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Static stretch promotes MEF2A nuclear translocation and expression of neonatal myosin heavy chain in C_2C_{12} myocytes in a calcineurin- and p38-dependent manner

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Rauch, Cyril, and Paul T. Loughna. Static stretch promotes MEF2A nuclear translocation and expression of neonatal myosin heavy chain in C_2C_{12} myocytes in a calcineurin- and p38-dependent manner. *Am J Physiol Cell Physiol* 288: C593–C605, 2005. First published October 13, 2004; doi:10.1152/ajpcell.00346.2004.—Although the effects of mechanical stimuli have been studied extensively in fully differentiated skeletal muscle and have been shown to promote changes in phenotype, including altered myosin heavy chain isoform expression, the effects of a change in mechanical environment have been poorly studied at earlier stages of skeletal muscle differentiation. In particular, the early events elicited by mechanical stimuli upon differentiating myocytes have not been investigated. In the present study, the effect of static stretch on the activation of transcriptional factors MEF2A and NFATc1, which have been shown to be involved in the differentiation and phenotype regulation of skeletal muscle, have been examined. Furthermore, putative second messenger signaling pathways that could be involved in the dephosphorylation and hence activation of these factors were also examined. We have demonstrated that static stretch application produces a robust increase in p38 phosphorylation preceding MEF2A, but not NFATc1, nuclear translocation as well as deactivation of GSK-3β via its phosphorylation. Using SB-203580 and cyclosporine A drugs to inhibit both p38- or/and calcineurin-dependent signals, respectively, we have shown that MEF2A phosphorylation and subsequent nuclear translocation are regulated by p38 and calcineurin in a biphasic, time-dependent manner. Moreover, we also present evidence for another kinase that is involved in the stretch-related signal triggering MEF2A hyperphosphorylation, impairing its nuclear translocation, and that is related to p38. Finally, we have shown that static stretch application overnight promotes neonatal myosin heavy chain expression, which is inhibited by an inactivation of both p38 and calcineurin.

differentiation; myocyte enhancer factor

MECHANICAL STIMULI ARE IMPLICATED as a major influence on a number of fundamental cellular processes, including differentiation, growth, and apoptosis (16, 22). Moreover, previous studies have highlighted the cytoskeleton and the focal adhesions as possible signal transducers (2, 19). During limb development, the elongation process could play an important role in the differentiation of skeletal muscle at both primary and secondary myotube stages. In this context, we recently showed that when differentiating C_2C_{12} myocytes are subjected to passive stretch over a 2-day period, the expression of adult myosin heavy chain (MHC) isoforms is upregulated, which is indicative of an acceleration of the differentiation process (unpublished observation). However, the early events elicited by mechanical stimuli, including second messenger pathways, and the activation of transcription factors known to promote differentiation and the development of a skeletal muscle phenotype have not been investigated.

There are two major stages in the differentiation of skeletal muscle cells: the withdrawal from the cell cycle of myoblasts and the subsequent induction of myotube-specific gene expression (4, 8, 20, 30). Once fully differentiated, skeletal muscle myofibers can still undergo changes in phenotype in response to a variety of stimuli, including mechanical stretch (26). Although this process is still poorly understood, recent studies have implicated a number of second messenger signaling pathways and transcription factors involved in the regulation of muscle fiber phenotype. For example, both the myocyte enhancer factor 2 (MEF2) and nuclear factor of activated T cells (NFAT) families of transcription factors have been shown to be involved in the alteration of the fiber phenotype through the Ca^{2+}-dependent phosphatase calcineurin by motor nerve stimulation or work overload (9, 43). Furthermore, these changes can be inhibited by the use of cyclosporine A (CsA), a known inhibitor of calcineurin activity, suggesting the involvement of calcineurin in the regulation of both MEF2 and NFAT mediating phenotypic alteration.

Recent studies have shown that α- and β-isoforms of the mitogen-activated protein kinase (MAPK) p38 can promote MEF2 expression during muscle differentiation and that they also are involved in the transcriptional activation of MEF2 proteins through their phosphorylation (10, 44, 47, 49). Two members of the MEF2 family are known to be responsive to p38, namely, MEF2A and MEF2C that bind to the DNA consensus sequence $CTA(A/T)_4TA(G/A)$ (6, 49). This sequence is found in the promoter region of numerous muscle-specific genes, especially those related to skeletal muscle fiber specificity (9). Although NFAT has been shown not to be involved in certain specific components of the differentiation process (17), this transcription factor is necessary in combination with MEF2A to allow a change in fiber specificity post-differentiation (9, 14, 43).

In the present study, we have examined, for the first time, the effect of short-term static stretch on C_2C_{12} myocytes upon NFATc1 and MEF2A phosphorylation states and the second putative messenger signaling pathway involved in this activation.

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MATERIALS AND METHODS

Cells and materials. C_2C_{12} cells were cultured on silicon-based plates purchased from Flexcell, precoated with collagen type 1 (Sigma, St. Louis, MO), and grown until 50% confluence in DMEM-Glutamax-I containing penicillin (50 μU/ml) and streptomycin (50 μg/ml) supplemented with 10% FBS (GIBCO-BRL/Life Technologies, Grand Island, NY) and maintained at 37°C in 5% CO_2. The medium was replaced with differentiation medium (2% horse serum, GIBCO-BRL/Life Technologies) 2 days before the application of static stretch. NFATc1 (sc-1789), MEF2A (sc-313), GSK-3β-p(Ser^9) (sc-11757), GSK-3β (sc-9166), Akt1-p(Ser^{473}) (sc-7585), Akt1 (sc-1618) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and p38 (S9212) and phosphorylated p38 (p38-p) (S9216) were obtained from New England Biolabs (Beverly, MA). Secondary antibodies purchased from Sigma were horseradish peroxidase (HRP)-conjugated antigoat (PI-9500), HRP-conjugated antirabbit (PI-1000), biotinylated antigoat (BA-5000), and antirabbit (BA-1000) were purchased from Vector Laboratories (Burlingame, CA). Tris·HCl, β-mercaptoethanol, bromophenol blue, SDS, glycerol, leupeptin, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), orthovanadate, and Triton X-100.

Static stretch application. The medium was renewed with fresh serum on the day before the initiation of experiments. The next day, CsA (Sigma) and SB-203580 (Calbiochem) were added as necessary to achieve final concentrations of 4 and 1 μM, respectively. To allow stretch of the C_2C_{12} cells, we used specific six-well plates containing a silicon membrane coated with collagen. This system was previously used for stretching skeletal muscle cells in vitro (13, 23). A continuous stretch of the silicon basal membrane of a well was produced by placing centrally, under the well, a glass bead of radius 1.6 cm as described previously (unpublished results). To maintain a constant global deformation, we placed a lead weight on top of the plate to maintain pressure on the well to ensure constant, steady deformation of the silicon basal membrane. In that way, the surface stretched can be separated into two areas, denoted S_1 and S_2 (see Fig. 1). On the basis of the geometry of this system (i.e., symmetry around an axis perpendicular to the base of the well passing through the center of the bead), calculations using trigonometry and differential geometry showed that the deformation applied on S_1 was constant and did not depend on the distance between the center and the periphery of the well (as is obvious on the basis of examination of the lateral view of the system, in which S_1 is represented by a line with a constant rate). Because the bead produced a parabolic shape, the last result for S_1 did not apply to the area S_2 at the top of the bead, meaning that the deformation in this region depended on the distance between the center and the circumference of S_2. Nonetheless, taking into account the dimensions of the well, we calculated that such a nonhomogeneous local deformation represented only 5% [$S_2/(S_1 + S_2) \times 100 = 5\%$] of the total relative increase in the surface area, suggesting a minor contribution to the global stretch. Finally, a calculation showed that the relative increase in the total surface area, e.g., the global deformation, equal to $(S_1 + S_2 - S_0)/S_0$, was ~8–9% (where S_0 is the surface area of the silicon membrane without stretch).

