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$P2Y_2$ Receptor Activation Regulates the Expression of Acetylcholinesterase and Acetylcholine Receptor Genes at Vertebrate Neuromuscular Junctions

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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 $P2Y_1$ receptor is localized at the nmj. Here, we extend the findings to show that another nucleotide receptor, $P2Y_2$, is also localized there and with $P2Y_1$ jointly mediates trophic responses to ATP. The $P2Y_2$ receptor mRNA in rat muscle increased during development and peaked in adulthood. The $P2Y_2$ receptor protein was shown to become restricted to the nmjs during embryonic development, in chick and in rat. In both rat and chick myotubes, $P2Y_1$ and $P2Y_2$ are expressed, increasing with differentiation, but $P2Y_4$ is absent. The $P2Y_2$ 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 $P2Y_1$-specific antagonist MRS 2179 (2′-deoxy-N^6-methyl adenosine 3′,5′-diphosphate diammonium salt). In differentiated myotubes, $P2Y_2$ 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 $P2Y_2$ receptor and also in that initiated at the $P2Y_1$ 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 $P2Y_1$ and $P2Y_2$ 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 transcriptionally 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 nonsynaptic 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

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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; IP_3, 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 $P2Y_1$ 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, $P2Y_1$ 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 Ca^{2+} 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 $P2Y_1$ 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 $P2Y_1$ 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 $P2Y_1$ receptor co-occurs with and acts cooperatively with the G_i-linked $P2Y_{12}$ receptor (Dorsam and Kunapuli, 2004). Here, we examine whether the two other PKC-linked P2Y receptors that are responsive to ATP, namely, $P2Y_2$ and $P2Y_4$, could be localized with $P2Y_1$ 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 Mg^{2+} 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 Mg^{2+} 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 $P2Y_1$ or $P2Y_2$ receptor were generated by electroporation of the respective plasmid constructs ($rP2Y_1$/pcDNA 3.1, $rP2Y_2$/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 $P2Y_1$ or $P2Y_2$ 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% CO_2) 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 [^{3}H]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 [α-^{32}P]dCTP-labeled probes against mouse AChE catalytic subunit cDNA, rat AChR α-subunit cDNA, or rat $P2Y_1$ receptor cDNA, all as detailed by Choi et al. (2001). For rat $P2Y_2$ receptor the ~2.0-kb probe of Chen et al. (1996) and for rat $P2Y_4$ 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 ^{32}P-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 $P2Y_1$ (Tokuyama et al., 1995) and $P2Y_2$ (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 or both of those two segments in their Elk-1 site, as defined under *Results*, and again used with the luciferase reporter to generate the $pAChE_{\Delta Elk\text{-}1[1]}$-Luc, $pAChE_{\Delta Elk\text{-}1[3]}$-Luc, and $pAChE_{\Delta Elk\text{-}1[1,3]}$-Luc constructs.

Myoblasts from 11-day chick embryos were cultured at 37°C for 2 days and transiently transfected with the plasmid constructs (2 μg plasmid per 35-mm dish or 1 μg 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 μg/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 μl) 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 (12,000*g*, 10 min) at 4°C. The cultured neurons were homogenized in 100 μl 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-μg sample per lane) and immunoblotted with all procedures as described previously (Choi et al., 2001), probing with anti-rat $P2Y_1$ or anti-rat $P2Y_2$ antibodies (Alomone Labs, Jerusalem, Israel; 1:400–1:1000 dilutions). The selectivity of the $P2Y_1$ and the $P2Y_2$ antibodies in immunoblotting was verified by incubation of each (2 μg) with 10 μg 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 Na_3VO_4/phosphate-buffered saline (PBS), followed by addition of 400 μl of lysis buffer containing 10 mM HEPES, pH 7.5, 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 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 μm) 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-$P2Y_1$ antibody was applied in 6% normal serum (goat or human, without difference) plus 2% BSA. The anti-$P2Y_2$ 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 $P2Y_1$ 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× 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 [^{3}H]inositol trisphosphate ([^{3}H]IP_3) was separated from other labeled inositol species by standard chromatographic procedures. The [^{3}H]IP_3 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 $P2Y_2$ Receptor mRNA in Developing Rat Muscles and Spinal Cord. A single transcript (~3.2 kb) for the $P2Y_2$ receptor was found in Northern blots of RNA isolated from rat gastrocnemius muscles (Fig. 1A). Up to embryonic day 14 (E14) muscle, the $P2Y_2$ 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 $P2Y_2$ transcript content further increased at birth, but its major increase was shown to occur around P4 (Fig. 1B). The expression profile of the $P2Y_1$ receptor mRNA (~4.2 kb) in the same muscles, from E14 to P31, was similar to that of the $P2Y_2$ 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 $P2Y_1$ receptor (Fig. 1A). The $P2Y_4$ 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 $P2Y_2$ 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 $P2Y_1$ and $P2Y_2$ receptors. The expression of $P2Y_1$ receptor, however, occurs noticeably earlier in the embryonic spinal cord than that of $P2Y_2$. 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 $P2Y_2$ receptor mRNA in muscle than in the spinal cord. In addition, the amount of the $P2Y_2$ receptor mRNA in adult (P31) rat muscle was higher (~5-fold) than that of $P2Y_1$ receptor.

