

412 **DISCUSSION**

413       The importance of satellite cell proliferation activity in skeletal muscle repair and  
414 hypertrophy has been appreciated for a considerable time. It is well documented that one  
415 of the earliest events, triggering the activation of quiescent satellite cells, is initiated by  
416 mechanical or chemical stimuli and enables satellite cells to migrate and enter the cell  
417 proliferation cycle. Bischoff (11) was among the first to explore the biochemical link  
418 between satellite cell activation and mechanical perturbation of muscle tissue. His  
419 classical experiments used a crushed-muscle extract, which he found could stimulate  
420 satellite cells associated with isolated fibers to synthesize DNA and divide. Johnson and  
421 Allen (30) later reported that the same crushed-muscle extract could stimulate adult rat  
422 satellite cells in culture to enter the cell cycle earlier than adult rat cultures in control  
423 medium. Shortening of the lag phase observed with freshly isolated adult satellite cells  
424 has since been used as an assay for satellite cell activation (1, 2, 58) and this assay was  
425 employed in experiments that first demonstrated that HGF was unique in its ability to  
426 activate satellite cell division in vitro (1). In subsequent experiments, the active agent in  
427 crushed-muscle extract was shown to be HGF, which was present in un-injured muscle and  
428 located primarily in the extracellular matrix (58, 62). In further experiments, Tatsumi et al.  
429 (59-61, 64) showed that satellite cells were stimulated to enter the cell cycle when subjected  
430 to mechanical stretch in primary culture and living muscle. We also demonstrated that  
431 satellite cell activation was due to rapid release of HGF from its extracellular tethering and  
432 the subsequent presentation to the receptor c-met, and later verified that HGF release is  
433 dependent on calcium-calmodulin formation and the down-stream NO radical-mediated  
434 activation of matrix metalloproteinases (MMPs) (47, 67, 75, 76).

435       These observations suggest that the stretch-activation of satellite cells is a cascade of  
436 sequential events including (in order) an influx of calcium ions and their binding to  
437 calmodulin, NO radical production by cNOS, MMP activation, HGF release from the matrix  
438 and presentation of HGF to the signaling receptor c-met (see a review of Ref. 66). In this  
439 model, a complex of HGF and proteoglycan extracellular domain would be shed from the  
440 matrix and presented to the receptor c-met. In fact, HGF associated with heparan sulfate  
441 moieties has a greater affinity for c-met relative to HGF alone, and therefore, has enhanced  
442 HGF signaling activity (20). Such a complex has been detected in PBS extracts from  
443 crushed muscle and intact muscle incubated in the NO donor, sodium nitroprusside (SNP)  
444 (Allen et al., unpublished data), supporting the above insight. In related experiments by

445 Wozniak and Anderson (73), released HGF may induce c-met expression as an  
446 immediate-early gene, thus also enhance HGF-c-met signaling in the satellite cell activation  
447 process. They additionally observed that the level of satellite cell activation was increased  
448 in un-stretched fibers from mdx and nNOS-knockout mice and in un-stretched normal fibers  
449 by treatment with a competitive NOS-inhibitor L-NAME (N<sup>G</sup>-nitro-L-arginine methylester),  
450 suggesting that a NO-independent HGF/c-met signaling pathway, and possibly other  
451 signaling pathways, control activation in muscle deficient in dystrophin and nNOS (73).

