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Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism

Jun-ichi Sadoshima and Seigo Izumo[1]

Molecular Medicine and Cardiovascular Divisions, Beth Israel Hospital, and Department of Medicine and Program in Cell and Developmental Biology, Harvard Medical School, 330 Brookline Ave, Boston, MA 02215, USA
[1]Corresponding author

It is well known that external load plays a critical role in determining cardiac muscle mass and its phenotype, but little is known as to how mechanical load is transduced into intracellular signals regulating gene expression. To address this question we analyzed the 'mechano-transcription' coupling process using an *in vitro* model of load-induced cardiac hypertrophy, in which a stretch of rat cardiac myocytes, grown on a deformable substrate, causes a rapid induction of immediate-early genes followed by growth (hypertrophic) response. We report here that cell stretch rapidly activates a plethora of second messenger pathways, including tyrosine kinases, $p21^{ras}$, mitogen-activated protein (MAP) kinases, S6 kinases ($pp90^{RSK}$), protein kinase C, phospholipase C, phospholipase D, and probably the phospholipase A_2 and P450 pathways. In contrast, the cAMP pathway is not activated significantly by stretch. The signals generated by these second messengers appear to converge into activation of the $p67^{SRF}-p62^{TCF}$ complex via the serum response element, causing induction of c-*fos*. The stretch response may involve an autocrine or paracrine mechanism, because stretch-conditioned medium, when transferred to non-stretched myocytes, mimicked the effect of stretch. These results indicate that mechanical load causes rapid activation of multiple second messenger systems, which may in turn initiate a cascade of hypertrophic response of cardiac myocytes.

Key words: immediate-early genes/MAP kinases/phospholipases/protein kinase C/tyrosine kinases

Introduction

In living animals, many types of cells are normally exposed to a variety of internal and external forces. Although it is well known that mechanical forces have a variety of effects on the structure and function of the cells, little is known as to how mechanical stimuli are converted into intracellular signals of gene regulation (reviewed in Ingber, 1991; Vandenburgh, 1992). This contrasts with a wealth of literature concerning the mechanisms of action of soluble growth factors, such as platelet derived growth factor (PDGF), epidermal growth factor (EGF) and bombesin (Williams, 1989; Ullrich and Schlessinger, 1990; Rozengurt, 1991), or that concerning the mechanism of action of intracellular regulators, such as the products of the *ras*, *src*, *myc* and *jun* oncogenes (Bishop, 1991; Cantley *et al.*, 1991).

External load plays a critical role in determining muscle mass and its phenotype in both cardiac and skeletal muscles *in vivo* (reviewed in Morgan and Baker, 1991; Vandenburgh, 1992). The first direct evidence that muscle cells are able to sense the external load, in the absence of neuronal or hormonal factors, came from a study by Vandenburgh and Kaufman (1979) who demonstrated that cultured chick skeletal muscle cells grown on an elastic substrate underwent hypertrophy (increase in cell size without cell division) in response to static stretch of the substrate. A similar phenomenon was observed in adult cardiocytes (Mann *et al.*, 1989). More recently, it has been shown that applying stretch to neonatal cardiac myocytes in culture results in a transcriptional activation of c-*fos* proto-oncogene (Komuro *et al.*, 1990) and many other immediate-early (IE) genes (Sadoshima *et al.*, 1992a) and this was followed by the appearance of the hypertrophic phenotype (Komuro *et al.*, 1991; Sadoshima *et al.*, 1992a). Interestingly, stretch of primary cardiac non-muscle cells (a mixture of fibroblasts, endothelial cells and smooth muscle cells) causes a rapid induction of IE genes followed by cell hyperplasia, instead of the hypertrophy seen in myocytes (Sadoshima *et al.*, 1992a). These results suggest that cell stretch stimulates the growth of many cell types.

Several studies have reported changes in intracellular second messengers in response to a variety of mechanical stimuli. These include increases in inositol monophosphate (IP_1) and bisphosphate (IP_2) in cardiac myocytes (von Harsdorf *et al.*, 1989; Komuro *et al.*, 1991) and in inositol trisphosphate (IP_3) and tetrakisphosphate (IP_4) in smooth muscle (Kulik *et al.*, 1991), an elevation of intracellular Ca^{2+} in lung epithelial cells (Wirtz and Dobbs, 1990) and in vascular endothelium (Geiger *et al.*, 1992), and increases in cyclic AMP (cAMP) formation in S49 lymphoma cells (Watson, 1989) and prostaglandin release in skeletal myotubes (Vandenburgh *et al.*, 1990). However, these studies were performed in diverse cell types using different means of applying mechanical stimuli. Therefore, it is not known whether a defined mechanical stimulus causes activation of a single or multiple second messenger systems in a given cell type.

We have begun a systematic analysis to identify the signal transduction pathways of the 'mechano-transcription coupling' process using an *in vitro* model of stretch-induced cardiac hypertrophy (Sadoshima *et al.*, 1992a,b). Because this model allows us to apply a simple and controlled mechanical stimulus to cardiac myocytes cultured in a defined serum-free medium, it is a suitable system to dissect the signal transduction pathways of mechano-transcription coupling. In this study, we elected to examine the stretch-induced mechano-transcription coupling process in a reverse way, using c-*fos* as a model. We chose c-*fos* gene expression

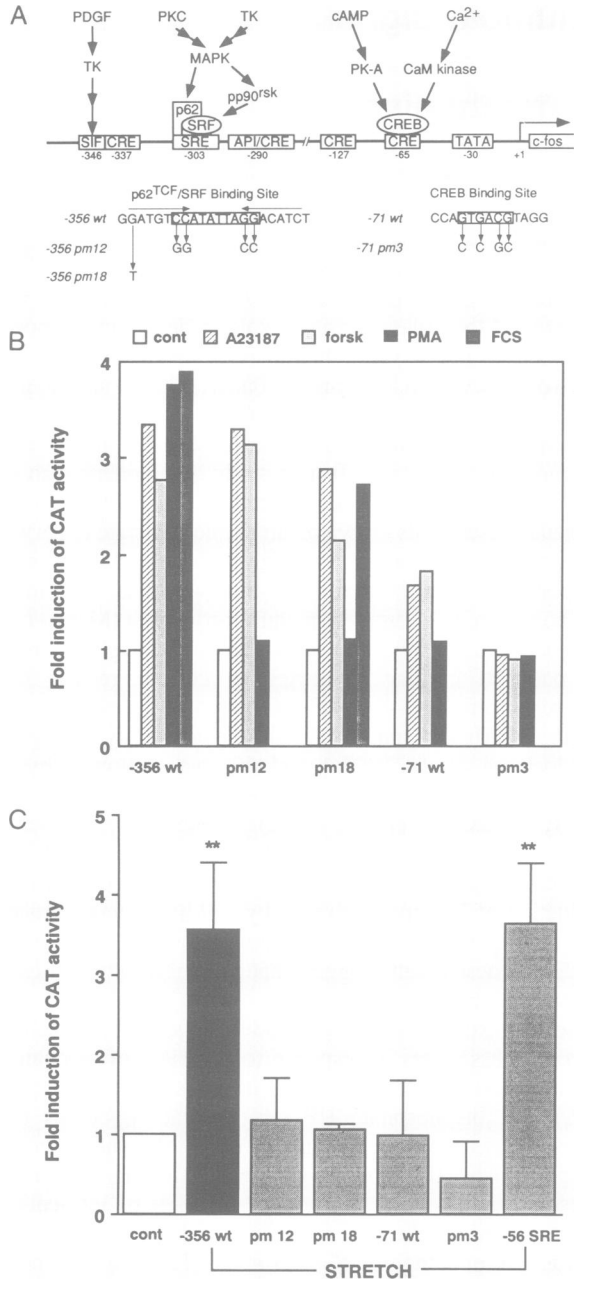

Fig. 1. Stretch-induced reporter gene activity of various c-*fos* CAT constructs. (**A**) Schematic representation of the mouse c-*fos* promoter region. Point mutations at the serum response element (SRE) and the Ca^{2+}/cAMP response element (CRE) are shown. Abbreviations: CAT, chloramphenicol acetyltransferase; PK-C, protein kinase C; PK-A, protein kinase A; CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; PDGF, platelet derived growth factor; SIF, *sis*/PDGF-inducible element; TK, tyrosine kinases; MAPK, MAP kinases; $pp90^{rsk}$, p90 ribosomal S6 kinases; $p62^{TCF}$, p62 ternary complex factor; TATA, TATA box; SRF, serum response factors; CREB, CRE binding proteins. (**B**) Cardiac myocytes were transfected with the indicated *fos*−CAT plasmids. Forty-eight hours after transfection, myocytes were stimulated with A23187 (1 μM), forskolin (forsk, 10 μM), PMA (1 μM) or fetal calf serum (FCS, 20%) for 2 h. For each construct, CAT activity was normalized to that of control without stimulation (cont). Data are the means of two or three transfection experiments. (**C**) Forty-eight hours after transfection, a 2 h stretch was applied. For each construct, stretch-induced CAT activity was normalized to that of control without stretch (cont). Data are mean ± SE of three to five independent transfection experiments. **, $P < 0.01$ versus non-stretched control.

as a 'nuclear marker' for load-induced signal transduction because: (i) the transcriptional activation of c-*fos* is one of the earliest nuclear responses of cardiac myocytes in response to mechanical load *in vivo* and *in vitro* (Izumo *et al.*, 1988; Komuro *et al.*, 1988, 1990; Sadoshima *et al.*, 1992a), and (ii) regulation of c-*fos* gene expression has been studied in detail in other cell systems and serves as a paradigm of gene regulation by second messengers generated by a variety of growth stimuli (Sheng and Greenberg, 1990). First, we identified the critical *cis*-acting DNA element necessary for stretch-responsiveness; second, we measured a number of intracellular second messengers in response to stretch; and third, we examined the roles of these second messengers in mediating c-*fos* induction by stretch.

