

The Response of Arterioles in Skeletal Muscle Grafts to Vasoactive Agents

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Arteriolar responses to the vasodilator adenosine and to the vasoconstrictor norepinephrine (NE) were examined in small bundles of extensor digitorum longus muscle grafted onto the cheek pouches of hamsters. Responses of arterioles to topically applied adenosine or NE were measured from 30 to 180 days after grafting and compared with the response of arterioles in control cheek pouches. Verapamil and potassium chloride (KCl) were applied to 120- and 180-day grafts to determine the response of arterioles to vasoactive agents not mediated by receptors. Arterioles in grafted muscle did not respond to adenosine until 60 days. The response increased with time but was significantly less than the control value even after 180 days. Arterioles in grafts did not respond to NE until 90 days. At both 90 and 120 days the degree of constriction in response to NE was not uniform along the length of a given arteriole (punctate response). By 180 days, the response of a given arteriole to NE was uniform but significantly less than the control value. In 120- and 180-day grafts the responses of arterioles to verapamil and KCl were similar to the responses of arterioles to adenosine and NE, respectively. We conclude that the diminished response of arterioles in small EDL muscle grafts to vasoactive agents may be caused by either a structural or a functional impairment in the smooth muscle layer of arterioles. © 1987 Academic Press, Inc.

INTRODUCTION

When skeletal muscles are autografted by standard operative procedures, all neural and vascular connections are severed and the transplanted muscle revascularizes and reinnervates spontaneously (Carlson *et al.*, 1979). Because the graft is ischemic for a period of days or weeks (Hansen-Smith *et al.*, 1980; White *et al.*, 1981), the muscle fibers degenerate and subsequently regenerate from a population of satellite cells (Snow, 1977). The revascularization of grafted skeletal muscle has been described previously (Hansen-Smith *et al.*, 1980; Faulkner *et al.*, 1983), but the reactivity of blood vessels in grafts to vasoactive agents has not been examined.

In extensor digitorum longus (EDL) muscle grafts in cats, White *et al.* (1981) reported an increase in resting blood flow, and then a decrease to control levels. Apparently, the regenerated blood vessels had a decreased vasomotor tone initially but this deficiency disappeared as the graft reached maturity. In response to

twitch contractions, maximum blood flow in the mature grafts was reached at a lower twitch frequency and was of a greater magnitude when normalized per 100 g of contractile protein. Thus, arterioles in mature standard muscle grafts appear to vasodilate maximally in response to low-frequency twitch contractions. The abnormal function may be due to either a decreased smooth muscle tone or an increased sensitivity of arterioles to locally released vasodilators. Irregularities observed in the smooth muscle layers of regenerated arterioles of grafted muscle (Hansen-Smith *et al.*, 1980) may also contribute to the inability of arterioles in grafts to control blood flow.

The purpose of this study was to examine, in grafted skeletal muscles, the effect of two vasoactive agents, adenosine and norepinephrine (NE), that act on arteriolar diameter through specific receptors. Two hypotheses are tested: compared with control arterioles in the hamster cheek pouch, arterioles in grafted EDL muscles have (1) a greater sensitivity to topically applied adenosine and (2) a decreased sensitivity to NE. In addition, the hypothesis that impairments in vascular reactivity are due to modifications in the function of the smooth muscle layer of arterioles is tested by comparing the maximum response of arterioles in grafted muscle to adenosine and NE with the response to the nonspecific vasoactive agents verapamil and potassium.

MATERIALS AND METHODS

A model in which small bundles of skeletal muscle were grafted onto the hamster cheek pouch was selected for these experiments. This model provided adequate video resolution for the measurement of changes in the diameter of arterioles in grafts following the topical application of vasoactive agents. Syrian Golden Hamsters weighing 100–110 g were anesthetized with a sterile intraperitoneal injection of sodium pentobarbital (0.1 mg/g body wt). A total of 20 control and 52 experimental animals were used in this study.

