insulin action in isolated nuclear envelopes. In contrast, the nonmitogenic lectin wheat germ agglutinin is ineffective in this system. Our data therefore support those of Beachy *et al.* (*14*) showing that Con A and PHA, but not wheat germ agglutinin, stimulate the production of a mediator substance in lymphocytes that activates mitochondrial pyruvate dehydrogenase.

It has been suggested that the binding of insulin to the plasma membrane receptor may be sufficient to initiate all of insulin's actions (*25*). However, since agents that mimic insulin are internalized and act on the nuclear envelope, it is also possible that insulin and agents that mimic it have direct effects in the cell interior. After internalization these agents may bind to the nuclear envelope insulin receptor, inhibit nuclear envelope phosphorylation, and thus initiate a series of events leading to enhanced mRNA transport from the nucleus into the cytoplasm.

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Calcium-Dependent Stress Maintenance Without Myosin Phosphorylation in Skinned Smooth Muscle

Abstract. *Stress development depended on calcium-stimulated myosin phosphorylation in an arterial smooth muscle preparation in which the concentration of calcium was controlled. However, developed stress was maintained at a concentration of calcium that did not support phosphorylation. These results, in conjunction with other evidence, suggest that the interaction of two regulatory mechanisms with different calcium sensitivities regulate both stress and the rate and energetics of contraction.*

Biochemical studies of contractile proteins isolated from vertebrate smooth muscle have provided strong evidence that phosphorylation of the 20,000-dalton myosin light chain (LC 20) by myosin light chain kinase enables cross-bridges to attach to the thin filament and cycle (*1*). The hypothesis that cross-bridge phosphorylation is necessary and sufficient to regulate contraction is strongly supported by the dependence of force development on phosphorylation in smooth muscle in which the cell membranes are disrupted ("skinned") so that the concentration of calcium can be controlled in contractions produced by exogenous Mg^{2+} and adenosine triphosphate (ATP) (*2*).

Murphy and co-workers (*3*) found that cross-bridge phosphorylation preceded contraction after stimulation of intact smooth muscle. However, Ca^{2+}-dependent steady-state stress was maintained after phosphorylation decreased during sustained contractions. The steady-state stress was associated with low cross-bridge cycling rates, as shown by isotonic shortening velocities, and was termed "latch" (*3*). Further studies led to our hypothesis that a second Ca^{2+}-dependent regulatory mechanism controls the formation of attached noncycling or slowly cycling cross-bridges characteristic of latching and that this system has a higher sensitivity to Ca^{2+} than does myosin light chain kinase and phosphorylation (*4*).

The apparent absence of a latch state in skinned tissues (*2*) may reflect loss or inactivation of a Ca^{2+}-dependent regulatory mechanism producing latching. Alternatively, myosin phosphorylation may be required for Ca^{2+} binding to a second regulatory site. We report Ca^{2+}-dependent stress maintenance at concentrations of Ca^{2+} below those required for myosin phosphorylation in skinned smooth muscle. Mild skinning procedures (*5*) were briefly applied to tissues whose contractile capacity had been determined. The preparation of thin (338 ± 18 μm) strips from the tunica media of swine carotid arteries, determination of their optimum length (L_0) for isometric stress generation (S) in response to K^+ depolarization, and measurement of phosphorylation after quick freezing have been described (*4, 6*). The tissues were treated for 60 minutes at 22°C with a solution containing 5 m*M* EGTA, 20 m*M* imidazole (*p*H 6.7), 50 m*M* potassium acetate, 0.5 m*M* dithio-

threitol, 150 mM sucrose, and 0.5 percent Triton X-100. The sucrose and Triton X-100 were then replaced with 6 mM $MgCl_2$ and ATP for approximately 20 minutes (relaxing solution). Stress and phosphorylation were measured in the first contraction-relaxation cycle produced by changes in the concentration of Ca^{2+} induced by a buffer system with 5 mM EGTA and varied $CaCl_2$ (*7*).

