*NOREPINEPHRINE-INDUCED DEPOLARIZATION OF BROWN FAT CELLS**

By B. A. Horwitz, J. M. Horowitz, Jr., and R. Em. Smith

DEPARTMENTS OF PHYSIOLOGICAL SCIENCES AND ANIMAL PHYSIOLOGY, UNIVERSITY OF CALIFORNIA (DAVIS)

Communicated by Horace W. Magoun, July 2, 1969

Abstract.—Intracellular potentials of brown fat cells in lightly anesthetized cold-acclimated rats were measured *in vivo*. The effects of adrenergic agonists and antagonists on these potentials were examined in an attempt to relate the electrical activity of the cells to the adrenergic-induced stimulation of brown fat thermogenesis.

Norepinephrine, the physiological mediator of brown fat heat production, significantly depolarized the membrane of these cells *in vivo*. This was effected either upon norepinephrine administration (3–100 μg/kg body wt) or excitation of the transsected nerve trunk to the interscapular fat pad and appreciably inhibited (55%) by doses of propranolol (1 mg/kg) sufficient to abolish the temperature increase of the tissue. Since theophylline (325 μm/kg) did not depolarize the cells, although it stimulated thermogenesis in the tissue, the depolarizing effect of norepinephrine is interpreted as being at least partially associated with biochemical events terminating in the activation of adenylate cyclase. However, the norepinephrine-induced electrical changes and the ensuing increase in brown fat thermogenesis appear to be causally independent and experimentally separable. On the other hand, our data do not preclude the speculation that the membrane phenomenon, if accompanied by increased intracellular Na^+, may serve partially to regulate the metabolic rate of brown fat during long-term physiological stimulation (e.g., cold stress) by increasing the rate of ATP utilization via the Na^+/K^+ pump.

Introduction.—Brown adipose tissue, in its primary role of thermogenesis,[1–5] is evidently activated through norepinephrine (NE) released from the sympathetic nerve endings[6, 7] serving the tissue. Preliminary data on tissue systems *in vivo*[8] and work with cells *in vitro*[9, 10] have indicated that NE depolarizes the membrane of the brown fat cell. On the postulate that this may effect increases of intracellular Na^+, it has been suggested that norepinephrine-induced depolarization may account for the increase in the rate of oxygen consumption of the brown fat by stimulating the Na^+/K^+ pump and the turnover of ATP.[9, 10]

However, previous work has suggested that *in vivo*[11] the metabolic rate of "resting" brown fat is controlled by substrate availability, and further, that the norepinephrine stimulation of the heat production of brown fat reflects initially the increased availability of oxidizable substrate rather than ADP.

The present study, therefore, was undertaken to examine in the brown fat the relationship between its norepinephrine-mediated thermogenesis and changes of the transmembrane potential.

Methods.—Twenty male Long-Evans hooded rats (350–400 gm), which had been cold acclimated (4° ± 1°C) for three to four weeks, were anesthetized with sodium pentobar-

bital (60 mg/kg body weight). The left jugular vein was cannulated for the administration of drugs, including sustaining doses of pentobarbital; the right carotid artery was cannulated for monitoring blood pressure. The interscapular brown fat pad was separated along its frontal plane to leave intact the thoracodorsal vessels, the central unpaired 4th thoracic vein of Sulzer,[12] and the innervation to the tissue. A plastic base plate and frame (Fig. 1*A*) was employed to immobilize the fat pad *in situ* (Fig. 1*B*). The central vein ran through the medial slot of the plate, and copper-constantan thermocouples, via the base plate (Fig. 1*A*), were in contact bilaterally with the ventral aspect of the fat pad. The rats were secured in a heavy stereotaxic frame by ear bars and a clamp fixed to the dorsal spinous process of T_{10}. The base plate was attached to the stereotaxic instrument. A rectangular plastic frame lined with polystyrene (Fig. 1*A*) was placed over the interscapular area so that gentle pressure was applied to the edges of the fat pad, while the major portion of the interscapular pad remained exposed (Fig. 1*B*). A small area of tissue was prepared for impaling by removing the overlying white fat and connective tissue. Saline was applied frequently over the exposed area to avoid surface dehydration.