The procedure for grafting EDL muscle bundles onto the hamster cheek pouch was a modified version of that described by Faulkner *et al.* (1983). The EDL muscle was removed from the left hindlimb under sterile conditions and divided into six to eight bundles of approximately 20 to 30 intact fibers. Two muscle bundles were grafted onto the left cheek pouch of the donor animal and onto the cheek pouches of two to three additional animals. To prepare the cheek pouch for grafting, a plastic disc, 3 cm in diameter, was attached to a spring-loaded holder (Greenblatt *et al.*, 1969) and inserted into the cheek pouch. An incision, approximately 4 cm in length, was made through the skin over the plastic disc in an anteroposterior direction. The incision was spread apart and the skin was retracted over the disc so that the surface of the cheek pouch was exposed. The mucoareolar membrane was removed. Each bundle of EDL muscle fibers was soaked in 0.75% bupivacaine for 10 min to ensure that all fibers were destroyed and that the muscle would regenerate from surviving satellite cells (Carlson, 1976). Bupivacaine is a myotoxic agent that causes degeneration of the muscle fiber but leaves the satellite cells and the original basal lamina of the fiber intact. Grafts were sutured onto the cheek pouch on top of, and perpendicular to, the skeletal muscle fibers located in the proximal region of the cheek pouch (Duling, 1973). This orientation permitted differentiation between fibers in the

graft and in the cheek pouch. The holder and plastic disc were removed and the incision over the cheek pouch was closed with 5-0 suture. Betadine solution was applied to the area of the incision.

Responses of arterioles in the grafts to topical application of either 10^{-4} M adenosine or 10^{-6} M NE were measured in different groups of animals at either 30, 60, 90, 120, or 180 days postgrafting. Grafts in each of eight animals were studied at each time period. These results were compared with the average response of arterioles in the proximal region of the cheek pouch in a group of control hamsters ($n = 15$). In a separate experiment, the response of arterioles in grafts to 10^{-6} M verapamil and 10^{-1} M KCl was measured at 120 and 180 days ($n = 6$) and compared with the response of arterioles in control cheek pouches ($n = 5$). To determine whether vascular reactivity of control arterioles varied during the 180-day experimental period, the response of two arterioles to 10^{-4} M adenosine and to 10^{-6} M NE was measured in the cheek pouches of each of four experimental hamsters 180 days after grafting. No significant difference was observed between the responses of control arterioles at these two time periods.

Concentrations of 10^{-4} M adenosine, 10^{-5} M NE, 10^{-6} M verapamil, and 10^{-1} M KCl produced the maximum responses in control arterioles. The arterioles returned to their original diameter within 15 to 20 min after the application of either adenosine or KCl and within times ranging from 25 to 45 min after the application of 10^{-6} M verapamil or 10^{-5} M NE. Arterioles tended to lose their vascular tone after only a few applications of 10^{-5} M NE. For this reason a concentration of 10^{-6} M NE was used to examine arteriolar response in all grafts and a concentration of 10^{-5} M NE was used only occasionally at each time period. A concentration of 10^{-6} M NE elicited an average decrease in control arteriolar diameter of approximately 60% and the vessel diameter returned to initial levels within 15 to 20 min.

To examine the responses of arterioles to the vasoactive agents, a hamster was anesthetized and cannulae were inserted into the trachea and the right femoral vein. The cheek pouch was prepared for viewing by intravital microscopy as described previously except that the plastic disc was left inside the cheek pouch. The hamster was transferred to the stage of an intravital microscope (Wayland and Frasher, 1971). Before each experiment, the muscle grafts were checked for viability either through direct electrical stimulation or through stimulation of the skeletal muscle layer underneath the graft. By touching the muscle layer with a pair of metal forceps, a twitch could be elicited in the graft. Only those grafts that responded to stimulation by twitching were used in this study. In 11 of the 52 experimental animals, neither of the two grafts responded to stimulation. No data were collected on these grafts.