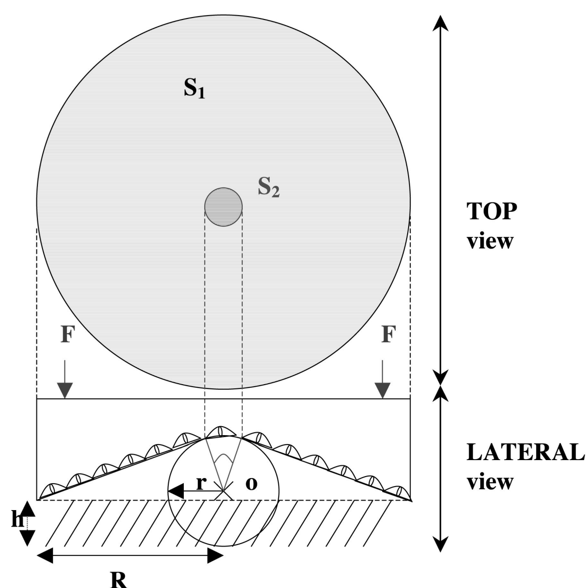

Fig. 1. Schematic representation of the apparatus used to apply static stretch (S) to C_2C_{12} myocytes. To allow continuous stretch, we simply stretched the silicon basal membrane by placing it centrally under the well of radius *R*. A glass bead of radius *r* was placed on a plastic support of thickness *h*. Also, to maintain a constant global deformation, we placed a lead weight on top of the plate to maintain constant pressure (force, *F*) on the well to deform the silicon basal membrane.

Immunohistochemistry. The cells were fixed with ice-cold paraformaldehyde 4%. After fixation, PBS-Triton (1% vol/vol)-BSA (0.5% wt/vol) was added for 1 h at room temperature to permeabilize the cells and was subsequently used to wash cells after antibody incubation. Primary antibody was incubated at a constant dilution of 1:100 (vol/vol) for 1 h in PBS-Triton-BSA. After being washed, biotinylated secondary antibody was incubated at a constant dilution of 1:100 (vol/vol) for 1 h at room temperature in PBS-Triton-BSA. The signal was amplified using the peroxidase-tyramide amplification kit (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instructions. For double labeling, after an overnight wash in PBS-Triton-BSA, the subsequent labeling was performed by following the same methodology. Observations were made using laser scanning confocal microscopy (Zeiss LSM510; Carl Zeiss, Thornwood, NY).

Measurement of fluorescence intensity. Fluorescence intensity was measured using dedicated software (EMBL-Zeiss). For each experiment, 70 cells were randomly selected in different fields, with each field containing five to seven cells. The fluorescence intensity of each nucleus was examined on a scale of intensity between 0 (no intensity) and 250 (saturating intensity). Subsequently, the number of nuclei corresponding to a given intensity was plotted as a function of intensity. These data produced Gauss-like curves determining the mean intensity of each experiment.

Western blotting. Incubations were terminated by washing with ice-cold PBS containing orthovanadate (Na_3VO_4) at 0.4 mM and whole cell lysates prepared in lysis buffer [63.5 mM Tris·HCl, pH 6.8, 10% glycerol (vol/vol), 2% SDS (wt/vol), 1 mM Na_3VO_4, 1 mM AEBSF, 50 μg/ml leupeptin, 5% β-mercaptoethanol (vol/vol), and 0.02% bromophenol blue (wt/vol)]. The protein content of the cell lysate was measured using the Bradford test (Sigma) and a spectrophotometer (Spectronic Genesys 2; Thermo Electron, Waltham, MA), and equal quantities of protein (30–60 μg/lane) were resolved using SDS-PAGE (10 or 7%). The gel was then transferred onto Hybond-P membrane (Amersham, Piscataway, NJ) that was then blocked with nonfat dry milk at 5% (wt/vol) in PBS-Tween (1:1,000 vol/vol). For immunodetection, the following primary antibodies were used at a concentration of 1:1,000 (vol/vol) in PBS-Tween for 1 h: NFATc1, MEF2A, GSK-3β-p(Ser^9), Akt1-p(Ser^{473}), GSK-3β, Akt1, p38-p, and p38. The membrane was subsequently washed five times for 10 min each in PBS-Tween. HRP-conjugated antibody was added at a concentration of 1:10,000 (vol/vol) in PBS-Tween for 1 h, and the membrane was washed five times for 10 min each in PBS-Tween before the chemiluminescence reaction was performed using ECL Plus (Amersham). Protein levels were examined using Hyperfilm (Amersham). The measurement of the protein expression levels was performed with an imaging densitometer (GS-690; Bio-Rad, Hercules, CA). To ensure equal loading of the proteins, the blots were

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stripped and reprobed using either the nonphosphorylated form of the protein studied when necessary or Coomassie blue staining.

Data analysis. Each experiment was repeated six times. All data are expressed as means ± SD. Paired *t*-test analysis was performed, and significance was accepted at $P < 0.05$.

RESULTS

Effect of static stretch on calcineurin-dependent cytoplasmic mediators MEF2A and NFATc1 and calcineurin-independent cytoplasmic mediators p38, GSK-3β, and Akt1. C_2C_{12} cells were allowed to differentiate for 2 days in differentiation medium and stretched for periods of 5 min to a maximum of 3 h to enable a time course of an intracellular event to be examined. We initially examined the effect of this stretch on the transcription factors MEF2A and NFATc1 (9, 18, 43). Figure 2*A* shows that within 5 min of static stretch application, an increase of ~50% dephosphorylation of NFATc1 ($P < 0.05$) was observed. This dephosphorylation was sustained for up to 1 h, after which it declined to control levels by 3 h.

In contrast to the dephosphorylation of NFATc1, there was no significant change in the phosphorylation state of MEF2A for the first 30 min of passive stretch. However, after 1 h, a dramatic dephosphorylation of this protein was observed ($P < 0.005$), and this was maintained up to the 3-h time point (Fig. 2*B*). Recent studies performed on different cell types, including skeletal muscle cells (44, 48) and cardiomyocytes (7, 21), have shown that p38 α- and β-isoforms are potential regulators of MEF2A and NFAT phosphorylation. Moreover, it also has been shown that phosphatidylinositol 3-kinase (PI3-K) can be involved in MEF2 activation (41). We therefore measured the levels of p38-p and of Akt1 phosphorylated on Ser^{473} (Akt-p), with the latter known to be strongly related to PI3-K activity. Moreover, because deactivation of GSK-3β via its phosphorylation on Ser^{9} is also known to positively regulate NFAT nuclear translocation in cardiomyocytes and skeletal muscle myotubes (5, 42), we also measured the level of GSK-3β-p under the same static stretch conditions. Within 5 min of static stretch application, a significant increase ($P < 0.005$) (Fig. 3*A*) of ~200% of p38-p was observed. Interestingly, GSK-3β was also significantly phosphorylated ($P < 0.005$ or 0.05) (Fig. 3*B*) in a manner similar to that observed for p38. In contrast to p38 and GSK-3β, Akt was significantly dephosphorylated ($P < 0.005$) (Fig. 3*C*).

p38, an upstream signaling mediator involved during static stretch application: effect of the inhibitor SB-203580. Activation of p38 through phosphorylation has been shown to promote the nuclear translocation of MEF2A in skeletal muscle cells as well as in cardiomyocytes (47, 49). Moreover, p38 also has been shown to antagonize NFAT nuclear translocation induced by calcineurin in cardiomyocytes (7). Therefore, to investigate the stretch-induced role of p38 phosphorylation on MEF2A and NFATc1, we used the drug SB-203580, which is known to specifically inhibit the downstream signaling events induced by both the α- and β-isoforms of p38 (1, 7). To observe any endogenous effect promoted by the incubation of this drug alone, we also measured the phosphorylation of MEF2A and NFATc1 with SB-203580 but without static stretch application. Incubation of SB-203580 alone not only induced NFATc1 dephosphorylation (~30%, $P < 0.05$) (Fig.

