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Crossbridge Attachment, Resistance to Stretch, and Viscoelasticity in Resting Mammalian Smooth Muscle

Abstract. *There exists a calcium-dependent resistance to stretch in resting mammalian smooth muscle that is not caused by depolarization of the cell membrane or release of calcium from intracellular sites. The similarity of the resistance to stretch in the resting state to that in rigor suggests that most, if not all, crossbridges are attached and thus able to resist stretch in noncontracting smooth muscles. When the muscle is stretched the breaking and subsequent reformation of links in nonstrained positions accounts for most of the so-called viscoelasticity, except at extreme lengths.*

When smooth muscles are rapidly stretched they exhibit a sharp rise in tension, which then decays nearly exponentially over a period of time. The initial rise in tension (resistance to stretch) and decay of tension (stress relaxation) have been attributed to a passive viscoelastic property of the muscle (*1*). In the course of experiments in which the effects of different ions on such relationships as length-tension and force-velocity were examined in various smooth muscles, it was noted that resistance to stretch and the resulting stress relaxation were markedly affected by the presence or absence of calcium in the bathing medium.

Tension was monitored in relation to change in length or stimulation (induced electrically or by potassium) in strips of rabbit taenia coli (N = 56), portal vein (N = 8), myometrium (N = 6), and aorta (N = 4), and of guinea pig taenia coli (N = 8). The muscles were bathed in a flowing Krebs-bicarbonate solution gassed with 95 percent O_2 and 5 percent CO_2, which had the following composition (millimolar): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.18; KH_2PO_4, 1.2; $CaCl_2$, 1.9; $NaHCO_3$, 25; and glucose, 11. Calcium-free Krebs solution contained 2 mM EGTA [disodium salt of ethylene glycol-*bis*(β-aminoethylether)-N,N'-tetraacetic acid, Sigma] and no added calcium. In sodium-free solutions NaCl was replaced with 118 mM dimethyldiethanol ammonium chloride (DDA, Eastman Organic Chemicals), $NaHCO_3$ was replaced with 25 mM choline bicarbonate, and atropine (1 μM) was added (*2*). 5-[(3,4-Dimethoxyphenethyl) methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile (D600, a gift from Knoll, AG) was used at 1 to 10 μM. For stimulation by potassium, KCl was added to a concentration of 100 mM in the Krebs solution. Solutions for caffeine contractures included either 4 or 8 mM caffeine. Thymol (Fisher Scientific) was used at 1 mM in Krebs solution. 1-Fluoro-2,4-dinitrobenzene (FDNB, Eastman Organic Chemicals) was used at 0.38 mM in glucose-free, calcium-free (2 mM EGTA) Krebs solutions gassed with 95 percent N_2 and 5 percent CO_2.

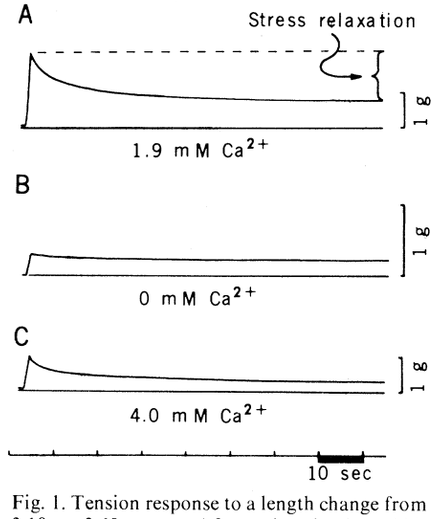

Fig. 1. Tension response to a length change from 3.10 to 3.60 mm at 1.0 mm/sec in the rabbit taenia coli at 21°C. The muscle was bathed in a 1.9 mM Ca^{2+}-Krebs solution (A), then 0 mM Ca^{2+} (2 mM EGTA)-Krebs solution for 0.5 hour (B), and finally 4 mM Ca^{2+}-Krebs solution for 1 hour (C). Note that record in (B) is amplified twice compared to (A) and (C). The muscle gave maximal active tension with tetanic stimulation at a length of 4.8 mm in 1.9 mM Ca^{2+}-Krebs solution. The solid line below each trace represents zero tension. Stress relaxation was calculated as the difference between the peak and the subsequent steady-state tension reached following the stretch.

The muscles used for measurement of adenosine triphosphate (ATP) were frozen by use of a device (Auto-pulverizer, Biochem Instruments) which quickly flattened the muscles between two stainless steel blocks precooled to −190°C with liquid nitrogen. The muscles were extracted as described previously (*3*). Adenosine triphosphate was assayed using a Varian 4200 Liquid Chromatograph with a Variscan detector and an NH_2 column (Varian Associates). Measurement was accomplished using a mobile phase of 0.35M KH_2PO_4 flowing at 60 ml/hour and by comparing the absorbance of samples and standards at 259 nm.