Transcripts for the $P2Y_2$ but not for the $P2Y_4$ receptors were also found in mouse C2C12 myotubes. We had previously found (Choi et al., 2003) that those cells during differentiation strongly express $P2Y_1$ receptors. Here, they showed a 5-fold increase in $P2Y_2$ transcript level from day 1 to day 5 during differentiation (Fig. 1D). This profile of $P2Y_2$ 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 $P2Y_2$ and ~4.0 kb for $P2Y_4$ 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 $P2Y_1$ or $P2Y_2$ or $P2Y_4$ 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 $P2Y_1$ or $P2Y_2$ or $P2Y_4$ 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-$P2Y_1$ antibody recognized in immunocytochemistry the $P2Y_1$ receptor, but also the $P2Y_2$ receptor (Fig. 2, A and B). In the same conditions, the anti-$P2Y_2$ antibody was specific for the $P2Y_2$ receptor, neither reacting with the $P2Y_1$ receptor (Fig. 2, A and B) nor (not shown) staining $P2Y_4$-expressing cells. Pretreatment with the $P2Y_1$ or the $P2Y_2$ 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-$P2Y_1$ antibody became specific but the intensity of its reaction at the $P2Y_1$ receptors was greatly decreased (Fig. 2, C and E). In that medium the anti-$P2Y_2$ 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

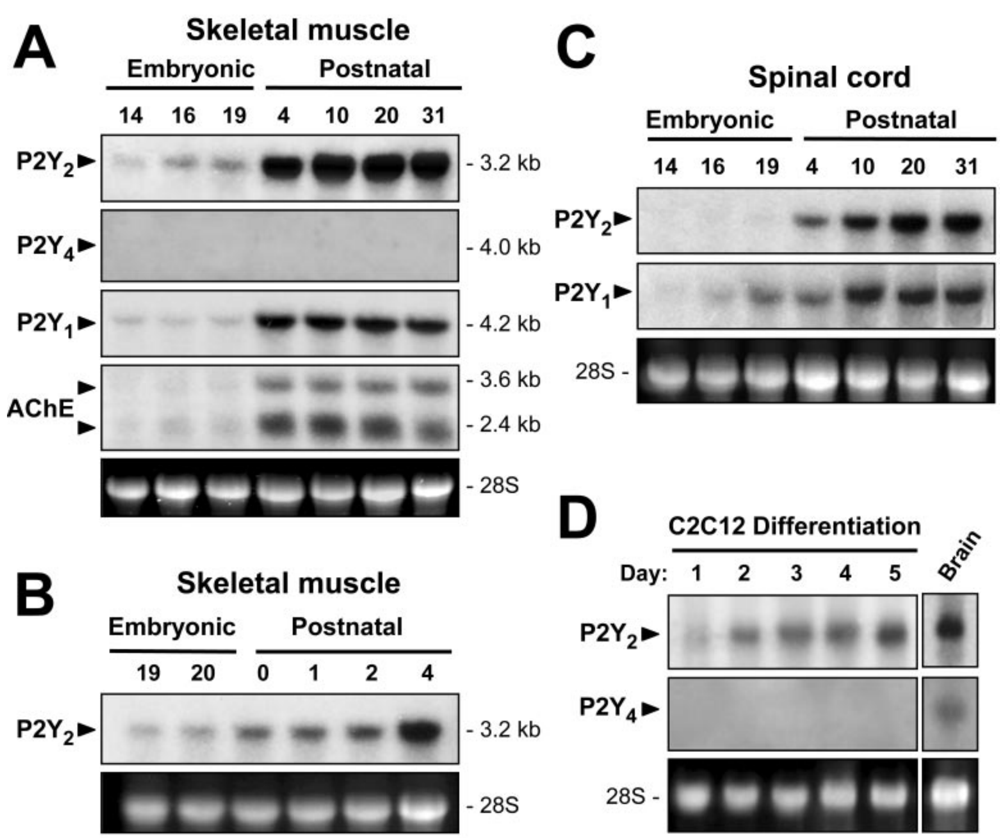

Fig. 1. Developmental changes of $P2Y_2$ receptor mRNA levels in rat or muscle and spinal cord. A, a single $P2Y_2$ receptor transcript at ~4.2 kb was detectable in rat gastrocnemius muscle, from E14 to P31. Transcripts of expected sizes encoding the $P2Y_1$ receptor (~3.2 kb) and AChE catalytic subunit (~2.4 and ~3.6 kb) were also detected, as shown. The $P2Y_4$ 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 $P2Y_2$ receptor transcript. C, in rat spinal cord $P2Y_2$ receptor mRNA increases similarly during development, and $P2Y_1$ 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 $P2Y_2$ transcript. The mouse $P2Y_4$ 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 $P2Y_2$ and $P2Y_4$ receptors. It is known that the $P2Y_4$ receptor is expressed in rodent brain but at a much lower level than $P2Y_1$ or $P2Y_2$, 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 $P2Y_1$ and $P2Y_2$ 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-$P2Y_1$ antibody was obtainable in the presence of 6% normal goat serum and 2% BSA, where the intensity at $P2Y_1$ receptors is adequate (Fig. 2D), whereas the cross-reactions are negligible to $P2Y_2$ (Fig. 2F) and to $P2Y_4$ (data not shown). That medium was, therefore, used in the muscle $P2Y_1$ immunostainings. For the anti-$P2Y_2$ 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 $P2Y_2$ immunostaining.