Results

The 'stretch-responsive element' of the c-fos promoter maps to the serum response element

We first examined *cis*-acting elements necessary for stretch-responsiveness of the c-*fos* promoter. The c-*fos* promoter is known to contain two major inducible *cis*-acting elements, the serum response element (SRE) and the calcium/cAMP response element (Ca^{2+}/CRE) (Sheng and Greenberg, 1990). The SRE is located ~300 bp upstream of the mRNA initiation site and is the binding site of the SRF and its associated factor $p62^{TCF}$ (Figure 1A). The SRE mediates transcriptional activation by the protein kinase C (PKC) pathway as well as by PKC independent growth factor signaling pathways (Gilman, 1988; Treisman, 1990). The major Ca^{2+}/CRE is located ~65 bp upstream of the mRNA start site, but weaker yet functional CRE-like motifs also exist at −337, −290 and −127 bp upstream of the mouse c-*fos* promoter, (Berkowitz *et al.*, 1989). The CREs are the binding sites of CRE binding proteins (CREBP) and mediate transcriptional activation by cAMP and Ca^{2+} (Berkowitz *et al.*, 1989; Sheng and Greenberg, 1990). Although it has been shown that the sequence between −404 and −227, which contains multiple *cis*-acting elements [such as the c-*sis* response element, SRE, AP-1, E box and CRE-like motifs (Figure 1A)], was necessary for the stretch-induced c-*fos* expression (Komuro *et al.*, 1991), the precise location of the stretch-responsive element has not been determined.

To address this question, six c-*fos*−CAT reporter constructs were used. The −356wt and −71wt constructs contain −356 and −71 bp upstream of the mouse c-*fos* promoter, respectively. Point mutations in the SRE of the −356wt construct and in the Ca^{2+}/CRE of the −71wt construct were designated as pm12 and pm3, respectively (Figure 1A). Point mutation of these sites abolishes the response to serum or to cAMP, respectively, in BALB/c 3T3 fibroblasts (Gilman, 1988; Berkowitz *et al.*, 1989) and in cardiac myocytes (Figure 1B). Another point mutation designated as pm18 contains a single base change at the 5′ side of the SRE sequence (Figure 1A). The mutated SRE in pm18 has been shown to bind SRF but to fail to form the ternary complex with $p62^{TCF}$, resulting in the loss of response to PKC while retaining the response to serum through PKC-independent pathways in BALB/c 3T3 fibroblasts (Graham and Gilman, 1991). We have confirmed that in the cardiocytes, the pm18 construct also failed to respond to a direct PKC activator, phorbol 12-myristate

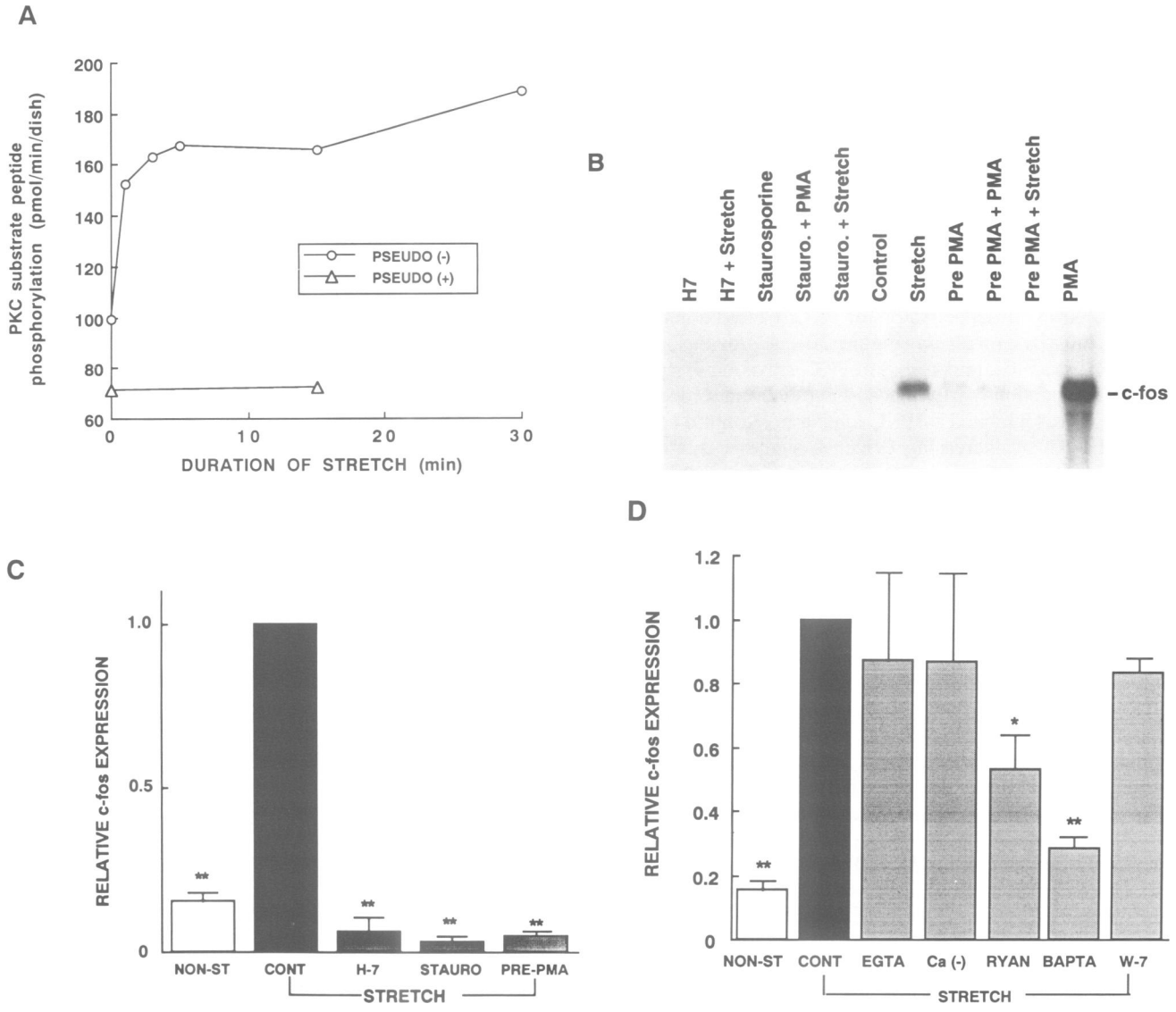

Fig. 2. Effects of PKC and Ca^{2+} on stretch-induced c-*fos* expression. (**A**) Stretch-induced protein kinase C (PKC) activation. PKC activity was measured by phosphorylation of a PKC-specific synthetic peptide substrate in a permeabilized cell assay in the presence (triangle) or absence (circle) of PKC pseudosubstrate peptide (30 μM, PSEUDO). Data are from a representative experiment performed in triplicate in each time point. (**B** and **C**) Effects of protein kinase inhibitors on stretch-induced c-*fos* expression as determined by Northern blot analysis. Cells were pretreated with inhibitors for 30 min (H-7 or staurosporine) or 24 h (PMA) and subsequently stretched for 30 min. A representative Northern blot is shown in (B). Densitometric analysis of multiple Northern blots is shown in (C). The result is expressed as a ratio to the value obtained from control stretch without drugs, which was set as 1. For each analysis, the hybridization signal was normalized to the signal obtained with a GAPDH probe to correct for possible variability of loading and transfer. (**D**) Effects of Ca^{2+} modulators on stretch-induced c-*fos* expression. Cells were pretreated for 30 min (EGTA, Ca^{2+}-free, or W-7), 1 h (BAPTA-AM) or 12 h (ryanodine) and were subsequently stretched for 30 min. The Ca^{2+}-free medium was applied after three washes with the same Ca^{2+}-free medium. Densitometric analysis of Northern blots was done as in (C). Abbreviations and drug concentrations used are as follows; NOT-ST, non-stretch; CONT, Control stretch; H-7 (10 μM); STAURO, staurosporine (1 μM); PMA, phorbol 12-myristate, 13 acetate (1 μM); Pre PMA, pre-treatment with PMA (1 μM) for 24 h; EGTA, 4 mM; Ca (-), Ca-free + EGTA (1 mM); RYAN, ryanodine (10 μM); BAPTA, BAPTA-AM (100 μM); W-7 (10 μM). Results are mean ± SE obtained from three to seven separate experiments. *, $P < 0.05$; **, $P < 0.001$ versus control stretch.

13-acetate (PMA), while retaining the response to stimulation by serum, forskolin (cAMP) and Ca^{2+} ionophore (Figure 1B).

