Myosin Synthesis Increased by Electrical Stimulation of Skeletal Muscle Cell Cultures

Abstract. *When cultures of skeletal muscle cells of the chick embryo are subjected to repetitive, electrical stimulation, the contractions increase the amount of protein produced by these cells. The increase is greater for contractile proteins such as myosin heavy chain than for total cellular protein. This demonstrates that in a culture system of skeletal muscle cells that have differentiated in the absence of innervation, one can elicit the protein synthetic response associated with skeletal muscle hypertrophy in vivo.*

Increased rates of work of skeletal muscle result in hypertrophy. Hypertrophy is characterized by an increased rate of contractile protein synthesis (*1–4*), a decrease in protein degradation (*2, 4, 5*), an increase in the transport of neutral amino acids (*6*), and an enhancement of RNA content (*2, 7*). The DNA content of hypertrophied muscle increases as a result of enhancement of the connective tissue elements of tissue rather than from proliferation of skeletal muscle cells (*2, 7*). Recent studies in vivo indicate that changing rates of stimulation of skeletal muscle can also result in the synthesis of different species of contractile protein (*8–13*), suggesting that it is the rate of contraction in vivo which results in differential gene activation. We have attempted to develop in vitro a model of skeletal muscle hypertrophy that is free of innervation so that the rate of contraction of the muscle cells formed in cell cultures can be controlled by driving them electrically. Using a single rate of stimulation, we have found that the electrical stimulation of skeletal muscle cells in culture results in increased rates of myosin synthesis.

We used skeletal muscle cell cultures established from 12- to 13-day embryonic chick breast muscle (*14*). Cells were plated in gelatin-coated wells (16 mm in diameter, Linbro 24-well plastic culture trays) at a density of 1.5×10^5 cells per well, and permitted to undergo differentiation into large, multinucleated muscle cells (myotubes). When the muscle cultures were 4 to 5 days old, those in 12 of the culture wells were stimulated for 14 to 48 hours with a 0.6-second train of 10- to 25-msec biphasic pulses delivered every 4 seconds by way of agar saline bridges (Fig. 1). The ratio of the period of stimulation to the period of inactivity was 0.15, such that for a total stimulation period of 48 hours, the actual time of stimulation would be 7 hours. Spontaneous contractions were rarely observed in these young cultures. The myotubes in the wells were observed under phase contrast microscopy both at the beginning and end of stimulation to make sure that they were contracting. Usually, most of the myotubes contracted with each stimulus train. We found that cultures coupled with platinum instead of agar bridges appeared unhealthy and did not produce increased amounts of contractile proteins, despite the occurrence of electrically stimulated contractions. High cell densities and high percentages of myogenic cell fusion contributed to our being able to obtain reproducible results in the system; variability in the system could be attributed to differences in myotube sensitivity to electrical stimulation and to myotubes pulling themselves from the dish during electrically induced contraction.

Rates of myosin synthesis and accumulation were determined by means of pyrophosphate extraction (20 m*M* sodium pyrophosphate, 1 m*M* $MgCl_2$, *p*H 9.5) (*15*) and SDS-polyacrylamide gel electrophoresis (*16*). Immediately after electrical stimulation, the cultures were exposed for 3.5 to 4 hours to L-[4,5-^{3}H]-leucine (Schwarz/Mann, specific activity 50 c/m*M*, 5 to 7 μc/ml) in leucine-free Eagle's minimal essential medium (MEM) and Hanks salts containing 3 percent horse serum. Protein was determined by the Lowry method (*17*) and

Table 1. Protein and myosin heavy chain synthesis in muscle cell cultures subjected to long-term electrical stimulation (S) or no stimulation (U). The cultures were 5 to 6 days old. Leucine incorporation was determined in the presence of leucine-free MEM. Each value is the average of eight duplicate determinations performed on eight culture wells, ± 1 S.D. The amount of pyrophosphate extract was determined as micrograms of protein per well. Specific activity was measured as counts per minute (cpm) per microgram of protein. In all experiments there was a statistically significant difference between stimulated and unstimulated cultures, at least at the $P < .05$ level (Student's *t*-test).

