Regulation of Dihydropyridine and Ryanodine Receptor Gene Expression in Skeletal Muscle

ROLE OF NERVE, PROTEIN KINASE C, AND cAMP PATHWAYS*

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The dihydropyridine (DHP) and ryanodine (RY) receptors play a critical role in depolarization-induced calcium release in skeletal muscle, yet the factors which govern their expression remain unknown. We investigated the roles of electrical activity and trophic factors in the regulation of the genes encoding the α₁, α₂, and β subunits of the DHP receptor as well as the RY receptor in rat skeletal muscle in vivo. Muscle paralysis, induced by denervation, had no effect on the DHP receptor mRNA levels while the RY receptor mRNA was decreased. In contrast, chronic superfusion of tetrodotoxin onto the sciatic nerve resulted in a marked increase in mRNA levels and transcriptional activity of both DHP and RY receptor genes. Since nerve can induce changes in second messenger pathways which modulate muscle gene expression, we attempted to identify factors which regulate DHP and RY receptor expression using cultured myotubes. Elevated cAMP levels specifically inhibited the expression of RY receptor mRNA while 12-O-tetradecanoylphorbol-13-acetate, an activator of protein kinase C, increased the transcripts encoding the RY receptor and the α₁ subunit of the DHP receptor. Changes in the level of mRNAs were paralleled by altered receptor numbers. Neither cAMP nor protein kinase C altered transcriptional activity of the DHP and RY receptor genes. These results demonstrate that neuronal factor(s) regulate DHP and RY receptor mRNA levels in vivo via transcriptional mechanisms while protein kinase C and cAMP can modulate DHP and RY receptor transcript levels by a transcription-independent process.

Nerve-induced muscle contraction is preceded by depolarization of the sarcolemmal membrane and elevation of intracellular calcium levels in muscle cells. Several sarcolemmal proteins, namely the nicotinic acetylcholine receptor (nAChR), and the voltage-gated sodium channel, play important roles in nerve-induced depolarization of the sarcolemma. The depolarization of the sarcolemmal membrane is transmitted via the transverse tubules across the triad junction to the sarcoplasmic reticulum. The dihydropyridine (DHP) receptors of transverse tubules respond to changes in membrane polarity by acting as voltage sensors/voltage-sensitive calcium channels and trigger calcium release by interacting with the ryanodine (RY) receptors/calcium release channels of the sarcoplasmic reticulum.

A large body of information is available regarding the structure, function, and subunit composition of DHP and RY receptors in adult skeletal muscle (reviewed in Fleischer and Inui (1989), Catterall (1991), McPherson and Campbell (1993), and Meissner (1994)). The cDNAs of all five subunits of the DHP receptor (Tanabe et al., 1987; Ellis et al., 1988; Ruth et al., 1988; J ay et al., 1990) and the RY receptor (Takeshima et al., 1989; Zorzato et al., 1990) in skeletal muscle have been cloned. However, very little is known about the factor(s) that induce and regulate the expression of the genes encoding these receptors.

Expression of many muscle-specific genes, like the nAChR subunits (Buonanno and Merlie, 1986), voltage-sensitive sodium channel (Offord and Catterall, 1989), and more recently the DHP receptor subunits and RY receptor (Kyselovic et al., 1994), are switched on when mononucleated myoblasts fuse to form multinucleated myotubes. As myotubes mature and acquire nerve supply, nerve-induced electrical activity and nerve-derived trophic factors play important roles in the regulation of muscle-specific gene expression. Electrical stimulation of developing muscle and myotubes in culture represses the expression of mRNA for the nAChR and the α-subunit of the voltage-sensitive sodium channel (Witzemann et al., 1991; Chahine et al. 1993). On the other hand, expression of the ε- and γ-subunit genes of the nAChR is regulated by neurotrophic factors (Witzemann et al., 1991; J o et al., 1995). Thus, while nerve-induced electrical activity and nerve-derived factors play an important role in the regulation of genes of at least two of the main proteins involved in neuromuscular transmission, the contribution of these processes in the regulation of DHP and RY receptor genes remains unknown. Furthermore, muscle-specific gene expression can be modulated by second messengers such as cAMP and calcium (Winter et al., 1993; Chahine et al., 1993). The role of second messenger pathways in modulating the expression of the DHP and RY receptors remains elusive. In a recent study it was shown that there was a temporal difference in the induction of the DHP receptor subunit mRNAs and the RY receptor mRNA in developing muscle, suggesting

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TPA, phorbol-12-myristate-13-acetate; RY, ryanodine; TTX, tetrodotoxin; PAGE, polyacrylamide gel electrophoresis.
that these genes may be under the control of distinct endoge-

nous factors (Kyselovic et al., 1994). The objective of the pres-

ent investigation was to elucidate the mechanisms that regu-

late the expression of DHP and RY receptor genes, the two
cellular molecules involved in electrical signal transmission at the
triad junction.