Intracellular potentials were measured with glass microelectrodes filled with 2.5 *M* KCl and having an impedance of 12–40 megohms (as determined by the ramp generator of a Winston S-857 preamplifier). The electrodes, initially positioned with the aid of a dissecting microscope, were advanced vertically into the brown fat tissue with a hydraulic microdrive (Kopf). A silver silver-chloride wire served as the reference electrode. The potentials were recorded with a Winston S-857 preamplifier (input impedance greater than 10^{14} ohms) and displayed on a Tektronix 565 oscilloscope and a Varian G-2000 recorder.

The nerve bundle innervating the right side of the interscapular pad was isolated and sectioned. Stainless steel (28-gauge) stimulating electrodes were placed around the distal end of the nerve bundle and insulated from the surrounding tissue with polystyrene. These nerves were stimulated with pulses of constant voltage (4–8 v, 2–10 pulses/sec, 20-msec duration) by means of a Grass SM-6 pulse generator in series with a Bioelectric ISA 100 isolator.

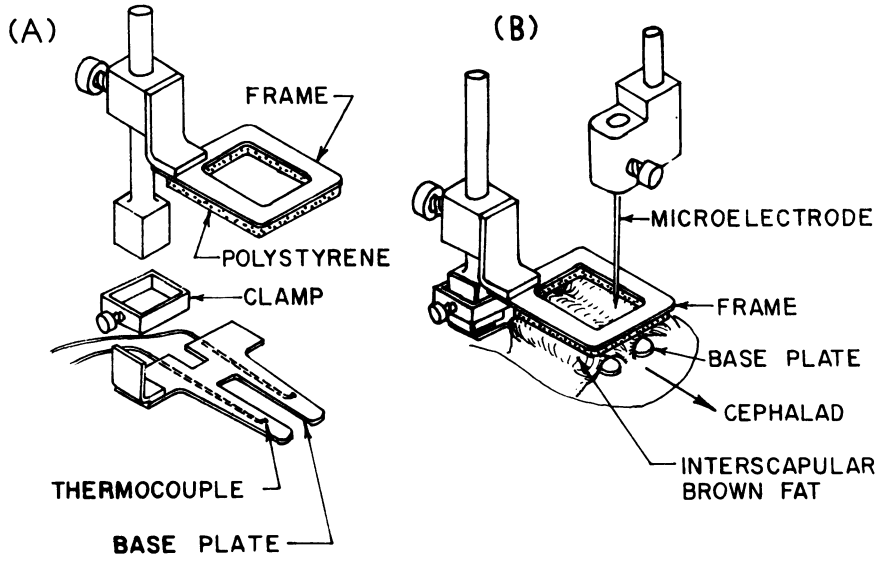

FIG. 1.—Diagram of the mechanical support of the interscapular fat pad for intracellular potential measurement. (*A*) Expanded view of individual components; (*B*) relationship of components to fat pad *in vivo*.

Norepinephrine (L-arterenol bitartrate (Sigma), 40 μg/ml) was freshly prepared for each experiment by dilution with saline (pH 7.4) from a concentrated (1–3 mg/ml) stock solution. The stock solution, which had been acidified to pH 3.4, was maintained at 2–5°C away from light and replaced after three days. Theophylline (Merck, 40 μg/ml), propranolol (Ayerst, 1 mg/ml), and phentolamine HCl (Ciba, 10 mg/ml) were prepared in saline and kept refrigerated until used. All drugs were briefly warmed before administration.

The temperatures of the brown fat, ambient air (23–24°C), and at 6 cm into the colon were continually monitored with a Leeds and Northrup 12-point Speedomax H recorder. Following completion of the experiment, the fat pad was examined for adhering muscle. The potentials of cells from areas where muscle was noted were not included in this study.

Results.—Of 865 cells in the interscapular brown fat pad of 16 cold-acclimated, anesthetized rats, the resting intracellular potential averaged −47.4 ± 0.4 mv (SE) with a range from −25.0 to −69.6 mv (Fig. 2). The transmembrane potentials were independent of the depth from the tissue surface. Injection of 1 to 2 ml of saline or 0.05 to 0.10 ml sodium pentobarbital (6%) had no effect on the membrane potentials.