For the anti-$P2Y_4$ antibody, no conditions were found in which it could show a specific reaction with the $P2Y_4$ receptor. At the highest serum levels tested, it still reacted significantly with both $P2Y_1$ and $P2Y_2$ (Fig. 2, C and E) as well as with $P2Y_4$ 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 $P2Y_4$ peptide fully blocked the reaction with the anti-$P2Y_4$ antibody on the $P2Y_4$ cell line (data not shown) but also greatly reduced its cross-reaction on the $P2Y_1$ or $P2Y_2$ cell lines, whereas failure of the peptide to block was seen for $P2Y_4$ peptide in the anti-$P2Y_1$ antibody cross-reaction on $P2Y_4$ and also for $P2Y_1$ peptide in the anti-$P2Y_4$ antibody cross-reaction on $P2Y_1$ (not shown). Inspection of the relevant sequences showed that there are three dipeptide matches between the C-terminal epitope used for $P2Y_4$ and other regions in $P2Y_1$ and likewise in $P2Y_2$. There is also one tripeptide match within the epitopes used for $P2Y_1$ and for

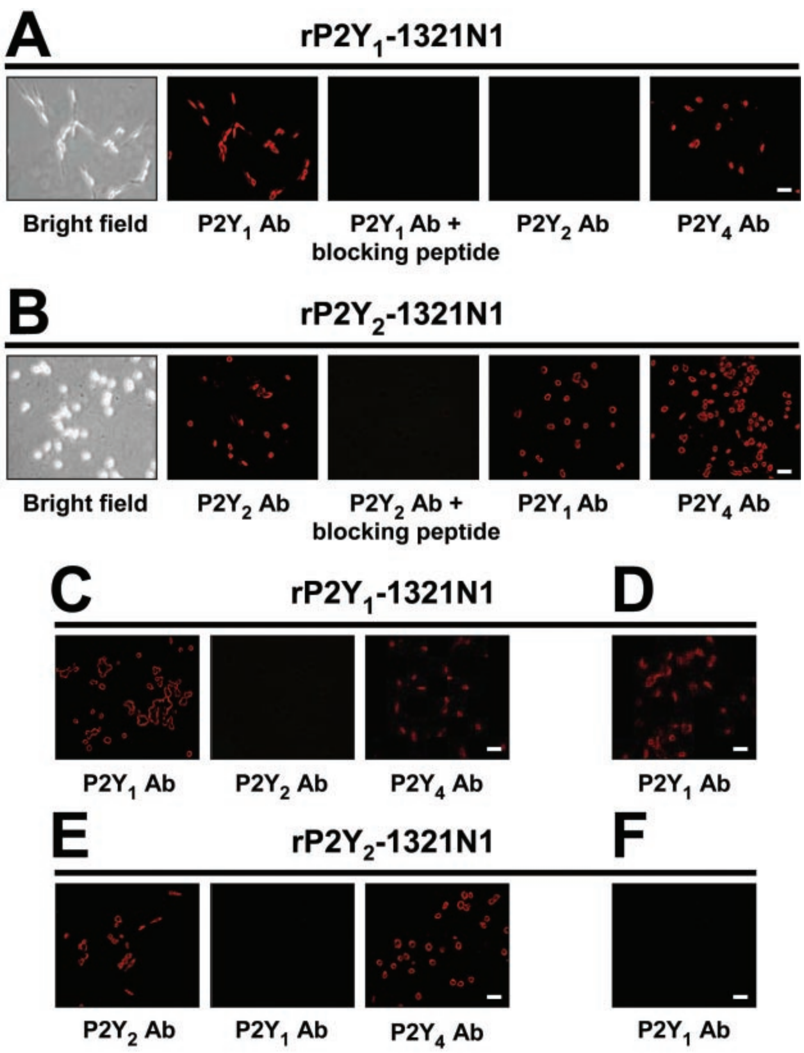

Fig. 2. Specificity of antibodies used in detecting P2Y receptor subtypes on muscle cells. In each case, 1321N1 cells transfected to stably express the single rat P2Y subtype indicated were reacted with each antibody raised against a given human P2Y subtype. The antibody incubation medium was, as stated, 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 $P2Y_1$ receptor is recognized (A) by the anti-$P2Y_1$ antibody (reaction blocked by the presence of the $P2Y_1$ peptide antigen, 1 μg in 50 μl of incubation medium), but also by the anti-$P2Y_4$ antibody and *not* by the anti-$P2Y_2$ antibody. The $P2Y_2$ receptor is recognized (B) by the anti-$P2Y_2$ antibody (reaction blocked by the presence of the $P2Y_2$ peptide antigen, 1 μg in 50 μl), but also by the anti-$P2Y_1$ antibody and by the anti-$P2Y_4$ antibody. C and E, reactions in high serum medium. The $P2Y_1$ receptor is recognized (C) by the anti-$P2Y_1$ antibody though with very low intensity, but also by the anti-$P2Y_4$ antibody and *not* by the anti-$P2Y_2$ antibody. The $P2Y_2$ receptor is recognized (E) by the anti-$P2Y_2$ antibody, although with very low intensity, but also by the anti-$P2Y_4$ antibody and *not* by the anti-$P2Y_1$ antibody. D and F, reactions in intermediate serum medium. In this medium, the anti-$P2Y_1$ antibody stains the $P2Y_1$ receptor (D) but its cross-reactivity on the $P2Y_2$ receptor is again abolished (F).