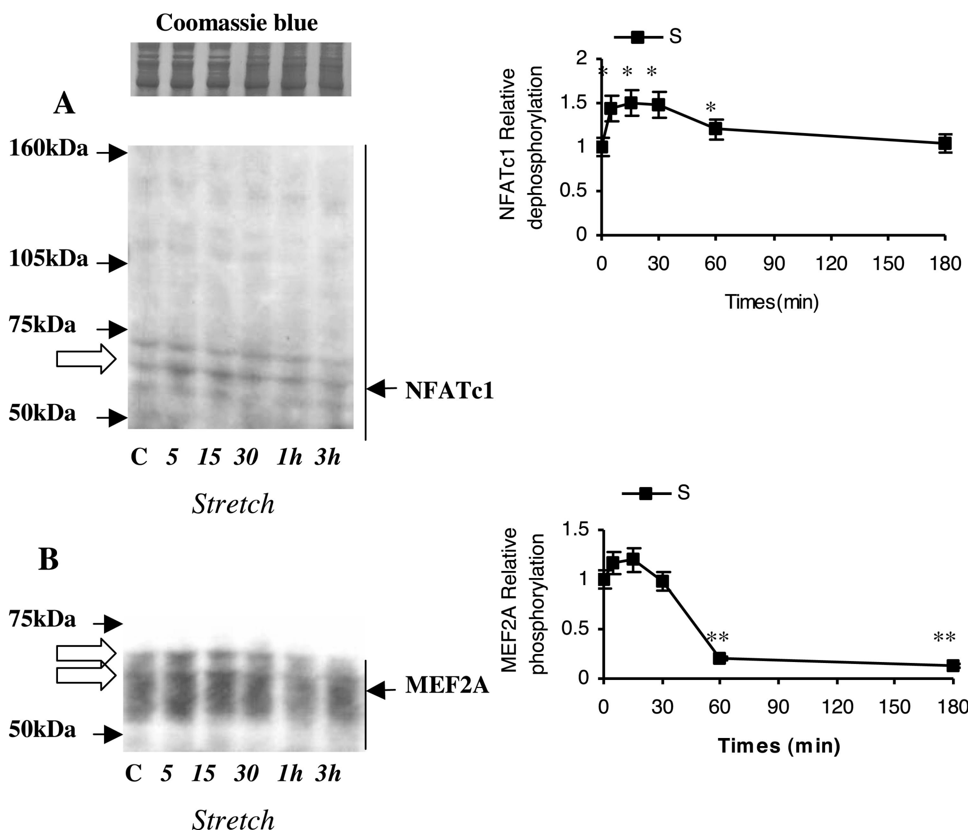

Fig. 2. Effect of static stretch application to nuclear factor of activated T cells (NFAT)c1 and myocyte enhancer factor (MEF)2A phosphorylation states. Static stretch application in C_2C_{12} cells was performed for 5 min–3 h. Subsequently, cells were lysed and Western blotting was performed, and antibodies against all of the proteins were used to visualize shift in protein migration characteristic of the phosphorylated form of NFATc1 (*A*) and MEF2A (*B*). On the representative NFATc1 Western blot, the open arrow indicates the dephosphorylated form of NFATc1, whereas the phosphorylated form of MEF2A is indicated by the *top* open arrow (*bottom* open arrow represents nonphosphorylated form). For each experiment, a native control experiment was conducted (*lane C*). Coomassie blue stain was used to verify equal amount of protein loaded, and densitometry was performed to plot the relative increase or decrease in phosphorylated proteins. *$P < 0.05$. **$P < 0.005$.

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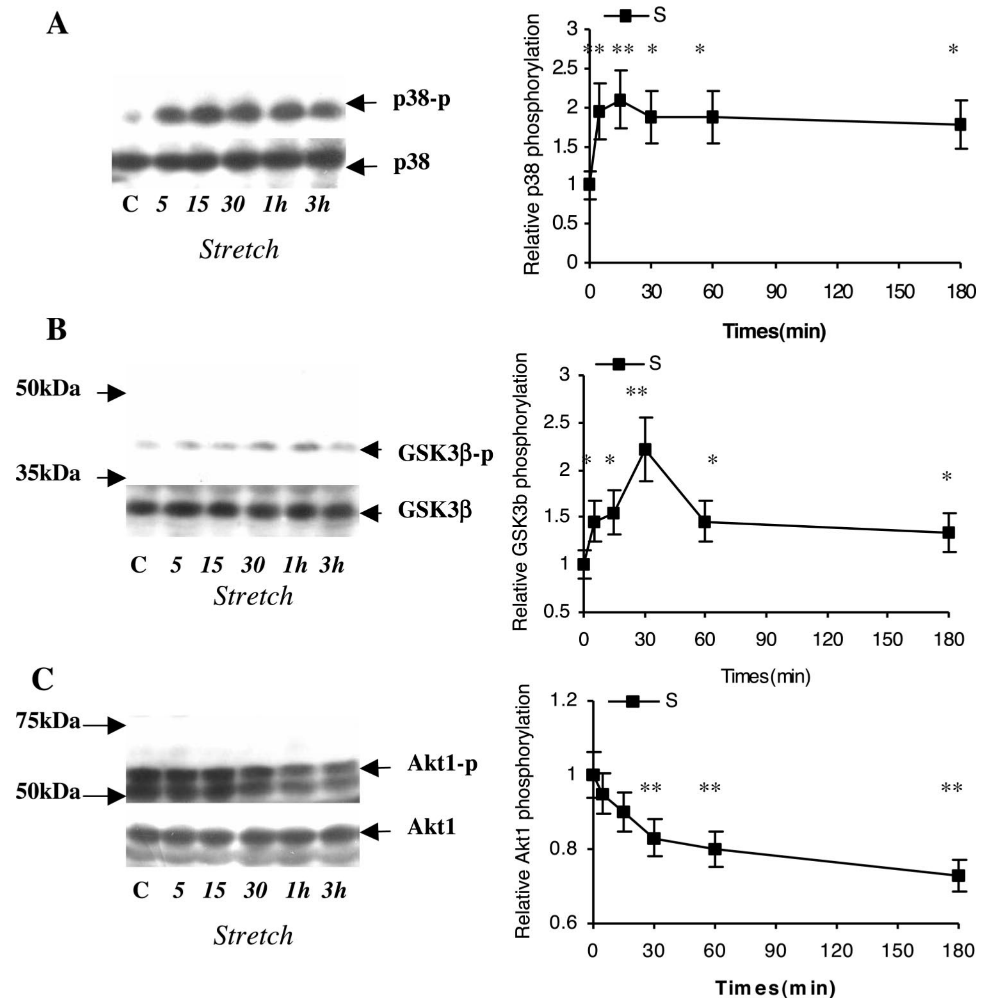

Fig. 3. Effect of static stretch application on p38, GSK-3β, and Akt1 phosphorylation states. Static stretch application was performed for 5 min–3 h in C_2C_{12} cells. Subsequently, cells were lysed and Western blot analysis was performed. Antibodies against specific phosphorylated forms of p38 (*A*), GSK-3β (*B*), and Akt1 (*C*) [denoted p38-p, GSK-3β-p (Ser^9), and Akt1-p (Ser^{473}), respectively] were used to visualize the level of phosphorylation for each protein. In addition, antibodies against all p38, GSK-3β, and Akt1 proteins were used to verify equal amounts of proteins loaded. For each experiment, a native control (*lane C*) experiment was conducted, and densitometry was performed to plot the relative increase or decrease in phosphorylated proteins. $*P < 0.05$. $**P < 0.005$.

4*A1*) at 30 min but also promoted an even more rapid (within 15 min) phosphorylation of MEF2A ($P < 0.05$) (Fig. 4*B1*). Interestingly, SB-203580 alone had no significant effect on the rapid NFATc1 dephosphorylation (within 5 min) observed in response to static stretch (Fig. 4*A*). Nonetheless, whereas stretch alone promoted the rephosphorylation of NFATc1 after 30 min, the presence of SB-203580 inhibited this process, suggesting an involvement of p38 in this rephosphorylation (Fig. 4*A*). Interestingly, whereas stretch alone produced a biphasic effect on MEF2A phosphorylation, the presence of SB-203580 in conjunction with stretch induced an inverted biphasic response, e.g., a strong dephosphorylation between 5 and 30 min ($P < 0.005$) (Fig. 4*B*), followed by a rephosphorylation that reached a plateau after 60 min ($P < 0.05$) (Fig. 4*B*). The difference between the effects of SB-203580 alone and SB-203580 with static stretch on MEF2A phosphorylation suggests the involvement of one or more signaling factors other than p38 kinase that are triggered by static stretch promoting MEF2A dephosphorylation. Similarly to MEF2A, a comparison of the effect of static stretch with or without SB-203580 on NFATc1 phosphorylation suggests that p38 activity is involved in the rephosphorylation of this protein in the latter part of the period studied.

To determine whether the incubation of SB-203580 with and without stretch could interfere with the phosphorylation of GSK-3β or Akt, we also measured the phosphorylation levels of these entities (Fig. 5, *B* and *C*). Whereas incubation of SB-203580 alone did not significantly change the profile of phospho-Akt or phospho-GSK-3β (Fig. 5, *B1* and *C1*), the application of static stretch in the presence of SB-203580 inhibited the decrease of phospho-Akt as well as the increase of phopho-GSK-3β (Fig. 5, *B2* and *C2*) observed in response to stretch alone (Fig. 3, *B* and *C*). These data suggest that stretch-induced alterations in the phosphorylation states of both GSK-3β and Akt are p38-p dependent.