452 It has not been determined how quiescent satellite cells sense mechanical stimuli to  
453 generate biochemical signals that elevate intracellular calcium ion concentrations; therefore,  
454 in the present work, the involvement of MS- and VGC-channels in the  
455 mechano-transduction system was examined. When satellite cell cultures were incubated  
456 with the MS-channel inhibitor GsMTx-4, the L-VGC-channel inhibitor nifedipine, or a less  
457 specific inhibitor, gadolinium chloride, stretch-induced HGF release from the matrix and the  
458 resulting cell activation were abolished completely in a dose-dependent manner. This was  
459 revealed by western blotting of conditioned media and a BrdU-incorporation assay (Fig. 2).  
460 By contrast, the T- VGC-channel inhibitor NNC55-0396 did not have inhibitory effects on  
461 the activation index. Addition of the calcium-chelator EGTA to culture media also  
462 inhibited the stretch-activation response and HGF release, indicating the significance of  
463 extracellular calcium ions in the activation cascade (Fig. 1 and Fig. 2 panel B). This  
464 means that free calcium ions stored in mitochondria may now be excluded from a role in  
465 this activation pathway. Calcium-imaging analyses verified these results, and therefore  
466 provide direct evidence that both MS- and L-VGC-channels are essential in calcium ion  
467 influx in response to mechanical stretch and that calcium ions may flow in through  
468 L-VGC-channels (Fig. 4). A possible explanation for these observations can be addressed  
469 by a possible model of MS-/L-VGC-channel coupling, in which the MS-channel responds  
470 to mechanical stimuli by gating cations; this would result in local depolarization of the  
471 plasma membrane, a signal that is then quickly sensed by L-VGC-channels (Ca<sub>v1.1</sub>, Ca<sub>v1.2</sub>)  
472 that allow a significant amount of calcium ions to permeate the cell and lead to  
473 calcium-calmodulin formation (see Fig. 5 panel B for details). In the nifedipine treatment,  
474 in which MS-channels are expected to retain biological activity to gate selective cations,  
475 significant calcium influx was not observed, suggesting that MS-channels concerned in the  
476 activation pathway allow entry of cations other than calcium ions and/or gate an  
477 undetectable level of calcium ions that is not sufficient to activate calmodulin-NOS

478 signaling directly but is enough to depolarize the cell membrane.

479       Considering the possible implication of MS-channels in the mechano-sensing  
480 mechanism of satellite cell activation, it is worth noting the valuable study by Formigli et al.  
481 (25), which provided the first experimental evidence that the transient receptor potential  
482 canonical channel 1 (TRPC1) represents an essential component of stretch-activated cation  
483 channel signaling in the mouse C2C12 myoblast cell line, as assayed by whole-cell  
484 patch-clamp and atomic force microscopic pulling. That study indicated that TRPC1 plays  
485 a crucial role in mechano-transduction during regulation of the early phases of myoblast  
486 differentiation. TRPCs (TRPC1-7) are a family of cation channels that assemble as homo-  
487 or hetero-tetramers to form voltage-independent, non-selective cation- ( $\text{Na}^+$  and  $\text{Ca}^{2+}$ )  
488 permeable pores (16, 46). Although numerous studies have shown that TRPC1 may be  
489 store-operated calcium-ion entry channels (SOCs) in various cell types (23, 74), there is  
490 also evidence that this channel is able to translate membrane stretch into cation currents  
491 across the plasma membrane (17, 37, 52). TRPC5 and 6 were also shown to be  
492 stretch-gated and GsMTx-4-sensitive cation-channels and TRPC6 plays an essential role of  
493 mechano-sensing in smooth muscle cells (26, 51; reviewed in Ref. 13), therefore, at least at  
494 this time, TRPC1, 5, and 6 may be reasonable candidates for the MS-channel in the satellite  
495 cell activation pathway concerned here. Our rat satellite cell cultures expressed TRPC1  
496 message, as revealed by RT-PCR, while TRPC6 was not detected at 24-hr post-plating (Fig.  
497 3 panel B), which is the time-point at which the stretch treatment was applied in the present  
498 and our previous experiments (65, 75-77); this provides a supportive background for the  
499 implication of TRPC1 in the above model of stretch-activation. TRPA1 (18), TRPV4 (10)  
500 and TRPM7 (41) are also reported to be stretch-sensitive channels and therefore can not be  
501 excluded from the list of candidate MS-channels at the present time, and the field awaits a  
502 study to examine their GsMTx-4 sensitivity and expression in quiescent satellite cells.

503       Another important observation by Formigli et al. (25) is that TRPC1 activity and  
504 stretch-induced cation influx in C2C12 myoblasts were modulated by  
505 sphingosine-1-phosphate (S1P), an important bioactive sphingolipid-metabolite that mainly  
506 acts through G-protein-coupled receptors present on mammalian cells, and thereby regulates  
507 numerous cell functions including cell proliferation, differentiation and apoptosis (78). In  
508 line with this, TRPC1 expression is reported to be significantly up-regulated during  
509 myogenesis, especially in the presence of S1P, supporting the physiological relevance of the  
510 sphingosine-S1P-TRPC1 axis in regulating C2C12 cell growth and differentiation (24, 25,

