Electrical activity regulates AChR gene expression via JNK, PKCζ and Sp1 in skeletal chick muscle

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Abstract Electrical activity of myotubes represses nicotinic acetylcholine receptor (AChR) gene expression. This effect is mimicked by okadaic acid and blocked by tetrodotoxin (TTX) or staurosporine in cultured myocytes [Altiok et al., EMBO J. 16 (1997) 717–725]. In this study, we investigated the mechanism of this repression. We show that addition of exogenous phospholipase D (PLD) and C inhibits AChR expression in a manner which parallels that of okadaic acid. Furthermore, okadaic acid caused an increase of the threonine phosphorylation of protein kinase Cζ (PKCζ) and activator of transcription factor (ATF2) and a decrease of the phosphorylation of Sp1. All these effects were reversed by staurosporine, and TTX also abolished ATF2 phosphorylation. These data reveal a possible involvement of PLD, c-jun N-terminal kinase, PKCζ and Sp1 in the repression of AChR genes by electrical activity. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: c-jun N-terminal kinase; Phospholipase D; Protein kinase C; Skeletal muscle; Sp1

1. Introduction

Neurally elicited electrical activity represses nicotinic acetylcholine receptor (AChR) gene expression in the extrajunctional areas of skeletal muscle fibers during development, and in the adult [1]. In vitro, the spontaneous, or neurally elicited, electrical activity of cultured myotubes represses AChR synthesis, whereas treatment of myotubes with the Na^+ channel blocker tetrodotoxin (TTX), or with Ca^{2+} channel blockers, increases AChR α-subunit mRNA levels by preventing Ca^{2+} entry via L-type channels [2–4]. Earlier studies using phorbol esters have suggested the implication of a Ca^{2+} activated conventional protein kinase C (PKC), but a direct demonstration of its involvement has not been provided [5,6]. The studies mentioned so far have focused on conventional PKCs, whereas other Ca^{2+} activated pathways have not been considered. To address this question we have investigated the possible contribution of a serine/threonine kinase (Ser/Thr kinase) and of its activation pathway by Ca^{2+} influx in electrically active myotubes. Here, we provide evidence in favor of the activation of c-jun N-terminal kinase (JNK) and PKCζ together with the dephosphorylation of Sp1 – at least partially via the stimulation of phospholipase D (PLD) – in the transduction pathway mediating repression of AChR genes by electrical activity.

2. Materials and methods

2.1. Materials

[γ-^{33}P]ATP, [^{32}P]UTP and [methyl-^{3}H]choline chloride were from Amersham Corp. GF109203X was kindly provided by Dr. Jorge Kirilovsky (Lab. Glaxo, France). HA1004 was from Biomol Research Lab, USA. Staurosporine was from Research Biochemicals, USA. Okadaic acid was from Upstate Biotechnology Inc. Anti-PKCζ antibody was from Santa Cruz Biotechnology Inc., anti-phosphothreonine, anti-phospho-activator or transcription factor (ATF2) and anti-ATF2 were from New England Biolabs. All other reagents including phospholipase D (*Streptomyces chromofuscus*, type VII) and phospholipase C (PLC, *Bacillus cereus*, type V), were from Sigma Chemical Co.

2.2. Primary culture of chick myotubes

Myoblasts were obtained from the hindlimbs of 11 day old chick embryos by mechanical dissociation and seeded at a density of 2×10^4 cells/mm^2 in gelatin coated, plastic dishes and grown in medium containing 4% horse serum and 2% embryonic extract, as described previously [2].

2.3. PKC activity assay

PKC activity was determined using a PKC assay kit (Upstate Biotechnology). Synthetic peptides corresponding to amino acids 4–14 of myelin basic protein (MBP, provided with the kit) or the ε-peptide (LC Laboratories) were used as PKC substrates. After treatment with the drugs, as described in the figure legends, the cells were washed once with ice cold phosphate buffered saline (PBS), and lysed in 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM β-glycerophosphate, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin. Insoluble material was removed by centrifugation at $13\,000\times g$ for 30 min at 4°C. Aliquots of crude extracts were incubated at 30°C in the presence of the substrates, 20 mM MOPS, 1 mM $NaVO_4$, 1 mM dithiothreitol, 1 mM $CaCl_2$, 75 mM $MgCl_2$, 500 μM ATP, 200 μCi/ml of [γ-^{33}P]ATP (>1000 Ci/mmol, Amersham), 2 μM protein kinase A inhibitor peptide, 20 μM compound R24571, lipid activators. The reactions were stopped by rapid centrifugation, and the aliquots were spotted on to P-81 phosphocellulose paper, washed with 75 mM orthophosphoric acid. Incorporation of ^{33}P was determined by liquid scintillation.