Condition	Pyrophosphate extract		[^{3}H]Leucine incorporation		S/U ratio for specific activity (%)	Myosin heavy chain*	
	Amount (μg/well)	S/U ratio (%)	Amount (cpm/well)	S/U ratio (%)		Incorporation (cpm)	S/U ratio (%)
			Experiment 1. Stimulation for 24 hours				
Unstimulated	19.2 ± 1.8	146	10,806 ± 3,270†	153	105	210 ± 15	146
Stimulated	28.2 ± 6.4		16,580 ± 4,161†			306 ± 43	
			Experiment 2. Stimulation for 24 hours				
Unstimulated	48.0 ± 1.5	112	249,580 ± 55,023	132	118	5,781 ± 1,208	151
Stimulated	53.8 ± 4.3		330,599 ± 51,666			8,768 ± 1,598	
			Experiment 3. Stimulation for 17.5 hours				
Unstimulated	53.0 ± 6.5	116	96,709 ± 14,714	128	110	2,058 ± 296	128
Stimulated	61.6 ± 11.0		124,438 ± 38,437			2,604 ± 248	
			Experiment 4. Stimulation for 14 hours				
Unstimulated	66.3 ± 4.0	124	98,762 ± 8,492	108	87	2,357 ± 576	134
Stimulated	82.2 ± 7.1		106,237 ± 8,535			3,165 ± 382	
			Experiment 5. Stimulation for 16.5 hours				
Unstimulated	95.7 ± 19.7	117	165,066 ± 13,620	121	103	3,219 ± 560	135
Stimulated	111.9 ± 10.7		200,666 ± 3,018			4,347 ± 554	
			Experiment 6. Stimulation for 37.5 hours				
Unstimulated	66.4 ± 10.2	112	122,437 ± 18,123	133	118	4,094 ± 931	142
Stimulated	74.4 ± 3.6		163,236 ± 28,882			5,843 ± 735	
Mean		121 ± 12.9		129 ± 14.9	107 ± 11.6		139 ± 8.5

*Values expressed as counts per minute in the myosin heavy chain band cut from polyacrylamide gels. †Leucine incorporation in complete MEM instead of leucine-free medium.

DNA by the diaminobenzoic acid reaction (*18*).

Table 1 contains data on the effects of electrical stimulation on the rates of contractile protein synthesis in electrically driven skeletal muscle cultures. The quantity of protein extracted from such cultures with a buffer of high ionic strength (pyrophosphate buffer) was increased by 12 to 46 percent (mean, 21 percent) above unstimulated control cultures. The amount of tritiated leucine incorporated into the pyrophosphate-extractable protein remained little changed from the controls, suggesting that increased transport of amino acids or changes in pool size alone do not explain the increments in radioactive leucine incorporation. Myosin heavy chain was then isolated from the pyrophosphate extracts by polyacrylamide gel electrophoresis. The amount of radioactive leucine in myosin heavy chain was increased by 28 to 51 percent (mean, 39 percent).

To determine whether the effects of electrical stimulation were relatively greater on contractile proteins than on muscle cell proteins in general, the amount of protein in the pyrophosphate extract was compared to that of the total protein in stimulated and control cultures completely solubilized in sodium hydroxide (Table 2). Whereas there was a mean increase of 29 percent in pyrophosphate-extractable protein, there was only a 7 percent increase in total cellular protein, indicating that the effect was relatively greater on the contractile proteins. To check for the prerequisite of active muscle contraction increasing protein synthesis, a subthreshold stimulus intensity was used in some experiments. No differences between stimulated and control cultures occurred in the absence of electrically stimulated contractions.