MATERIALS AND METHODS

Female Sprague-Dawley rats (16–17 days gestation) were obtained from Charles River Laboratories (St-Constant, Quebec). Animals were housed in temperature-controlled rooms and maintained on a 12-h light/12-h dark cycle. Care and treatment of animals were conducted in accordance with guidelines presented by the Canadian Council on Animal Care.

Cell Culture—Skeletal myocytes were isolated from hindlimbs of 4-day-old rat pups by trypsinization (0.25%, w/v). Cells were suspended in Dulbecco's modified Eagle's medium containing 10% horse serum, 0.5% chick embryo extract, 100,000 units of streptomycin, 100,000 units of penicillin, and 50 mg of gentamicin. Approximately 3 million cells were plated in gelatin-coated Petri dishes and incubated for 4 days at 37°C in an atmosphere of 95% oxygen and 5% carbon dioxide. On the fourth day, medium was changed and cytosine (β-D-arabinofuranoside (10 μM) was added. Cells were maintained in culture for a maximum of 7 more days. Whenever necessary, drugs were added on day 4 and cells were incubated with drugs for 72 h.

Tissues—Left hindlimb muscles of adult rats (180–200 g) were denervated by cutting and removing a 4–5 mm segment of the sciatic nerve 10-mm distal to the sciatic notch as described (Jasmin et al., 1990). Skeletal muscle from these animals was then homogenized in 10 mM Tris-HCl, 0.3 M sucrose, pH 7.4, with a glass-tissue homogenizer.

Muscle RNA Isolation and Northern Blot Analysis—

RNA Extraction—Total RNA from adult muscle and cultured myotubes were isolated by the lithium chloride urea method as described earlier (Kyselovic et al., 1994). Twenty micrograms of total RNA were size fractionated in triplicate on formaldehyde-agarose gels and blots were transferred to nitrocellulose membranes (MSI MAGNA) containing 10 μM NaCl, 0.015 M sodium citrate, pH 7.0. The blots were hybridized at 44°C overnight with randomly primed labeled cDNA probes and were washed twice (1 × SSC; 0.1% SDS) at room temperature. Films were exposed for autoradiography at −70°C for 1–3 days using two intensifying screens. After hybridizing with one probe, each membrane was stripped and sequentially reprobed with a different probe. The expression of each mRNA species was quantitated from the autoradiograms and the contribution of the 4°C overnight wash to the observed expression was calculated.

RESULTS

Electrical Activity/Neurotrophic Factor(s) Modulate DHP and RY Receptor Gene Expression—Since electrical activity and trophic factors can modulate expression of muscle nAChR and the sodium channel in vivo, we investigated whether these
influences would also affect DHP and RY receptor genes. In
order to investigate the contribution of nerve-induced electrical
activity on DHP and RY receptor gene expression, we studied
the effect of surgical denervation of the soleus muscle on re-
ceptor mRNA levels in adult rat. We also investigated the role
of nerve-derived trophic factors by using chronic superfusion of
TTX onto the sciatic nerve because this approach allows axonal
transport and nerve-muscle contacts to be maintained in the
absence of any electrical activity (Lavole et al., 1976; Witze-
mann et al., 1991). The efficacy of denervation and TTX treat-
ment was verified as described previously (Michel et al., 1994;
Jasmin et al., 1995).
RNA isolated from skeletal muscle were size fractionated,
transferred to nylon membranes, and hybridized with CDNA
probes for the subunits and the RY receptor following denervation and TTX
paralysis of adult rat muscle.