2.4. Northern blot analysis

After treatment of the cells, RNA was purified. Northern blots were made with 15 μg of total RNA in each lane. Blots were hybridized with a riboprobe synthesized from a pGEM3 vector (Promega, Madison, WI, USA) containing a 2.3 kb fragment of the AChR α_1-subunit cDNA which overlaps exons 2–6. [^{32}P]UTP (800 Ci/mmol, Amer-

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Abbreviations: AChR, nicotinic acetylcholine receptor; ATF2, activator of transcription factor; HR, Heregulin; JNK, c-jun N-terminal kinase; PKCζ, protein kinase C ζ; PLC, phospholipase C; PLD, phospholipase D; Ser/Thr, serine/threonine; SRF, serum response factor; TTX, tetrodotoxin

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sham) was used as the label. The same blots were rehybridized with a muscle creatine kinase (MCK) specific RNA probe, as described previously [3].

2.5. Quantification of surface AChRs

Surface AChR levels were measured by ^{125}I labeled α-bungarotoxin (α-BTG; ~200 Ci/mmol, Amersham) binding to myotube cultures as previously described [2].

2.6. Separation of extracellular [^{3}H]choline and [^{3}H]phosphorylcholine

To label phosphatidylcholine pools, cells were exposed to 10 μCi of [methyl-^{3}H]choline chloride for 24 h in culture medium. After labeling, cells were washed four times, new minimal essential medium was added, and cells were incubated for a further 24 h with indicated drugs. At various times, the incubation medium was removed to tubes on ice and centrifuged at 200×*g* at 4°C for 10 min. In order to separate the choline and phosphocholine cell medium was extracted with tetraphenylboron in heptanone [7]. With this technique, [^{3}H]choline is extracted into the organic phase while [^{3}H]phosphorylcholine (Pcholine) remains in the aqueous phase. Aliquots of each layer were counted for tritium content.

2.7. Diacylglycerol (DAG) mass determination

Cells were extracted by a modification of the method of Bligh and Dyer [8]. Cellular DAG mass was quantitated employing the method of Preiss et al. [9] using the DAG assay reagent system (Amersham).

2.8. Western blot analysis

After treatment with the drugs, as described in the figure legends, the cells were washed once with ice cold PBS, and lysed in a buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM sodium orthovanadate, 10 mM β-glycerophosphate, 10 mM NaF, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin. Insoluble material was removed by centrifugation at 13 000×*g* for 20 min at 4°C. The supernatants were subjected to electrophoresis on SDS–PAGE gels and transferred to nitrocellulose using a Bio-Rad apparatus. Protein blots were probed overnight at 4°C with primary antibodies as indicated. Blots were analyzed by using anti-rabbit IgG conjugated to horseradish peroxidase and visualized by ECL.

3. Results and discussion

In this study we investigated the signal transduction pathway involved in the repression by electrical activity of AChR gene expression in chick myotubes. We have previously shown that okadaic acid blocks the increase of AChR protein and α-subunit mRNA levels caused by TTX treatment and that this effect is reversed by staurosporine [10]. Okadaic acid, a specific inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A), has been shown to block the differentiation of muscle cells by abolishing the expression of the genes encoding the myogenic factors myoD1 and myogenin [11]. Okadaic acid, at 2.5 nM concentration which inhibits AChR protein and α-subunit mRNA levels in myotubes, selectively inhibits PP2A [12]. The regulation of Ser/Thr phosphorylation via PP2A has been implicated in the transcriptional control of cell differentiation and proliferation in several cell types [13,14].