511 39). Notably, Nagata et al. (40) clearly showed that S1P can induce satellite cells to enter  
512 the cell cycle, whereas inhibiting the sphingolipid signaling cascade (which generates S1P  
513 mainly from sphingomyelin and sphingosine by N-SMase, ceramidase and sphingosine  
514 kinase) reduces the number of satellite cells able to divide in response to mitogen  
515 stimulation. A similar observation was reported by Sassoli et al. (48); S1P promotes  
516 satellite cell renewal and differentiation in damaged muscle. Although the S1P-mediated  
517 activation pathway is not well elucidated at the present time, considering the regulative role  
518 of S1P in TRPC1 gating in C2C12 myoblasts (25), one possible hypothesis may be that S1P  
519 associates with TRPC1 (and/or TRPC5, 6) directly or indirectly and can trigger satellite cell  
520 activation even without stretch stimuli. Alternatively, S1P could drastically decrease the  
521 threshold intensity of stretch that is required for TRPC gating; this would be followed by  
522 L-VGC-channel activation and lead to influx of extracellular calcium ions and their  
523 subsequent presentation to calmodulin to form calcium-calmodulin complexes as the cNOS  
524 activator. In line with this, it is worth noting the excellent study by Sbrana et al. (49),  
525 which showed that S1P activates the cytoskeleton contraction that in turn stretches the cell  
526 membrane and increases its stiffness; therefore such changes may improve the  
527 mechano-sensitivity of un-stretched C2C12 myoblasts. This S1P-TRPC scenario was  
528 recently addressed by some focused studies on TRPC functions related to sphingolipid  
529 biology, and therefore does not exclude other possibilities including that the S1P pathway is  
530 not dependent on either TRPC signaling or the activation cascade concerned here.

531 In summary, the present experiments bridge the gap between mechanical stretch  
532 stimuli and the increase in intracellular calcium-ion concentration by demonstrating that  
533 inhibition of MS-channels and L-VGC-channels in vitro, prior to mechanical stretch,  
534 prevents the influx of extracellular calcium ions, HGF-release from the matrix and re-entry  
535 of adult satellite cells into the cell cycle. The physiological significance of the  
536 MS-channel gating-initiated pathway described using cultured satellite cells remains to be  
537 verified in muscle fibers, as does the crucial role of calcium-calmodulin formation in the  
538 NO-radical production by cNOS that was described recently in isolated satellite cells in  
539 culture (67). Nonetheless, the current report demonstrated that in isolated satellite cells, a  
540 functional coupling of MS-channel and L-VGC-channel gating plays the central role in  
541 mechano-sensing machinery that instigates the activation of satellite cells.

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793

794 **FIGURE LEGENDS**

795 Fig. 1. Significance of extracellular calcium ions in stretch-activation of satellite cells in  
 796 vitro. Satellite cells from adult rat skeletal muscle were stretched in culture (*black bars*)  
 797 for 1 hr beginning at 24-hr post-plating in FlexerCell FX-2000 System (25% stretch at  
 798 12-sec intervals, as originally optimized in Tatsumi et al. (59) and shown in panel *A* again)  
 799 in the presence (*bar d*) or absence (*bar c*) of 1.8 mM ethyleneglycol tetraacetic acid (EGTA)  
 800 in DMEM-10% normal horse serum (pH 7.2), followed an assay to detect activation using  
 801 bromodeoxyuridine (BrdU) at 48-hr post-plating. This experiment also included the  
 802 following control cultures: *bar a*, un-stretched culture; *bar b*, 2-hr stretch culture  
 803 (equivalent to our regular period of stretch, originally described in Ref. 59); *bar e*, stretched  
 804 culture receiving 1.8 mM EGTA for 24-48 hr post-plating; *bar f*, positive control culture  
 805 supplemented with 2.5 ng/ml recombinant mouse hepatocyte growth factor (HGF) for 24-48  
 806 hr; *bar g*, HGF culture receiving 1.8 mM EGTA for 24-48 hr; *bars h* and *i*, control cultures  
 807 with 1.8 mM EGTA for 24-25 hr and 24-48 hr, respectively. Bars depict mean and  
 808 standard error for four cultures per treatment; double asterisks indicate that the treatment  
 809 mean was significantly different from the mean of positive control cultures (*bar f*) at  $p <$   
 810 0.01. *NS*, no significant difference ( $p > 0.05$ ) in the activation index.

