Communication

Protein-tyrosine Phosphatases Specifically Regulate Muscle Adult-type Nicotinic Acetylcholine Receptor Gene Expression*

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Innervation of skeletal muscles results in expression of adult-type nicotinic acetylcholine receptors (αβδc) beneath the neuromuscular junction. This local expression is a result of selective induction of adult-type nicotinic acetylcholine receptor (nAChR) genes in endplate-associated myonuclei. The molecular mechanism by which the nerve induces gene expression in these nuclei is not known. We have shown previously that ionophore-induced calcium influx across the plasma membrane preferentially decreases expression from the adult-type specific nAChR ε-subunit gene (Walke, W., Staple, J., Adams, L., Gnegy, M., Chahine, K., and Goldman, D. (1994) J. Biol. Chem. 269, 19447–19456). Here we provide evidence that the genes encoding adult-type nAChRs are specifically regulated by protein-tyrosine phosphatase activity. Orthovanadate, a specific protein-tyrosine phosphatase inhibitor, caused increased expression of the ε-subunit gene in rat primary myotubes and was able to completely block the suppressive effects of increased calcium influx on ε-subunit RNA expression. Overexpression of protein-tyrosine phosphatases selectively decreased expression from the adult-type nAChR genes with no effect on the embryonic-type specific γ-subunit gene. These results demonstrate that protein-tyrosine phosphatases regulate mammalian adult-type nAChR gene expression and suggest a mechanism by which muscle innervation selectively regulates gene expression in endplate-associated myonuclei.

Synaptic transmission between nerve and muscle is mediated by the muscle nicotinic acetylcholine receptor (nAChR) (1). Prior to muscle innervation or after denervation of adult muscle, embryonic-type nAChRs (αβδβ) are expressed throughout the muscle fiber. Following innervation, these receptors disappear. This is because nerve-induced muscle activity suppresses the expression of genes encoding them (2–5). Innervation also induces the local expression of adult-type nAChRs (αβδc) at the neuromuscular junction (NMJ). This is largely a result of selective expression of these genes in endplate-associated myonuclei (6). The replacement of a γ by an ε-subunit to constitute an adult-type nAChR results in different gating and ion conductance properties of the receptor (7).

Muscle activity-dependent suppression of extrasynaptic nAChR gene expression has been linked to a calcium-dependent protein kinase C pathway in chick (8–10) and a CaM-dependent pathway in rat (11). However, very little is known about the signaling mechanisms mediating the restricted expression of adult-type nAChRs at the NMJ. There are suggestions that motor neuron-derived factors may mediate this local expression. One such candidate factor is ARIA, a protein purified from chick brain (12). When added to myotube cultures, ARIA stimulates nAChR synthesis, in part by increasing nAChR subunit mRNA levels (13). While ARIA is known to promote rapid tyrosine phosphorylation of a 185-kDa muscle transmembrane protein (15, 16), there is no evidence indicating that this phosphorylation mediates ARIA-induced increases in nAChR subunit mRNAs. Since the ε-subunit is unique to adult-type nAChRs, it serves as a marker for molecules that may regulate synapse-specific gene expression. ARIA preferentially induces (13), while calcium influx preferentially decreases, ε-subunit RNA (14). Here we report that the genes encoding the adult-type nAChRs are selectively regulated by protein-tyrosine phosphatase activity and have linked this activity to mediating the effects of increased calcium influx on ε-gene expression. Based on these results, we propose that muscle innervation induces synapse-specific gene expression by modulating protein-tyrosine phosphatase activity.

EXPERIMENTAL PROCEDURES

Myotube Cultures—Primary rat muscle cell cultures were prepared as described previously (11). On day 4 after plating, cultures used for RNase protection assays were treated with cytosine arabinoside (2.8 μg/ml) for 48 h to prevent fibroblast proliferation. On day 6, the primary medium was replaced by low serum medium (Dulbecco's modified Eagle's medium containing 5% horse serum and 2 μg/ml tetrodotoxin) and treated with calcium ionophore A23187 and/or sodium orthovanadate, a protein-tyrosine phosphatase inhibitor. For transfections, differentiated myotube cultures on day 4 after plating were used.