Specific inhibitors of conventional PKC, GF109203X, Go6976 [15], and protein kinase A (PKA), HA1004 [16], failed to antagonize the repression of AChR α-subunit mRNA and protein biosynthesis caused by okadaic acid (not shown) or by spontaneous electrical activity, while the broad spectrum kinase inhibitor staurosporine and TTX completely reversed these inhibitions (Fig. 1A,B). These data suggest that conventional PKC isoforms or PKA are not involved in the activity elicited repression of AChR gene expression in myotubes. This conclusion was further supported by studies with the general Ser/Thr inhibitor H-7, which inhibits PKC and PKA with a half maximal concentration of ~10 μM [15]. In this range of concentrations, H-7 did not affect the basal α-subunit mRNA levels in spontaneously contracting myotubes (not shown).

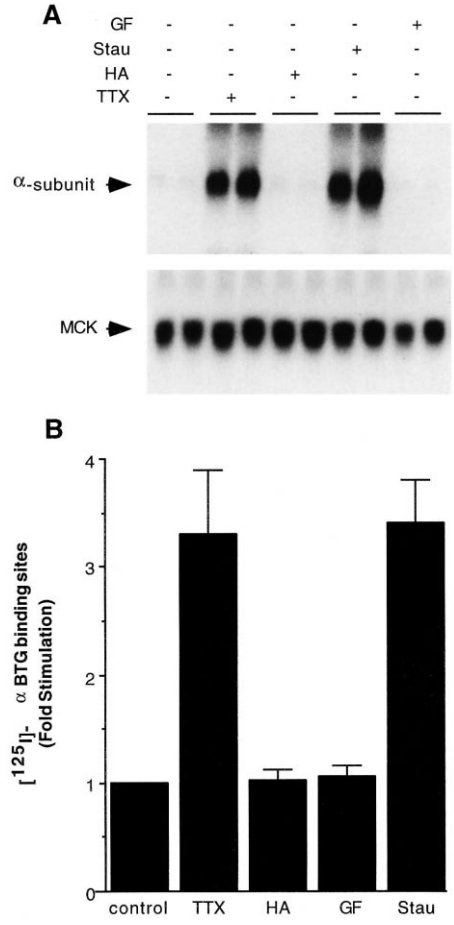

Fig. 1. Effect of 1 μM TTX, 2.5 nM staurosporine (stau), 5 μM HA1004 (HA), and 1 μM GF109203X (GF) on AChR (A) α-subunit mRNA and (B) surface ^{125}I labeled α-BTG binding sites in myotubes. A: Autoradiograms of Northern blot hybridization analysis of mRNA in chick myotubes treated with indicated drugs. Cells were treated between days 4 and 6 after plating and total RNA was extracted. AChR α-subunit and MCK mRNAs were analyzed using their cRNA probes. The same blot was used for hybridization with the two probes. The arrows show the position of AChR α-subunit and MCK mature mRNAs. −, absence; +, presence of drug. B: After the indicated treatments between days 4 and 7 surface ^{125}I labeled α-BTG binding sites were quantitated as described in Section 2. The results are expressed as fold increase over untreated value of cells, and are the means of four separate experiments (means ± S.D.).

Ca^{2+} influx has been shown to mediate repression by electrical activity [2–4]. We thus tested FK506, a Ca^{2+}/calmodulin dependent Ser/Thr phosphatase (PP2B = calcineurin) inhibitor [17]. FK506 had no effect on AChR protein and α-subunit mRNA levels either in the absence or in the presence of TTX or okadaic acid, excluding a role of calcineurin in the activity induced repression of AChR genes (not shown).

DAG or another lipid mediator derived from phosphatidylcholine (PC) has been implicated in the sustained activation of Ca^{2+} independent atypical isoforms of PKCs [15,18]. We have examined whether the PC degradative pathway, or any of the

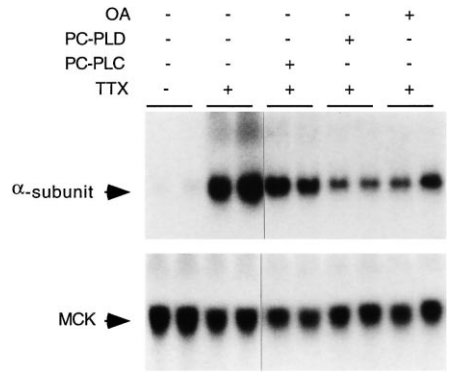

Fig. 2. Inhibition of TTX increased AChR α-subunit mRNA levels by okadaic acid (OA), PC-PLD and PC-PLC. On day 5 after plating, myotubes were treated with 1 μM TTX, 2.5 nM okadaic acid, 1 U/ml PC-PLD and 1 U/ml PC-PLC as indicated, then further incubated for 2 days. AChR α-subunit and MCK mRNAs were analyzed as described in Fig. 1A. The arrows show the position of AChR α-subunit and MCK mature mRNAs. −, absence; +, presence of drug.