811

812 Fig. 2. Dose-dependent effect of cation-channel inhibitors on stretch-activation of satellite  
 813 cells and up-stream HGF release. Panel *A*, satellite cells were subjected to an environment  
 814 of cyclic stretch for 1 hr beginning at 24-hr post-plating in the presence of various  
 815 concentrations of individual cation-channel inhibitors, GsMTx-4 (*closed circle*), nifedipine  
 816 (*closed square*), gadolinium chloride (*open circle*), and NNC55-0396 (*open square*), and  
 817 assayed for BrdU-incorporation at 48-hr post-plating. This experiment also included  
 818 control cultures as follows: *bar a*, un-stretched culture (*white bar*); *bar b*, 1-hr stretch  
 819 culture (*black bar* marked *Positive Control*); *bars c-g*, control cultures receiving 2.5 ng/ml  
 820 HGF at 24-48 hr post-plating along with none, 0.1  $\mu$ M GsMTx-4, 10  $\mu$ M nifedipine, 100  
 821  $\mu$ M gadolinium chloride, and 100  $\mu$ M NNC55-0396, respectively (*grayed bars*); *bars h-j*,  
 822 un-stretched cultures with individual cation-channel inhibitors (each at the optimal  
 823 concentration). Data points and bars depict mean and standard error of four cultures per  
 824 treatment; single and double asterisks indicate that the treatment mean was significantly  
 825 different from the mean of positive control cultures (*bar b*) at  $p < 0.05$  and  $p < 0.01$ ,  
 826 respectively. Panel *B*, a typical data set of ECL-western blotting of HGF in two or three

827 independent experiments; conditioned media (serum-free DMEM) from 1-hr stretch  
 828 cultures with none (*lane m*), 0.1  $\mu\text{M}$  GsMTx-4 (*lane n*), 10  $\mu\text{M}$  nifedipine (*lane o*) or 1.8  
 829 mM EGTA (*lane p*), were analyzed for HGF release from the extracellular tethering by  
 830 ECL-western blotting standardized with internal  $\beta$ -actin in cell lysates that were harvested  
 831 after stretch. *Lane q*, un-stretch medium; *k*, molecular weight standards (STD); *l*, mouse  
 832 recombinant HGF (12 ng) to show the band at 60-kDa  $\alpha$ -chain for active HGF (*upper row*)  
 833 and 42-kDa  $\beta$ -actin in mouse C2C12 myoblasts (*lower row*) as positive controls (PC); *r*,  
 834 negative controls (NC) of stretch medium (*upper row*) and whole cell lysate (*lower row*)  
 835 blotted without the primary antibody and with secondary antibody.

836

837 Fig. 3. Detection of mRNA for the L-type voltage-gated calcium channel  
 838 (L-VGC-channel) and transient receptor potential canonical channels 1 and 6 (TRPC1, 6) in  
 839 satellite cells. Satellite cell cultures were maintained in DMEM-10% HS and assayed for  
 840 mRNA expression of  $\alpha_{1S}$ ,  $\alpha_{1C}$ ,  $\alpha_{1D}$ ,  $\alpha_{1F}$  subunits of the L-VGC-channel (panel *A*) and  
 841 TRPC1, 6 (panel *B*) at 24-hr post-plating by RT-PCR. Bands were visualized with  
 842 Gel-Red staining after agarose gel electrophoresis. *NC*, negative controls (satellite cell  
 843 mRNA without reverse transcription); *SCs*, satellite cells; *PC*, positive controls (rat skeletal  
 844 muscle for  $\alpha_{1S}$  and TRPC1, cardiac muscle for  $\alpha_{1C}$ , brain for  $\alpha_{1D}$  and TRPC6, and eye for  
 845  $\alpha_{1F}$ ).

846

847 Fig. 4. Effect of cation-channel inhibitors and EGTA on the increase in intracellular  
 848 calcium ion concentration in response to stretch. Calcium-imaging analysis was conducted  
 849 under the StageFlexer Jr. system using Fluo3-loaded satellite cells that were treated with  
 850 individual cation-channel inhibitors (0.1  $\mu\text{M}$  GsMTx-4, 10  $\mu\text{M}$  nifedipine) for 30-min just  
 851 prior to adding two cycles of stretch at 24-hr post-plating. Row +*EGTA*, cells were  
 852 incubated with 1.8 mM EGTA in DMEM-10% HS just prior to the addition of the stretch.  
 853 Companion cells were treated with 3  $\mu\text{M}$  A23187 (a calcium ionophore) and served as a  
 854 positive control (row *A23187*) (67). Fluorescence intensity was assigned from blue to red  
 855 in color-coded images; the normal resting free calcium-ion level was represented by blue,  
 856 and gradual increases were demonstrated by a change to yellow and then red. After  
 857 calcium imaging, cells were visualized by immunostaining to confirm the presence of c-met,  
 858 a marker molecule for myogenic cells in our primary cell cultures (DAB-stained images not  
 859 shown).