To maintain body temperature at approximately 37° the hamster was placed on a heated pad. The plastic disc was secured to a plexiglass holder, forming a well 0.5 cm deep and 2.5 cm in diameter, with the cheek pouch at the bottom of the well. A 0.5-cm-wide groove in the side of the well allowed the suffusate to run off the cheek pouch. Suffusate at a temperature of 37° and a pH of 7.4 was delivered from a 50-ml reservoir through an 18-gauge blunted needle to bathe the surface of the cheek pouch. The suffusate was a bicarbonate-buffered salt solution gassed with 5% CO_2 in nitrogen. The suffusate had a millimolar composition

of NaCl 130.9, KCl 4.7, CaCl₂ 2.0, MgSO₄ 1.2, and NaHCO₃ 20.0. The flow rate of the suffusate of 5–6 ml/min was adjusted to provide a cheek pouch temperature of approximately 35°, measured by a bead thermistor placed directly on the cheek pouch. The suffusate was withdrawn by aspiration through the groove in the side of the well. Anesthesia was maintained at a constant level by infusion of a dilute solution of pentobarbital sodium (9 mg/ml) into the femoral vein at a rate of 0.5 ml/hr. The hamster breathed a free-flowing gas mixture of 30% oxygen in nitrogen spontaneously through a tracheal cannula. The high oxygen concentration of the inspired gas ensured that arterial P_{O_2} remained at a level that would not affect vascular tone. Arterial P_{O_2} of hamsters breathing air is 50–60 mm Hg, which may affect vascular tone of blood vessels (Robert Hester, University of Mississippi, personal communication).

During four preliminary experiments blood pressure and blood gases were recorded from the right carotid artery. Blood pressure was recorded every 30 min and a blood sample of 0.5 cc was withdrawn every 60 min for 4 hr. The mean blood pressure of 108 ± 3 mm Hg did not vary significantly from the beginning to the end of the experiment. Blood P_{O_2} was 87 ± 4 mm Hg at the beginning and 80 ± 3 mm Hg at the end of the 4-hr period. This range of arterial P_{O_2} is slightly higher than the values of 74 ± 5 mm Hg measured in unanesthetized hamsters (O'Brien *et al.*, 1979; Lucey *et al.*, 1980). The difference is unlikely to affect resting arteriolar diameter (Duling, 1972).

Experimental protocol. In each experiment, the vasoactive agent was added to the 50-ml reservoir to give the designated final concentration. Changes in arteriolar diameter were measured directly from a video monitor and the maximum change in diameter was recorded. The initial and final diameters of arterioles were calculated based on prior calibration using a stage micrometer. After each administration of either adenosine or NE, the reservoir was flushed three times with suffusate. Enough time, at least 15 min, was allowed between administrations to allow the arteriole to return to its original diameter. Since smaller arterioles generally show a greater sensitivity to NE than do larger arterioles (Duling *et al.*, 1968), variability among arterioles was minimized by using arterioles between 10 and 30 μm in diameter.

Since vascular tone was not present in some preparations, each experiment began with an administration of 10^{-4} M adenosine to confirm that the arteriole was reactive. In experiments in which arterioles in muscle grafts were being examined, the test for reactivity was conducted using arterioles in the cheek pouch. If arterioles in the cheek pouch did not respond to 10^{-4} M adenosine with greater than an 80% increase in arteriolar diameter, the preparation was rejected. Data from 7 of the control animals and 10 of the experimental animals were rejected on the basis of a subnormal response to adenosine.

All data are presented as means \pm SEM. Significant differences between the mean responses of arterioles in grafts and the mean responses of arterioles in control animals were determined by a Student *t* test for small samples (Snedecor and Cochran, 1967).