$P2Y_2$ and another two between the $P2Y_1$ epitope and elsewhere on $P2Y_4$. 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-$P2Y_4$ 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 $P2Y_1$ or the $P2Y_2$ receptor proteins in muscle specimens.

Under conditions when both the $P2Y_1$ and $P2Y_2$ immunostaining are selective, the $P2Y_2$ 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 $P2Y_2$ receptor at the nmjs (Fig. 3A). Furthermore, serial sections (10 μm) were stained with anti-$P2Y_1$ and anti-$P2Y_2$ antibodies (1:500 dilution of each), and the colocalization of the two immunoreactivities was confirmed (Fig. 3B).

The extent of colocalization of $P2Y_2$ 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 $P2Y_2$ 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-$P2Y_2$ receptor antibody could still be observed in some extrajunctional areas.

Activation of $P2Y_2$ Receptors Induces IP_3 Accumulation and ERK Phosphorylation. We have demonstrated previously (Choi et al., 2001, 2003) that activation by adenosine tri- or diphosphates of the $P2Y_1$ receptors present in cultured chick myotubes mobilizes intracellular Ca^{2+} and also leads to an increase in the expression of AChE. Here, we have found that not only ATP but also UTP can stimulate IP_3 accumulation in mouse C2C12 myotubes and chick myotubes (Fig. 5A). UTP is inactive on rodent and avian $P2Y_1$ receptors, and agonist activity of UTP to increase IP_3 is characteristic of only two of the P2Y subtypes in the rat or mouse, the $P2Y_2$ and the $P2Y_4$ 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 $P2Y_4$ behaves likewise. However, as shown above, $P2Y_4$ mRNA is absent, whereas $P2Y_2$ mRNA and protein are amply present in the cells studied here. The increase in IP_3 accumulation was dose-dependent and at saturating (100 μM) UTP application it has risen in mouse myotubes to 6-fold over the basal level (Fig. 5A).

The activation of the endogenous $P2Y_2$ 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 μM 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 μM 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

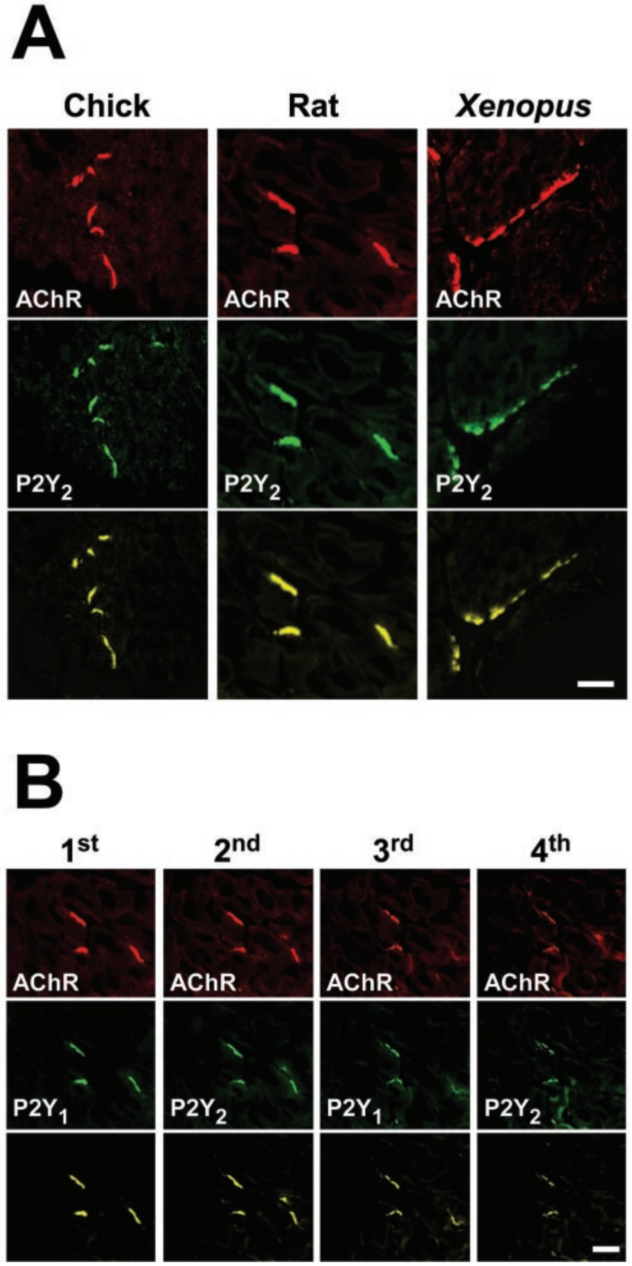

Fig. 3. Localization of $P2Y_2$ receptor in rat muscles. A, chicken, or rat, or *Xenopus* muscle sections (20 μm) were used. For each, the same field is shown stained by the anti-$P2Y_2$-receptor antibody (green) or for AChR (red) by TMR-BuTX (10 nM) or superimposed (yellow). B, serial sections (10 μm) were cut from rat muscles, and each of them was stained with anti-$P2Y_1$ and $P2Y_2$ antibodies alternatively or superimposed (yellow) on bottom panels. Scale bar, 20 μm.