These constructs were transiently transfected into cardiac myocytes and induction of CAT activity was examined after stretching cells for 2 h. As shown in Figure 1C, only the −356wt construct exhibited significant stretch-inducible CAT activity, and the point mutations in the SRE (pm12 and pm18) abolished the stretch responsiveness to the otherwise intact c-*fos* promoter. This suggests that the stretch response element maps to the SRE. Moreover, failure of induction of CAT activity in pm18, which contains only a single base mutation at position −318, suggests that the PKC- and $p62^{TCF}$-dependent pathway is essential. The −71wt, pm18 and pm12 constructs contain the intact Ca^{2+}/CRE motifs and all responded to Ca^{2+} ionophore stimulation (Figure 1B). The failure of induction by stretch in these constructs (Figure 1C) suggests that the Ca^{2+}/CRE alone is not sufficient to confer stretch-responsiveness on the c-*fos* promoter. To determine whether the SRE alone is sufficient to confer stretch-responsiveness, we transfected another construct (SRE-56) in which one copy of SRE was ligated to a minimum c-*fos* promoter containing −56 bp upstream sequence (Grueneberg *et al.*, 1992). This construct

responded to stretch to a similar degree as the −356wt construct (Figure 1C), confirming that the stretch response element maps to the SRE.

Stretch activates PKC and PKC is necessary for stretch-induced IE gene expression

The results of the DNA transfection experiments indicated that the stretch response element requires not only the inner core of the SRE but also the $p62^{TCF}$ binding site, a target site for PKC-dependent activation of c-*fos*. Therefore, we next examined the role of PKC in stretch-induced c-*fos* expression. PMA (1 μM) and phorbol 12,13-dibutyrate (PDBu, 2 μM), direct activators of PKC, caused a marked increase in c-*fos* expression (Figure 2B). A previous study suggested a potential importance of PKC activation in stretch signal transduction partly because stretch-induced c-*fos* expression was inhibited by PKC inhibitors (Komuro *et al.*, 1991). However, increasing evidence suggests that these drugs may in some cases act as general kinase inhibitors when used *in vivo* (Hannun *et al.*, 1991). Furthermore, the previous study did not observe an increase in IP_3 levels and did not measure diacylglycerol (DAG) content or PKC activity (Komuro *et al.*, 1991). This raises the question of whether PKC activation actually occurs in response to stretch. An alternative explanation for the suppressive effects of PKC inhibitors might be that PKC is not activated above baseline by stretch but that its basal level of activity plays a permissive role in stretch-induced c-*fos* expression. Therefore, we examined whether stretch causes activation of PKC, as measured by phosphorylation of a PKC-specific synthetic peptide substrate (GS peptide) in a permeabilized cell assay (Alexander *et al.*, 1990). As shown in Figure 2A, stretch caused a rapid doubling in PKC activity; this activation persisted for at least 30 min. Treatment of cells with PMA caused a 3-fold increase in PKC activity in the same assay (data not shown). Addition of a PKC pseudosubstrate peptide (House and Kemp, 1987) inhibited both basal and stretch-induced phosphorylation of the GS peptide (Figure 2A), indicating that phosphorylation of the GS peptide is specific to PKC.

We next examined whether PKC activation is necessary for c-*fos* induction. H-7 (10 μM) and staurosporine (1 μM), general inhibitors of protein kinases, completely suppressed stretch-induced c-*fos* expression (Figure 2B and C) in agreement with a previous result (Komuro *et al.*, 1991). A 24 h pretreatment of cardiac myocytes with PDBu (2 μM) or PMA (1 μM), which has been shown to downregulate PKC activity in the same cell type as the one used here (Henrich and Simpson, 1988), also significantly blocked induction of c-*fos* by PMA as well as by stretch (Figure 2B and C). Similar results were obtained using Egr-1/*zif*-268 gene, which also contains the SRE (Christy and Nathans, 1989), as a nuclear marker (data not shown). These results corroborate the transfection data (Figure 1) and suggest that stretch activates PKC and that PKC activation is necessary for stretch-induced expression of IE genes.

Role of Ca^{2+} in mediating stretch-induced c-fos expression

The DNA transfection experiments presented above suggested that the Ca^{2+}/CRE is not sufficient to confer stretch-responsiveness on the c-*fos* promoter. We next examined the role(s) of Ca^{2+} in mediating the stretch response. In non-stretched cardiac myocytes, treatment with the Ca^{2+} ionophore, A 23187 (0.3 μM), induced a 6.6-fold increase in c-*fos* expression, suggesting that Ca^{2+} alone can be a trigger of c-*fos* expression in cardiac myocytes. The addition of 4 mM EGTA (which gave a calculated extracellular Ca^{2+} concentration below 100 nM) or changing the culture medium to a Ca^{2+}-free EGTA (1 mM) medium (which gave a calculated extracellular Ca^{2+} concentration below 10 nM) showed no statistically significant reduction in the level of stretch-induced c-*fos* expression (Figure 2D). In contrast, prior depletion of the intracellular stores of Ca^{2+} by ryanodine (10 μM) (Meissner, 1986) or by buffering intracellular free Ca^{2+} by membrane-permeable BAPTA-AM (100 μM) (Tsien, 1980) significantly attenuated stretch-induced c-*fos* expression (Figure 2D). [Although stretch-induced release of IP_3 (see below) should result in Ca^{2+} release from intracellular stores, we do not know to what extent cell stretch caused an increase in intracellular Ca^{2+} concentration because of the technical difficulties in accommodating our stretch device to the fluorometer.] On the other hand, a calmodulin antagonist W7 at a concentration which fully inhibits Ca^{2+}/calmodulin-dependent protein kinase (Ca^{2+}/CaM kinase) activity in PC12 cells (Morgan and Curran, 1986) and in cultured cardiac cells (Sei *et al.*, 1991), failed to inhibit stretch-induced c-*fos* expression (Figure 2D). These results suggest that Ca^{2+} influx from the extracellular space or activation of Ca^{2+}/CaM kinase do not seem necessary for stretch-induced c-*fos* expression but certain levels of intracellular Ca^{2+} may play a permissive role in stretch-induced signal transduction.

Stretch activates phospholipase C

We next examined how PKC is activated by cell stretching. DAG has been shown to be an endogenous activator of PKC (Nishizuka, 1988). One of the pathways of DAG formation is hydrolysis of phosphatidylinositol bisphosphate (PIP_2) by activation of phospholipase C (PLC), which results in production of DAG and IP_3 (Berridge *et al.*, 1984). Previous reports have shown that stretch of cardiac muscle cells causes an increase in the production of IP and IP_2 but not IP_3 (von Harsdorf *et al.*, 1989; Komuro *et al.*, 1991). Therefore, we asked whether stretch causes PIP_2 hydrolysis by directly measuring IP_3 mass. Figure 3A shows stretch-induced accumulation of IP_3 which peaked around 1 min. An antitumoral xanthate compound, D609, has been shown to inhibit PLC both *in vitro* and *in vivo* (Schalasta and Doppler, 1990 and references therein). The accumulation of IP_3 by stretch was completely suppressed by D609 (100 μM, Figure 3A, triangles). We then examined whether PLC activation is necessary for the stretch response. Both D609 (100 μM) and neomycin (100 μM), which inhibits PLC by binding to phosphoinositides (Cockcroft and Gromperts, 1985; Kim *et al.*, 1989 and references therein), significantly suppressed stretch-induced c-*fos* expression (Figure 3B). These results are consistent with the notion that PIP_2 hydrolysis by PLC activation is an important step in mediating the stretch response, although we cannot rule out potential non-specific effects of D609 and neomycin on other signal transduction pathways.

Cell stretch also activates phospholipase D

Another major membrane phospholipid, phosphatidylcholine, is also hydrolyzed by phospholipases C, D and A_2