Involvement of the Ca^{2+}-dependent phosphatase calcineurin during static stretch application: effect of CsA. Whereas the changes in the phosphorylation states of both Akt and GSK-3β in response to static stretch were fully reversed by SB-203580 incubation, this was not the case for NFATc1 and MEF2A, suggesting the involvement of another stretch-induced signaling factor. Calcineurin has been shown to be involved in both NFAT and MEF2 nuclear translocation (9, 43), and CsA is a well-known inhibitor of calcineurin activation (28). Nevertheless, CsA is also known to block the activation of p38 (28). Therefore, we used the nonspecificity of CsA (blocking both

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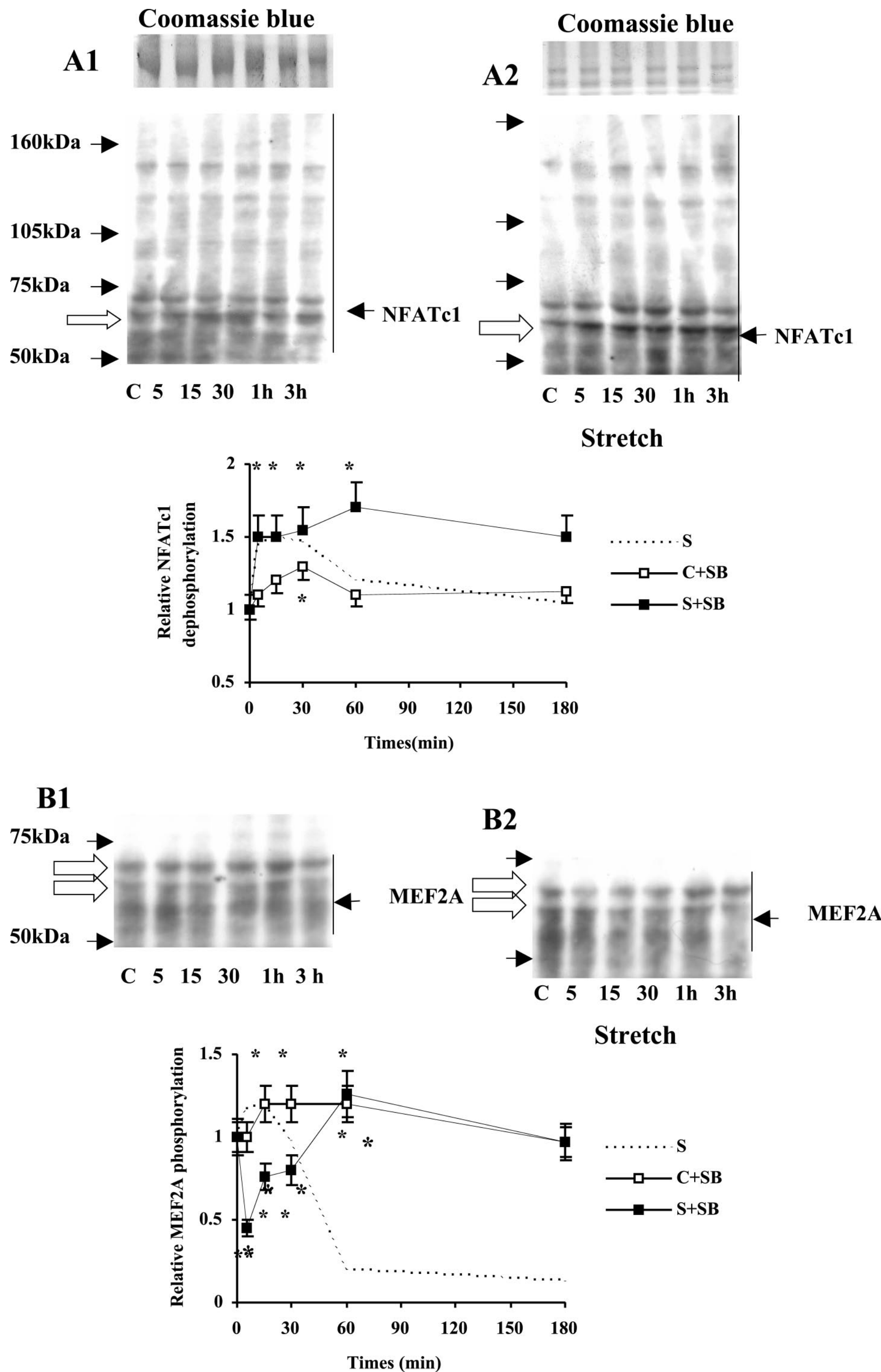

Fig. 4. Effect of static stretch application and p38 downstream inhibitor SB-203580 (SB) on NFATc1 and MEF2A phosphorylation states. Five minutes before static stretch application, SB was incubated at a final concentration of 1 μM. SB was incubated with control cells (unstretched) for 5 min–3 h (*A1*, *B1*). In a different experiment, static stretch was applied in conjunction with SB for the same time course (*A2*, *B2*). Subsequently, cells were lysed and Western blot analysis was performed, and antibodies against all of the proteins were used to visualize the shift in protein migration characteristic of the phosphorylated forms of NFATc1 (*A1*, *A2*) and MEF2A (*B1*, *B2*). For each experiment, a native control experiment (*lane C*) was performed without stretch and SB incubation. Coomassie blue was used to verify equal amount of protein loaded, and densitometry was used to plot the relative increase or decrease of phosphorylated proteins. S, static stretch only; C+SB, incubation of SB only; S+SB, static stretch application in the presence of SB incubation. $*P < 0.05$. $**P < 0.005$.

the p38 and calcineurin signaling pathways) to determine the role of calcineurin in the changes in phosphorylation of MEF2A and NFATc1 by comparing the effects of this drug with that of the specific p38 inhibitor SB-203580. CsA was used at a final concentration (4 μM) known to inhibit NFAT nuclear translocation induced by IGF-I through a calcineurin pathway in C_2C_{12} skeletal muscle cells (35).

Incubation of CsA prevented the stretch-induced changes in phosphorylation of both MEF2A and NFATc1 over the entire time course studied (Fig. 6, *A2* and *B2*). Furthermore, CsA incubation alone (without stretch) did not change the phosphorylation of these proteins (Fig. 6, *A1* and *B1*). Finally, to determine whether CsA impairs stretch-induced p38 phosphorylation, we also measured the level of p38-p when stretch was applied and found no differences between incubation of CsA with or without static stretch (Fig. 7*A*). These data suggest that under static stretch conditions, both p38 and calcineurin are involved in the changes in phosphorylation states of both

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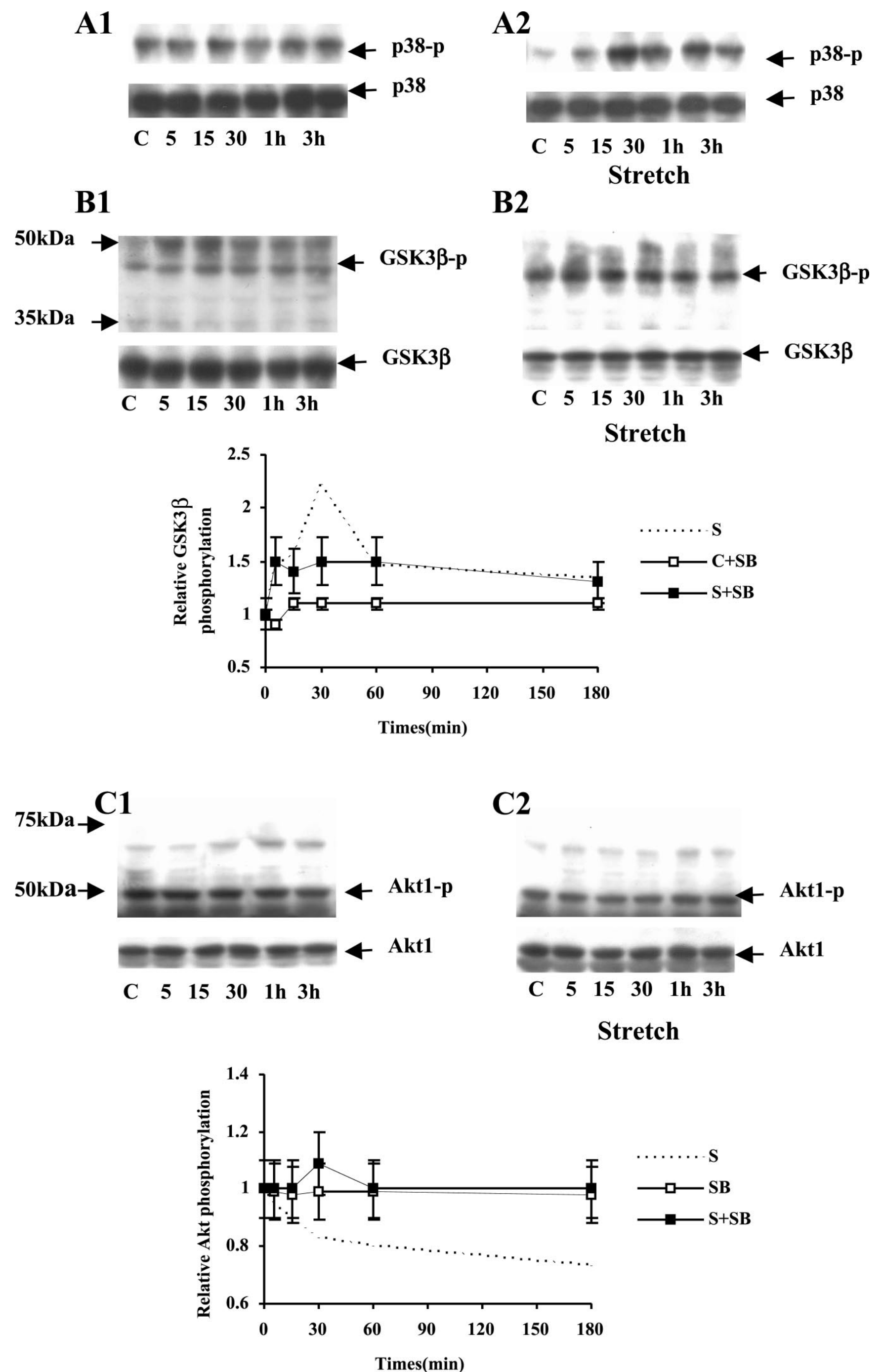