The apparatus and method used for electrically stimulating the muscles and for determining length-tension relationships was described by Gordon and Siegman (*4*). Length changes were made manually with a micrometer drive or with a servomotor (Gould Instruments). Temperature was kept at 21°C to eliminate spontaneous contractile activity of the tissue (*4*).

Qualitatively similar results were obtained in all of the muscle preparations studied, and Fig. 1A shows a typical tension response to a length change of 0.5 mm at the rate of 1 mm/sec in 1.9 mM calcium-containing Krebs solution. After incubation in calcium-free Krebs solution for 0.5 hour the resistance to stretch and resulting stress relaxation were dramatically reduced (Fig. 1B). The ATP content of the muscle in a calcium-free solution was the same as that of a control muscle in a calcium-containing medium. Reequilibration in 1.9 or even 4.0 mM calcium-containing Krebs solution began to restore the resistance to stretch within 2 minutes. Within 1 hour it had stabilized to 76 ± 3 percent [N = 7, mean ± standard error of the mean (S.E.M.) for all muscle lengths] of its original value (Fig. 1C). Increasing the calcium concentration of the Krebs solution from 1.9 to 4.0 mM had no effect on the passive tension, resistance to stretch, or stress relaxation. Although the passive tension of the muscle, measured as the steady-state force maintenance at a particular length, is slightly greater than the minimum tension reached upon release from longer lengths, neither was affected by the presence or absence of calcium in the bathing medium.

As shown in Fig. 1A, stress relaxation is measured as the difference between the peak tension response and the subsequent steady-state tension achieved after the

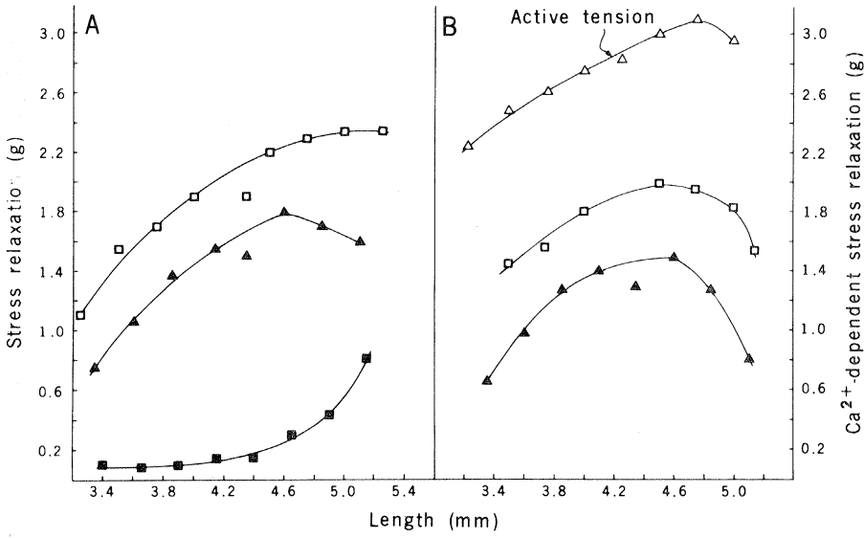

Fig. 2. (A) The stress relaxation (defined in Fig. 1) resulting from 0.5 mm stretch at 1 mm/sec to the length shown on abscissa in Krebs solutions containing 1.9 m*M* Ca^{2+} (□), then 0 m*M* Ca^{2+} (2 m*M* EGTA) for 0.5 hour (■), and finally 4 m*M* Ca^{2+} for 1 hour (▲), for the same muscle as shown in Fig 1. (B) Calcium-dependent stress relaxation calculated as the difference between stress relaxation in 1.9 m*M* Ca^{2+}-Krebs (□), or 4.0 m*M* Ca^{2+}-Krebs (▲), and 0 m*M* Ca^{2+} (2 m*M* EGTA)-Krebs solution based on values shown in (A). The active tension curve is from tetanically stimulated muscle in 1.9 m*M* Ca^{2+}-Krebs solution (Δ).