PKC isoforms, were involved in the repression of AChR genes by electrical activity. Exogenous application of PC specific phospholipase C (PC-PLC) and phospholipase D (PC-PLD) to TTX treated myotubes resulted in a decrease of α-subunit mRNA levels similar to that obtained by okadaic acid (Fig. 2). PC makes up the largest fraction of total plasma membrane phospholipids and growth factors, cytokines, phorbol esters and calcium ionophores can cause hydrolysis of PC, resulting in the sustained elevation of DAG concentration [19]. The hydrolysis of PC by PLC produces phosphorylcholine (Pcholine) and DAG, whereas PLD produces choline and phosphatidic acid (PA); however, the products of each reaction are interconvertible [20].

We labeled the choline phospholipids of cultured myotubes by overnight incubation with [^{3}H]choline and assessed the effect of spontaneous myotube activity on PC metabolism by treating [^{3}H]choline labeled cells with TTX overnight. Under control conditions there was a gradual accumulation of [^{3}H]choline in the cell medium. Inhibition of the electrical activity of the membrane by TTX reduced this accumulation by 50%, while [^{3}H]Pcholine accumulation was less affected (Fig. 3A) suggesting the involvement of PLD. The phorbol ester PMA also increased the accumulation of [^{3}H]choline in the cell medium of TTX treated cells, while [^{3}H]Pcholine generation was not affected (not shown), thus indicating the activation of PLD by PKC. Phorbol esters and calcium ionophores are known to activate PLD in many cell types by activating conventional PKCs and increasing cytosolic Ca^{2+} [21]. In myotubes, the PKC inhibitor GF109203X was not able to decrease the accumulation of [^{3}H]choline in the supernatant of electrically active cells, thus ruling out the direct involvement of a conventional PKC in PLD stimulation by electrical activity (not shown).

If electrical activity stimulates the hydrolysis of PC by PLD, then an increase in PA formation should lead to an increase in DAG concentration. Six days after plating, DAG levels were approximately 30% higher in control than in TTX treated myotubes (Fig. 3B). This suggests that chronic blocking of the electrical activity of myotubes by TTX inhibits DAG generation.

We next examined whether PKC activity is regulated by DAG or by PA produced by PLD activation resulting from electrical activity. In the in vitro kinase assay, using MBP_{4-14} as a substrate, the blocking of electrical activity by TTX did not inhibit the high basal PKC activity. On the other hand, the PKC inhibitor GF109203X totally inhibited PKC activity, and the general kinase inhibitor staurosporine partially inhibited it (Fig. 4A). Okadaic acid had no significant effect on basal PKC activity. These data suggest that, although a high level of conventional PKC activity is present in these myotubes, this activity is not regulated by TTX treatment under conditions which enhance AChR gene expression. In contrast, GF109203X, which did not affect AChR gene ex-