Fig. 5. Effect of static stretch application and p38 downstream inhibitor SB incubation on p38, GSK-3β, and Akt1 phosphorylation states. Five minutes before static stretch application, SB was incubated at a final concentration of 1 μM. SB was incubated with control cells (unstretched) for 5 min–3 h (*A1*, *B1*, *C1*). In a different experiment, static stretch was applied in conjunction with SB (*A2*, *B2*, *C2*). Subsequently, cells were lysed and Western blot analysis was performed. Antibodies against specific phosphorylated form of p38 (*A1*, *A2*), GSK-3β (*B1*, *B2*), and Akt1 (*C1*, *C2*) [denoted p38-p, GSK-3β-p(Ser9), and Akt1-p(Ser473), respectively] were used to visualize the level of phosphorylation for each protein. In addition, antibodies against all p38, GSK-3β, and Akt1 proteins were used to verify equal amount of proteins loaded. For each experiment, a native control (*lane C*) without stretch and SB incubation was conducted, and densitometry was performed to plot the relative increase or decrease in phosphorylated proteins. $*P < 0.05$. $**P < 0.005$.

NFATc1 and MEF2A. Moreover, a comparison of NFATc1 phosphorylation-dephosphorylation in the presence of SB-203580 when static stretch was applied suggests that p38 is involved in NFATc1 phosphorylation, whereas calcineurin is involved in its dephosphorylation. Interestingly, in the case of MEF2A, a comparison of Figs. 4*B* and 6*B* suggests that in response to short-term static stretch, p38 and calcineurin also have opposite effects on MEF2A phosphorylation. However, even if p38 promotes MEF2A phosphorylation in response to short-term static stretch, this kinase is also involved in the dephosphorylation of MEF2A over long-term static stretch (>30 min) in association with calcineurin, because CsA inhibits any change in MEF2A phosphorylation over the entire time course when static stretch is applied. Therefore, it seems that without p38 activation, stretch-induced MEF2A dephosphorylation cannot be sustained, suggesting that p38 is acting in a permissive manner.

Nuclear localization of MEF2A and NFATc1. In different cell types, movement of MEF2A and NFATc1 from the cytoplasm to the nucleus has been shown to be dependent on p38 and/or calcineurin (9, 18, 35, 43, 44, 47–49). By labeling both NFATc1 and MEF2A, the effect of static stretch on nuclear

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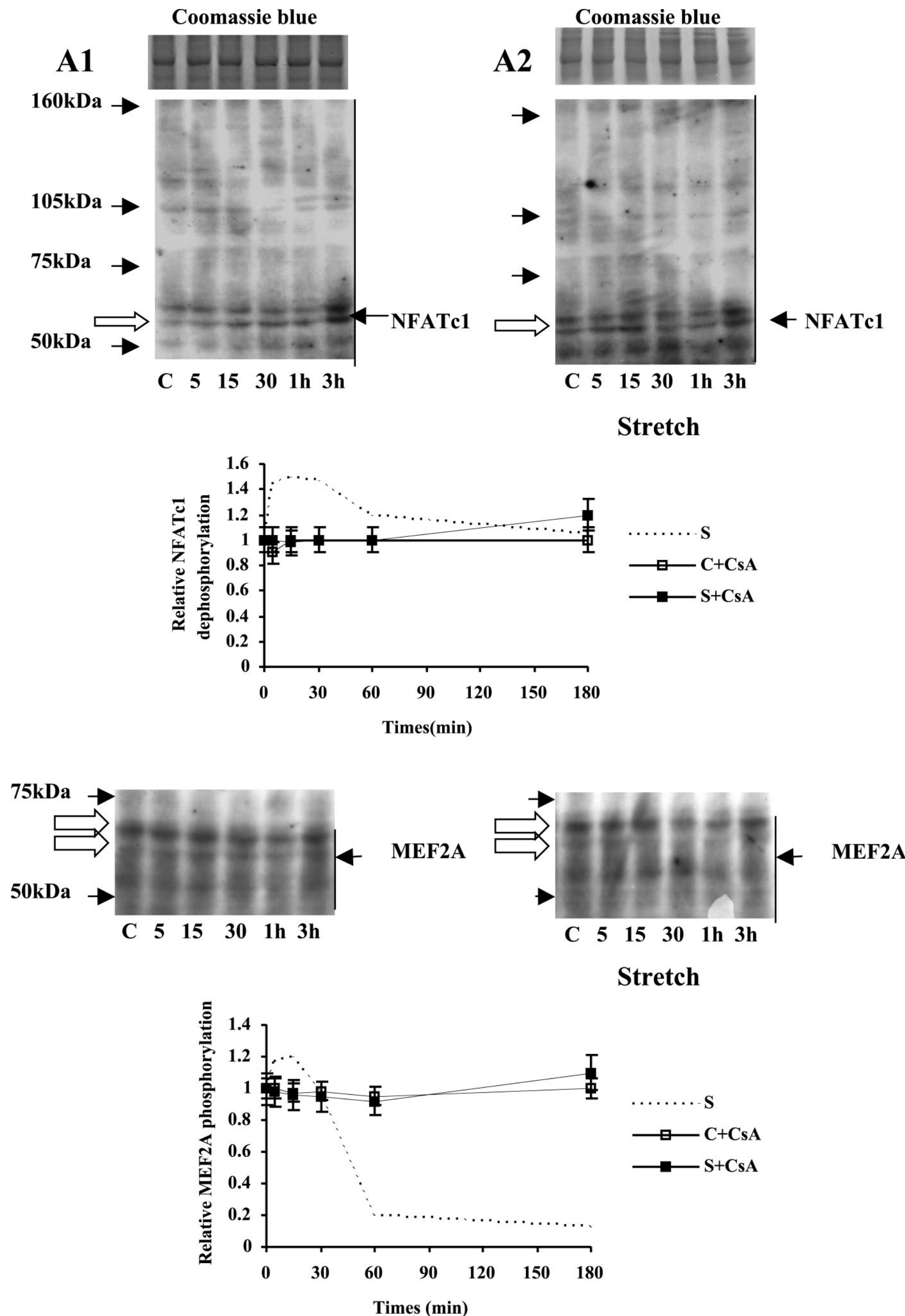

Fig. 6. Effect of static stretch application and p38 and calcineurin inhibitor cyclosporine A (CsA) incubation on NFATc1 and MEF2A phosphorylation states. Five minutes before static stretch application, CsA was incubated at a final concentration of 4 μM. CsA was incubated with control cells (unstretched) for 5 min–3 h (*A1*, *B1*). In a different experiment, static stretch was applied in conjunction with CsA for the same time course (*A2*, *B2*). Cells were then lysed, Western blotting was performed, and antibodies against all of the proteins were used to visualize shift in protein migration characteristic of the phosphorylated form of NFATc1 (*A1*, *A2*) and MEF2A (*B1*, *B2*). For each experiment, a native control experiment (*lane C*) without stretch and CsA incubation was conducted. Coomassie blue was used to verify equal amount of protein loaded, and densitometry was used to plot the relative increase or decrease of phosphorylated proteins. C+CsA, incubation of CsA only; S+CsA, static stretch application in the presence of CsA incubation. $*P < 0.05$. $**P < 0.005$.