RNA Isolation—RNA from primary muscle cultures was isolated as described previously (15). After the final precipitation, the RNA pellet was digested with proteinase K, followed by phenol-chloroform extraction and ethanol precipitation.

RNase Protection Assays—RNase protection and densitometric quantitation was essentially carried out as described previously (11, 19). Plasmids used to prepare probes for detecting nAChR α-, β-, γ-, and ε-RNAs have been described previously (19). Rat myosin light chain (MLC) antisense probe was used in all the assays, as a control for normalizing data. When normalized to total RNA loaded on the gel, MLC RNA showed no regulation under the conditions used in this report. A SacI/PstI fragment of rat MLC cDNA (18) subcloned into pSP73 vector was used to generate the MLC probe. This fragment spans part of exon 4, the entire exon 5, and a portion of exon 6 of the CDNA. Following hybridization of RNA with appropriate probes, RNase digestion was carried out using RNase T2 as described previously (19).

Plasmids—The chick ε-subunit promoter construct (pBSεCAT; Ref. 4) contains 850 bp of sequence 5’ to position +30 fused to the CAT gene.
This 850-bp fragment was subcloned into the luciferase expression vector, pXPl (20). The δ-promoter/luciferase expression vector has already been described (21). The γ-subunit promoter construct (pXP1γ 722) harbors a 722-bp fragment of γ-subunit gene spanning nucleotides -754 to -33 (relative to the translation initiation site), which is subcloned into the pXPl vector (22). A second γ-subunit promoter construct (pXP1γ 388) contains a 388-bp fragment of γ-subunit gene spanning nucleotides -2 to 388 (relative to the translation initiation site) subcloned into pXP1 vector. The ε-subunit promoter/luciferase expression construct, as described previously (14), contains about 5 kilobase pairs 5'-flanking DNA of ε-subunit gene subcloned into the pXP2 vector (20).

The protein-tyrosine phosphatase (PTP) expression constructs contain cDNAs of human PTP 1D (23), PTP 1B (24), or PTP CL100 (25), each subcloned into pCMV5 vector (26). PTP 1D, the catalytically inactive mutant of PTP 1D, is identical to PTP 1D except for mutation of cysteine 450 to serine in the catalytic domain. The minimal enkephalin promoter/chloramphenicol acetyltransferase expression vector (ENK72CAT) used to control for variability in transfection efficiency has been described previously (27).

Transfections—Myotube cultures were transfected by calcium phosphate precipitation (28, 29) with 10 μg/60-mm plate of ε-subunit promoter/luciferase expression construct and 20 μg/60-mm plate of ENK72CAT. Transfections for PTP overexpression studies were carried out using 5 μg/60-mm plate of test plasmid (α-, γ-, δ- or ε-subunit promoter/luciferase expression vector) along with 30 μg/60-mm plate of PTP 1D, PTP 1D, PTP CL100 and 20 μg/60-mm plate of ENK72CAT. Following 3-4 h of incubation with calcium phosphate precipitate, the cells were glycerol-shocked for 2 min and placed in primary culture medium containing tetrodotoxin (2 pg/ml) and cytosine arabinoside (2.5 μg/ml). To perform luciferase and CAT assays (30, 31), the cells were harvested either 24 h after transfection or following 48 h treatment with 20 μM sodium orthovanadate beginning on day 3 after transfection.

RESULTS

Orthovanadate (10–100 μM) has been widely used to specifically inhibit protein-tyrosine phosphatase activity (32, 33). Primary myotube cultures were exposed to varying concentrations of sodium orthovanadate for 24 h. As shown in Fig. 1, vanadate caused a concentration-dependent increase in ε-subunit RNA. The specificity of this effect is illustrated by the finding that the γ-subunit and MLC RNAs were unaffected by vanadate treatment. Furthermore, a 24-h incubation of cultured myotubes with okadaic acid (1 μM), a serine-threonine phosphatase inhibitor (34), had no effect on these same RNAs (data not shown). Higher concentrations (2–10 μM) of okadaic acid could not be tested because primary myotube cultures did not survive 24 h of exposure to the drug.