Initial contractions produced by high (7 μM) Ca^{2+} approximated the responses of the intact tissues. Isometric stress generation was 91.8 ± 5.7 percent (mean ± standard error; $N = 15$) of the control value elicited by K^+ depolarization. The lower temperature, coupled with dissection damage in the thin tissues (*4*), depressed maximum stress relative to previous results (*3*). Steady-state phosphorylation values (Fig. 1) were comparable to peak levels transiently attained in intact tissues (*3, 4*). The time courses of stress development and phosphorylation were similar (Fig. 1A), as was the Ca^{2+} dependence of steady-state stress and of phosphorylation (Fig. 2A) during contractions elicited by increasing the concentration of Ca^{2+}. These results confirm the earlier finding (*1, 8*) that myosin phosphorylation is associated with development of isometric stress. They also show that high stresses approximating those attained by intact vascular smooth muscle are associated with high values of phosphorylation [up to 0.8 mole of inorganic phosphate (P_i) per mole of LC 20 in some preparations]. In contrast to other studies (*8, 9*), there was no evidence for cooperativity in the contractile response to phosphorylation (Fig. 2A).

Dephosphorylation in $1.8 \times 10^{-8} M$ Ca^{2+} occurred more rapidly than the decline in stress (Fig. 1B). Although these rates were affected by diffusion of the Ca^{2+} buffer system, stress persisted at times when phosphorylation reached basal levels. Similarly, adenosinetriphosphatase activity falls far more rapidly on reduction of the concentration of Ca^{2+} than does stress (*10*). The possibility that stress could be maintained after the concentration of Ca^{2+} was reduced was tested under steady-state conditions. Skinned tissues were first contracted with 8 μM Ca^{2+}. The Ca^{2+} buffer concentration in the contracting solution was reduced 50-fold (total EGTA, 0.1 mM). This facilitated equilibration to a lower level of Ca^{2+}, and stress and phosphorylation attained steady-state values in 15 to 20 minutes, in contrast to the situation illustrated in Fig. 1B. The data plotted in Fig. 2B show the resulting dependence of stress and phosphorylation 30 minutes after Ca^{2+} was reduced from maximum levels.

The dependence of phosphorylation on the concentration of Ca^{2+}, characterized by the parameter K_{50}, was not significantly reduced ($P > 0.1$; t-test for means of two populations with increasing or decreasing Ca^{2+}) (Fig. 2, A and B). However, the K_{50} for the dependence of stress on Ca^{2+} concentration was significantly ($P < 0.005$) reduced if the tissue was first contracted at a high concentration of Ca^{2+} with concomitant high levels of phosphorylation.

In control experiments tissues were initially contracted with 7 μM Ca^{2+}, relaxed with 0.018 μM Ca^{2+}, and then stimulated a second time with successively higher levels of Ca^{2+}. The maximum stress obtained was 80 ± 11 percent ($N = 9$) of that generated in the first response after skinning, and the maximum phosphorylation (0.53 ± 0.06 mole of P_i per mole of LC 20; $N = 6$) was decreased proportionally, although this difference was not significant ($P = 0.1$). Deterioration of stress generation and a decreased Ca^{2+} sensitivity in skinned smooth muscle have been ascribed to a loss of calmodulin (*11*). However, the K_{50} for the Ca^{2+} dependence of stress under conditions of increasing Ca^{2+} in the second contraction ($6.5 \times 10^{-7} M$) was the same as that obtained in the first contraction (Fig. 2A). This rules out a time-dependent increase in the Ca^{2+} sensitivity for stress as an explanation for the results shown in Fig. 2B.

Figure 2B presents direct evidence for a regulatory mechanism with a high sensitivity for Ca^{2+} that allows stress maintenance without proportional phosphorylation (latching), as inferred from studies of intact tissues (*4*). Moreover, these results indicate that myosin phosphorylation or the resulting stress is a prereq-