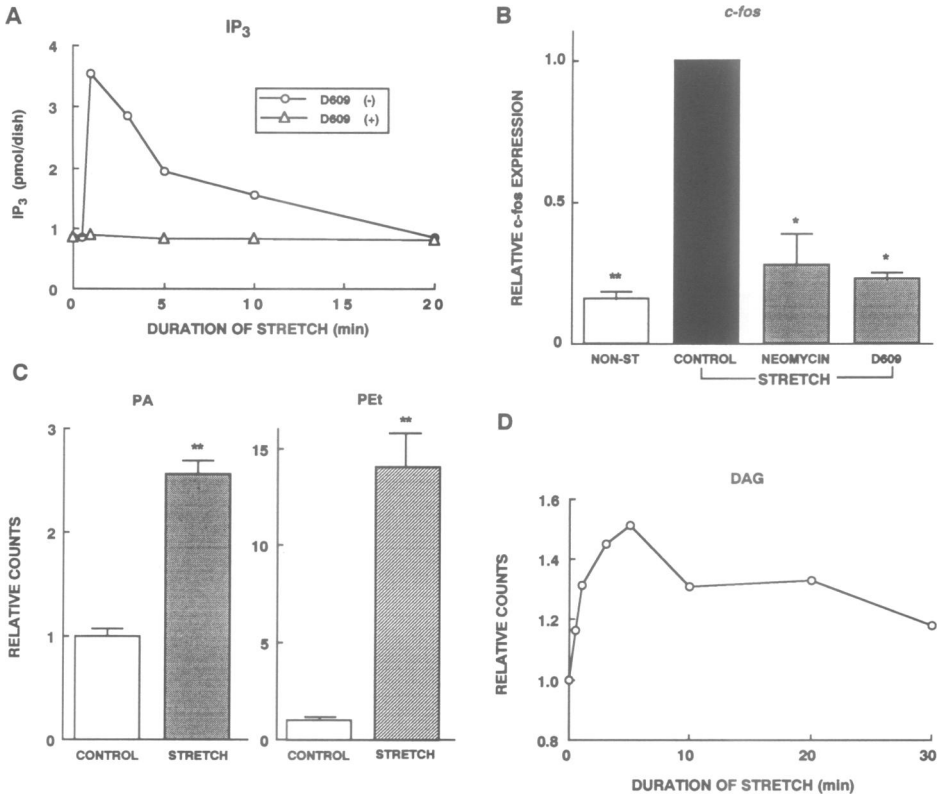

Fig. 3. Cell stretch activates PLC and PLD. (**A**) Stretch-induced accumulation of inositol phosphates. Cardiac myocytes were stretched for the indicated times in the presence (triangles) or absence (circles) of D609 (100 μM), an inhibitor of PLC. Cellular content of IP_3 was determined by a quantitative assay. Data are expressed in pmol/stretch dish (5×10^5 cells) and are from a representative experiment performed in quadruplicate. Non-stretched myocytes harvested with the same time course showed no significant change from time zero (not shown). A similar result was obtained when IP_3 measurement was done by the method of Berridge *et al.* (1984). (**B**) Effects of PLC inhibitors on stretch-induced c-*fos* expression. Densitometric analysis of c-*fos* mRNA was done as in Figure 2C. Each hybridization signal was expressed as a ratio to the signal obtained in a 30 min stretch without drugs. Results represent mean ± SE obtained from four separate experiments. Abbreviations and drug concentrations used are as follows; NON-ST, non-stretch; neomycin (100 μM); D 609 (100 μM). *, $P < 0.05$; ** $P < 0.001$ versus control stretch. (**C**) Stretch-induced hydrolysis of phosphatidylcholine. Cardiac myocytes were incubated in medium 199 containing [^{3}H]myristic acid for 24 h and stretched for 30 min in the presence of 0.5% ethanol. Phosphatidic acid (PA, left) and phosphatidylethanol (PEt, right) were separated by TLC. Data are expressed relative to the counts obtained in control non-stretched myocytes. Values represent mean ± SE from 10 samples from two separate experiments. ** $P < 0.001$ versus control. (**D**) Stretch-induced accumulation of DAG. Cardiac myocytes were stretched for the times indicated. DAG mass was determined from the Bligh−Dyer organic phase by the DAG kinase method. Data are expressed relative to the counts obtained in non-stretched myocytes (control). Data are from a representative experiment performed in quadruplicate.

in response to various growth stimuli, and their breakdown products also act as second messengers (reviewed in Nishizuka, 1992). Phosphatidic acid (PA), which is produced through activation of phospholipase D (PLD), is metabolized into DAG by PA phosphohydrolase, and can be the major pathway for the activation of PKC in some systems (Exton, 1990). PA itself can stimulate hydrolysis of phosphoinositides, mobilize Ca^{2+} from the intracellular stores and act as a growth factor (Moolenaar *et al.*, 1986). Therefore, we examined whether stretch also stimulates the hydrolysis of phosphatidylcholine. In cardiac myocytes prelabeled with [^{3}H]myristic acid for 24 h, a 30 min stretch resulted in a 2.6-fold increase in radiolabeled PA (Figure 3C). More importantly, in the presence of 0.5% ethanol, cell stretch caused a 14.1-fold increase in phosphatidylethanol (PEt) (Figure 3C), a specific marker of PLD activation (Exton, 1990).

Stretch causes a sustained increase in cellular content of DAG

The results presented above suggest that mechanical stretch of the cardiac myocytes activates both PLC and PLD. Since both enzymes can produce DAG through different pathways, we measured DAG content in stretched myocytes (Figure 3D). Although DAG is known to be rapidly metabolized in the cell, an increased cellular DAG content was observed at 1 min, reaching a peak (150% of control) at 5 min. The increase was still observed at 30 min. This sustained increase in DAG content is consistent with the activation of both PLC and PLD (Nishizuka, 1992).

Stretch-induced c-fos expression is not mediated by the cAMP pathway

We next examined the role of the cAMP−protein kinase A pathway in stretch-induced c-*fos* expression. In non-stretched cardiac myocytes, a 30 min treatment with 8-bromo cAMP (100 μM), a membrane permeable cAMP, or forskolin (10 μM), a direct stimulator of adenylate cyclase, caused 3.4- and 16.2-fold increases in the c-*fos* expression, respectively. This suggests that the cAMP pathway can directly induce c-*fos* in the cardiac myocytes (Figure 4A). However, measurement of intracellular cAMP content in response to stretch showed only a minimal increase compared with that induced by forskolin (Figure 4B). Furthermore, neither methacholine (200 μM), a muscarinic-cholinergic agonist which suppresses the adenylate cyclase activity

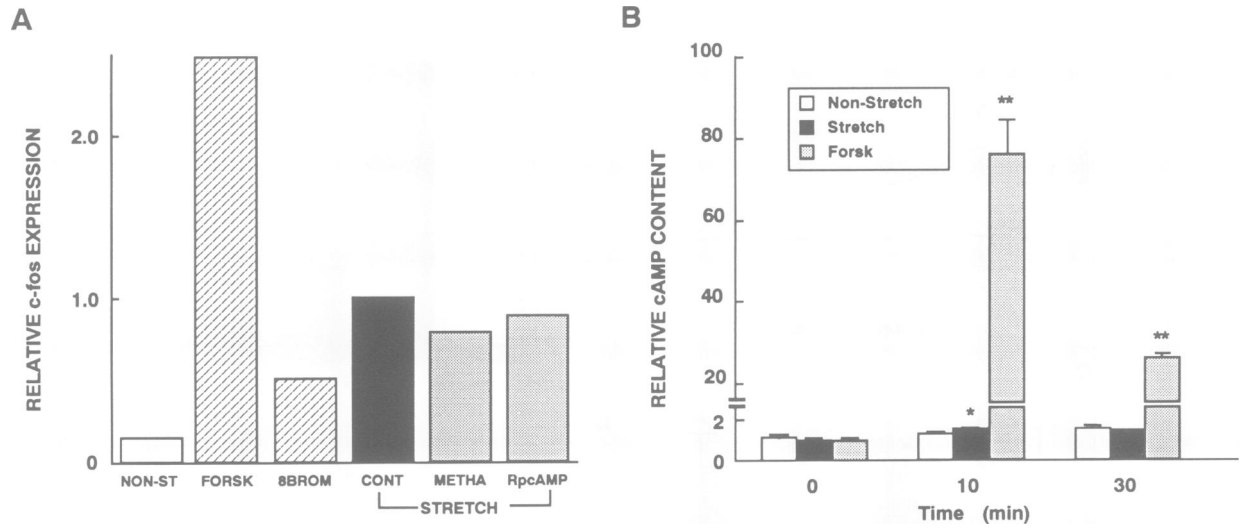

Fig. 4. The cAMP pathway is not essential in stretch response. (**A**) Effects of cAMP modulators on stretch-induced c-*fos* expression. Densitometric analysis of Northern blots was done as in Figure 2C. Each hybridization signal was expressed relative to the signal obtained in a 30 min stretch without drugs. Effects of forskolin and 8-bromo-cAMP were assessed in non-stretched myocytes. Data are from a representative experiment. Abbreviations and drug concentrations used are as follows; NON-ST, non-stretch; FORSK, forskolin (10 μM); 8BROM, 8-bromo cAMP (100 μM); CONT, control stretch; METHA, methacholine (200 μM); RpcAMP, Rp-adenosine 3′,5′-cyclic phosphorothioate (300 μM). (**B**) Stretch-induced accumulation of cAMP. Cyclic AMP was determined by radioimmunoassay. The concentration of forskolin used was 10 μM. Data are expressed as values relative to the cAMP content obtained in the non-stretched myocyte at time zero. *, $P < 0.05$; **, $P < 0.01$ versus non-stretched myocytes.

through G_i in cardiac myocytes (Xenophontos *et al.*, 1989), nor a high dose of Rp-cAMPS (300 μM), a membrane-permeable specific inhibitor of protein kinase A (Wang *et al.*, 1991, and references therein), prevented the stretch-induced c-*fos* expression (Figure 4A). These data corroborate the transfection data (Figure 1) and suggest that the cAMP pathway does not appear to be necessary in stretch-induced c-*fos* expression.