860

861 Fig. 5. A possible mechanism of satellite cell activation in response to mechanical stimuli.

862 Panel *A*, reproduced from Fig. 4 of Tatsumi and Allen (66) with some modifications.

863 Ca-CaM, calcium-calmodulin complex; L-Arg, L-arginine; NOS, NO synthase; MMP2,

864 matrix metalloproteinase 2. Panel *B*, calcium ion influx mechanism was examined in the

865 present study and the model was schematically presented. Steps 1 and 2: cations stream

866 into the cell through mechano-sensitive cation channels (MS-channel) to induce local

867 depolarization of the cell membrane (changes in membrane potential); step 3: depolarization

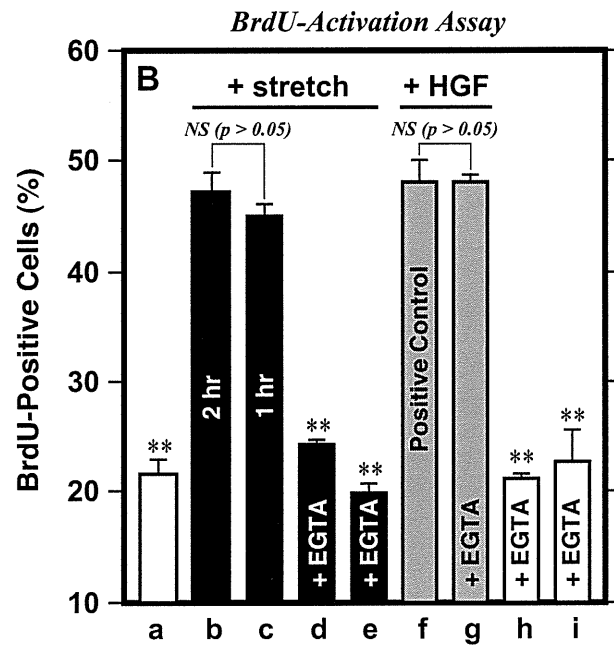
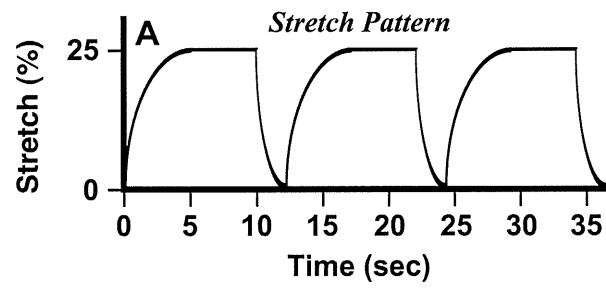
868 promotes gating of adjacent L-type voltage-gated calcium-ion channels (L-VGC-channel),

869 and the result is calcium ion influx that promotes formation of the Ca-CaM complex that