stretch. Figure 2A shows the dependence of stress relaxation on muscle length and on the presence or absence of calcium in the medium. In calcium-free Krebs solution the stress relaxation was very small at short muscle lengths, but a rather sharp rise in stress relaxation occurred at longer lengths, where the passive tension of the muscle also increased dramatically. At lengths where there was considerable stress relaxation in calcium-free media the stress-strain characteristics of the muscle were similar to those of collagen (*5*). Control experiments have shown that stress relaxation of nerve sheaths, rat tail collagen fibers, and reaggregated collagen are unaltered in calcium-free medium (*6*). The calcium-dependent stress relaxation, measured as the difference between the stress relaxation in 1.9 or 4.0 m*M* calcium-containing Krebs solution and that in calcium-free medium, is shown in Fig. 2B. The contour of the curve describing the relationship between the calcium-dependent stress relaxation and the muscle length was very similar to that of the active tension and muscle length, also shown in Fig. 2B. Studies correlating stiffness and tension in resting and activated skeletal muscle have related stiffness to the number of crossbridges attached, and hence muscle length, in accordance with the sliding filament model (*7*). This suggests that a sliding filament model may underlie both the calcium-dependent stress relaxation and active tension in smooth muscle. Similar results were found in all the smooth muscles studied. The fact that the calcium-dependent stress relaxation could only be restored to 76 ± 2 percent (N = 7, mean ± S.E.M. for all muscle lengths) of the original value on readdition of calcium is consistent with observations that restoration of calcium-dependent responses is not always complete (*8*).

The regulatory effects of intracellular calcium on the interaction of contractile proteins are well known (*9*). It is likely that the calcium-dependent resistance to stretch and stress relaxation is due to a calcium effect on the contractile elements. For example, stretch might depolarize the muscle membrane and elicit an action potential (*10*), thus causing influx or release of calcium from intracellular sites (*11*). In this manner there could be actin-myosin interaction causing resistance to stretch dependent on the extent of actin-myosin overlap. Further experiments on rabbit taenia coli of the type shown in Figs. 1 and 2 show that D600, which blocks inward calcium current in smooth muscle during the action potential (*12*), also blocked potassium contracture and the response to electrical stimulation, in both sodium-containing and sodium-free solutions. It had no effect, however, on the calcium-dependent resistance to stretch and stress relaxation in this muscle.

Thymol (1 m*M*) blocks potassium and acetylcholine contractures of depolarized intestinal smooth muscle, probably by suppressing calcium influx during the action potential and release of calcium from intracellular sites (*13*). We have found that in the presence of thymol (1 m*M*), calcium-dependent resistance to stretch and stress relaxation remained unchanged even though potassium and caffeine contractures were blocked. Our results thus suggest that the calcium-dependent resistance to stretch and stress relaxation is not caused by an influx of calcium during depolarization of the cell membrane or by release of calcium from intracellular sites, which result in the activation of the normal tension-developing mechanism. The calcium-dependent resistance to stretch thus depends on the small amounts of free calcium in the sarcoplasm of the resting muscle in the presence of normal amounts of calcium in the external medium.

The ATP content of the rabbit taenia coli was reduced from 1.35 ± 0.013 (N = 5) to 0.137 ± 0.003 μmole/g (N = 3) after 3.0 hours in 0.38 m*M* FDNB (*14*), glucose-free, and calcium-free (2 m*M* EGTA) Krebs medium gassed with 95 percent N_2 and 5 percent CO_2. Following this treatment, resistance to stretch was very similar to that in the resting state in a calcium-containing medium; the ratio of the peak resistance to stretch in rigor to that in an untreated muscle bathed in 1.9 m*M* calcium-Krebs solution was 0.96 ± 0.07 (N = 16). It was thus significantly greater than that of a muscle in a calcium-free medium with its full complement of ATP. In low-ATP (rigor) muscles the resistance to stretch was not dependent on the presence or absence of calcium in the bathing medium. It is thus unlikely that the calcium-dependent resistance to stretch can be associated with the mechanical properties of the cell membrane and connective tissue. The calcium-dependent resistance to stretch in low-ATP smooth muscles was similar to the high resistance to stretch due to the presence of rigor bonds in skeletal muscles with low ATP (*15*). Therefore, rigor characterized by very low levels of ATP and increased stiffness can exist in mammalian smooth muscle (*16*).

If it is assumed that all crossbridges are attached in the rigor state (*17*), then the observation that the resistance to stretch at rest in a calcium-containing medium is quite similar to that in rigor strongly suggests that most, if not all, crossbridges are attached and thus are able to resist stretch in resting smooth muscles. At levels of free intracellular calcium found at rest, these attached crossbridges are not generating a net force since their detachment, shown by loss of resistance to stretch, and hence viscoelasticity, in calcium-free media, does not affect the steady-state force maintained by the muscle. Thus, under these conditions such attached crossbridges are not involved in the maintenance of tone. Stretching the muscle at rest results in the straining of attached crossbridges, and stress relaxation reflects the temporary detachment of the strained crossbridges fol-

lowed by reattachment under unstrained conditions. Except at extreme lengths, where there is a large contribution from connective tissue, this could account for most of the viscoelasticity in resting smooth muscle. It follows that there appear to be three effects associated with different intracellular calcium concentrations: (i) at very low levels the calcium-dependent resistance to stretch disappears; (ii) at higher levels crossbridge attachment occurs, manifested as viscoelasticity; and (iii) at still higher levels there may be activation of actomyosin adenosine triphosphatase and crossbridge cycling manifested as tone or, following normal excitation-contraction coupling, tension development and shortening.