To demonstrate that the accumulation

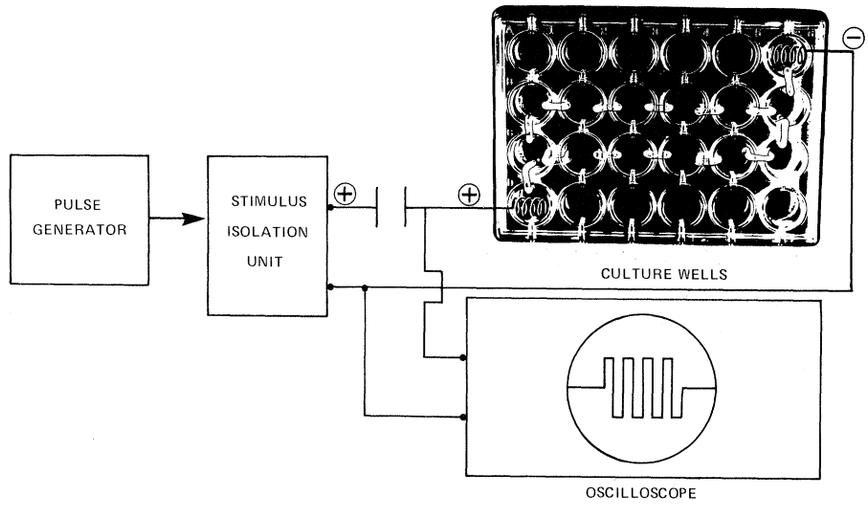

Fig. 1. Schematic diagram of culture stimulation. The electrical configuration consisted of two rows, each of six wells, connected electrically in parallel with each well also being connected electrically in series. Adjacent control rows of wells were also connected by agar bridges, but were not stimulated. A stimulus of 100 volts and 5 ma maximum (isolated from ground) was coupled to the tray by way of a 1 μfarad capacitor to provide biphasic pulses and to prevent the accumulatin of a net electrical charge. Electrical connections from the stimulator were made to silver–silver chloride wires, which were placed in the end wells which contained medium without cells.

Table 2. Protein and DNA content of stimulated (S) and unstimulated (U) muscle cell cultures. The NaOH and pyrophosphate extracts were measured as micrograms of protein per well. Each value is the average of the two determinations shown in parentheses. The mean is reported ± 1 S.D. Student's *t*-test: $P < .025$ for NaOH extract, $P < .0005$ for pyrophosphate extract, and $P < .35$ for DNA.

Condition	NaOH extract		Pyrophosphate extract		DNA	
	Amount (μg/well)	S/U ratio (%)	Amount (μg/well)	S/U ratio (%)	Amount (μg/well)	S/U ratio (%)
	Experiment 1. Stimulation for 40 to 48 hours, beginning on day 7					
Unstimulated	49.8 (55.5; 44.1)	92	21.7 (21.7)	136	0.43 (0.43; 0.43)	100
Stimulated	46.2 (46.2)		29.6 (35.8; 23.4)		0.43 (0.39; 0.47)	
	Experiment 2. Stimulation for 40 to 48 hours, beginning on day 5					
Unstimulated	74.7 (70.0; 79.4)	112	28.2 (26.2; 29.6)	118	0.58 (0.62; 0.53)	86
Stimulated	83.9 (91.80; 75.9)		33.3 (29.7; 36.8)		0.50 (0.43; 0.57)	
	Experiment 3. Stimulation for 40 to 48 hours, beginning on day 5					
Unstimulated	65.2 (60.7; 69.6)	105	32.5 (27.4; 37.6)	114	0.43 (0.43)	113
Stimulated	68.5 (69.6; 67.4)		37.0 (35.1; 38.8)		0.49 (0.47; 0.52)	
	Experiment 4. Stimulation for 40 to 48 hours, beginning on day 5					
Unstimulated	54.1 (44.9; 63.3)	100	22.8 (20.1; 25.5)	127	0.58 (0.62; 0.53)	93
Stimulated	54.0 (49.8; 58.2)		29.0 (32.9; 25.2)		0.54 (0.57; 0.50)	
	Experiment 5. Stimulation for 40 to 48 hours, beginning on day 5					
Unstimulated	56.6 (58.4; 54.8)	104	32.9 (32.9)	123	0.46 (0.40; 0.52)	97
Stimulated	58.8 (55.8; 61.8)		40.4 (39.9; 40.8)		0.44 (0.39; 0.51)	
	Experiment 6. Stimulation for 40 to 45 hours, beginning on day 5					
Unstimulated	174.6 (185.4; 163.9)	117	85.5 (77.0; 94.0)	142		
Stimulated	204.0 (215.9; 192.1)		121.8 (114.2; 129.3)			
	Experiment 7. Stimulation for 24 hours, beginning on day 7					
Unstimulated	112.4 (111.3; 113.6)	110	58.8 (55.6; 64.8)	118		
Stimulated	123.9 (131.5; 116.3)		69.6 (69.3; 70.1)			
	Experiment 8. Stimulation for 40 to 48 hours, beginning on day 5					
Unstimulated	121.7 (120.2; 123.3)	116	61.2 (57.8; 64.6)	155		
Stimulated	140.9 (138.9; 142.9)		95.0 (84.6; 105.4)			
Mean		107 ± 8.5		129 ± 14.1		98 ± 10

of protein in these experiments was due to enhanced protein synthesis rather than to cell proliferation, the DNA content of stimulated and unstimulated cultures was compared (Table 2). There was no stimulation of proliferative growth by electrically induced contractions.