A 20 µg of total RNA from muscle of control (lane C), denervated (lane D), and TTX-paralyzed (lane T) rats
were separated electrophoretically, transferred to a nylon membrane, and hybridized with 32P-labeled CDNA
probes complementary to skel-
etal muscle calcium channel a1, a2, and subunit mRNAs, ryanodine
receptor (RyR), and AChE mRNAs. Signals at 6.4 (a1), 8 (a2), 1.9 (β), 15
(RY receptor), 2.4 (AChE), and 1.8-kb gliceraldehyde-3-phosphate de-
hydrogenase (GAPD) were visualized by autoradiography. Bar
 Autoradiograms were quantitated by densitometry. Densitometry data
were normalized with respect to the gliceraldehyde-3-phosphate dehydro-
genase mRNA and expressed as percent of control message. Each bar
represents the mean ± S.E. of five independent experiments. * , repre-
sents significantly different from the control (p < 0.05).

Fig. 1. Gene expression of the DHP receptor a1, a2, and subunits and the RY receptor following denervation and TTX
paralysis of adult rat muscle. A, 20 µg of total RNA from muscle of control (lane C), denervated (lane D), and TTX-paralyzed (lane T) rats
were separated electrophoretically, transferred to a nylon membrane, and hybridized with 32P-labeled CDNA probes complementary to skeletal muscle calcium channel a1, a2, and β subunit mRNAs, ryanodine receptor (RyR), and AChE mRNAs. Signals at 6.4 (a1), 8 (a2), 1.9 (β), 15
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FIG. 1. Gene expression of the DHP receptor a1, a2, and β subunits and the RY receptor following denervation and TTX paralysis of adult rat muscle. A, 20 µg of total RNA from muscle of control (lane C), denervated (lane D), and TTX-paralyzed (lane T) rats were separated electrophoretically, transferred to a nylon membrane, and hybridized with 32P-labeled CDNA probes complementary to skeletal muscle calcium channel a1, a2, and β subunit mRNAs, ryanodine receptor (RyR), and AChE mRNAs. Signals at 6.4 (a1), 8 (a2), 1.9 (β), 15 (RY receptor), 2.4 (AChE), and 1.8-kb gliceraldehyde-3-phosphate dehydrogenase (GAPD) were visualized by autoradiography. Bar
 Autoradiograms were quantitated by densitometry. Densitometry data were normalized with respect to the gliceraldehyde-3-phosphate dehydro-
genase mRNA and expressed as percent of control message. Each bar represents the mean ± S.E. of five independent experiments. * , represents significantly different from the control (p < 0.05).

Fig. 2. Gene expression of DHP and RY receptors in development. RNA from developing myotubes at different days of development was examined in Northern blots for expression of the a1, a2, and β subunits of the DHP receptor, RY receptor, and tubulin mRNAs with specific CDNA probes (A). The mRNA levels were quantified by densitometry and normalized for RNA loading (B). The data shown is a typical representation of two different experiments.

Role of cAMP and Protein Kinase C Pathways in DHP and RY Receptor mRNA Expression—In skeletal muscle paralyzed by either in vivo denervation or addition of TTX to cultured myotubes, cAMP is believed to be the second messenger that

FIG. 2. Gene expression of DHP and RY receptors in development. RNA from developing myotubes at different days of development was examined in Northern blots for expression of the a1, a2, and β subunits of the DHP receptor, RY receptor, and tubulin mRNAs with specific CDNA probes (A). The mRNA levels were quantified by densitometry and normalized for RNA loading (B). The data shown is a typical representation of two different experiments.
Fig. 3. Gene expression of DHP receptor α1, α2, and β subunits and theRY receptor in myotubes treated with TTX and forskolin. A, myotubes on day 4 were exposed to TTX (1 μM) and forskolin (1 μM) for 72 h. On day 7, RNA was isolated from untreated control (lane C), TTX (lane T), and forskolin (lane F) pretreated myotubes. RNA was size separated electrophoretically, transferred to a nylon membrane, and hybridized with 32P-labeled cDNA probes complementary to the skeletal muscle 6.4-kb α1, 8-kb α2, and 1.9-kb β subunit mRNAs of the DHP, the 15-kb RY receptor (RYr) mRNA, and the 1.8-kb glyceroldehyde-3-phosphate dehydrogenase (GAPD) mRNA. Signals were visualized by autoradiography. B, effects of 8-bromo-cAMP and isobutyryl-methylxanthine (IBMX) on the expression of α1 subunit of the voltage-sensitive calcium channel in skeletal myotubes in culture. Myotubes on day 4 were exposed to 50 μM 8-bromo-cAMP and 50 μM isobutylmethylxanthine for 72 h. On day 7 RNA was isolated, size separated, transferred to nylon membrane, and hybridized with 32P-labeled cDNA probe complementary to the α1 subunit and glyceroldehyde-3-phosphate dehydrogenase mRNAs. Signals were visualized by autoradiography.