(data not shown). UDP, an agonist for the chick $P2Y_3$ (Webb et al., 1996) and for the similar rat $P2Y_6$ 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 $P2Y_2$ 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 $P2Y_2$ 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 $P2Y_1$-induced AChE expression (Choi et al., 2001, 2003), the activation of $P2Y_2$ 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 $P2Y_1$ 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 $P2Y_1$ and $P2Y_2$) producing a stronger effect than UTP. 2-MeSADP, a selective and more potent agonist for the $P2Y_1$ receptor (Filippov et al., 2000), also strongly induced this promoter activity; that action was completely blocked by a specific antagonist of the $P2Y_1$ 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 $P2Y_2$ receptor. All of these observations confirmed that not only $P2Y_1$ but also $P2Y_2$ receptor action can contribute to *AChE* gene activation here.

Our previous studies have shown that two binding site sequences (Table1, 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 $P2Y_1$ receptor-dependent *AChE* gene expression (Choi et al., 2003). Association of these Elk-1 binding sites on the *AChE* gene promoter with $P2Y_2$ receptor activation also was therefore considered here. Those two responsive elements, Elk-1[1] and Elk-1[3], which were

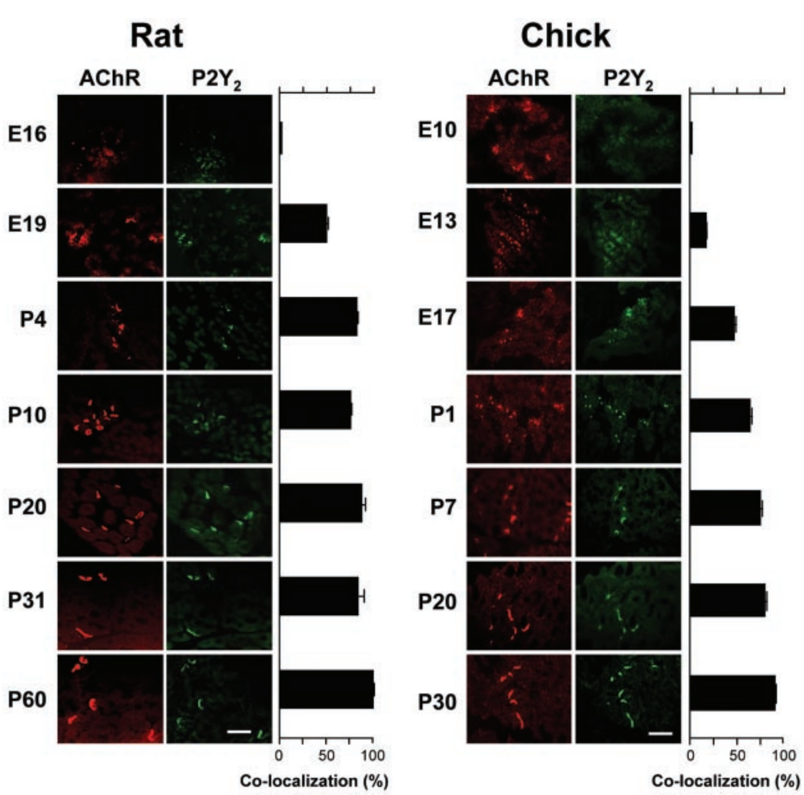

Fig. 4. Colocalization of $P2Y_2$ receptors and AChRs in muscle during development. Sections are double-stained as in Fig. 3. Rat gastrocnemius and chick pectoral muscles were used from E16 in rat or E10 in chick to adult (P30). The mean percentage of colocalization between $P2Y_2$ receptors and AChRs 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 $P2Y_2$ receptor immunostaining. Data are mean ± S.E.M. values, from counts over five or six fields from each of 10 sections from four animals. Scale bar, 10 μm.

