Receptors. Our results have further eliminated the possible involvement of other P2Y receptor subtypes in mediating the synaptic responses to ATP and UTP. The lack of UDP-mediated activation of ERKs in chick or mouse myotubes (Fig. 5, C and D) excluded the possible involvement of the ADP- and UDP-sensitive P2Y₃ and P2Y₆ receptors. Moreover, the expression of the P2Y₃ receptor, present in some other chicken tissues, was previously shown to be absent in embryonic or adult chicken skeletal muscle (Webb et al., 1996). The P2Y₄ receptor was a potential candidate, since in rodents it is known (as cited under *Results*) to respond to both ATP and UTP to produce IP₃ as seen here. However, using Northern blot analysis on

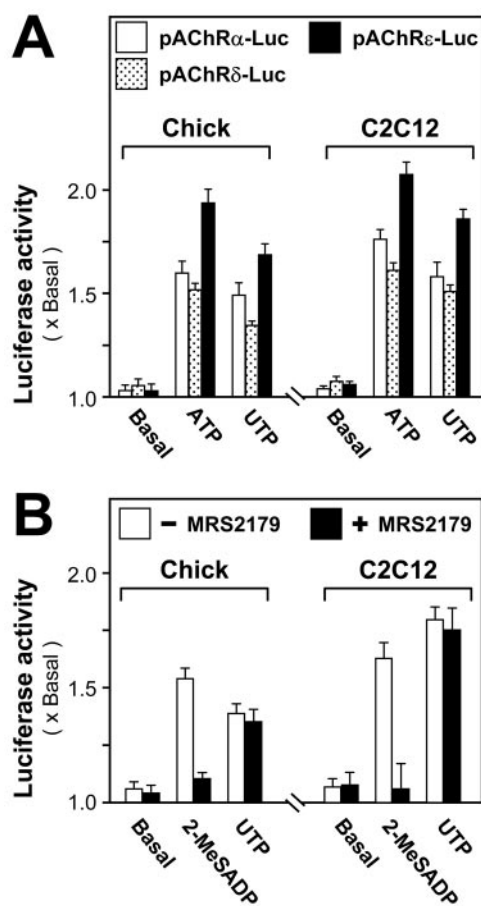


Fig. 8. The P2Y₂ receptor activation induces gene expression for three subunits of muscle AChR. A, pAChR α -Luc, pAChR δ -Luc, or pAChR ϵ -Luc were expressed singly in mouse and in chick myotubes, and each set was exposed to ATP or UTP (50 μ M), or medium alone, for luciferase analysis (with procedures as for Fig. 6B). B, likewise, pAChR ϵ -Luc was expressed in rat or chick myotubes, and the cultures were exposed to 2-MeSADP or UTP (50 μ M each), each with or without MRS 2179 (100 μ M). MRS 2179 blocked the 2-MeSADP-induced, but not the UTP-induced gene activation. The luciferase values are expressed as in Fig. 6.

either rat muscle or C2C12 mouse myotubes, the P2Y₄ receptor transcript was below the level of detection, a reproducible finding, whereas P2Y₁ and P2Y₂ transcripts were always found there (Fig. 1). The P2Y₄ receptor protein was reported by Cheung et al. (2003) by immunohistochemical staining in rat embryonic and postnatal skeletal muscles (although, anomalously, on peripherally but not centrally located postnatal fibers) but not at the nmjs. The same anti-P2Y₄ antibody (from Alomone Labs) was used in that study and in ours; in the conditions reported by Cheung et al. (2003), we have shown (Fig. 2) that serious cross-reactivity occurs with the P2Y₁ and P2Y₂ receptor subtypes. Conversely, in fact, P2Y₁ and P2Y₂ receptor immunoreactivities were found by Cheung et al. (2003) to be absent in rat skeletal muscle fibers and nmjs (E18 to 2 months) in contrast to our clear positive staining there in the rat (Figs. 3 and 4), again with the same antibodies in both studies. The main differences in the two cases is that Cheung et al. (2003) used in the antibody incubation no Triton X-100 addition, which we make to increase penetration at the nmjs, and also high serum (10%), which we found will block the reaction of these particular anti-P2Y₁ and anti-P2Y₂ (but not anti-P2Y₄) antibodies (Fig. 2). In support of our findings, a similar abundance, development and nmj location of the P2Y₁ receptors was found previously in chicken muscles using an independent antibody, which we raised to a different epitope in the chicken P2Y₁ receptor (Choi et al., 2001).