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Activation of Transplant Immunity: Effect of Donor Leukocytes on Thyroid Allograft Rejection

Abstract. *The survival of thyroid allografts in mice was prolonged by either holding the grafts in in vitro culture for 20 to 27 days or by cobalt-60 irradiation of the donor 2 days before transplantation with or without the intravenous injection of colloidal carbon just before removing the thyroid from the donor. In both cases the rejection process was restored by an intravenous injection of recipients with living peritoneal exudate cells (50 to 80 percent macrophages) syngeneic to the thyroid donor.*

The survival of both thyroid and ovarian transplants in allogeneic mice has been prolonged by first culturing the grafts for several weeks in vitro (*1, 2*). The finding by Summerlin and co-workers of a similar prolongation of cultured skin grafts (*3*) has not been confirmed partly because of failure of cultured skin grafts to revascularize even when syngeneic to the recipient (*1*). Two alternative mechanisms have been proposed for the prolonged survival of thyroid and ovarian grafts. (i) A modification of tissue antigens occurs during the culture in vitro resulting in a loss of tissue antigenicity. (ii) Some of the leukocytes trapped in the graft are essential for the activation of recipient T lymphocytes (thymus derived), and are gradually lost during the culture in vitro.

In relation to this second proposition, we have proposed that the activation of specific T lymphocytes is the function of a living cell with special properties (*4*) and have reported evidence that in mice the macrophage is the most efficient if not the only cell with this stimulating activity (*5*). Others have reported similar evidence for guinea pig and human macrophages (*6*). The importance of passenger leukocytes in transplant immunity has already been demonstrated (*7*).

We reasoned that, if the loss of tissue leukocytes were responsible for the prolonged survival of cultured organ grafts, then the injection into the recipient of leukocytes syngeneic to the graft should activate specific transplant immunity and cause graft rejection. In contrast, if the loss of tissue antigens was responsible for the prolonged survival, the injection of leukocytes would have little effect. We also used an alternative method of reducing the number of blood leukocytes in the graft: gamma radiation of the donor 2 days before removal of the organs. This treatment also prolonged allograft survival. With both the cultured and the irradiated grafts we found that the intravenous injection of living peritoneal exudate cells (rich in macrophages and lymphocytes), if syngeneic to the graft, stimulated its rejection.

Individual lobes of BALB/c (H-2d) mouse thyroid glands were placed on rafts in 60-mm organ culture dishes (Falcon) containing Eagle's minimum essential medium (MEM) supplemented with 10 percent fetal calf serum, penicillin, and streptomycin. The cultures were maintained for 20 to 27 days at 37°C in a humid atmosphere of 95 percent O_2 and 5 percent CO_2. The organ culture medium was changed three times each week.

Thyroids were transplanted by placing individual lobes under the left kidney capsule. Thyroidectomy was performed on recipients of cultured thyroids and their controls (Table 1), but not on the other recipients of a direct transplant (Tables 2 and 3). After several weeks in culture the ability of transplanted thyroid glands to take up iodine was considerably reduced compared to direct transplants, and thyroidectomy of the recipient was required to stimulate transplant function to detectable levels. The function of the transplanted thyroids was determined by an intraperitoneal injection of recipient mice with 0.25 μc of carrier-free ^{125}I. The recipient was killed 24 hours later, and both kidneys were removed and placed in counting tubes containing formalin in saline. The radioactivity in the left kidney, which contained the thyroid transplant, as well as that in the control right kidney, was then counted in a gamma counter. Some groups of recipients were placed on low iodine food for 3 days prior to the injection of ^{125}I. This diet increased the uptake of radioactivity in the grafted and control kidneys approximately fivefold, but the ratio of radioactivities in the grafted to the control kidneys was not significantly altered. All transplants of cultured thyroid were examined histologically after counting. Selected kidneys receiving direct transplants were similarly examined, including all grafted kidneys whose radioactivity was not significantly above its control.

Peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavities of normal mice with approximately 10 ml of MEM each. The cells were washed twice with MEM and counted. Electron micrographs showed that 50 to 80 percent of the cells so obtained had the typical lysosomes and ruffled membranes of macrophages. The other cells were mostly small lymphocytes.

All 11 uncultured BALB/c (H-2d) thyroids were completely rejected 15 days af-