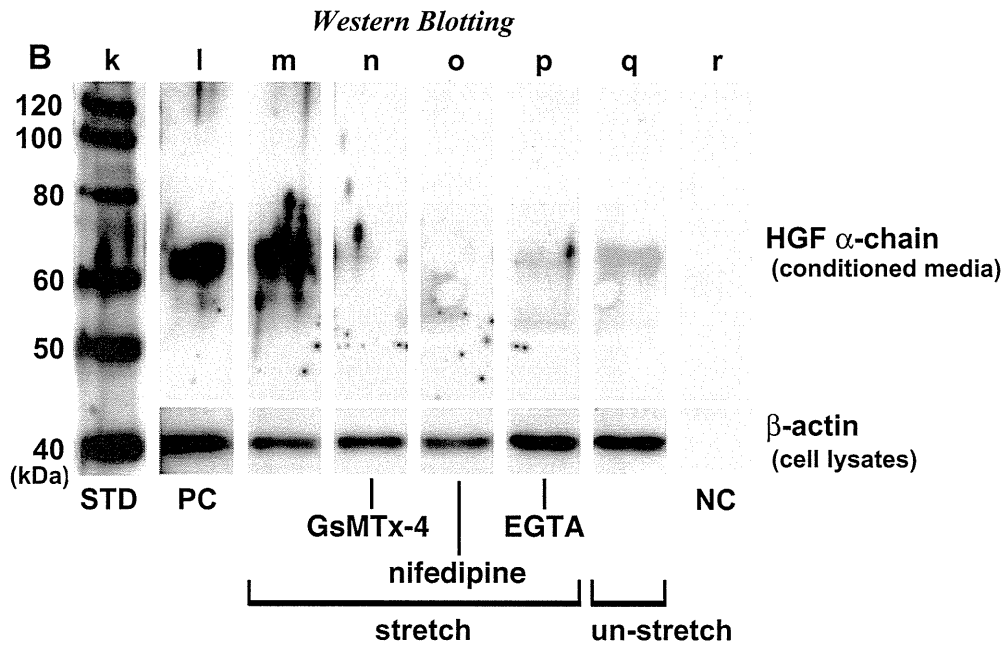
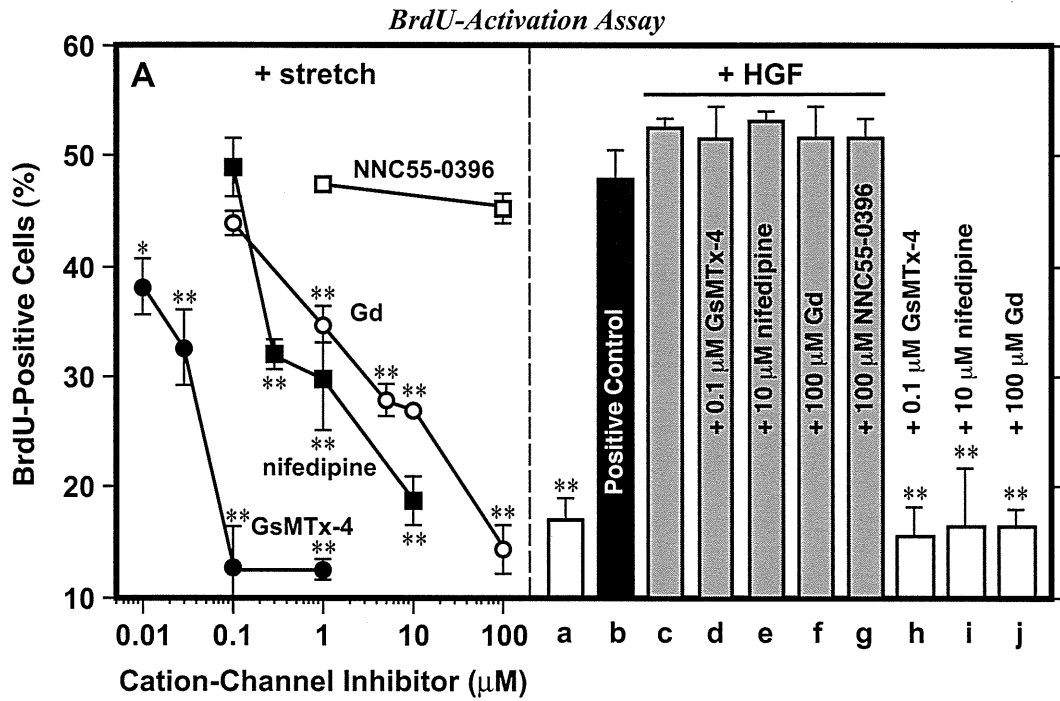
870 initiates NOS activity.