Induces the expression of many muscle-specific genes (Offord and Catterall, 1989; Chahine et al., 1993). We therefore examined the effect of forskolin, an activator of adenylate cyclase, on the expression of transcripts of α1, α2, and β subunits of the DHP receptor and the RY receptor. Exposing myotubes for 72 h to forskolin did not alter the mRNA expression of α1, α2, and β subunit of the DHP receptor (Fig. 3A, lane F). However, the RY receptor mRNA expression was potently inhibited by forskolin pretreatment of cultured myotubes (Fig. 3A, lane F). No change in the expression of α1 subunit message was seen when myotubes were either exposed to 50 μM isobutylmethylxanthine, a phosphodiesterase inhibitor, or 500 μM 8-bromo-cAMP, a cAMP analog (Fig. 3B).

We have reported that during development, the α1 subunit and RY receptor mRNAs and DHP and RY binding sites reached their respective peaks at a time when myotubes were spontaneously contracting (Kyselovic et al., 1994). Spontaneous contractile activity of skeletal myotubes is associated with membrane depolarization and activation of protein kinase C (Vergara et al., 1985). We investigated the effect of TPA, an activator of protein kinase C, and staurosporine, a protein kinase C antagonist, on the expression of DHP and RY receptor mRNAs. Staurosporine inhibited the basal level of expression of both the α2 subunit of the DHP and RY receptor, while TPA (200 ng) stimulated the expression of α2 subunit and the RY receptor mRNA (Fig. 4A). The stimulatory effect of TPA was antagonized by staurosporine (Fig. 4A). Staurosporine inhibited the expression of α2 subunit (27%) and RY receptor (31%), while TPA stimulated mRNA levels of both the α2 subunit and RY receptor by 175 and 145%, respectively (Fig. 4B). TPA and staurosporine did not affect the expression of α2 or β subunit mRNAs of the DHP receptor in cultured myotubes (not shown).

Neurotrophic Factor(s) Regulate Transcription of DHP and RY Receptor Genes—We investigated if alteration in the mRNA levels of the DHP and RY receptor genes in TTX-paralyzed muscle was due to altered transcriptional activity of the respective genes. We subjected the nuclei isolated from adult rat skeletal muscle to nuclear run-on assay. As shown in Fig. 5A, nuclei obtained from control skeletal muscle transcribed DHP receptor subunit genes and RY receptor gene along with those of tubulin and glyceroldehyde-3-phosphate dehydrogenase (CON pend). In TTX-paralyzed muscle the rate of transcription of the DHP receptor α1, α2, and β subunit and RY receptor genes was enhanced (Fig. 5A, TTX panel). When normalized with respect to glyceroldehyde 3-phosphate message and expressed as percent of control response, in TTX-paralyzed muscle the α1, α2, and β subunit genes of the DHP receptor were induced 1.7 ± 0.18-fold (n = 3), 1.6 ± 0.25-fold (n = 3), and 2 ± 0.25-fold (n = 3), respectively. The RY receptor gene was induced 1.3 ± 0.13-fold (n = 3) (Fig. 5B). Tubulin gene transcription was unaltered 1 ± 0.1-fold (n = 3).

Since forskolin and TPA altered the DHP and RY receptor mRNA level following 72 h exposure of myotubes, we attempted to investigate if changes in transcript levels could be attributed to enhanced transcription of the respective genes. We isolated nuclei from myotubes pretreated with forskolin (1 μM) and TPA (200 ng) for 72 h. Nuclei were subjected to in vitro transcription. As shown in Fig. 6A, TPA (TPA panel) and forskolin (Forsk panel) pretreatment of myotubes did not alter the rate of transcription of the DHP and RY receptor genes. No statistically significant difference in the expression of either calcium channel subunit gene was observed when data were normalized with respect to glyceroldehyde-3-phosphate dehydrogenase message and expressed as percent of control (Fig. 6B).