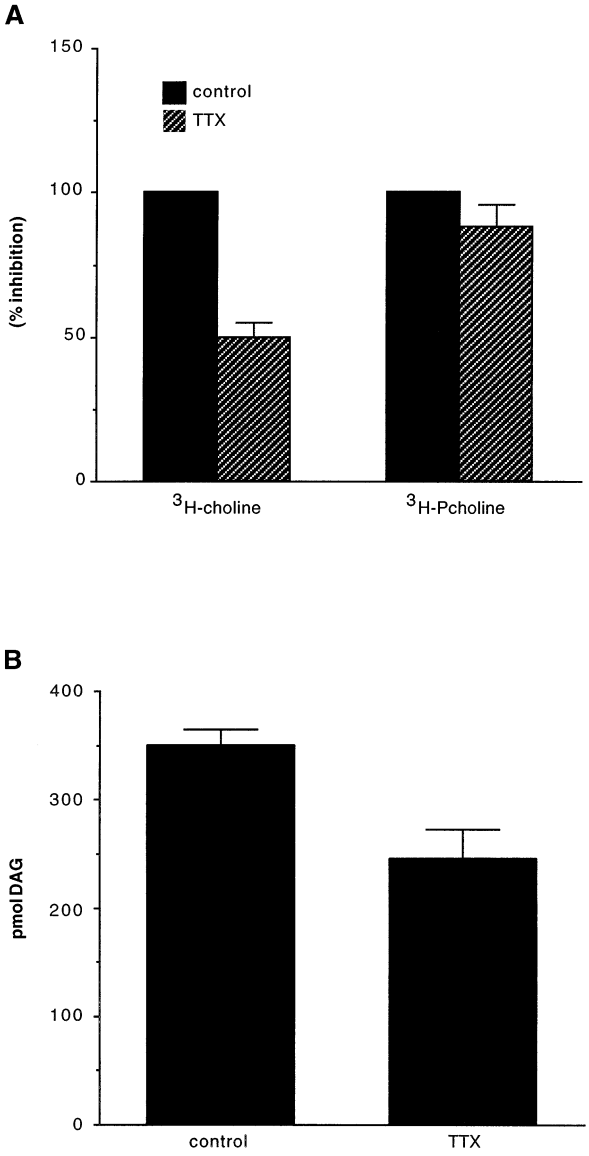

Fig. 3. Inhibition of (A) release of [^{3}H]choline and (B) formation of DAG by TTX. A: Between days 5 and 6 after plating myotubes were labeled with [methyl-^{3}H]choline chloride in the absence (control) and presence of 1 μM TTX. Cells were washed and and new medium was added with and without TTX as indicated. After 1 h medium was collected and measured for release of [^{3}H]choline and [^{3}H]Pcholine as described in Section 2. B: On day 6 after plating cells were extracted and cellular DAG mass was quantitated as described in Section 2. The results are expressed as percent inhibition of the value of control cells, and are the means of three separate experiments (means ± S.D.).

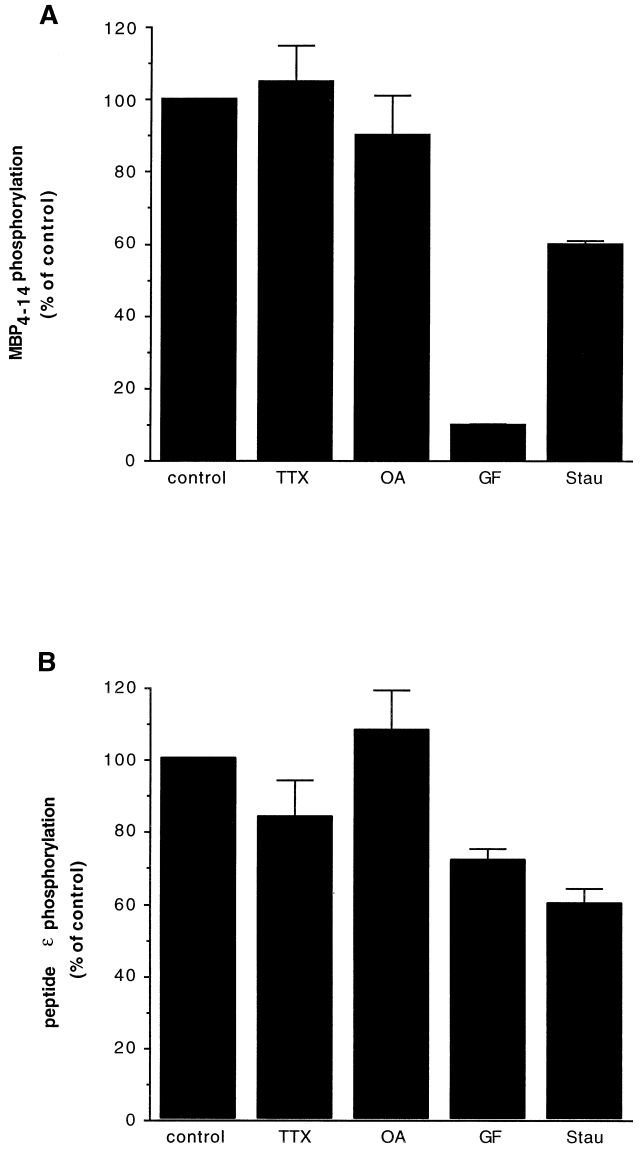

Fig. 4. Effect of 1 μM TTX, 2.5 nM okadaic acid (OA), 1 μM GF109203X (GF) and 2.5 nM staurosporine (stau) on PKC activities in myotubes. Between days 4 and 5 after plating the myotubes were treated with indicated drugs. Then they were lysed and PKC activity in the crude extracts was determined using (A) MBP_{4-14} and (B) peptide ε. Values obtained are expressed as a percentage of the activity present in untreated control cells, and are the means of three separate experiments (means ± S.D.).