Arachidonic acid metabolites play important roles in stretch-induced c-fos expression

Membrane phospholipid breakdown by phospholipases A_2, C or D has been shown to cause a release of arachidonic acid (Dennis *et al.*, 1991; Ferguson and Hanley, 1991). Arachidonic acid and its metabolites, produced through the cycloxygenase pathway, the lipoxygenase pathway and the cytochrome P450 pathway, act as second messengers and regulate the activity of ion channels and protein kinases in many systems (Piomelli and Greengard, 1990). In non-stretched cardiac myocytes, treatment with arachidonic acid (100 μM) caused a 5-fold increase in c-*fos* expression (Figure 5A, ARA). We examined whether stretch of cardiac myocytes causes release of arachidonic acid. Stretch caused the release of significantly more [^{3}H]arachidonic acid and its metabolites than in the non-stretched control (Figure 5B). Although arachidonic acid can be released by multiple phospholipases (Dennis *et al.*, 1991), increasing evidence suggests that PLA_2 may play a major role in growth factor-induced arachidonate production (Axelrod, 1990; Nishizuka, 1992). Stretch-induced release of [^{3}H]arachidonic acid was inhibited by pretreatment with quinacrine (Figure 5B), a putative PLA_2 inhibitor (Schweitzer *et al.*, 1990 and references therein). Stretch-induced c-*fos* expression was also attenuated by quinacrine (10 μM, Figure 5A) or oleyloxyethyl phosphorylcholine (OOPC, 10 μM, not shown), another putative blocker of PLA_2 (Magolda and Galbraith, 1989). These results are consistent with, though do not unequivocally prove, the hypothesis that cell stretch may result in the activation of PLA_2.

We next examined which pathways of arachidonic acid metabolism are important for stretch-induced c-*fos* expression. Indomethacin (10 μM), a cycloxygenase inhibitor (Piomelli and Greenguard, 1990), showed no significant effect on stretch-induced c-*fos* expression. On the other hand, NDGA (20 μM), an inhibitor for lipoxygenase as well as cytochrome P450 monoxygenase (Kurachi *et al.*, 1989; Piomelli and Greenguard, 1990; Haliday *et al.*, 1991; Sellmayer *et al.*, 1991), significantly suppressed stretch-induced c-*fos* expression (Figure 5A). Caffeic acid (30 μM), which has been shown to inhibit lipoxygenase but not cytochrome P450 monoxygenase (Piomelli and Greenguard, 1990; Sellmayer *et al.*, 1991), did not affect stretch-induced c-*fos* expression. In contrast, ketoconazole (20 and 100 μM), an inhibitor of cytochrome P450 monoxygenase (Piomelli and Greenguard, 1990; Sellmayer *et al.*, 1991), significantly suppressed stretch-induced c-*fos* expression (Figure 5A). These results suggest that arachidonic acid metabolites, especially cytochrome P450 monoxygenase metabolites, may play an important role in stretch-induced c-*fos* expression, although we cannot exclude potential non-specific effects of some of the pharmacological reagents used.

Stretch causes activation of tyrosine kinases

Many cell growth stimuli cause tyrosine-phosphorylation of various intracellular substrates, through receptors that are either directly or indirectly coupled to tyrosine kinases (Cantley *et al.*, 1991). To examine the possibility that stretch causes activation of tyrosine kinases, protein tyrosine phosphorylation was assessed by anti-phosphotyrosine immunoblotting using a polyclonal antibody (Kamps and Sefton, 1988). As shown in Figure 6A, a 1 min stretch induced a marked increase in the phosphotyrosine content of proteins with molecular mass of ~60 and 120 kDa. A reproducible, though less marked, increase in the

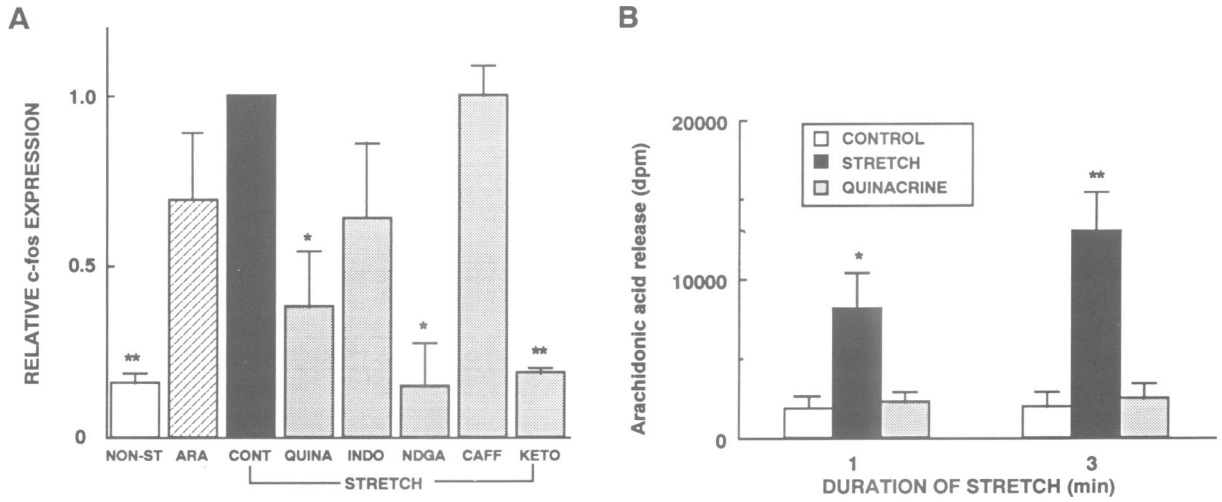

Fig. 5. Roles of the arachidonic acid metabolism in stretch response. (**A**) Effects of arachidonic acid metabolism modulators on stretch-induced c-*fos* expression. Densitometric analysis of c-*fos* mRNA was done as in Figure 2C. Each hybridization signal was expressed relative to the value obtained in a 30 min stretch without drugs. Effect of a 30 min treatment with arachidonic acid was assessed in non-stretched myocytes. Results represent mean ± SE of three to five separate experiments. Abbreviations and drug concentrations used are as follows; NON-ST, non-stretched; ARA, arachidonic acid (100 μM); CONT, control stretch; QUINA, quinacrine (10 μM); INDO, indomethacin (10 μM); NDGA, nordihydroguaiaretic acid (20 μM); CAFF, caffeic acid (30 μM); KETO, ketoconazole (100 μM). *, $P < 0.05$; **, $P < 0.001$ versus control stretch. (**B**) Effect of stretch on release of [^{3}H]arachidonic acid. The [^{3}H]arachidonic acid release during the indicated periods was obtained as the difference between the counts obtained before and after stretch. The medium was also collected from non-stretched cells (control) and stretched cells in the presence of quinacrine (10 μM) at the same time points. Quinacrine was applied 30 min before stretch. Each datum represents mean ± SE of nine samples. *, $P < 0.05$; **, $P < 0.01$ versus control.

phosphotyrosine content was observed in proteins with approximate molecular masses of 170, 85, 70, 42−44 and 30 kDa. These signals must be specific to phosphotyrosyl residues because an inclusion of 50 mM phosphotyrosine in the immunoreaction step abolished the signals (data not shown). To examine the role of tyrosine phosphorylation in the stretch response, we tested the effect of a tyrosine kinase inhibitor, genistein (Akiyama *et al.*, 1987; Mustelin *et al.*, 1990, and references therein), on stretch-induced c-*fos* expression. Genistein (20 μM) completely suppressed the stretch-induced c-*fos* expression (data not shown), suggesting that tyrosine kinase activation may be necessary for the stretch response. However, it remains to be determined whether the inhibitory effect of genistein was solely due to its effect on tyrosine kinases.

Stretch causes activation of MAP kinases and S6 kinases

It is of interest that stretch causes tyrosine phosphorylation of 42−44 kDa proteins and that MAP kinases (or extracellular signal-regulated kinases, ERKs) have molecular sizes of ~42 and 44 kDa. MAP kinases are a family of serine/threonine kinases, which are activated by both tyrosine- and threonine-phosphorylation in response to a variety of growth factors (reviewed in Blenis, 1991; Cobb *et al.*, 1991). MAP kinases have been shown to phosphorylate p62^{TCF} (Gille *et al.*, 1992) and Jun (Pulverer *et al.*, 1991) to activate their transcriptional activity.

In order to determine whether MAP kinases are activated by stretch, MAP kinase activity was measured by the immune complex 'in-gel kinase assay' (Gotoh *et al.*, 1990). Although we observed little MAP kinase activity in non-stretched cells, there was a significant increase in kinase activity migrating at 42 and 44 kDa after stretch or PMA treatment for 10 min (Figure 6B). Immunoprecipitates of non-immune sera had no kinase activity on myelin basic protein (data not shown). Figure 6C shows time courses of stretch-induced activation of 42 and 44 kDa MAP kinases. Both MAP kinases were activated within 1 min of stretch. Their activity reached a peak around 10 min and then gradually decreased to the control level within 1−2 h.

It is known that MAP kinases phosphorylate one of the S6 kinases, RSK (pp90^{RSK}), and regulate its activity (Sturgill *et al.*, 1988; Erikson, 1991). RSK may regulate expression of IE genes and their target genes by phosphorylating SRF, Fos and Jun (reviewed in Blenis, 1991). Therefore, we examined whether cell stretch also activates RSK. The kinase assay was performed under conditions inhibitory to calcium- and cyclic nucleotide-dependent kinases using S6 peptide (RRLSSLRA), designed based on the phosphorylation site sequence of the ribosomal S6 protein (Pelech and Krebs, 1987), as a substrate. As shown in Figure 6D, stretch also activated kinase activity to S6 peptide. The time course of activation of S6 peptide kinase followed that of MAP kinases, being activated within 1 min, reaching a peak around 10 min but decreasing more slowly than that of MAP kinases. To confirm that RSK is activated by stretch, the S6 peptide kinase assay was performed after immunoprecipitation with anti-RSK antibody (α-RSK) (Chen *et al.*, 1991). A 10 min stretch caused a 2.26- ± 0.29-fold ($n = 3$) increase in the RSK activity over non-stretched cardiac myocytes, a level similar to that observed by the direct kinase assay. Immunoprecipitates with non-immune sera had no significant kinase activity on S6 peptide (not shown). These results suggest that stretch activates RSK.