Electrical Activity/Neurotrophic Factor-induced Changes in DHP and RY Receptor Polypeptides—Since chronic superfusion of TTX had such a pronounced stimulatory effect on the expression of DHP and RY receptor genes, we examined the translation of the gene product with specific antibodies (De Jongh et al., 1989). Homogenates of skeletal muscle from control, denervated, and TTX-paralyzed muscle were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-α2, anti-β, and anti-RY receptor antibodies. Denervation did not significantly affect the expression of α2 subunit.
polypeptide compared with control, while the α1 subunit levels were elevated in TTX-paralyzed muscle (Fig. 6). Similarly, there was an increase in the level of the RY receptor polypeptide in the TTX-paralyzed muscle as compared with denervated and untreated control muscle (Fig. 7). The β subunit polypeptide levels were not significantly altered by any of these treatments. When expressed as percent of control response, we found that the α1 subunit of the DHP receptor and the RY receptor polypeptide levels were increased by 1.5 ± 0.1 (n = 3) and 1.4 ± 0.14-fold (n = 3), respectively (Fig. 7B).

cAMP and Protein Kinase C Modulate DHP and RY Receptor Numbers in Myotubes—As noted above, interference of protein kinase C activity in cultured myotubes with TPA and staurosporine had a pronounced modulatory effect on the mRNA expression of both the α1 subunit of the DHP receptor and the RY receptor. While agents that elevate cAMP levels down-regulated RY receptor mRNA in cultured myotubes, they had no effect on DHP receptor subunits. In order to determine if changes in mRNA levels translated into changes in DHP and RY receptor numbers, the number of DHP and RY binding sites in cultured myotubes pretreated with forskolin (1 μM), TPA (200 ng), and staurosporine (200 ng) were measured. [3H]PN 200-110 and [3H]ryanodine were used as DHP and RY receptor ligands, respectively. In untreated myotubes, the number of PN 200-110 and RY binding sites on day 7 were 210 ± 15 (n = 3) and 48 ± 10 fmol/mg (n = 3) of protein, respectively. TPA treatment resulted in an increase in DHP binding to 270 ± 27 (n = 3) fmol/mg of protein while ryanodine binding was increased to 80 ± 12 (n = 3) fmol/mg of protein in cultured myotubes (Fig. 8). Staurosporine (200 ng) inhibited the number of DHP binding sites to about 30 ± 6 (n = 3) fmol/mg of protein (Fig. 8). The RY binding sites were reduced to 10 ± 2 (n = 3) fmol/mg of protein in myotubes treated with forskolin.

**DISCUSSION**

The results of the present study demonstrate that nerve plays a critical role in regulating the expression of DHP and RY receptors in vivo. Our data also indicates that the cAMP and protein kinase C pathways can modulate the protein and mRNA levels but not the transcriptional activity of these important genes.

Formation of the neuromuscular junction is an important step in the course of development and maturation of skeletal muscle. Muscle-specific voltage-sensitive sodium channel and nAChR subunits undergo redistribution from the body of myotubes to the neuromuscular junction as well as subunit and/or isoform switching under the influence of nerve supply (Schuetze and Role, 1987; Hall and Sanes, 1993). A common approach to determine the contribution of nerve-induced electrical activity on muscle-specific gene expression is to surgically denervate the muscle. Denervation of adult muscle and blocking spontaneous contractility of cultured myotubes with TTX reverses myotubes back in time to the embryonic stage which is manifested by pronounced up-regulation of the gene expression of the embryonic isoforms of nAChR and TTX-insensitive sodium channel (Goldman et al., 1989; Kallen et al., 1990; Offord and Catterall, 1989; Witzemann et al., 1991; Chahine et al., 1993; reviewed in Schuetze and Role (1987) and Hall and Sanes (1993)). By contrast, denervation of adult skeletal muscle had no effect on the mRNA levels of α1, α2, and β subunits of the DHP receptor while the RY receptor and AChE messages were down-regulated. Similarly, blocking spontaneous contractility of cultured myotubes with TTX and bupivacaine did not have any effect on the DHP and RY receptor mRNA levels. This lack of effect of muscle inactivity on DHP and RY receptor transcript levels may be related to the fact that unlike muscle-specific nAChR and sodium channel proteins, the DHP and RY receptors do not appear to undergo isoform and/or subunit switching and redistribution from the body of myotubes to neuromuscular junction in response to nerve influence.