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

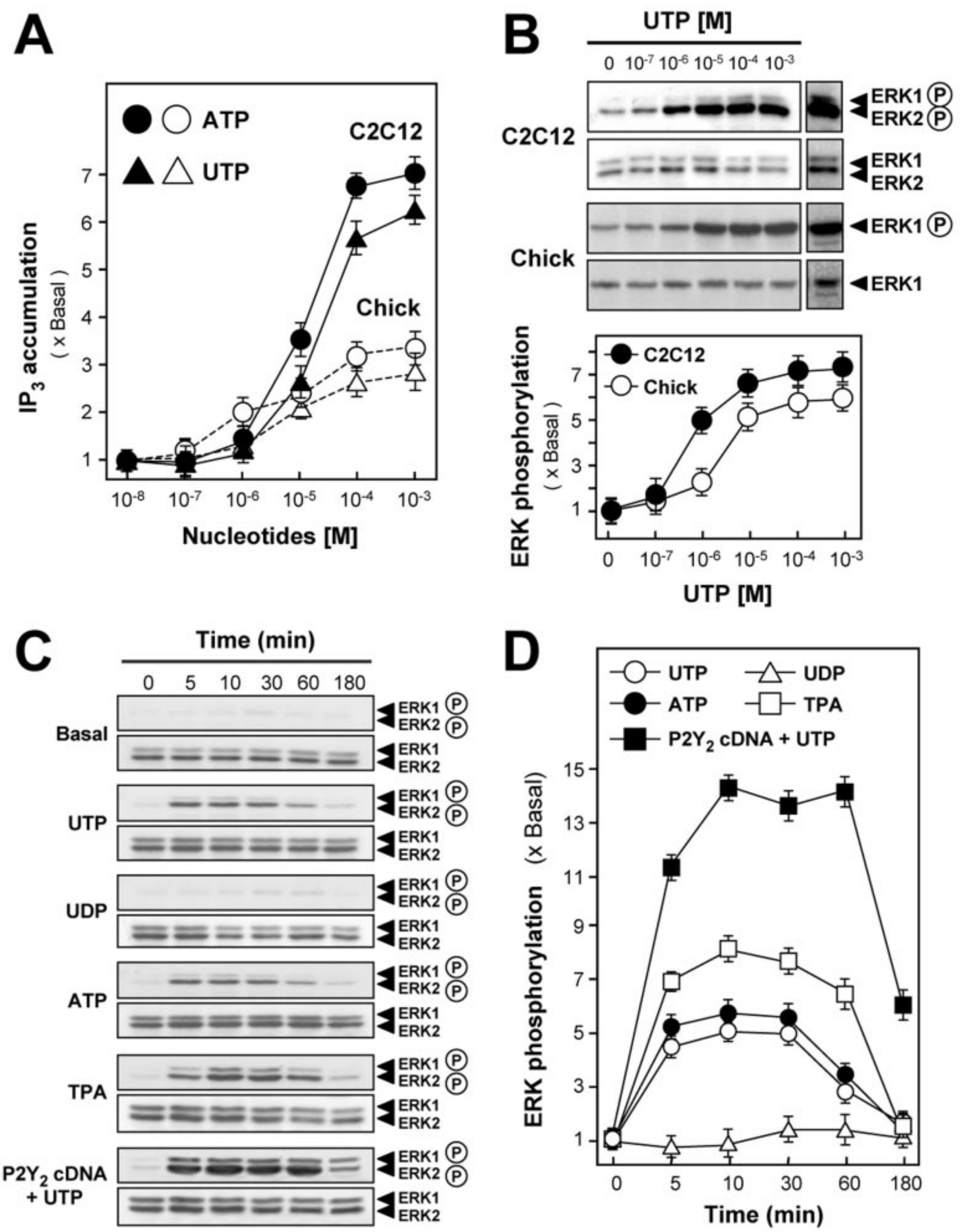

Fig. 5. Activation of $P2Y_2$ receptor induces total IP_3 formation and ERK phosphorylation in cultured myotubes. In each of the plots here, the ordinate shows the ratio of the stimulated over the basal level (no drug treatment). A, stimulation of IP_3 production in chick, or mouse myotubes, by application of $P2Y_2$ 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 $P2Y_2$ 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 μM each, or with the PKC activator TPA at 50 nM. Myotubes were also pretransfected with rat $P2Y_2$ 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 ± 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: $pAChE_{\Delta Elk\text{-}1[1]}$-Luc, $pAChE_{\Delta Elk\text{-}1[3]}$-Luc and $pAChE_{\Delta Elk\text{-}1[1,3]}$-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 $P2Y_2$ 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 $pAChE_{\Delta Elk\text{-}1[1,3]}$-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