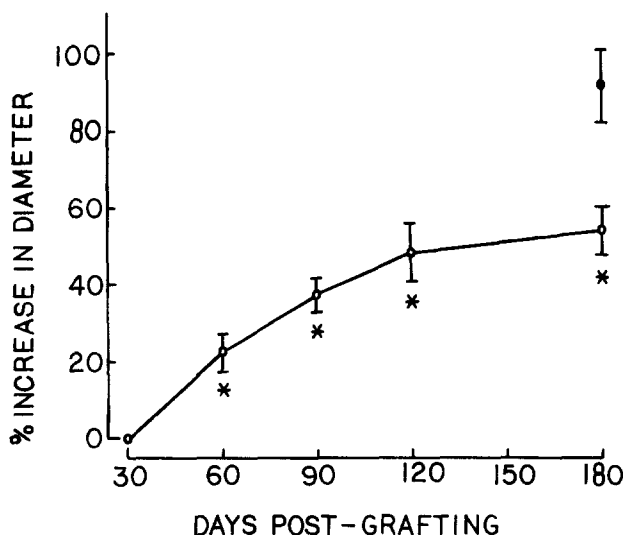


FIG. 1. Responses of arterioles in control cheek pouches (●) ($n = 12$) and of arterioles in grafted muscle between 30 and 180 days (○) when 10^{-4} M adenosine was suffused onto the hamster cheek pouch. Values given are means \pm SEM. For arterioles in grafts, $n = 4$ at 30, 60, and 90 days, $n = 6$ at 120 days, and $n = 12$ at 180 days. *Significantly different from control ($P < 0.01$).

RESULTS

The response of control arterioles in cheek pouches and of arterioles in grafted EDL muscles to 10^{-4} M adenosine is presented in Fig 1. Arterioles in control cheek pouches had an average initial diameter of $23 \pm 4 \mu\text{m}$ and increased in diameter by $93 \pm 8\%$ when adenosine was applied. Arterioles in grafted muscle did not respond to adenosine until 60 days postgrafting and the response was significantly less than the control value ($P < 0.01$). A gradual increase in the response to adenosine was observed as the number of days postgrafting increased, but the average increase in diameter at 180 days was only 54%. This response was significantly less than the control value ($P < 0.01$).

The responses of arterioles in control cheek pouches and arterioles in grafted muscle to 10^{-6} and 10^{-5} M NE are presented in Table 1. When arterioles in control cheek pouches reached their maximum response to 10^{-6} M NE, blood flow was either intermittent or stopped completely. Blood flow stopped completely in response to 10^{-5} M NE. Arterioles in 30- and 60-day grafts did not constrict when either 10^{-6} or 10^{-5} M NE was applied, but blood flow through the graft was affected by the constriction of arterioles in the cheek pouch. Under these circumstances, the control of blood flow resided in the cheek pouch arterioles outside of the graft. In 90- and 120-day grafts, NE caused a segmented or punctate response in most arterioles. For example, the diameter of one segment of an arteriole in a 120-day graft decreased by 40% of resting diameter while an adjacent segment decreased by 70%. This segmented appearance of the arteriole, produced by variable responses of different sections to NE, was present in 8 of 10 arterioles in 90- and 120-day grafts that were exposed to 10^{-6} M NE and in 6 of 7 arterioles in 90- and 120-day grafts that were exposed to 10^{-5} M NE. The response was uniform along the length of the other three arterioles. Arterioles in 90- and 120-

TABLE I
RESPONSE OF ARTERIOLES IN CONTROL CHEEK POUCHES AND OF ARTERIOLES IN GRAFTED MUSCLE TO 10^{-5} M AND TO 10^{-6} M NE (MEANS \pm SEM)