Effect of norepinephrine: Large doses of norepinephrine (57–100 μg/kg body wt), injected over 0.8 to 1.2 minutes into each of 11 rats, resulted in elevated brown fat temperatures and depolarization of 40 cells out of the 43 impaled. Of these the magnitude and rate of depolarization varied among the cells, and the membrane potentials fell to an average of 37.1 ± 3.8 per cent (SE) (range: 13.0–78.5%) of the preinjection values. In general, initiation of the depolarization occurred before the injection was completed and either prior to or concurrent with the increase of tissue temperature (Fig. 3). Moreover, compared to the temperature response of the brown fat, the norepinephrine-induced depolarization was relatively transient, with recovery usually beginning while the tissue temperature was still elevated (Fig. 3).

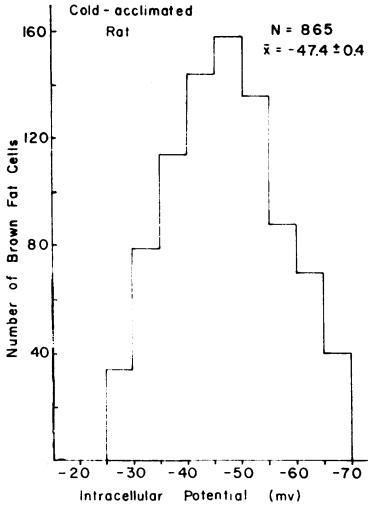

FIG. 2.—Distribution of resting potentials of interscapular brown fat cells.

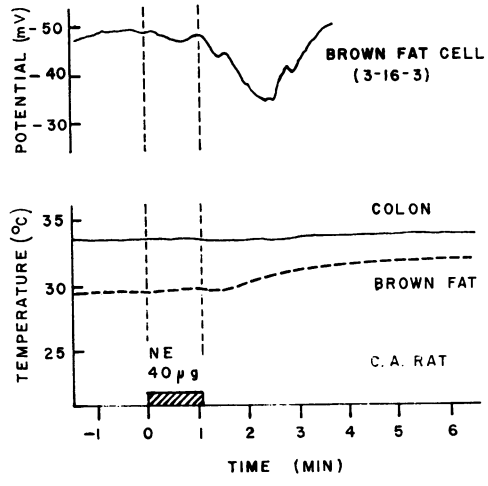

FIG. 3.—Effect of injected NE (100 μg/kg body wt) on the intracellular potential of a brown fat cell and the temperatures of the colon and fat pad.

Electrical changes were also observed with low doses of norepinephrine. Upon the infusion of 1–2 μg of norepinephrine per minute, there occurred an immediate fall of the transmembrane potential, which reversed when the infusion was halted (Fig. 4). On the other hand, an increase in the temperature of the brown fat was not always detectable under these conditions (Fig. 4).

Effect of nerve stimulation: Stimulation of the transsected nerve bundle innervating the fat pad resulted in depolarization of the brown fat cell membrane (24 cells, 5 rats) followed by an increase in brown fat temperature (Fig. 5). These responses were manifested beyond a threshold stimulation of 2 to 4 v. The magnitudes of the depolarizations and changes of brown fat temperature varied directly with intensities applied between 4 and 8 v and with frequencies from 2 to 10 pulses/sec. At a given strength of stimulus, however, the degree of depolarization tended to approach an asymptote even with extended duration of the stimulus. Repolarization of the membrane began shortly after cessation of the stimulation, while recovery of the tissue temperature proceeded more slowly (Fig. 5).

Effect of theophylline and adrenergic antagonists: Injection of theophylline (325 μmoles/kg body weight), whether before or after the administration of norepinephrine, was followed by an elevation of the temperature of the brown fat although no significant depolarization of the brown fat cells (12 cells, 7 rats) was noted (Fig. 6). That this lack of depolarization did not in fact reflect excessive membrane damage was suggested insofar as these cells remained sensitive to subsequent administration of norepinephrine.