translocation of these transcription factors was examined for a 1-h period with or without drug (SB-203580 or CsA) incubation (Fig. 8*A*). Application of a static stretch triggered MEF2A nuclear translocation in contrast to the control. Moreover, when SB-203580 or CsA was incubated with static stretch, no significant changes in MEF2A nuclear translocation were observed. Interestingly, we did not observe NFATc1 nuclear translocation under static stretch, and, unexpectedly, we did not observe NFATc1 nuclear translocation when cells were incubated with SB-203580 in the presence or absence of static stretch over a 1-h period, even though this transcription factor was dephosphorylated under these conditions (Fig. 4*B*). We also measured the fluorescence intensity and plotted the frequency of occurrence for a given intensity as a function of the intensity on a scale varying between 0 (no signal) and 250 (saturating signal), allowing us to accurately describe the profile of nuclear translocation of these transcription factors. Figure 8*B* shows that the treatments applied did not significantly change NFATc1 nuclear intensity as represented by Gauss-like curves concentrated in an intensity range of 25–50. However, in contrast to NFATc1, static stretch triggered a strong shift of MEF2A nuclear intensity (Fig. 8*B*), demonstrating that the quantity of MEF2A in the nuclei could be increased up to 10-fold compared with control values. Moreover, incubation of either SB-203580 or CsA inhibited

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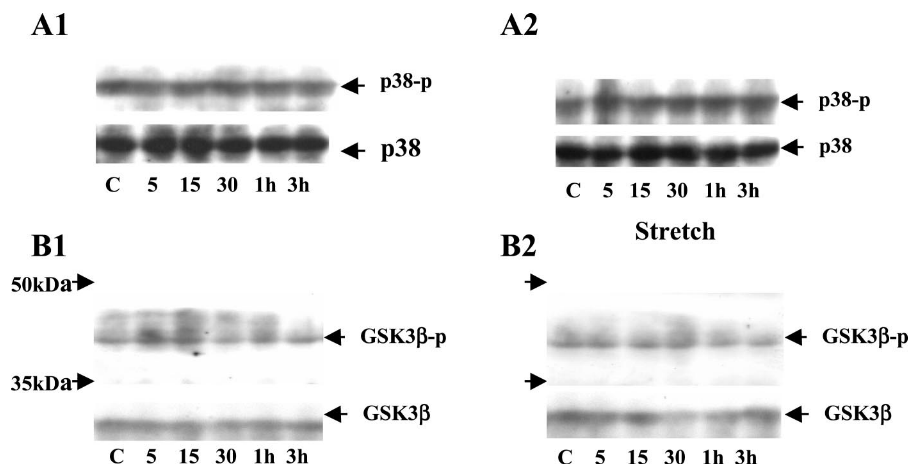

Fig. 7. Effect of static stretch application and p38 and calcineurin inhibitor CsA incubation on p38 and GSK-3β phosphorylation states. Five minutes before static stretch application, CsA was incubated at a final concentration of 4 μM. CsA was incubated with control cells (unstretched) for 5 min–3 h (*A1*, *B1*). In a different experiment, static stretch was applied in conjunction with CsA for the same time course (*A2*, *B2*). Cells were then lysed, and Western blotting was performed. Antibodies against the specific phosphorylated form of p38 (*A*) and GSK-3β (*B*), denoted p38-p and GSK-3β-p (Ser^9), respectively, were used to visualize the level of phosphorylation for each protein. In addition, antibodies against all p38 and GSK-3β proteins were used to verify equal amount of proteins loaded. For each experiment, a native control experiment (*lane C*) without stretch and CsA incubation was conducted.

this stretch-induced effect, recentering Gauss-like curves in an intensity range of 25–50, similar to control values.

Neonatal MHC expression. MEF2 transcription factors are known to regulate different isoforms of MHC during exercise or electrical stimulation in fully differentiated skeletal muscle. After 2 days in differentiation medium, C_2C_{12} cells are committed to differentiation. Because MEF2A is translocated into the nucleus of C_2C_{12} cells when they are submitted to a static stretch and MEF2A is involved in muscle differentiation (3, 18, 24, 29, 31, 32), we examined whether static stretch application could promote the initially expressed neonatal form of neonatal MHC (MHCneo). It was observed that static stretch stimulated an ~30% increase in MHCneo compared with the control when applied overnight, e.g., corresponding to 18 h (Fig. 9) ($P < 0.05$). However, both SB-203580 and CsA inhibited this increase (Fig. 9).

DISCUSSION

We have recently shown that 2 days of static stretch applied to C_2C_{12} myocytes accelerated their differentiation as exemplified by an accelerated rate of adult MHC expression and alterations in myogenic regulatory factor levels (unpublished observations). A number of studies have shown that stretch of muscle cells both in vivo and in vitro can induce expression of growth factors that may act in an autocrine-paracrine manner (11, 25, 33, 45, 46). To date, there has been little investigation into the early signaling events directly elicited by mechanical stimuli in skeletal muscle. In this study, we examined some of the potential second messenger signaling factors that could be involved in mediating early stretch-induced responses and how these factors might play a role in the activation of the MEF2 and NFAT transcription factors.

Role of p38 in stretch-induced NFATc1 and MEF2A phosphorylation. p38 is a member of the stress-activated kinase family, and p38 α- and β-isoforms have been shown to be involved in myogenic differentiation (8, 12, 44, 48). Moreover, p38 isoforms α and β also are involved in the transcriptional regulation of MEF2 proteins via direct phosphorylation (10, 44, 47, 49). In addition, MEF2 and NFAT proteins in association with calcineurin are known to be involved in the regulation of muscle fiber types in adult skeletal muscle (14, 43). Furthermore, because both p38 and MEF2A have been shown to play a role in muscle differentiation, it might be expected that cross talk between these factors could occur during the earlier stages of the differentiation process.

We initially examined the effect of static stretch on the phosphorylation states of the potential modulators of muscle phenotype and/or differentiation MEF2A, MEF2C, and NFATc1. Whereas we observed no changes in MEF2C phosphorylation during the time course studied in response to static stretch (data not shown), a transitory dephosphorylation of NFATc1 was observed during the initial 1-h period. In contrast, the same stretch stimulus triggered no change in MEF2A phosphorylation during the initial 30 min, but this was followed by almost complete dephosphorylation. Different second messenger signaling pathways have been shown to be involved in these phosphorylation processes. Our results demonstrate a decrease in Akt phosphorylation in response to stretch during the time course studied, suggesting that PI3-K activation is not involved (41). In contrast, we found an increase in both p38 and GSK-3β phosphorylation.

To further examine the role of p38, we used SB-203580, a specific downstream inhibitor of activated p38-p (1, 7). Incubation with the drug in the presence of static stretch resulted in sustained NFATc1 dephosphorylation for almost the entire time course studied, as opposed to stretch alone, in which this dephosphorylation was transient. Under the same conditions, the profile of MEF2A phosphorylation-dephosphorylation was the reverse of that seen in response to static stretch alone. Interestingly, we observed that both GSK-3β and Akt phosphorylation were altered with SB-203580 incubation. Therefore, we can presume that cross talk may exist between Akt and p38 on the one hand and GSK-3β and p38 on the other when the stretch is applied.

The mechanism by which a mechanical signal is transduced into intracellular phosphorylation events is at present unclear. However, Aikawa et al. (1) suggested that in cardiac myocytes, mechanical stress is directly sustained by integrins, which in turn activate focal adhesion kinase (FAK), an integrin-associated kinase. It was suggested that phosphorylation of FAK, in turn, leads directly to phosphorylation of p38. Among other putative mechanosensors, such as the involvement of ion channels, this one could explain our observations. However,