	10^{-5} M NE				10^{-6} M NE			
	<i>n</i>	Initial diameter (μ m)	% Initial diameter	Range	<i>n</i>	Initial diameter (μ m)	% Initial diameter	Range
Control	8	21.7 \pm 2	0	—	10	23.3 \pm 4	40 \pm 3	25-50
Days postgrafting								
30	3	19.4 \pm 5	No response		4	19.8 \pm 4	No response	
60	3	23.9 \pm 4	No response		4	24.6 \pm 3	No response	
90	3	21.8 \pm 4	Segmented	45-70	4	21.4 \pm 4	Segmented	50-100
120	4	21.4 \pm 3	Segmented	28-60*	6	20.1 \pm 2	Segmented	30-70†
180	6	22.0 \pm 3	39 \pm 3	23-48	10	22.6 \pm 2	53 \pm 5	25-65

* One arteriole constricted uniformly along its length to 32% of the initial diameter.

† Two arterioles constricted uniformly to 61 and 53% of the initial diameters, respectively.

day grafts showed a wide range of responses, but the constriction of arterioles in the 120-day grafts was generally greater than that in 90-day grafts. Although the segmented arteriolar response to NE was absent in 180-day grafts, the average decrease in diameter was only 79% of the control response to 10^{-6} M NE ($P < 0.05$) and 61% of the control response to 10^{-5} M NE ($P < 0.01$).

The responses of control arterioles and of those in 180-day grafts to different concentrations of adenosine and NE are presented in Figs. 2 and 3. At a con-

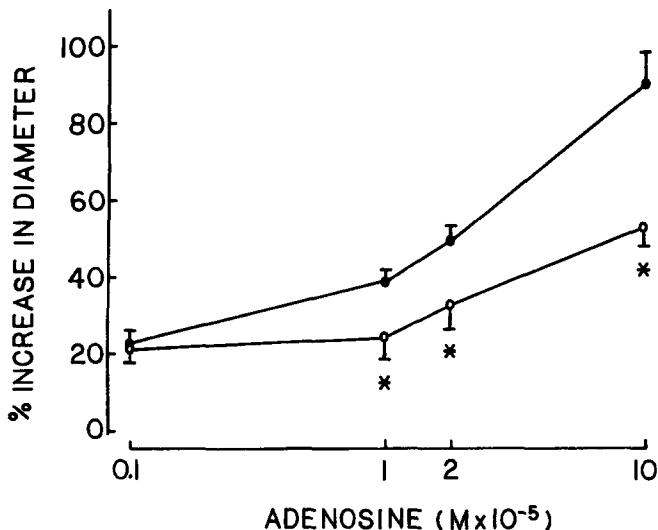


FIG. 2. Responses of arterioles to different concentrations of adenosine observed in control cheek pouches (●) and in 180-day grafts (○). Each dose was applied randomly. At each concentration of adenosine, $n = 8$ for both arterioles in control cheek pouches and arterioles in grafts, except for 10^{-4} M adenosine where $n = 12$. Values given are means \pm SEM. *Significantly different from control ($P < 0.01$).

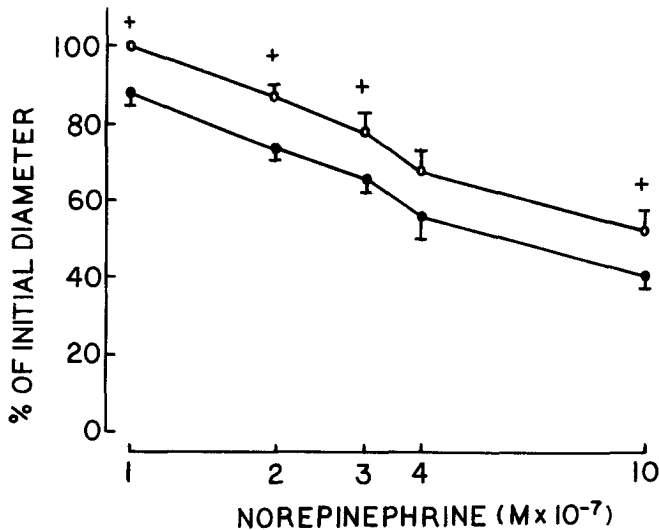


FIG. 3. Responses of arterioles to different concentrations of NE observed in arterioles in control cheek pouches (●) and in 180-day grafts (○). Each dose was applied randomly. At each concentration of NE, $n = 8$ for both control arterioles and arterioles in grafts, except for the control 10^{-6} M NE where $n = 10$. Values given are means \pm SEM. *Significantly different from control ($P < 0.05$).