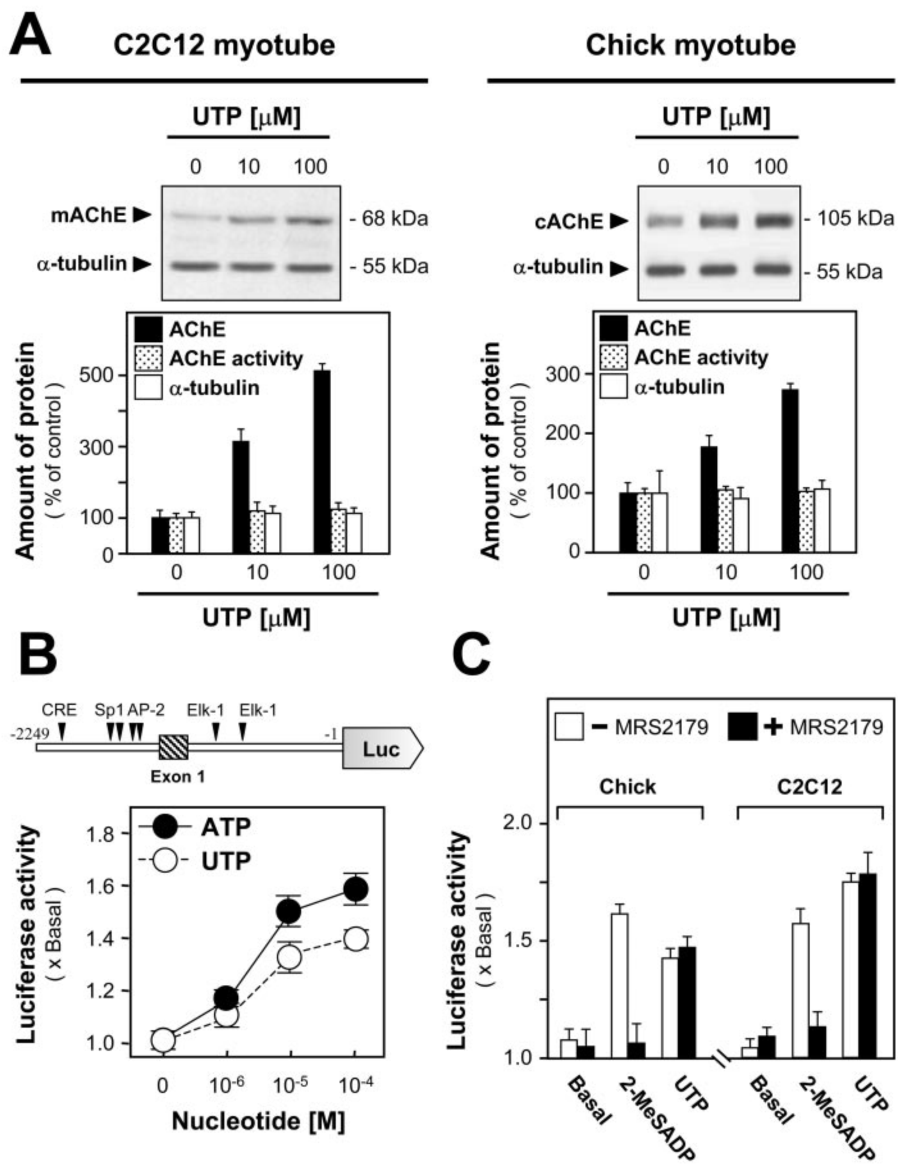

Fig. 6. $P2Y_2$ receptor agonists stimulate the expression of the AChE catalytic subunit. A, UTP at increasing concentrations (0–100 μM) was applied onto cultured rat or chick myotubes for 24 h (with three changes of medium and the appropriate ligand-regenerating enzyme system present, during the incubation 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 mytoubes. 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 $P2Y_1$-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 μM) or the $P2Y_1$-selective 2-MeSADP (50 μM), was applied, each with or without MRS 2179 (100 μM).

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 pAChRα-Luc, pAChRδ-Luc and pAChRϵ-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 AAG **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 ACG **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.