Stretch activates p21^{ras}

Recently, p21^{ras} has been shown to play a role in signal transduction pathways induced by serum, nerve growth factor (NGF), phorbol ester, PDGF, insulin and T-cell antigen receptor stimulation (Downward *et al.*, 1990; Satoh

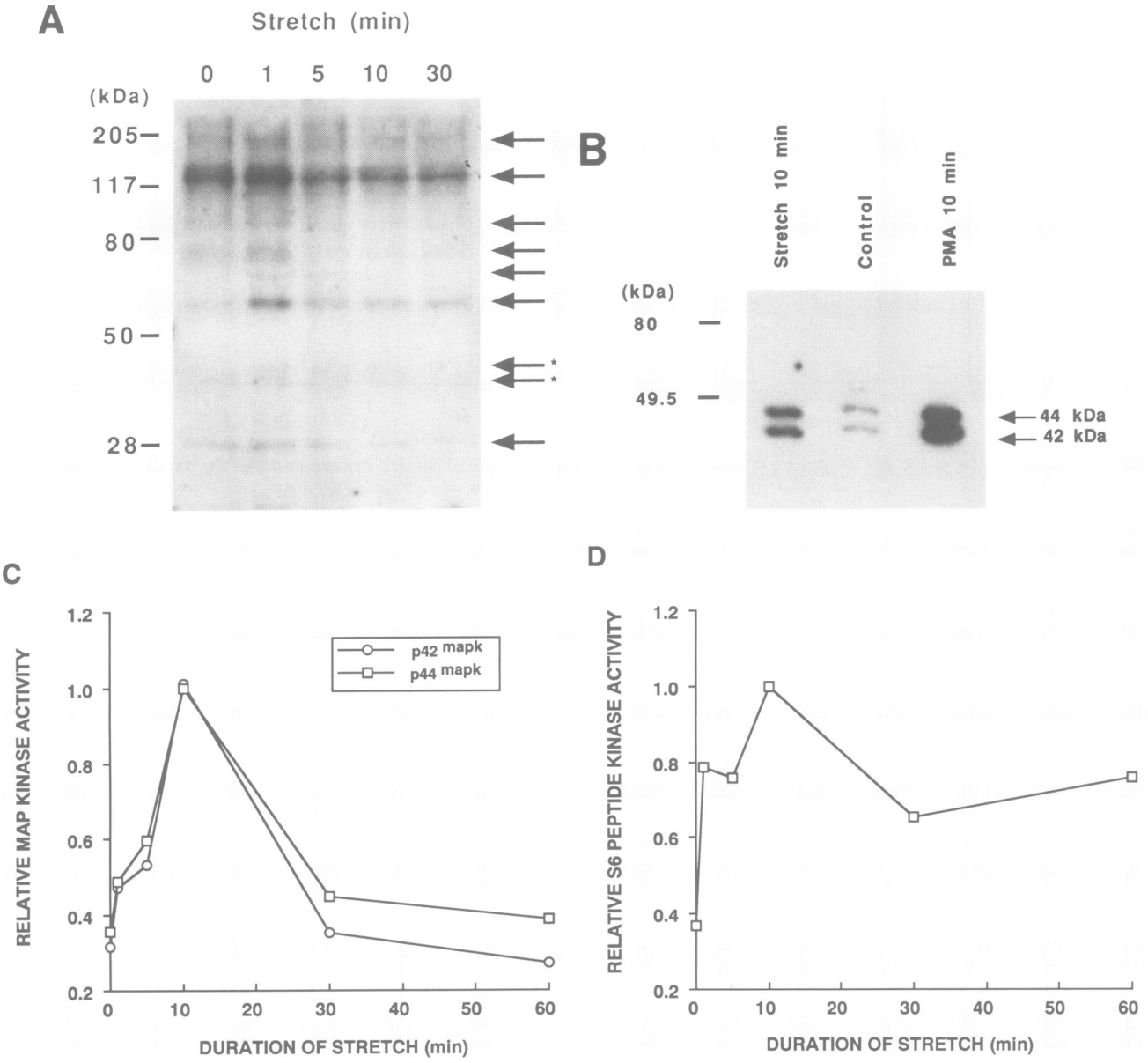

Fig. 6. Stretch-induced activation of tyrosine kinases, MAP kinases and S6 peptide kinases. (**A**) Anti-phosphotyrosine immunoblotting of cardiac myocytes. Cells were stretched for the times indicated and tyrosine phosphorylation was assessed by anti-phosphotyrosine immunoblotting after SDS−PAGE on 10% gels. Ponceau S staining of the membrane showed equal loading of protein in each lane (not shown). Cell stretch induced a marked increase in the phosphotyrosine content of p120 and p60 and to a lesser degree of p170, p85, p70, p42−p44, and p30 as well (arrows). Asterisks show p42 and p44, which are further characterized in Figures 6B and C. The autoradiograph shown is representative of five separate experiments. (**B**) Representative immune complex in-gel kinase assay of MAP kinases. Intensity of both 42 and 44 kDa bands corresponds to MAP kinase activity using MBP as a substrate. PMA (1 μM) was used as a positive control. (**C**) Time courses of stretch-induced activation of MAP kinases (p42 and p44). MAP kinase activity was assessed as in (B). Phosphorylation bands at 42 and 44 kDa were quantified by laser densitometry in the linear response range of the X-ray film. Data are from representative experiments. The maximum activity during the time course is designated as 1.0. (**D**) Time course of stretch-induced activation of S6 peptide kinases. S6 peptide kinase activities were obtained using S6 peptide as a substrate. Phosphorylated S6 peptide was absorbed onto Whatman P81 phosphocellulose paper and counted for radioactivity after washing. Similar results were obtained by the immune complex kinase assay using anti-pp90^{RSK} antibody (see Results section).

et al., 1990; Medema *et al.*, 1991; Thomas *et al.*, 1992; Wood *et al.*, 1992). In particular, p21^{ras} was found to be essential in MAP kinase activation by NGF and phorbol ester in PC12 cells (Thomas *et al.*, 1992; Wood *et al.*, 1992). Therefore, we examined whether p21^{ras} was activated during the early phase of the stretch response. The activity of p21^{ras} is normally regulated by a cycle of binding to GTP to give the biologically active form, followed by hydrolysis of bound GTP to GDP to form an inactive state. Activation of p21^{ras} was assessed in cardiac myocytes by immunoprecipitating p21^{ras}, followed by measurement of the amount of GTP bound to it relative to GDP (Downward *et al.*, 1990). In control cardiocytes, GDP-bound p21^{ras} was much more abundant than the GTP-bound form (Figure 7). Stretch caused a significant increase in GTP binding to p21^{ras}, detectable as early as 1 min after stretch (Figure 7 and data not shown). The rapid kinetics of p21^{ras} activation were also observed in stimulation of other cell types (Downward *et al.*, 1990; Satoh *et al.*, 1990). At present, we do not know how p21^{ras} is activated by stretch.

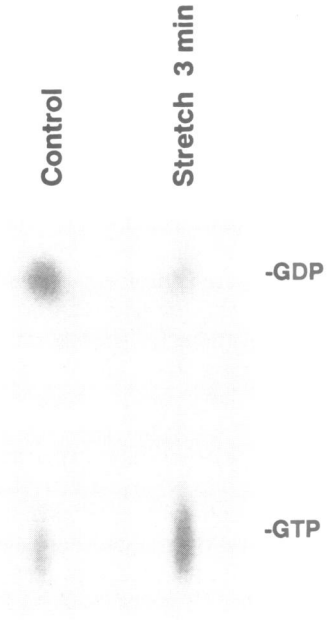

Fig. 7. Effect of stretch on guanine nucleotide binding to p21^{ras}. Cardiac myocytes were labeled with [^{32}P]orthophosphate. A thin layer chromatogram of the nucleotides eluted from immunoprecipitates of p21^{ras} from non-stretched and stretched (3 min) myocytes is shown. The positions of GTP and GDP standards are indicated. In this example, the amount of GTP bound to p21^{ras} as a proportion of total nucleotides was 28% in non-stretched and 68% in stretched myocytes.

It is of interest that arachidonic acids released by mitogen stimulation have been reported to activate p21^{ras} by inhibiting GTPase activating proteins (GAP) (Tsai *et al.*, 1989). It also remains to be determined whether p21^{ras} activation is required for the stretch-induced c-*fos* expression.