To determine whether the vanadate-induced increase in ε-subunit RNA is at least in part mediated through transcriptional mechanisms, we examined the effect vanadate had on ε-subunit gene promoter activity. Myotubes were co-transfected with ε-promoter/luciferase and ENK72CAT expression vectors. Three days after transfection, cells were treated with sodium orthovanadate (20 μM) for 48 h, followed by harvesting the cells for luciferase and CAT assays. Vanadate treatment increased ε-subunit gene promoter activity over 2-fold (Fig. 2).

To directly study the PTP-mediated regulation of nAChR promoter activity, different PTP expression vectors were co-transfected with the reporter plasmid (α-, γ-, δ-, or ε-subunit promoter/luciferase expression vector) and ENK72CAT. For control transfections, pCMV5 vector without the PTP insert was co-transfected with the reporter plasmid and ENK72CAT. As shown in Fig. 3, PTP overexpression decreased the promoter activity of α-, δ-, and ε-subunit genes without affecting that of the embryonic-type specific γ-subunit gene. In addition to using pXP1γ 388 vector, we repeated the experiments with pXP1γ 722 construct and obtained similar results.

Previously, we observed that calcium ionophores caused a preferential decrease in adult-type specific ε-subunit RNA levels as a result of increased calcium influx across the plasma membrane (14). Here we tested whether this calcium-dependent regulation could be mediated by protein-tyrosine phosphatases. We thus treated myotubes with A23187 (1 μM) for 48 h in the presence and absence of sodium orthovanadate (20 μM). As revealed by RNase protection assays, vanadate completely blocked the A23187-induced decrease in ε-subunit RNA (Fig. 4).
particular protein-tyrosine phosphatases (PTP lD, PTP lDM, PTP subunit-encoding genes. Myotube cultures were co-transfected with a reporter plasmid (α-, γ-, δ-, or ε-subunit promoter/luciferase expression vector), ENK72CAT, and a pCMV expression vector that harbors a particular protein-tyrosine phosphatase (PTP lD, PTP lDM, PTP lB, or PTP CL100). In addition to the catalytically inactive mutant, PTP lDM, we also used the pCMV vector lacking a PTP insert, as a control (− in figure). The cells were harvested for luciferase and CAT assays 24 h after transfection. Experiments were repeated a minimum of three times. Bar graphs represent the average of triplicate transfections normalized to CAT activity; error bars are ± standard deviation.

**FIG. 3.** Protein-tyrosine phosphatase overexpression specifically down-regulates promoter activity of adult-type nAChR subunit-encoding genes. Myotube cultures were co-transfected with a reporter plasmid (α-, γ-, δ-, or ε-subunit promoter/luciferase expression vector), ENK72CAT, and a pCMV expression vector that harbors a particular protein-tyrosine phosphatase (PTP lD, PTP lDM, PTP lB, or PTP CL100). In addition to the catalytically inactive mutant, PTP lDM, we also used the pCMV vector lacking a PTP insert, as a control (− in figure). The cells were harvested for luciferase and CAT assays 24 h after transfection. Experiments were repeated a minimum of three times. Bar graphs represent the average of triplicate transfections normalized to CAT activity; error bars are ± standard deviation.

**FIG. 4.** Orthovanadate blocks calcium ionophore A23187-induced down-regulation of adult-type specific ε-subunit RNA expression. Cultured myotubes were treated with ionophore A23187 (1 μM) for 48 h in the presence and absence of orthovanadate (20 μM) prior to harvesting the cells for RNA isolation. The levels of ε-subunit and MLC RNAs were determined by RNase protection assays. Top portion of figure is a representative autoradiogram from an RNase protection assay. Bottom portion of figure represents quantitation of RNase protection assays by scanning densitometry. Data were normalized to MLC RNA levels. Experiments were repeated three times. Error bars represent ± the standard error of the mean.