The results reported here demonstrate that direct electrical stimulation of contraction of embryonic skeletal muscle cells in vitro enhances the amount of protein produced by myotubes. This increase is greater for the contractile proteins (myosin heavy chain) selectively extracted by pyrophosphate and isolated by SDS-polyacrylamide gel electrophoresis than for total protein within the cell. Furthermore, this increase is not due to enhanced amino acid transport alone, because the specific activity of the proteins is little changed; nor is it due to a selective uptake of leucine per se, because there is a quantitative increase in the protein content of the cells after they have been stimulated. Although electrical stimulation could result in changes in rates of myosin degradation, this alone could not explain the increase in protein content because the increase in the rate of incorporation of radioactive amino acids is comparable to the amount of protein which accumulates.

The role of activity or contraction in the later maturation of muscle fiber is not completely resolved by these or other electrical stimulation experiments in vitro. Cohen and Fischbach (*19*) have demonstrated a decrease in acetylcholine sensitivity and in numbers of receptors in electrically stimulated fibers compared to tetrodotoxin-treated fibers. Shainberg *et al.* (*20*), using the same system, demonstrated that electrically stimulated cultures and unstimulated control cultures (which had spontaneously contracting myotubes) have the same number of acetylcholine receptors, whereas tetrodotoxin increases receptor synthesis. Electrical stimulation is also reported to produce a 30 to 90 percent decrease in the acetylcholinesterase activity of fibers (*21*).

Electrical stimulation that produces an increase in the amount of myosin could lead to the synthesis of the same or different types of myosins within these cells. During work-induced hypertrophy in vivo in the adult, Jablecki and Kaufman (*3*) have shown that the same kind of myosin is synthesized. However, in cross-reinnervated skeletal muscle and in muscle subjected to long-term stimulation through intact innervation in vivo, there can be changes in the physiological and biochemical properties of the muscle cells. For example, during cross-reinnervation both fast and, to a lesser extent, slow muscle acquire the characteristics of the other type with respect to contractile speed (*22*), myosin adenosine triphosphatase activity (*11, 23*), protein subunit pattern (*11–13*), content of metabolic enzymes (*22*), and transport of calcium ions into the sarcoplasmic reticulum (*24*). Moreover, fast muscle electrically stimulated at a rate characteristic of slow muscle acquires contractile speed (*8, 25*), myosin adenosine triphosphatase activity (*8, 10*), and types of myosin light chains characteristic of slow muscle (*8–10*).

The experiments reported here indicate that innervation is not a prerequisite for changing rates of myosin synthesis because the muscle cells we used had never been innervated. They suggest that contraction itself, or electrical stimulation, are sufficient to change the rate of myosin synthesis and accumulation. Although we stimulated these cultures at frequencies consistent with slow muscle (*25*), we do not know the type of light chains synthesized in our stimulated cultures. This system permits modification of the rates of stimulation over a variety of frequencies, so that it should be possible to determine whether or not it is the rate of contraction which results in differential gene activation and the consequent production of different myosin light chains.

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Need for a Better Solar Radiation Data Base

In 1965 Bennett (*1*) completed his analysis of the available solar radiation data in the United States and warned about the problems in using these data. And in August 1972 the National Weather Service ceased publishing solar radiation records because of the poor quality of the data (*2*). Despite these warnings, highly questionable data have been used in studies involving the location and design of solar electrical power plants.

On the basis of the data available in 1962, the National Oceanic and Atmospheric Administration (NOAA) prepared insolation tables and maps that appeared in the *Climatic Atlas of the United States* (*3*). The station at China Lake–Inyokern, California, stood out in this publication as receiving far more solar radiation than any other location in the United States. This was duly noted and accepted by most climatologists. In 1965 Sellers (*4*) said, "Inyokern, California, at an elevation of 744 m in the arid rain shadow on the east side of the southern Sierra Nevada Mountains receives more solar radiation during the year than any other point in the United States for which data are available."

However, in 1966 the National Weath-