Stretch-conditioned medium mimics the effect of stretch

The above results indicate that activation of multiple signal transduction pathways seems to occur within 1 min of stretch. Therefore, we next examined whether a continuous stretch is necessary for c-*fos* induction. Figure 8A shows the effects of a short duration stretch on c-*fos* expression. In this experiment, cardiac myocytes were stretched for short periods (1, 5 and 10 min) and were returned to the resting length. Cells were then harvested at the 30 min time point. The results indicate that a stretch as short as 1 min was enough to elicit c-*fos* expression 30 min later. One potential explanation of this result could be that release of a humoral factor(s) occurs within a few minutes of cell stretch and is responsible for the subsequent activation of the c-*fos* gene. To test this hypothesis, we examined the effects of 'stretch-conditioned' medium on non-stretched cells. Cardiocytes were stretched for short periods and the stretch-conditioned medium was transferred to non-stretched cardiocytes. Although transferring the medium from the non-stretched cardiocytes did not elicit a c-*fos* response, the media conditioned by 1, 5 and 10 min of stretch induced 3.0-, 9.3- and 9.6-fold increases respectively in c-*fos* expression in non-stretched cardiocytes (Figure 8B). The results indicate that stretch may cause release of a factor(s) into the culture medium, which in turn induces c-*fos* expression. Stretch-conditioned medium also activated MAP kinases in recipient cells (data not shown).

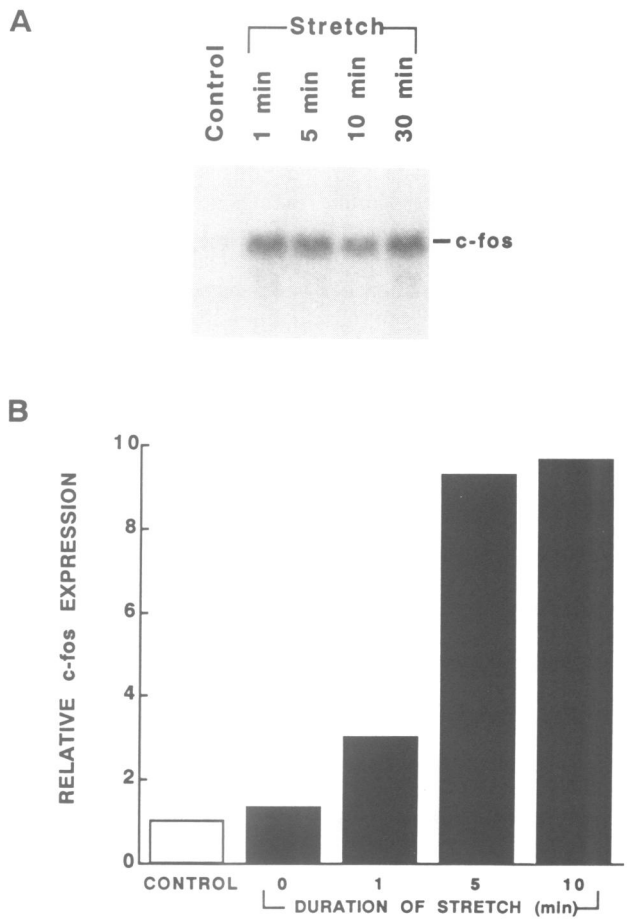

Fig. 8. (**A**) Effects of short duration stretch on c-*fos* expression. Cardiac myocytes were stretched for the times indicated and harvested at 30 min. A representative Northern blot is shown. Similar results were obtained from three additional experiments. (**B**) Effects of 'stretch-conditioned' media on c-*fos* expression in non-stretched cardiocytes. Cardiocytes were stretched for the times indicated. The culture medium of the stretched cells was transferred to non-stretched cardiocytes. The cells were incubated in the stretch-conditioned medium for 30 min and c-*fos* expression was examined by Northern blot analysis. Hybridization was assessed as a relative to the value obtained from non-treated cells. Zero min stretch indicates the medium transfer from non-stretched cardiocytes. Data are from a representative experiment. Similar results were obtained from four additional experiments.

Discussion

In order to elucidate how mechanical forces are transduced into intracellular signals regulating gene expression, we have investigated the roles of various signal transduction pathways in stretch-induced c-*fos* gene expression in cardiac myocytes *in vitro*. Our results suggest that the stretch response causes a rapid activation of multiple second messenger systems, including tyrosine kinases, p21^{ras}, MAP kinases, S6 kinase (RSK), PLC, PLD, PKC and arachidonic acid metabolism (particularly the P450 pathway), many of which seem to contribute to c-*fos* gene induction by stretch. On the other hand, the cAMP pathway does not seem to be essential for stretch response, and intracellular Ca^{2+} may play a permissive role. The stretch-induced signal transduction pathways of the c-*fos* gene transcription seem to converge into activation of p62^{TCF}–SRF complex on the SRE. The stretch response may involve an autocrine/paracrine

mechanism, because the stretch-conditioned medium mimics the effect of stretch.

How cardiac and skeletal muscle convert mechanical stimuli into growth signals has been a longstanding question. To our knowledge this study represents the most comprehensive analysis to date addressing this issue. At present we do not know whether similar findings apply to non-cardiac cells because comparable studies have not been done in other cell types. The importance of mechanical forces regulating cell growth and function has been suggested in many cell types, including smooth muscle, chondrocytes, osteocytes, vascular endothelium, epidermal cells, lung epithelial cells and fibroblasts (Ingber, 1991; Vandenburgh, 1992). It is possible that different cell types have different mechanisms of mechanotransduction because the nature of mechanical stimuli to which these cells are normally exposed differs considerably.

How does cell stretch lead to the activation of multiple intracellular second messengers? One possibility is that mechanical stress directly activates signal transducing molecules, such as growth factor receptors (in the absence of the ligand), G proteins, phospholipases or protein kinases. It has been postulated that mechanical forces applied to the cell surface might generate direct conformational changes in the molecules associated with the plasma membrane, which may activate the downstream second messenger systems (Watson, 1991). However, at present, there is little direct evidence for this hypothesis.

The second possibility is that mechanical stretch activates putative cellular mechanotransducers, such as mechanosensitive ion channels (Sachs, 1989), cytoskeleton or integrins (Ingber, 1991), which in turn activate multiple second messengers. Our previous study argues against the possibility that stretch-activated cation channels (gadolinium-sensitive types), actin microfilaments, microtubules or RGD-binding integrins are involved in the stretch-induced c-*fos* induction (Sadoshima *et al.*, 1992b). However, there remains a distinct possibility that non-RGD binding integrins and other cell adhesion molecules may play important roles in mechanotransduction of cardiac myocytes. In CH3 10T1/2 fibroblasts, integrin $\alpha_5\beta_1$ can behave similarly to a growth factor receptor to activate the Na^+/H^+ antiporter implicated in growth control (Schwartz *et al.*, 1991).

The third possibility, which we favor most, is that mechanical stress causes release of some growth factor(s) and that this factor activates its receptor and subsequent second messenger cascades. Our results indicate that transferring the culture medium conditioned by stretched cells mimicked the effect of stretch. Thus, there appears to be a soluble factor(s) whose release is induced by cell stretch, which in turn may act as a signal for c-*fos* induction. However, it is also possible that the initial mechanotransduction occurs via certain extracellular matrix molecules and integrin receptors, which subsequently could trigger the release of soluble factors capable of activating growth factor receptors. For example, platelet adhesion causes rapid activation of tyrosine kinases, phospholipases and PKC, and elevation of cytoplasmic Ca^{2+}, with subsequent release of vasoactive substances (reviewed in Hynes, 1992; Shattil and Brugge, 1991). It is noteworthy that cardiac myocytes are known to secrete atrial natriuretic factor in response to mechanical stretch (Lang *et al.*, 1985; Kinnunen *et al.*, 1992). However, atrial natriuretic factor is not known to cause c-*fos* induction or cardiac hypertrophy. It is likely that other substances may be released by mechanical loading to act as growth factors for cardiac myocytes (Hammond *et al.*, 1982). However, it is necessary to identify the molecular nature of such factors in order to establish the role of autocrine and/or paracrine mechanisms in cardiac hypertrophy.

In this study, we have used c-*fos* expression as an early nuclear marker for stretch-induced signal transduction. At present, however, there is no formal proof that early c-*fos* expression is necessary for later cardiac hypertrophy. In other cell systems, though, prevention of Fos protein translation by antisense RNA and microinjection of Fos-specific antibodies have been shown to prevent DNA synthesis in response to growth stimuli (Holt *et al.*, 1986; Riabowol *et al.*, 1988). A similar experiment has yet to be reported in cardiac myocytes. Even if increased c-*fos* expression is not essential for development of load-induced hypertrophy, the results obtained here should serve as a prototype of the signal transduction pathways by mechanical stimuli, because other IE genes, such as Egr-1, were also activated by stretch in a similar manner as c-*fos* (data not shown).