centration of 10^{-6} M adenosine, the response of arterioles in grafts was not different from the control value. The responses of arterioles in grafts was significantly less than control responses at all other concentrations ($P < 0.01$). At each concentration of NE except 4×10^{-7} M, the change in diameter of arterioles in grafts was significantly less than the control response ($P < 0.05$).

To determine whether the diminished response of arterioles in grafts to adenosine and NE was the result of impaired receptor function, the nonspecific vasodilator verapamil and the nonspecific vasoconstrictor K^+ (added to the suffusate as KCl) were topically applied to 120- and 180-day grafts. These results are presented in Table 2. In two arterioles in each of four cheek pouches, the average increase

TABLE 2

COMPARISON OF THE MAXIMUM RESPONSES OF ARTERIOLES IN CONTROL CHEEK POUCHES AND IN 120- AND 180-DAY GRAFTS TO VERAPAMIL, ADENOSINE, POTASSIUM CHLORIDE, AND NOREPINEPHRINE (MEANS \pm SEM)

	Control			Graft (120 days)			Graft (180 days)		
	<i>n</i>	Initial diameter	% Initial diameter	<i>n</i>	Initial diameter	% Initial diameter	<i>n</i>	Initial diameter	% Initial diameter
Verapamil (10^{-6} M)	8	20.6 \pm 2	197 \pm 8	6	22.0 \pm 2	140 \pm 7	6	21.8 \pm 3	162 \pm 6
Adenosine (10^{-4} M)	12	23.0 \pm 4	193 \pm 8	6	20.2 \pm 3	145 \pm 6	12	23.6 \pm 4	154 \pm 5
KCl (10^{-1} M)	8	23.6 \pm 3	0	4	22.2 \pm 2	Segmented	6	21.8 \pm 3	45 \pm 7
NE (10^{-5} M)	8	21.7 \pm 2	0	4	21.4 \pm 3	Segmented	6	22.0 \pm 3	39 \pm 3

in diameter in response to 10^{-6} M verapamil was $97 \pm 8\%$. The increase in diameter in response to verapamil was not significantly different from the increase in diameter of arterioles in control cheek pouches in response to 10^{-4} M adenosine. Similarly, the responses of arterioles in 120- or 180-day grafts to 10^{-6} M verapamil and 10^{-4} M adenosine were not significantly different. There was no further increase in the response of arterioles in 180-day grafts when the concentration of either adenosine or verapamil was increased, indicating that maximum responses had been reached at concentrations of 10^{-4} M adenosine and 10^{-6} M verapamil.

Seven of eight arterioles in control cheek pouches were completely occluded by addition of 10^{-1} M KCl to the suffusate. In the other arteriole the diameter was decreased to 12% of the initial diameter. There was no difference between the responses of arterioles to 10^{-5} M NE and 10^{-1} M KCl in either 120- or 180-day grafts. There was no further increase in the response of arterioles in 180-day grafts when the concentration of either NE or KCl was increased.

DISCUSSION

Skeletal muscles that are transplanted to a different site without vascular or neural anastomoses adapt to the innervation and functional requirements of that site (Gutmann and Carlson, 1975). In our experiments, small bundles of EDL muscle fibers were transplanted onto the cheek pouch. Under these circumstances, the skeletal muscle fibers that regenerate are skeletal muscle fibers with the characteristics of fibers in the cheek pouch. Some of the endothelial cells survive this grafting procedure but smooth muscle cells do not (Faulkner *et al.*, 1983). Revascularization of the small grafts in the cheek pouch appear to result from growth of endothelial cells out from the graft with subsequent anastomosis with a vessel in the cheek pouch (Faulkner *et al.* 1983; Weiss and Faulkner, 1983). Conversely, the smooth muscle cell layer of blood vessels in the graft must develop from the migration and division of smooth muscle cells that were originally from the cheek pouch. Based on this series of events, arterioles in the cheek pouch were used as controls rather than arterioles in EDL muscle.