The P2Y₁₁ receptor can also respond to both ATP and UTP, but UTP cannot activate it to produce IP₃ (White et al., 2003) as occurs here. The P2X receptors, ion channels that operate fast signaling, are also activated by ATP but they are insensitive to UTP (North, 2002). It has been reported that the P2X₇ receptor occurs at nmjs, but exclusively in the presynaptic nerve terminal (Deuchars et al., 2001). Expression of other P2X receptor subtypes at the nmj has been examined in the rat and was reported to be absent (Deuchars et al., 2001; Ryten et al., 2001). Thus, we conclude that the ATP- and UTP-induced signaling to the *AChE* and *AChR* genes in skeletal muscles depends upon the P2Y₁ and the P2Y₂ receptors.

ATP and UTP at the nmj. ATP released from the motor terminal would be a native agonist at both P2Y₁ and P2Y₂ receptors, having EC₅₀ of the order of 1 μM at each in recombinant expressions (Palmer et al., 1998; Wildman et al., 2003), increasing in potency with increasing receptor density (Filippov et al., 2000). The P2Y₁ and P2Y₂ receptors located at the nmjs could both, therefore, become activated by the released ATP, the concentration of which in the synaptic cleft reaches ~300 μM (Silinsky and Redman, 1996). Thus, the action of ATP in directing the post-synaptic gene expression is, from the present results, presumed to be mediated by both P2Y₁ and P2Y₂ receptors. The extent of the presence of UTP, however, at active nmjs has never been determined. Nevertheless, the presynaptic cholinergic vesicles there are predicted (Zimmermann, 1994) to contain UTP at ~10% of their content of ATP, on the basis of that ratio as determined in the similar vesicles of chromaffin cells containing transmitter and ATP. This is to be expected since in cytosol in general the proportion of free UTP to free ATP is ~10% (Anderson and Parkinson, 1997), and the ATP loading system of vesicles, where studied, does not distinguish between ATP and UTP (Bankston and Guidotti, 1996). Hence, signif-

icant synaptic UTP levels can be expected at the vertebrate nmj during impulse trains. In addition, conversion can also occur of ATP to UTP via ectoenzymes and endogenous UDP (Lazarowski et al., 1997), which might add a further slower phase to the activation. The presence of the P2Y₂ receptor at the nmjs could have arisen to use UTP as an additional transmitter source, in line with the rationale suggested above for the appearance of a second P2Y receptor there.

Our results have demonstrated that both the P2Y₁ and the P2Y₂ receptors in muscle cells activate specific signaling pathways, which stimulate gene expression of the postsynaptic *AChE* and *AChR* (Figs. 5–8; Choi et al., 2001, 2003). Concurrently, we have shown that 2-MeSADP likewise stimulates muscle cell gene expression of the additional collagen-tail subunit required for the anchoring of the asymmetric form of *AChE* at mature nmjs (Lee et al., 2004). However, we should note that these various effects on transcription are not the only route involved, since the increase of *AChE* levels in myogenesis in C2C12 cells is known to arise also in part by stabilization of its mRNA (Luo et al., 1999, and references therein).

Acknowledgments

We thank Prof. H. Soreq (Hebrew University of Jerusalem, Jerusalem, Israel) for providing human *AChE* promoter DNA and Dr. D. Goldman (University of Michigan, Ann Arbor, MI) for providing rat *AChR* subunit δ and ε promoter DNAs.

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