TTX paralysis of the sciatic nerve markedly induced the α1, α2, β subunit transcripts of the DHP receptor as well as the RY receptor mRNA. Increases in the DHP receptor mRNAs were accompanied by corresponding increases in transcriptional activity of the respective genes in TTX-paralyzed skeletal muscle. This suggested that increases in the DHP and RY receptor transcripts were due, at least in part, to induction of the respective genes. The increase in the mRNA levels of α1 subunit and the RY receptor were accompanied by changes in the two polypeptide levels when assayed with specific antibodies. In TTX-paralyzed skeletal muscle, there is no neuronal evoked action potentials and transmitter release but axonal transport and spontaneous quantal release of neurotrophic factors is maintained at the neuromuscular junctions (see Witzemann et al. (1991)). Thus, induction of the DHP and RY receptor genes may be attributed to the release of some nerve-derived fac-
tor(s). In this regard several nerve-derived factors, including calcitonin gene related peptide (New and Mudge, 1986), acetylcholine receptor inducing agent (Jo et al., 1995), and Schwann cell-derived maturation factor (Chapron and Koenig, 1989) are capable of inducing the expression of muscle nAChR genes. In addition, it has been shown that growth factors including transforming growth factors (Shih et al., 1990; Giannini et al., 1992) and fibroblast growth factor (Marks et al., 1991) can regulate the DHP and RY receptor message levels. We do not know if any of these aforementioned factors contribute toward the induction of DHP and RY receptor genes observed in TTX-paralyzed muscle. We did not observe any inducing effect of brain-derived neurotrophic factor, ciliary neurotrophic factor, or nerve growth factor on the DHP receptor mRNA level in primary culture of skeletal myotubes.  

Previous studies have shown that muscle inactivity and denervation lead to increased cAMP levels and activation of the cAMP-dependent protein kinase pathway in muscle (Chahine et al., 1990). Furthermore, the induction of nAChR and TTX-insensitive sodium channel genes are believed to be mediated via the cAMP pathway in inactive and denervated muscle (Chahine et al., 1993; Offord and Catterall, 1989). In the present study, we did not see any effect of forskolin treatment of myotubes were represented as percent of control response. Each bar represents mean ± S.E. of three separate experiments.

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**FIG. 6. Effects of forskolin and TPA on transcriptional activity of DHP and RY receptor genes.** A, myotubes on day 4 were exposed to buffer (CON), TPA (200 ng), and 1 µM forskolin (FORSK). Nuclei were isolated on day 7 and transcribed at 28 °C using 32P-labeled UTP as described under “Materials and Methods.” Labeled RNA (1.5 x 10^6 cpm) were hybridized for 48 h at 42 °C to nylon membranes containing 10 µg each of linearized α1, α2, β subunits, RY receptor (RYR), and glyceraldehyde-3-phosphate dehydrogenase (GAPD) plasmids and the non-linearized tubulin (TB) plasmid. Signals were visualized by autoradiography. Data is the representative of three similar experiments. B, densitometry data in control, TPA, and forskolin-treated cultured myotubes were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase message. Changes in transcriptional activity due to TPA or forskolin treatment of myotubes were represented as percent of control response. Each bar represents mean ± S.E. of three separate experiments.

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**FIG. 7. Dihydropyridine and RY receptor polypeptide levels in denervated and TTX-paralyzed adult skeletal muscle.** A, muscle proteins (200 µg) from control (C), denervated (D), and TTX-paralyzed (T) muscles were separated in SDS-PAGE and subjected to Western blot analysis with specific antibodies raised against the α1, β subunits of the voltage-sensitive calcium channel (De Jongh et al., 1989) and the RY receptor. The apparent molecular mass of the immunoreactive polypeptides were 170-, 56-, and 450-kDa, respectively. The results are a typical representation of three independent experiments. B, densitometry data were expressed as percent of control response. Each data point represents mean ± S.E. of three independent experiments. * represents significantly different from control (p < 0.05).