pression, totally abolished conventional PKC activity. We may note that we have previously shown that conventional PKCs interfere with the initial step of the stimulatory pathway triggered by HRG in these myotubes [22].

In examining whether novel or atypical PKC isoforms were stimulated by electrical activity, we used a synthetic peptide based on the sequence of the pseudo substrate region of PKCε, which is a substrate of some of the novel and atypical PKC isoforms [23]. The basal phosphorylation of ε-peptide was slightly reduced by TTX. GF109203X and staurosporine also partially though significantly reduced this activity, while okadaic acid slightly increased it (Fig. 4B). This is consistent with the notion that Thr phosphorylation is required for the activation of all PKCs and that PP2A directly regulates the activity of atypical PKCs [24]. PKCα also phosphorylates the ε-peptide, and this is possibly a reason why the inhibition by TTX was so slight. But staurosporine inhibited ε-peptide phosphorylation more strongly than GF109203X, in contrast to MBP_{4-14} phosphorylation. These results suggest that the stimulation of PLD by electrical activity does not lead to the activation of a conventional PKC isoform, but rather to the activation of a novel, or atypical, PKC isoform. Our finding that PKCζ is phosphorylated (Fig. 5) and activated (Fig. 4B) by electrical activity in cultured myotubes is consistent with previous reports indicating that PLD derived PA activates an atypical PKC or a novel Ser/Thr kinase [25,26]. However, despite the observation that okadaic acid and staurosporine regulate the phosphorylation and the activity of PKCζ in a manner similar to the regulation of AChR gene expression, TTX treatment did not modify the phosphorylation of PKCζ (Fig. 5).

We have further shown that ATF2 was phosphorylated by electrical activity, and that this phosphorylation was blocked by TTX treatment (Fig. 5). ATF2 phosphorylation was also enhanced by okadaic acid, and this effect was prevented by staurosporine (Fig. 5). JNK/p38 kinase dependent N-terminal phosphorylation of ATF2 at Thr-69 and Thr-71 in response to cellular stress has been shown to be regulated by protein phosphatases sensitive to okadaic acid [27]. The involvement of p38 kinase in the repression of AChR genes by electrical activity was excluded by the fact that the specific inhibitor of p38 kinase, SB202190, was not able to reverse the inhibitory effect of okadaic acid or of spontaneous electrical activity on AChR genes in these myotubes (not shown). Furthermore, high basal JNK activity in contracting myotubes was blocked by TTX in in vitro kinase assays using GST-c-jun as a sub-

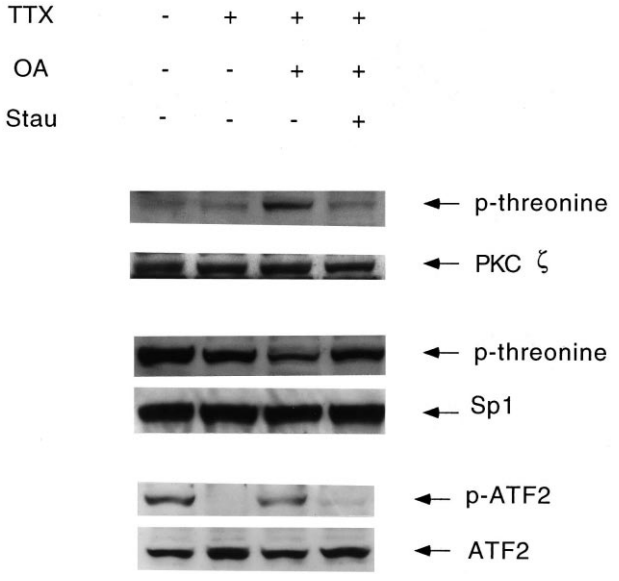

Fig. 5. Regulation of threonine phosphorylation of PKCζ, ATF2 and Sp1 by 1 μM TTX, 2.5 nM okadaic acid (OA), and 2.5 nM staurosporine (stau), in myotubes. Myotubes were treated with drugs as indicated between days 5 and 7. Phosphorylated PKCζ and Sp1 were determined by Western blot analysis using antibodies specific for phosphothreonine (p-threonine), and phosphorylated ATF2 (p-ATF2) on separate blots (upper panels). Specificities of the bands were determined by using respective antibodies against non-phosphorylated PKCζ, Sp1 and ATF2 on stripped membranes (lower panels).

strate (not shown), indicating that JNK most likely phosphorylates ATF2.