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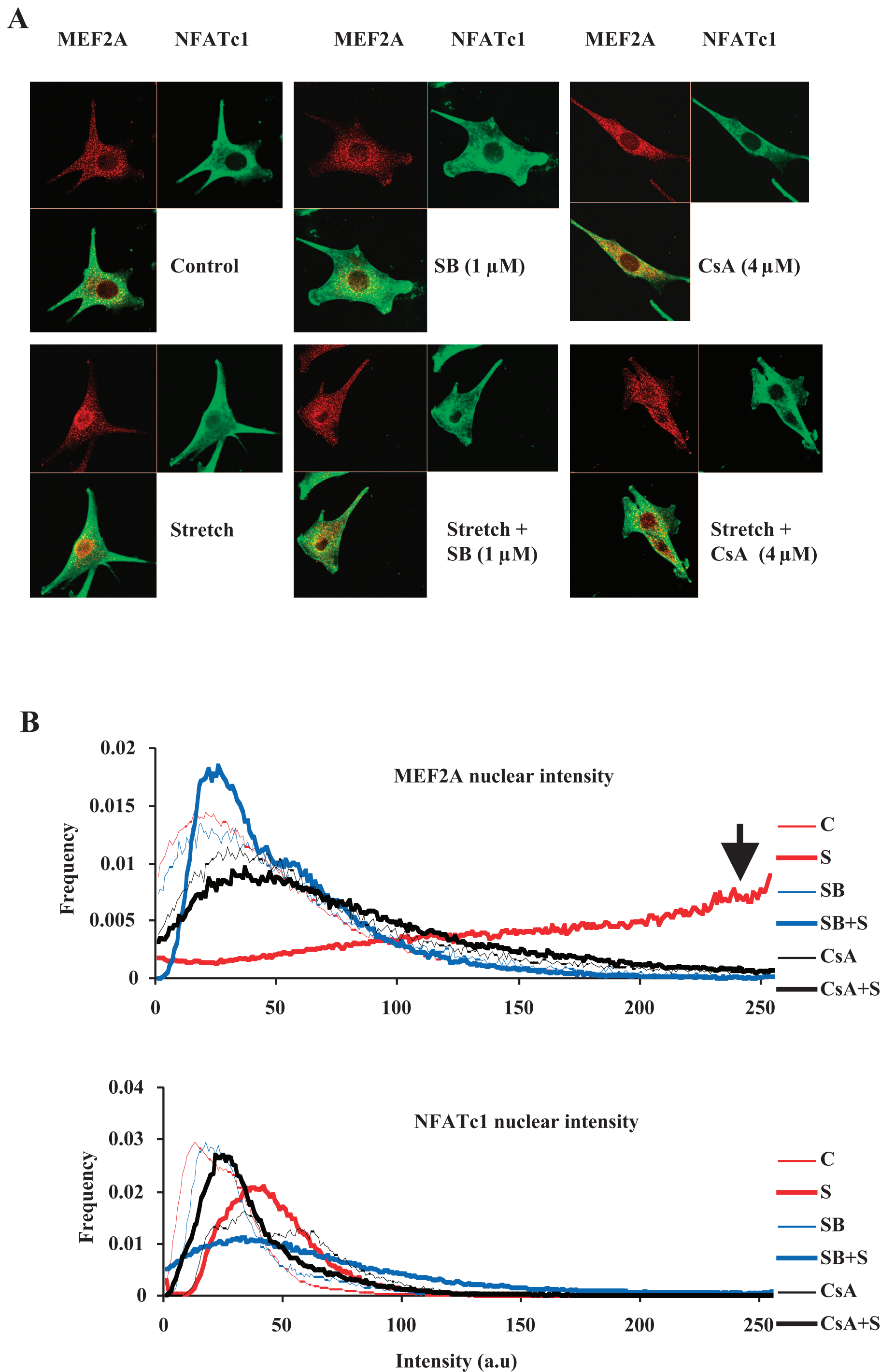

Fig. 8. Effect of CsA or SB incubation on NFATc1 and MEF2A nuclear translocation induced by application of static stretch. Five minutes before static stretch application, CsA or SB was incubated at a final concentration of 4 or 1 µM, respectively. When necessary, C_2C_{12} cells were stretched for 1 h. Labeling of both MEF2A and NFATc1 was performed to observe nuclear localization under the different treatments (*A*). Measurement of nuclear fluorescence intensities was performed to determine the level of both proteins in the nuclei (*B*). C, control.

whether a similar mechanism is involved in the response of C_2C_{12} cells to static stretch remains to be elucidated.

Effects of calcineurin on stretch-induced phosphorylation of NFATc1 and MEF2. The Ca^{2+}-dependent phosphatase calcineurin is known to be involved in the regulation of muscle phenotypes via NFAT and MEF2 activation (9, 14, 43) as well as during differentiation (17, 18, 35). Therefore, using CsA, we decided to investigate how the inhibition of p38 and calcineurin activities would change the profile of NFATc1 and MEF2A phosphorylation-dephosphorylation (28). The addition of CsA in conjunction with passive stretch inhibited change in both MEF2A and NFATc1. Moreover, comparison of our data regarding the effects of SB-203580 and CsA incubation suggests the existence of competition between p38 and calcineurin with regard to MEF2A and NFATc1 phosphorylation states. NFATc1 dephosphorylation occurs via calcineurin, in contrast to p38, which seems to promote its phosphorylation. With regard to MEF2A, calcineurin seems to promote its dephosphorylation during the short exposure to static stretch, whereas p38 tends to promote its phosphorylation. However, during a

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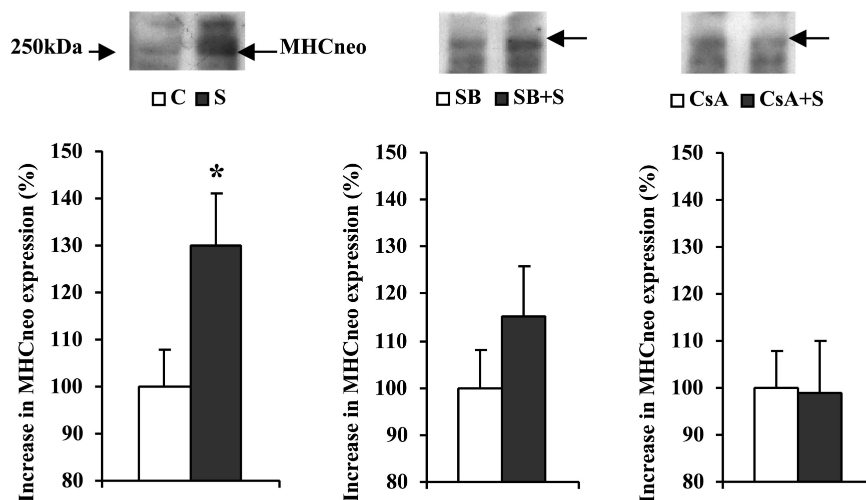

Fig. 9. Effect of static stretch application overnight and/or CsA or SB incubation on the neonatal myosin heavy chain (MHC) isoform (MHCneo) expression. Five minutes before static stretch application, CsA or SB was incubated at a final concentration of 4 or 1 μM, respectively. When necessary, C_2C_{12} cells were stretched overnight. Protein lysates were used for Western blot analysis to examine the expression of MHCneo. Densitometry was used to plot the changes in the expression of this protein. $^*P < 0.05$.

longer-term, 3-h static stretch, it seems unlikely that calcineurin alone is involved in a biphasic effect on MEF2A phosphorylation, mainly because the Ca^{2+}-dependent calcineurin is a phosphatase. This result suggests that another protein might be involved during long-term stretch that is upregulated (or activated) by SB-203580 administration and sensitive to CsA. That different signaling factors (p38 and calcineurin) are recruited to the differentiation process indicates that both second messengers could be involved in a specific manner in stretch-induced acceleration of skeletal muscle differentiation. Because this process is multifaceted and involves altered gene expression, cell fusion, and growth, it is likely that a number of parallel pathways are involved.

Nuclear translocation of NFATc1 and MEF2. MEF2A and NFATc1 are transcription factors, and we examined whether they were translocated to the nucleus in response to stretch. After stretching cells for 15 min and 1 h, we stained both NFATc1 and MEF2A with or without drug incubation. At 15 min, we observed no stretch-induced change in nuclear translocation for either factor (data not shown). After a 1-h static stretch, MEF2A, but not NFATc1, nuclear translocation was demonstrated. In addition, both SB-203580 and CsA treatment inhibited MEF2A nuclear translocation. Our observations concerning NFATc1 are in contrast to previous results obtained in cardiac muscle, which showed continuous dephosphorylation of NFATc1 using a dominant negative of p38 to trigger its nuclear translocation and cell hypertrophy in a calcineurin-dependent manner (7). A number of possible explanations concerning the absence of NFATc1 nuclear translocation can be suggested. First, when stretch is applied without drug incubation, NFATc1 is only temporarily dephosphorylated by calcineurin, and this might be insufficient to promote its nuclear import at the myocyte stage compared with fully differentiated myotubes. Second, GSK-3β activity has been shown to promote NFAT nuclear export (5, 42). Therefore, after 1 h of static stretch application, GSK-3β may not be sufficiently deactivated, because it is maximally deactivated at 30 min after the initiation of the stretch stimulus. Third, dephosphorylation of NFATc1 at different sites can occur and might be highly complex with regard to import and export to the nucleus.