871

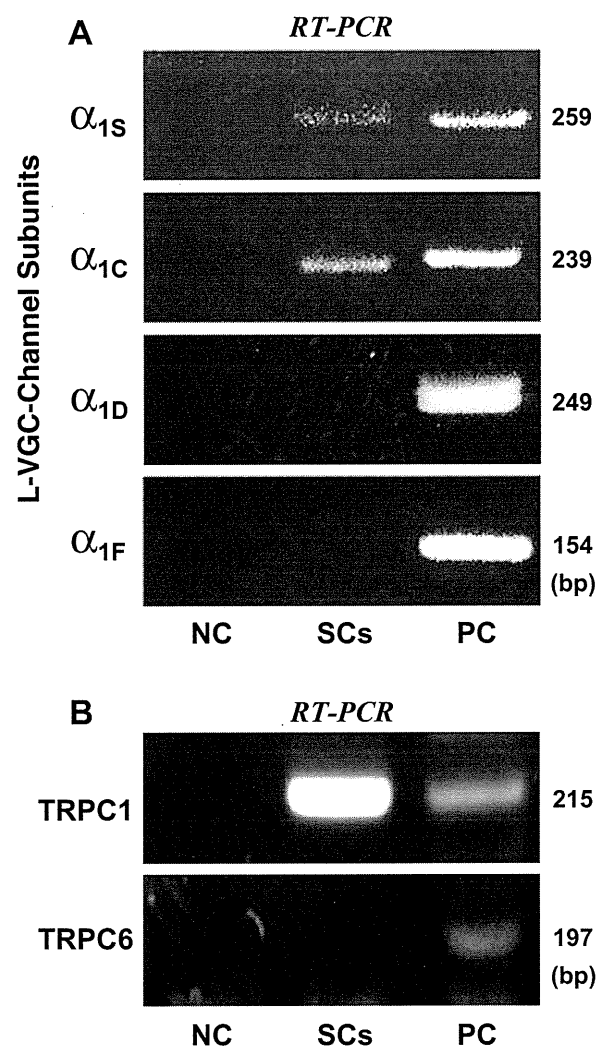
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**Fig. 1, Hara et al.**



**Fig. 2, Hara et al.**



**Fig. 3, Hara *et al.***



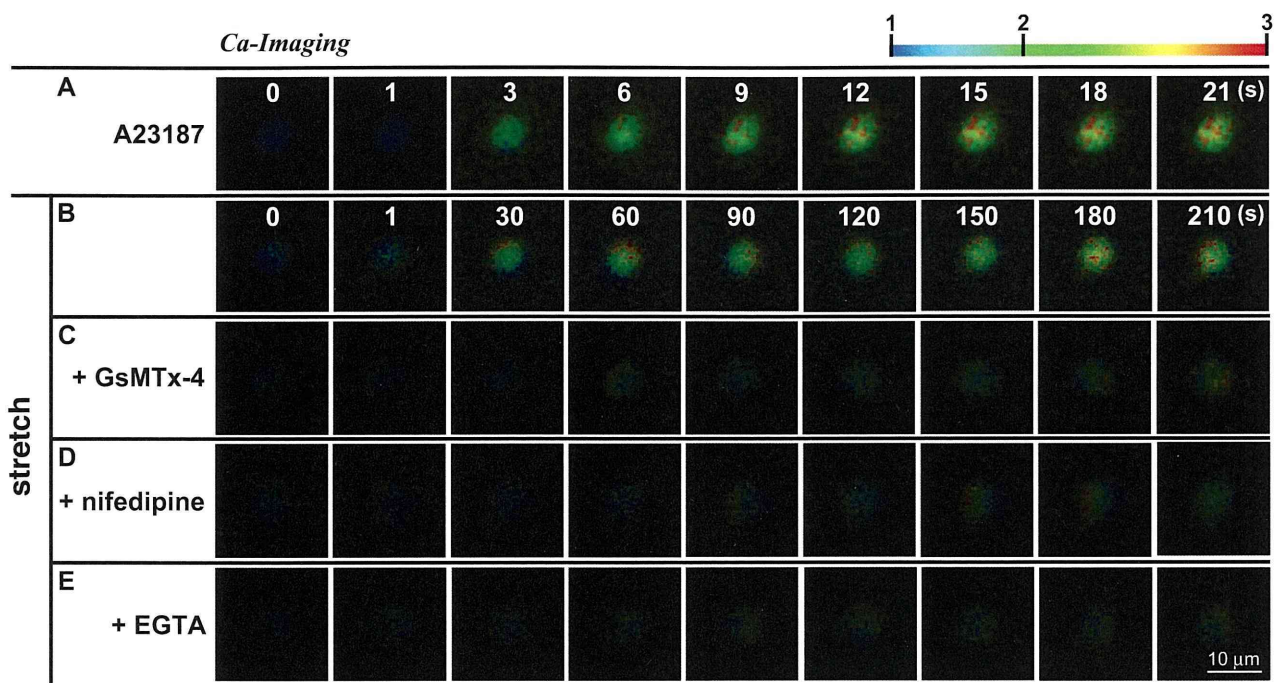


Fig. 4, Hara *et al.*