Compared with arterioles in the proximal region of the cheek pouch, arterioles in bundles of muscle grafts have a diminished response to the vasodilators adenosine and verapamil and to the vasoconstrictors NE and K^+ . This indicates an impaired ability of the vascular bed of standard free muscle grafts to regulate blood flow. Verapamil and K^+ are nonspecific vasoactive agents that change the lumen diameter of arterioles by acting directly on the smooth muscle, whereas adenosine and NE interact with specific receptors with subsequent activation of second messenger systems. The similarity of the responses of arterioles in 120- and 180-day grafts to verapamil and adenosine and to K^+ and NE, respectively, indicates that the diminished response of arterioles in grafts may be due to abnormalities in the vascular smooth muscle layer rather than a dysfunction of the receptor-mediated responses. A broad spectrum of structural abnormalities has been observed in the smooth muscle coat of blood vessels in EDL muscle grafts in rats (Hansen-Smith *et al.*, 1980).

Direct evidence is lacking, but the punctate or segmented arteriolar response to NE at 90 and 120 days may have resulted either from a discontinuity in the smooth muscle layer or from irregularities in the organization of the smooth

muscle cell layer (Hansen-Smith *et al.*, 1980). At 180 days postgrafting, arterioles constricted uniformly along the length of the arteriole when NE was applied, indicating that the abnormal response to NE had disappeared.

White *et al.* (1981) reported that the resting blood flow of standard EDL muscle grafts in cats increased fourfold over control values during the first months after grafting but eventually returned to control levels. The grafts used in the present study were much smaller, but the results indicate that the increase in resting blood flow may have been due to a loss of vasomotor tone caused by either a complete absence of a response, or a diminished response, to NE. Although the time course of events is different, our observation that the response of arterioles in the cheek pouch grafts to NE returned gradually and was near control values by 180 days postgrafting is consistent with the gradual return of resting blood flow to control values observed in the larger EDL grafts in cats. Whether the response of small grafts in the hamster cheek pouch to NE returns to control levels at any time past 180 days cannot be determined at this time.

White *et al.* (1981) also observed that blood flow in EDL grafts in cats was poorly regulated in response to twitch contractions. A maximum blood flow was reached at a frequency of only 1 Hz, whereas in control EDL muscles blood flow reached a maximum value at 4 Hz. The hypothesis that the high flow rate in grafts in response to a low metabolic challenge was due to an increased sensitivity to locally released vasodilators is not supported by the present data. Rather, the present study indicates a diminished response of arterioles to the vasodilators adenosine and verapamil. Decreased vascular tone resulting from a decreased sensitivity to NE may be responsible for the diminished response of arterioles to vasodilators and may contribute to the high blood flow rate observed in grafts in response to low-frequency twitch contractions.

Since arterioles in grafted EDL muscle in this study showed a decreased response to adenosine, even after 180 days postgrafting, we reject the hypothesis that arterioles in grafts are more sensitive to adenosine than are those in controls. The hypothesis that arterioles in grafts are less sensitive to NE was accepted. Although the exact mechanism remains uncertain, the decreased response of arterioles in grafted muscle to NE appears related to a dysfunction in the smooth muscle layer. The decreased response of arterioles to adenosine may be caused by a decreased vasomotor tone and a reduction in the capacity to vasodilate. The response of arterioles to a variety of vasoactive substances needs to be examined before arteriolar function in grafted skeletal muscle can be fully elucidated.

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