PKCζ dependent activation of JNKs has been reported in cardiac myocytes during ischemia [28]. In our studies, phorbol ester treatment did not stimulate the phosphorylation of PKCζ but caused phosphorylation of ATF2 (not shown), suggesting that JNK probably lies downstream of PKCζ. Interestingly, our findings parallel the observations made in cultured rat ventricular myocytes where the specific isoenzymes of PKC and JNK were activated either by electrically stimulated contractions or by Ca^{2+} channel agonists [29,30]. JNK is also activated by intracellular Ca^{2+} in other systems involving Ca^{2+} stimulated gene expression [31,32]. As shown in these studies, calcium activation of JNK, with subsequent phosphorylation of c-jun, activates gene transcription via interaction between c-jun, the serum response factor (SRF), and the transactivation domain of Sp1. Together with these studies, our results suggest the possibility that a similar interaction may occur at the level of transcription factors interacting with skeletal muscle AChR gene promoters. Two *cis*-regulatory elements of the AChR α-subunit gene promoter have been shown to cooperate with the E-boxes in the denervation response, and one of these regions binds the Sp1 and Sp3 zinc finger transcription factors [33]. In agreement with this observation, it has been shown that activation of the human cardiac α-actin promoter in skeletal muscle requires the integrity of DNA binding sites for the SRF, Sp1 and for the myogenic factors of the basic helix-loop-helix (bHLH) family [34].

On the basis of these observations, we would like to propose the hypothesis that: (1) the regulation of muscle specific genes transcription involves the concerted action of several transcription factors, including SRF, Sp1 and myogenic bHLH; and (2) the modification of one of these factors by electrical activity stimulated pathway represses AChR genes transcription.

Consistent with this hypothesis is the observation by Alroy et al. [35] that a Sp1 containing complex is formed on the N-box (NDF/heregulin response element) [36] on the promoter of the AChR ε-subunit, and the phosphorylation of Sp1 by NDF positively regulates the transcription of AChR ε-subunit [35]. Our finding (Fig. 5) that Sp1 phosphorylation is prevented by okadaic acid, which mimics repression by electrical activity, further supports the proposed hypothesis. Also, Alroy et al. [35] have reported that the activation of the AChR ε-subunit by NDF is blocked by okadaic acid, which prevents Sp1 phosphorylation. These authors have further suggested that okadaic acid acts on a kinase upstream of Sp1 in the NDF signalling pathway and that the increased phosphorylation of that kinase blocks Sp1 phosphorylation.

Our results might be interpreted in a similar framework. An electrically stimulated kinase may well prevent the phosphorylation of Sp1 in the same manner as okadaic acid, and thus may repress AChR genes transcription. It is tempting to speculate that JNK or PKCζ may phosphorylate a kinase (or phosphatase) upstream of Sp1 and, as a consequence, prevent Sp1 phosphorylation and thus AChR gene transcription. Accordingly, electrical activity and growth factor action may share common pathways which, at some stage, would converge on Sp1. It is interesting to note that N-box specific Ets transcription factors such as GABPα and β are already known to contribute to the regulation of AChR gene transcription, in addition to the factors mentioned [36]; moreover, the synergism between the Ets family of transcription factors and Sp1 is well known in other systems [37].

These studies, however, do not exclude other interactions in the anticipated complex network of signal transduction pathways present in the muscle cell. For example, a direct interaction between myogenic factors and JNK may take place. Indeed, on one of them, Myf5, conserved putative JNK phosphorylation sites have been identified, and the JNK pathway negatively regulates skeletal muscle myogenesis at their level [38]. Further work is needed to fully understand the network of interactions involved in the complex regulation of AChR gene transcription by electrical activity.

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