Potential relationship between p38 and calcineurin due to static stretch. Studies of different cell types have shown that p38 activation induces MEF2A phosphorylation and subsequent nuclear translocation, allowing MEF2A/DNA interaction and transcription (10, 27, 43, 47–49). However, other studies performed notably on C_2C_{12} myoblasts have shown that calcineurin promotes MEF2A dephosphorylation, which increases its transcriptional activity (27, 43). In the present study, MEF2A phosphorylation seemed to be tightly controlled by both p38 and calcineurin. Although our results have shown MEF2A hypophosphorylation and nuclear location after 1 h of static stretch, this was prevented by SB-203580 incubation, which promoted its phosphorylation and inhibited its nuclear localization. These results suggest that both p38 and calcineurin activation are necessary to trigger and maintain MEF2A nuclear translocation, allowing its hypophosphorylated state to potentially modulate transcription. Interestingly, however, SB-203580 incubation alone seems to promote MEF2A phosphorylation similar to that observed during longer-term stretch (1–3 h). This suggests that p38 might indirectly antagonize MEF2A hyper-phosphorylation, impairing its nuclear import or participating in its nuclear export. At this stage, the signaling factors responsible for this effect are unknown. However, the recent study of MEF2A phosphorylation by Cox et al. (10) using mass spectroscopy demonstrated that MEF2A is phosphorylated at different sites in response to different kinases. They proposed that among these kinases, GSK-3β activity is probably involved in the phosphorylation of MEF2A on Ser^{255}. It is also noteworthy that studies have shown that GSK-3β activation negatively regulates NFAT nuclear translocation (42) and that, conversely, deactivation of GSK-3β via phosphorylation on Ser^{9} promotes NFAT nuclear translocation and hypertrophy in both skeletal myotubes and cardiac muscle in vivo (5, 42). Because MEF2A and NFAT are transcription factors involved in expression of contractile proteins and are also implicated in change of phenotype as well as hypertrophy (14, 21), we may expect a common regulation of these proteins, probably involving GSK-3β. This hypothesis is supported by the fact that under static stretch application, our results show cross talk between p38 and GSK-3β, because incubation of SB-203580 can alter increases of GSK-3β phos-

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phorylation on Ser[9] (Fig. 5*B*). It seems, therefore, that both p38 and calcineurin, certainly in association with at least one other signaling protein, play a complex role in MEF2A nuclear translocation in response to a mechanical stimulus. A summarized view containing this hypothesis is shown in Fig. 10.

The present study emphasizes the important role of p38 in MEF2 activation in response to a mechanical stimulus in skeletal muscle cells at an early stage of differentiation. Furthermore, this activation is rapid and transient. However, recent studies have shown that this pathway may also be activated in fully differentiated skeletal muscle in response to mechanical stimuli. Al-Khalili et al. (3) demonstrated that contraction of isolated rat epitrochlearis muscles causes activation of MEF2 DNA binding that is inhibited by SB-203580.

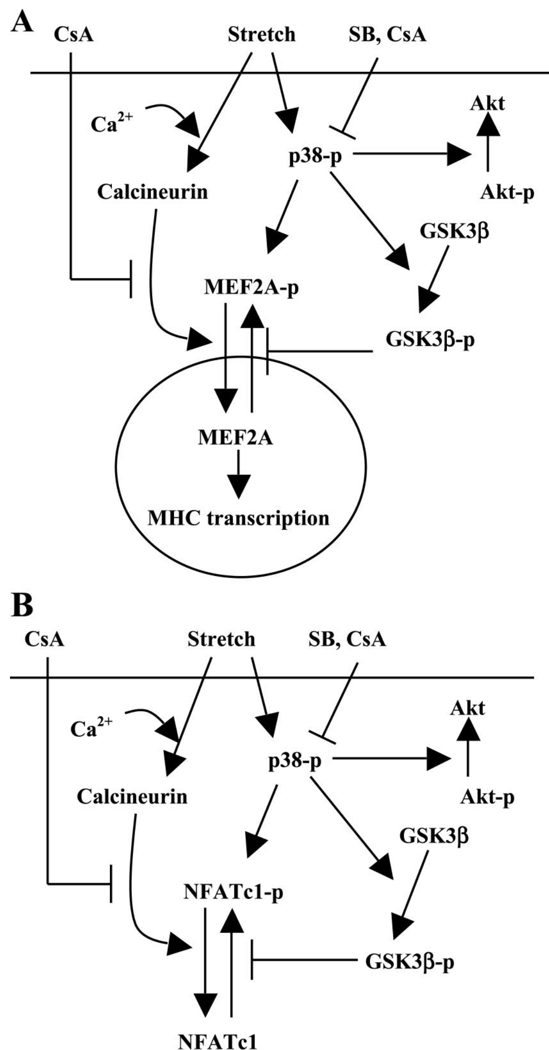

Fig. 10. Diagrams showing the signaling pathway elicited by a short-term application of static stretch on NFATc1 and MEF2A. *A*: summary of MEF2A potential activation, including both calcineurin and p38 as necessary to promote the final hypophosphorylated form of MEF2A, allowing its translocation in the cell's nucleus and inducing potential expression of contractile proteins such as MHCneo. This diagram also proposes the potential involvement of another kinase, such as GSK-3β, that could increase the MEF2A hyperphosphorylated form, impairing its ability to be maintained in nuclei. *B*: summary of NFATc1 potential activation, including both calcineurin and p38, and involvement in the change of phosphorylation of NFATc1. Nonetheless, in contrast to MEF2A, results have not shown significant nuclear accumulation of NFATc1.

Stretch-induced upregulation of neonatal MHC. The transcription factor of MEF2 is known to regulate the expression of MHC isoforms in striated muscle cells (9, 14, 15, 43). Moreover, increase in MEF2 activity leads to MHC expression in rhabdomyosarcoma (34). Therefore, when we applied an overnight static stretch, we observed an increase in the protein level of MHCneo, suggesting an accelerated rate of differentiation of the C_2C_{12} myocytes. Both SB-203580 and CsA prevented this. However, at this stage, we cannot rule out that cofactors are also triggered by static stretch and are sensitive to the inhibitors used. For example, the possible expression of growth factors such as IGF-I as a specific response to overnight passive stretch could be involved in accelerated MHCneo expression. Studies are ongoing to clarify this point. Therefore, expression of MHCneo might not be triggered by MEF2A nuclear translocation alone, because possible involvement of other cellular elements cannot be ruled out.

CONCLUSIONS

Environmental changes involved in biological tissues are suspected to take part at many levels, occurring spontaneously during embryogenesis, in association with morphogenetic movements, or in direct relation with the physical environment in developing or completely differentiated tissues (9, 14, 16, 36–40, 43).

More specifically, environmental changes are known to trigger adaptive responses in muscle phenotype via expression of contractile proteins such as MHC isoforms. In the present study, we aimed to define whether static stretch application on myocytes could change the rate of the differentiation process with regard to MHC expression as well as which short-term signaling pathways might be involved. We focused our study on myocytes to determine how MEF2A and NFATc1 are regulated in response to mechanical strain. We showed that the involvement of calcineurin and p38 are crucial, but also complex, in promoting MEF2A nuclear translocation. We also demonstrated that neonatal MHC expression is influenced by static stretch. The rapid activation of signaling pathways in response to a mechanical stimulus suggests that even a short-term stimulus may elicit a signaling cascade that could lead to an acceleration of differentiation. This could lead to an accelerated expression of contractile proteins such as MHC that in turn could accelerate sarcomere formation with concomitant earlier functional contractility within the myotube. During development, muscle fibers are formed in waves, with the majority of secondary fibers forming on a scaffold of earlier-formed primary fibers. Mechanical stimuli elicited by either gradual limb elongation or movement of the embryo in utero may play a crucial role in the rate of development of these fibers. There is also evidence that mechanical stimuli may play an important role in maintaining the differentiated state in the postnatal animal (16). Our laboratory (26) previously showed that stretch induces a slow phenotype in adult muscle, whereas the opposite is the case when stretch is prevented. Recent in vivo and in vitro studies have suggested that mechanical stimuli in adult muscle also induce MEF2 activation in a p38-dependent manner (3). Whether these signaling pathways

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are crucial to mechanical regulation of postnatal muscle phenotype is at present unclear, however. Therefore, we support the network of evidence that exogenous stimuli such as static stretch contribute to MHC expression even during the early stages of skeletal muscle development, potentially changing the rate of differentiation.

GRANTS

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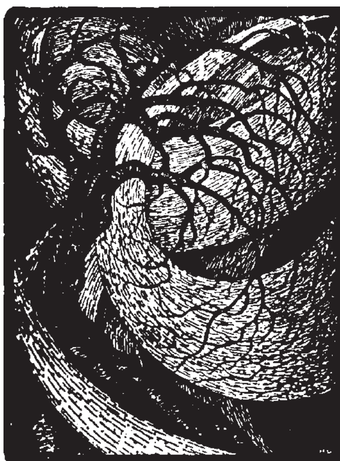

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