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**FIG. 8. Forskolin, TPA, and staurosporine affect the number of DHP and RY receptor binding sites in cultured myotubes.** Cultured myotubes were exposed on day 4 to forskolin (1 µM), TPA (200 ng), and staurosporine (200 ng) for 72 h. On day 7 myotubes were homogenized and DHP and RY binding were determined using [3H]PN200-110 and [3H]Ryndinole (Murphy and Tuana, 1990). Each bar represents the mean ± S.E. of three independent experiments.
Forskolin had a pronounced inhibitory effect on the level of RY receptor mRNA and expression of the sarcoplasmic reticulum Ryanodine binding sites, suggesting that the inhibitory effect of forskolin on the RY receptor binding sites was mediated through down-regulation of the transcript. An inhibition of the RY receptor mRNA level was also observed in denervated muscle. Thus elevated cAMP levels may contribute toward the regulation of RY receptor message level. Forskolin, however, did not have any effect on the induction of the RY receptor gene. This suggests that forskolin, and consequent elevated cAMP levels, might be acting by destabilizing the RY receptor transcript. A similar mechanism has been suggested in the down-regulation of the AChE mRNA in skeletal myotubes in culture (Luo et al., 1994). In cultured myotubes, where the electrical activity was depressed with TTX or bupivacaine, there was no change in RY receptor mRNA levels. While we do not know the precise reason for these results, it is possible that the elevation in cAMP levels in TTX-treated myotubes was not sufficient to inhibit RY receptor mRNA levels. It should be noted that, unlike the nAChR and voltage-sensitive sodium channel, the expression of RY receptor mRNA was not induced by blocking sarclemmal electrical activity and elevated cAMP levels, but was repressed instead.

It has been shown that spontaneous and electrical stimulation-induced muscle contractions are preceded by membrane depolarization and activation of protein kinase C (Vergara et al., 1985; Huang et al., 1992), which can modulate the expression of several muscle-specific genes (Klarsfeld et al., 1989; Huang et al., 1992; Walke et al., 1994). Our results demonstrated that TPA, the protein kinase C activator, increased the level of both DHP receptor subunit and RY receptor transcripts and the respective protein levels. Since staurosporine, a protein kinase C antagonist, antagonized TPA-induced responses, we can rule out the possibility that TPA was acting by down-regulating protein kinase C. Staurosporine also inhibited the mRNA and protein levels of $\alpha_1$ subunit and RY receptor in developing cultured myotubes, at a time when the endogenous protein kinase C is maximally activated, suggesting that protein kinase C may play a role in regulation of these transcripts. Since TPA did not increase transcriptional activity of the DHP receptor subunit and RY receptor genes, and both TPA and staurosporine responses were limited only to the $\alpha_1$ subunit message of the DHP receptor, it is possible that TPA was acting by modulating the stability of the $\alpha_1$ subunit and RY receptor transcripts. Consistent with this notion, TPA response elements were not detected in the proximal region of the RY receptor gene. In this regard, mRNA stabilization has been suggested to play an important role in the regulation of the AChE mRNA level both during myogenesis and development (Fuentes and Taylor, 1993).

Despite having a similar onset of induction, the DHP and RY receptor transcripts follow temporally distinct courses to reach their respective peaks. While the DHP receptor subunits reached their peak before the appearance of spontaneous contractility, the RY receptor message began to accumulate when myotubes were spontaneously contracting. This may imply that mRNAs of these receptors are regulated by distinct endogenous factors. Endogenous cAMP can be one such factor. We have shown that the RY receptor transcript level was lower in denervated muscle where basal cAMP levels have been reported to be elevated (Chahine et al., 1993). We have also found that elevation of cAMP levels in myotubes markedly inhibited the RY receptor message, without altering transcriptional activity of the gene. Thus it is possible that at the early stages of muscle development the RY receptor transcript is stabilized by high cAMP levels. Subsequently, as myotubes mature and begin to contract spontaneously, activation of protein kinase C (Vergara et al., 1985; Huang et al., 1992) and/or down-regulation of cAMP may stabilize the RY receptor message. Consistent with this we have found that TPA increased RY receptor message level.

In conclusion, in this study we show that nerve plays an important role in the regulation of DHP and RY receptor genes. Nerve-derived factor(s) can enhance transcriptional activity of DHP and RY receptor subunit genes and their respective mRNA levels. DHP receptor subunit messages are insensitive to muscle denervation and muscle inactivity while RY receptor mRNA is down-regulated by denervation of skeletal muscle. TPA and cAMP can also modulate transcript levels of the $\alpha_1$ subunit of DHP receptor and RY receptor by a transcription independent mechanism.

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