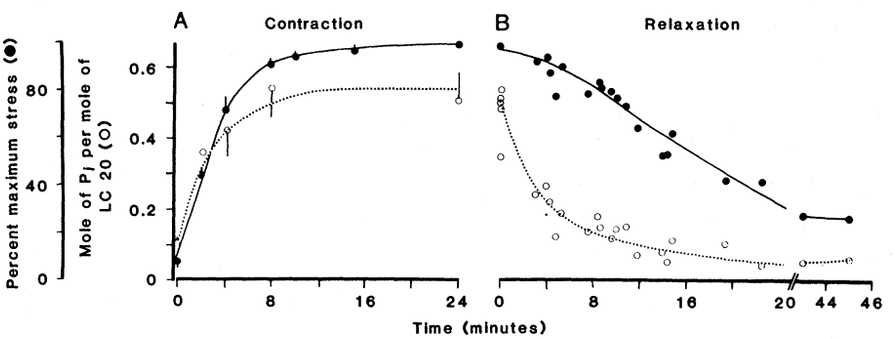

Fig. 1. (A) Time course of stress development (●) and of myosin phosphorylation (○) in carotid media strips when Ca^{2+} was increased from 1.8×10^{-8} to $7 \times 10^{-6} M$ (means ± standard errors; $N = 4$ to 5). (B) Stress and phosphorylation in individual tissues relaxing in a solution containing $1.8 \times 10^{-8} M$ Ca^{2+} after 15 to 20 minutes in $7 \times 10^{-6} M$ Ca^{2+}. The maximum stress was $6.9 \pm 0.7 \times 10^4$ N/m^2 ($N = 12$).

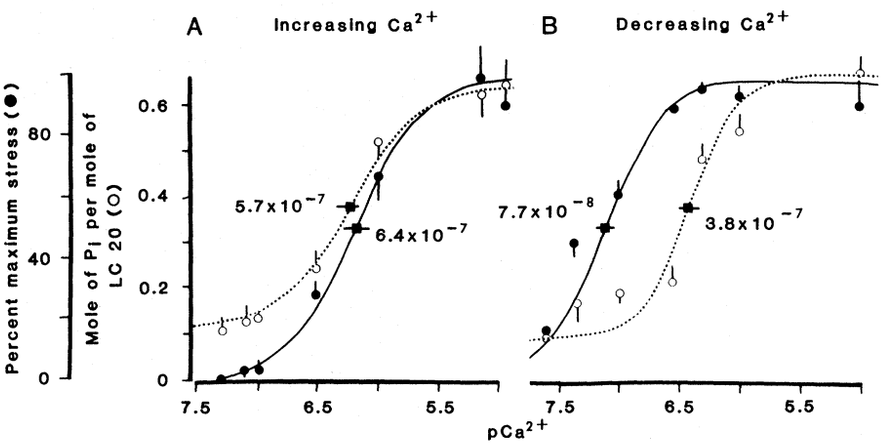

Fig. 2. Steady-state Ca^{2+} dependence of stress and myosin phosphorylation when Ca^{2+} was increased from $1.8 \times 10^{-8} M$ (A) or decreased from $8 \times 10^{-6} M$ (B) (means ± standard errors; $N = 4$ to 6). Maximum stress was $9.3 \pm 1.2 \times 10^4$ N/m^2 ($N = 10$). The curves were calculated for the individual data points with a nonlinear curve-fitting program for the relation: relative stress $= 1/1 + (K_{50}/[Ca^{2+}])^n$, where K_{50} and n are curve-fitting parameters (*5, 17*). The calculated concentrations of Ca^{2+} for the half-maximal change in the responses (K_{50}) are shown with their 65 percent confidence intervals (■). Values of n (65 percent confidence limits) are (A) stress, 1.56 (1.32 to 1.77); phosphorylation, 1.57 (1.23 to 1.92) and (B) stress, 1.83 (1.55 to 2.14); phosphorylation, 2.34 (2.06 to 3.19).

uisite for latching, because no increase in tissue stiffness or stress was observed without phosphorylation when the level of Ca^{2+} was increased from $1.8 \times 10^{-8} M$.

The Ca^{2+} binding site regulating latching is unknown. The regulatory light chains of all myosins have Ca^{2+} binding sites, some of which have a regulatory role in many invertebrate muscles (*12*). Such sites are attractive candidates because phosphorylation of the regulatory light chain could alter its conformation and Ca^{2+} binding characteristics. It is also possible that cross-bridge attachment to the thin filament after phosphorylation enables a thin-filament protein to bind Ca^{2+} (*1*), leading to maintained cross-bridge attachment. A small hysteresis in the stress-Ca^{2+} relation in skinned striated muscle has been attributed to an increased affinity of the regulatory site for Ca^{2+} because of active stress in the myofilaments (*13*).