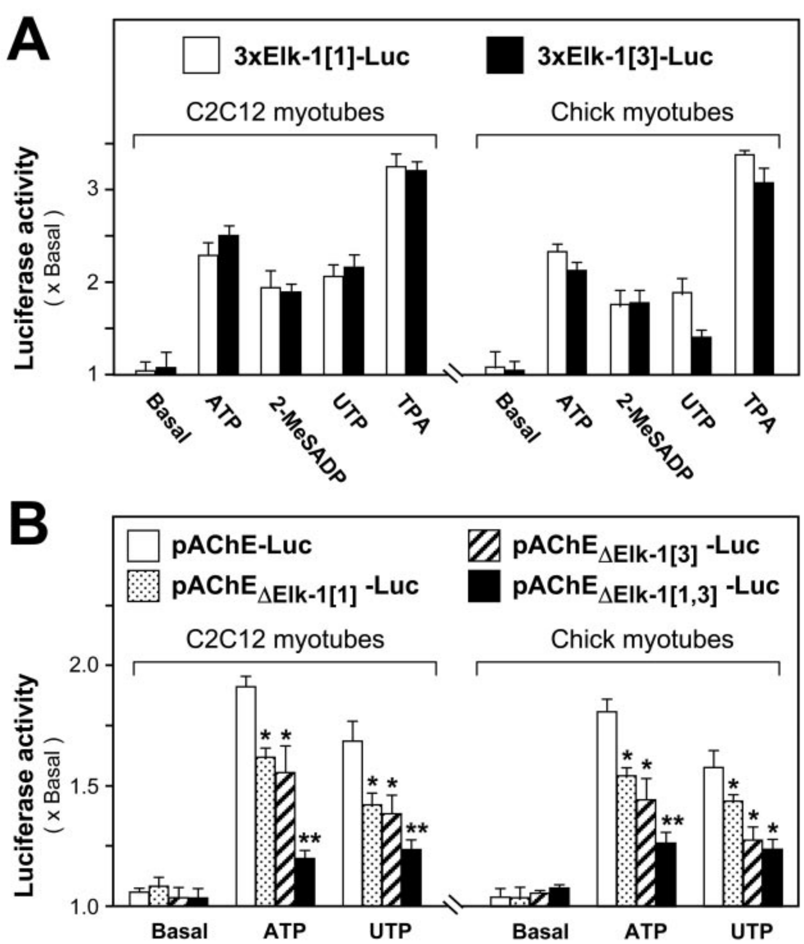

Fig. 7. Two identified binding sites for Elk-1 in the human AChE promoter region exert promoter activity and respond to $P2Y_2$ receptor activation. A, reporter gene constructs of Elk-1[1] or Elk-1[3], each carrying one of the identified Elk-1 binding sites from the AChE promoter, were transfected into mouse or chick myotubes as for Fig. 6. These cultures were exposed (16 h; 37°C) to the agents shown (50 μM 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$_{\Delta Elk-1[1]}$-Luc, pAChE$_{\Delta Elk-1[3]}$-Luc and pAChE$_{\Delta Elk-1[1,3]}$-Luc, were used likewise, here the incubation being with 50 μM 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 $P2Y_1$ and $P2Y_2$ 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 $P2Y_1$ and $P2Y_2$ 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 $P2Y_1$ and $P2Y_2$ receptors at the nmjs. First, immunocytochemistry on rat muscle with anti-$P2Y_1$ and anti-$P2Y_2$ 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 $P2Y_1$, 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 $P2Y_1$ in skeletal muscle. Fourth, UTP, an agonist at the $P2Y_2$ but not the $P2Y_1$ 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 $P2Y_1$-specific antagonist MRS 2179. Finally, the UTP-induced ERK phosphorylation was strongly potentiated by overexpression of $P2Y_2$ receptors in the myotubes (Fig. 5).

A cooperative action of the $P2Y_1$ and $P2Y_2$ 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 $P2Y_1$ and $P2Y_2$ receptors with postsynaptic density proteins (N. L. Siow et al., unpublished data).

Confinement of Postsynaptic Nucleotide Trophic Actions to the $P2Y_1$ and $P2Y_2$ 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 $P2Y_3$ and $P2Y_6$ receptors. Moreover, the expression of the $P2Y_3$ 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 $P2Y_4$ receptor was a potential candidate, since in rodents it is known (as cited under *Results*) to respond to both ATP and UTP to produce IP_3 as seen here. However, using Northern blot analysis on

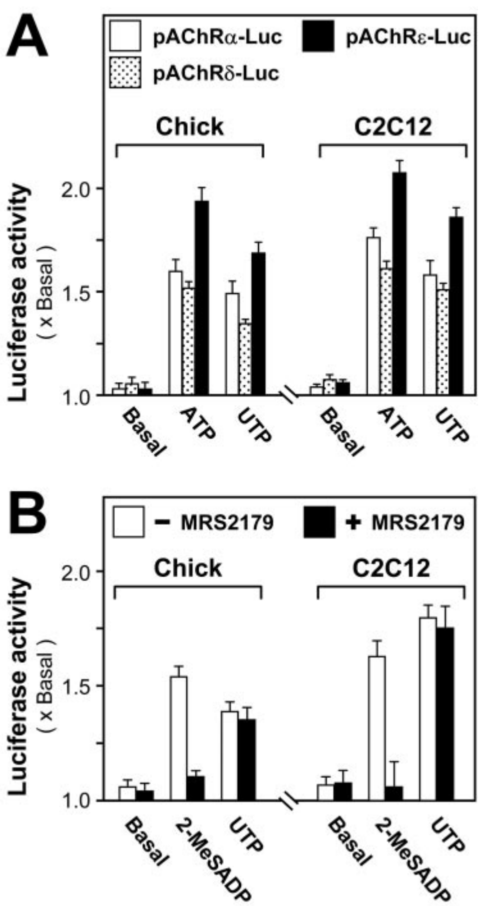

Fig. 8. The $P2Y_2$ receptor activation induces gene expression for three subunits of muscle AChR. A, pAChRα-Luc, pAChRδ-Luc, or pAChRε-Luc were expressed singly in mouse and in chick myotubes, and each set was exposed to ATP or UTP (50 μM), or medium alone, for luciferase analysis (with procedures as for Fig. 6B). B, likewise, pAChRε-Luc was expressed in rat or chick myotubes, and the cultures were exposed to 2-MeSADP or UTP (50 μM each), each with or without MRS 2179 (100 μM). 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 $P2Y_4$ receptor transcript was below the level of detection, a reproducible finding, whereas $P2Y_1$ and $P2Y_2$ transcripts were always found there (Fig. 1). The $P2Y_4$ 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-$P2Y_4$ 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 $P2Y_1$ and $P2Y_2$ receptor subtypes. Conversely, in fact, $P2Y_1$ and $P2Y_2$ 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-$P2Y_1$ and anti-$P2Y_2$ (but not anti-$P2Y_4$) antibodies (Fig. 2). In support of our findings, a similar abundance, development and nmj location of the $P2Y_1$ receptors was found previously in chicken muscles using an independent antibody, which we raised to a different epitope in the chicken $P2Y_1$ receptor (Choi et al., 2001).

The $P2Y_{11}$ receptor can also respond to both ATP and UTP, but UTP cannot activate it to produce IP_3 (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 $P2X_7$ 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 $P2Y_1$ and the $P2Y_2$ receptors.

ATP and UTP at the nmj. ATP released from the motor terminal would be a native agonist at both $P2Y_1$ and $P2Y_2$ receptors, having EC_{50} 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 $P2Y_1$ and $P2Y_2$ receptors located at the nmjs could both, therefore, become activated by the released ATP, the concentration of which in the synaptic cleft reaches $\sim$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 $P2Y_1$ and $P2Y_2$ 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 $\sim$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 $\sim$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, significant 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 $P2Y_2$ 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 $P2Y_1$ and the $P2Y_2$ 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).

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