**DISCUSSION**

This report presents the first evidence that protein-tyrosine phosphatases specifically regulate muscle adult-type nAChR gene expression. Based on these results, we propose a role for protein-tyrosine phosphatases in mediating synapse-specific expression of nAChR genes beneath the NMJ.

The development of the NMJ is marked by the postnatal switch in the expression of nAChRs from an embryonic-type (α2β7δ) to an adult-type (αβδ). This switch in expression is dependent upon muscle innervation. Nerve-induced muscle activity suppresses expression of embryonic-type nAChR genes throughout the muscle fiber (2-5), while nerve-derived factors appear to locally induce adult-type nAChR gene expression selectively in endplate-associated nuclei (35, 36). Therefore, the ε-subunit gene serves as a marker for characterizing synapse-specific expression, while the γ-subunit gene is indicative of extrasynaptic and activity-dependent regulation.

Viewed in this context, a striking feature of the present data is that unlike the other nAChR subunit genes, γ-subunit gene expression appears unresponsive to regulation by protein-tyrosine phosphatases (Figs. 1 and 3). However, vanadate causes over a 2-fold increase in ε-subunit RNA levels (Fig. 1). The finding that vanadate also induces greater than a 2-fold increase in the ε-subunit gene promoter activity (Fig. 2) illustrates that the effects of vanadate are mediated through transcriptional mechanisms. Since we observed no changes in ε- or γ-subunit RNA levels following treatment with okadaic acid (data not shown), it would be reasonable to conclude that the observed effects of vanadate are specifically mediated via inhibition of PTPs. However, it may be argued that in tetrodotoxin-treated myotube cultures, the vanadate-induced increase in γ-subunit RNA was not apparent due to its high level of expression in inactive myotubes. In contrast, the ε-subunit gene exhibits a relatively low basal expression in these cultures, and thus may be further induced upon vanadate treatment. Therefore, we performed transient co-transfection experiments to study promoter regulation of nAChR subunit-encoding genes in response to PTP overexpression. PTP overexpression causes a marked reduction of α-, δ-, and ε-subunit gene promoter activity without affecting expression from the γ-subunit promoter (Fig. 3). These results are consistent with our finding that inhibition of PTP by vanadate differentially regulates γ- and ε-subunit-encoding gene expression (Figs. 1 and 2). The specificity of this response is further illustrated by the fact that overexpressing PTP lDM, a catalytically inactive mutant of PTP lD, does not regulate promoter activity of any of the nAChR subunit-encoding genes (Fig. 3).

Significant advances have recently been made in our understanding of PTPs as an extensive family of transmembrane and intracellular proteins that are involved in a number of pathways of cellular signal transduction (37). For the present PTPs overexpression studies, we used several non-transmembrane PTPs (PTP lD, PTP lB, and PTP CL100) with different structural...
ARIA-induced increase in nAChR subunit mRNA, which occurs that, like other neurotrophins, ARIA may actually activate sev-
oncogene (16). Nerve-derived factors such as ARIA have been
tase NMJ. There is precedence for this type of regulation by EGF
sine kinase inhibitors fail to block ARIA-induced phosphoryla-
tion (13). Furthermore, ARIA-induced phosphorylation of 185-
gene expression, the relative contributions of kinase
vanadate can completely block this effect of calcium (Fig. 4).
endplate-associated myonuclei. Precedence for this type of
h  despite the continued presence of ARIA (15). There is no
expression vector, Dr. J. Dixon for providing ITP expression vectors,
em biting as nerve-derived factors or calcium influx across the
plasma membrane may regulate the local expression of these
genes beneath the NMJ via modulations of protein-tyrosine
phosphatase activity. Finally, it should be noted that identi-
fication of a specific PTP that may be involved in this regulation
has not been possible using PTP overexpression experiments.
We are currently using alternative strategies to isolate a physi-
ologically relevant PTP that may regulate synapse-specific ex-
pression of adult-type nAChRs beneath the mammalian NMJ.
Possible candidates may include calcium-dependent nuclear

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