Vascular smooth muscle normally opposes stress due to blood pressure with a rate of energy consumption some one three-hundredths of that required by frog striated muscle to maintain a comparable stress (*14*). Stimulation of arterial smooth muscle by agonists produces a transient increase followed by a decrease in myoplasmic Ca^{2+}, as estimated from light emission by aequorin-loaded cells (*15*) and from myosin phosphorylation (*3, 4*). In previous studies (*3*) it was suggested that stress maintenance by nonphosphorylated cross-bridges is associated with reduced cycling rates, as estimated from isotonic shortening velocities. On the basis of this study, we suggest that the high economy of stress maintenance in smooth muscle is due to an unidentified regulatory mechanism with a high sensitivity to Ca^{2+}. This system appears to function only after the initial activation and Ca^{2+} transient that lead to myosin phosphorylation and stress development. Relaxation is the result of further reductions in cell Ca^{2+} below the threshold for latching (*16*).

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Electrical Synapse Formation Depends on Interaction of Mutually Growing Neurites

Abstract. *A neuron's competence to form electrical synapses depends on its growth status. Experiments in situ and in cell culture with identified neurons of the snail* Helisoma *demonstrate that active neurite outgrowth from both potential partners must be spatially and temporally coincident for electrical synapse formation to occur.*

Growing neurons can establish highly predictable sets of connections during both development and adult neuronal plasticity. Equally important, many potential connections are never formed.

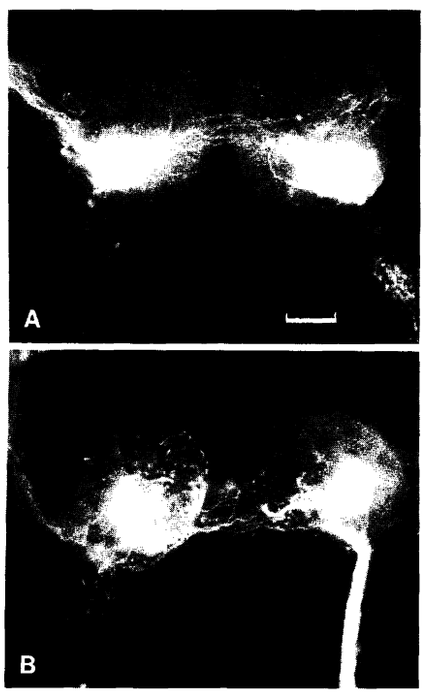

Fig. 1. Buccal ganglia with Lucifer yellow–injected (*14*) pairs of neuron 5's, shown after selective "zap axotomy" (where axon exits ganglion) of both neurons (A) or a single neuron (B). Mean coupling coefficients are 0.29 ± 0.05 ($N = 8$) and 0.03 ± 0.01 ($N = 8$) for (A) and (B), respectively. The unperturbed neuron 5 (on the right in B) retained its normal morphology. Cultures were 5 days old (*4, 5*). Scale bar, 100 μm.

Specific connectivity could depend on many mechanisms, including those governed by intrinsic properties of neurons or by the nature of interactions between neurons. Because highly reproducible sets of new electrical synapses can be triggered to form between neurons of the snail *Helisoma* (*1, 2*), it is now possible to determine why certain identified neurons reliably form connections while others do not.

We tested the hypothesis that formation of new electrical synapses requires spatially and temporally coincident neurite outgrowth from each of the potential partners. This growth-dependence hypothesis was initially suggested by experiments in which nerve crushing evoked neurite growth in buccal ganglia in situ (*3*). We now report two higher resolution experimental approaches which independently demonstrate that only growing neurons readily form electrical connections.

Our first approach was to restrict treatment to individual neurons while preserving the in situ environment. We used a cultured ganglionic preparation known to have little or no central neuronal sprouting in the absence of axotomy near the cell body (*3–6*). Individual axons were selectively severed by localized photoirradiation of injected fluorescent dye ("zap axotomy") (*6*). When neurons 5R and 5L were zap-axotomized, they grew profusely and formed electrical connections that were qualitatively indistinguishable from those formed when bilateral nerve crushing evoked growth (*6*). To test the require-