A question arises as to which of the signal transduction pathways is activated first by stretch. Our pharmacological experiments indicate that inhibitors of several different signal transduction pathways seem almost equally effective in suppressing c-*fos* expression. Four possibilities may be considered to explain this phenomenon. (i) The inhibitors used could have affected multiple signaling pathways. Staurosporine, for example, might have affected tyrosine kinases in addition to PKC. (ii) Each pathway could be activated independently but contribute synergistically to c-*fos* expression. 'Cross-talk' among signaling pathways seems to be a general phenomenon (Cantley *et al.*, 1991; Nishizuka, 1992). (iii) There may be a hierarchy in the activation of different pathways. For example, activation of tyrosine kinases by stretch may lead to activation of PLC, $p21^{ras}$, PKC and MAP kinases, as has been reported for NGF stimulation of PC12 cells (Thomas *et al.*, 1992; Wood *et al.*, 1992). Precise kinetic analysis of each pathway, especially that of a very early time course after stretch, is necessary to determine temporal and hierarchical relationships of activation of each pathway. (iv) Modulation of multiple transcription factors by multiple second messengers might be necessary to transactivate c-*fos* by stretch. Recently a number of factors have been identified that form a complex with SRF (Rivera and Greenberg, 1990; Treisman, 1990). SRF is phosphorylated by $pp90^{RSK}$ after growth factor stimulation (Blenis, 1991) and phosphorylation of $p62^{TCF}$ by MAP kinase increases the ternary complex formation on the SRE (Gille *et al.*, 1992). Other SRF co-factors may also be targets for modulation by multiple second messenger systems and much work is needed to establish the link between the signaling pathways and the SRE binding factors.

In conclusion, we have demonstrated that a brief stretch of cardiac myocytes causes rapid activation of multiple intracellular signal transduction pathways, which may be mediated by release of growth factors. The activation of multiple signal transduction pathways by stretch is highly reminiscent of the complex cellular response to growth factors (Williams, 1989; Ullrich and Schlessinger, 1990;

Rozengurt, 1991). In retrospect, it is not surprising that the stretch response resembles the growth factor response of quiescent cells, because the hypertrophic response of cardiac myocytes in response to mechanical overload *in vivo* and *in vitro* closely resembles the mitogenic response of other cell types to growth factor stimulation (Izumo *et al.*, 1988; Komuro *et al.*, 1988, 1990; Chien *et al.*, 1991; Parker and Schneider, 1991; Sadoshima *et al.*, 1992a). It remains to be determined what is (are) the factor(s) released in response to stretch and how mechanical stretch leads to release of such factor(s).

Materials and methods

Cell culture

Primary cultures of neonatal rat cardiac myocytes were prepared as described by Sadoshima *et al.* (1992a). We routinely obtained myocyte cultures containing 90–95% myocytes, as assessed by microscopic observation of cell beating and by immunofluorescence staining with a monoclonal antibody (MF20) against sarcomeric myosin heavy chain (Bader *et al.*, 1982). All experiments were done in serum-free conditions 24–48 h after changing to serum-free medium. The stretch culture dishes used have been previously described (Sadoshima *et al.*, 1992a). Uniaxial strain was applied by stretching the silicone sheet (Dow Corning) by 20%. The stretch did not increase the release of lactate dehydrogenase or creatine kinase, indicating that the stretch did not cause cell injury (Sadoshima *et al.*, 1992a).

Plasmid constructs and DNA transfection

Mouse c-*fos*–CAT (chloramphenicol acetyltransferase) constructs containing the c-*fos* gene 5′ flanking sequence were generously given by Dr M.Gilman. (Gilman, 1988; Berkowitz *et al.*, 1989; Grueneberg *et al.*, 1992). Transfection was performed by the $CaPO_4$ precipitation method (Sambrook *et al.*, 1989). 15 μg of c-*fos*–CAT reporter plasmid DNA and 5 μg of a RSV β-galactosidase expression plasmid was transfected per dish. 48 h after transfection, cells were stretched for 2 h and harvested. Cell extracts were made and CAT assay was performed as described by Kingston and Sheen (1990). To correct for transfection efficiency, β-galactosidase activity of cell extracts was measured as described by Sambrook *et al.* (1989). CAT activity was also normalized to the protein content of each extract determined by the method of Lowry *et al.* (1951).

Isolation and Northern blot analysis of RNA

Isolation of total cellular RNA and Northern blot analysis were performed as described previously (Sadoshima *et al.*, 1992a). The relative amounts of a specific mRNA were quantified by laser densitometry of the corresponding autoradiograms in the linear response range of the X-ray films. The hybridization signals of specific mRNAs were normalized to those of GAPDH mRNA to correct for differences in loading and/or transfer. The levels of GAPDH mRNA were not affected by stretch (not shown).

Protein kinase C assay

PKC assay in permeabilized cells was performed using 200 μM PKC-specific substrate peptide, PLSRTLSVAAKK (Bachem), as described by Alexander *et al.* (1990). In some experiments, 30 μM PKC pseudosubstrate peptide (House and Kemp, 1987) was included in the reaction. After 10 min of incubation at 37°C kinase reactions were terminated with 40% (w/v) trichloroacetic acid (TCA). In this assay condition, kinase activities to this peptide were linear. The amount of ^{32}P-labeled peptide on phosphocellulose P81 filter (Whatman) was counted using a liquid scintillation counter.

Inositol phosphates and diacylglycerol measurement

For IP_3 measurement, myocytes were stretched and harvested with 1 ml of ice-cold 10% TCA. TCA was removed with five diethylether extractions and the final extract was neutralized with NaOH. Cellular IP_3 level was determined by a quantitative assay (Amersham). Determination of inositol phosphates was also done by the method described by Berridge *et al.* (1984). Cellular DAG content was determined from the dried Bligh–Dyer organic phase (Bligh and Dyer, 1959) by the DAG kinase method (Preiss *et al.*, 1987) using DAG kinase (Sphinx Biotechnologies).

Phosphatidylcholine hydrolysis

Cardiac myocytes were incubated in medium 199 containing [^3H]myristic acid (3 μCi/ml) for 24 h. Myocytes were stretched in the presence of 0.5% ethanol, and harvested with 0.5 ml of ice-cold methanol. Cells were scraped and extracted by the method of Bligh and Dyer (1959). The organic phase of the Bligh–Dyer extract was dried under N_2 and analyzed for [^3H]PEt and [^3H]PA by TLC (Whatman) using a method described by Wrighton *et al.* (1983). PEt and PA were identified using unlabeled standards (Avanti) visualized by exposure to iodine vapor.

Cyclic AMP assay

Experiments were performed in the presence of IBMX (500 μM), which was added 10 min before stretch. After stretching myocytes, 0.5 ml of ice-cold 6% TCA was added. After a 10 min incubation at 4°C, the supernatants were extracted five times with 5 vol of ether. Extracts were subjected to lyophilization. The concentration of cAMP was determined by radioimmunoassay (Biomedical Technology Inc.). Cyclic AMP content was normalized with the protein content of the sample.

Arachidonic acid release

Release of [^3H]arachidonic acid was measured using a variation of the method described by Takuwa *et al.* (1991). Cells were labeled with [^3H]arachidonic acid (0.5 μCi/ml) in a serum-free culture medium for 4 h. Cells were washed three times with the culture medium, incubated in the same medium and then stretched. An equal number of cultures were treated identically without stretch. Medium was collected before and after stretch at the indicated time points and spun for 1 min. The radioactivity of the supernatant was counted in a liquid scintillation counter. [^3H]arachidonic acid release during the period was obtained as the difference between the counts before and after stretch.

Anti-phosphotyrosine immunoblotting

Cell stretch was terminated by a rapid aspiration of the medium and an addition of 200 μl ice-cold radioimmune precipitation (RIPA) buffer (Kamps and Sefton, 1988). Cell extracts were solubilized by addition of 50 μl of 5× Laemmli SDS–PAGE sample buffer and were then electrophoresed on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Blots were probed by sequential incubation with affinity-purified rabbit anti-phosphotyrosine antibodies (Kamps and Sefton, 1988) at a concentration of 2 μg/ml and [^{125}I]protein A (100 μCi/μg) followed by autoradiography.

MAP kinase and S6 kinase assays

Cell-free lysates were prepared in buffer A (Gotoh *et al.*, 1990). For the measurement of MAP kinase activity, immune complex was prepared by adding 5 μl of polyclonal antiserum raised against C-terminal MAP kinase peptide (α-cMAPK) (Chen *et al.*, 1992) to lysates containing equal amounts of protein (300 μg). Pansorbin was then added, and the immunoprecipitates were electrophoresed on 10% SDS–polyacrylamide gels containing 0.5 mg/ml myelin basic protein. Kinase assays in MBP-containing polyacrylamide gel were performed with the method described by Gotoh *et al.* (1990). S6 peptide kinase activity was measured under conditions inhibitory to Ca^{2+}- and cyclic nucleotide-dependent kinases as described by Pelech and Krebs (1987), using S6 peptide (RRLSSLRA) as a substrate. S6 peptide kinase assay was also performed by immune complex kinase assay using an anti-$pp90^{RSK}$ antibody, α-RSK (Chen *et al.*, 1991).

Determination of guanine nucleotides bound to $p21^{ras}$

Cells were labeled with [^{32}P]P_i (NEN) at 0.5 mCi/ml in phosphate-free DMEM (GIBCO) for 12 h. $p21^{ras}$-associated guanine nucleotides were determined by an immunoprecipitation assay described by Downward *et al.* (1990). For immunoprecipitation, anti-$p21^{ras}$ antibody precoupled to protein A–agarose (Y13-259, Oncogene Science) was used.

Statistics

Data are given as mean ± SEM. Statistical analysis was performed using analysis of variance or unpaired Student's *t*-tests as appropriate. Significance was accepted at the $P < 0.05$ level.

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