Intravenous injection of the β-adrenergic blocking agent, propranolol (0.5 mg or 1.0 mg/kg body wt) did not noticeably depress the norepinephrine-induced elevation of arterial blood pressure. Either dose, however, abolished the stimulation of brown fat temperature and significantly diminished the magnitude of the membrane depolarization (18 cells, 3 rats) which followed electrical stimulation of the transsected nerve bundle. The blocking effect of 0.5 mg propranolol/kg was approximately 68 per cent of that seen with 1.0 mg/kg (55% in-

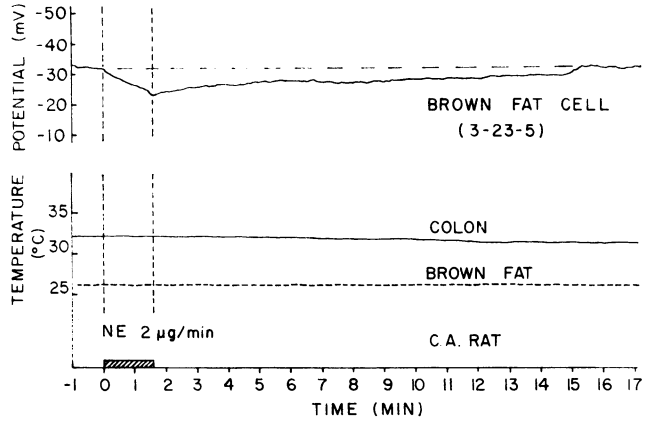

FIG. 4.—Effect of infused NE (2 μg/min for 1.5 min) on the intracellular potential of a brown fat cell and the temperatures of the colon and fat pad. Rat weighed 390 gm.

hibition with a dose of 1.0 mg propranolol/kg). Administration of the α-adrenergic antagonist, phentolamine (10 mg/kg body wt) also blocked the depolarization (about 73%) following nerve stimulation (16 cells, 2 rats). On the other hand, this dose of phentolamine (sufficient to abolish the norepinephrine-induced pressor response) did not prevent the increase in brown fat temperature following nerve excitation.

Discussion.—Histologically, the interscapular fat pad from rats, cold-acclimated as described here, is composed predominantly of brown adipocytes, heavily vascularized, and virtually without white (unilocular) fat cells.[13] Hence, the cells of the interscapular fat pad described in this study have been considered to be brown fat cells.

The resting intracellular potentials of these cells are similar to those previously obtained *in vivo* in cold-acclimated rats.[8] The fact that the mean of these is considerably higher (~20 mv) than those measured *in vitro*[9, 10] probably reflects the differing experimental conditions. In both systems, however, the cell range of resting membrane potentials was quite large, suggesting possible damage. In view of this possibility, it should be pointed out that the effects of the various treatments employed in the present study were observed in cells with membrane potentials falling within the entire range measured.

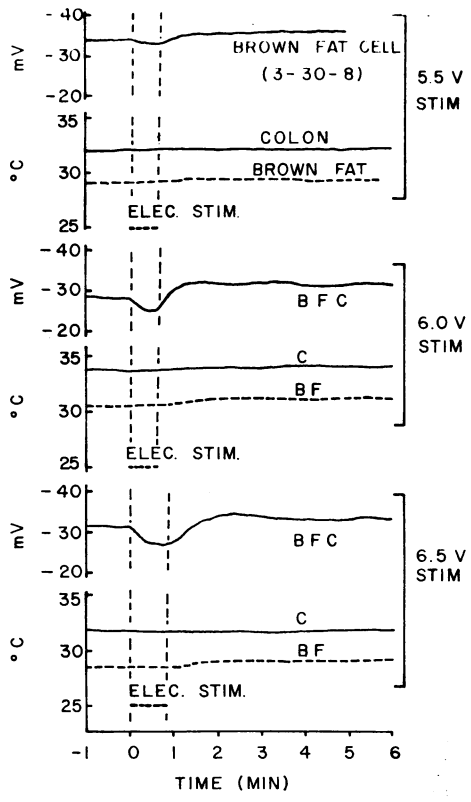

FIG. 5.—Effect of nerve stimulation on the intracellular potential of a brown fat cell and the temperatures of the colon and fat pad. Stimulus duration = 20 msec; pulse rate = 10/sec.

Our observation that the membrane potentials of brown fat cells become less negative during the administration of norepinephrine is consistent with the results previously obtained *in vivo*[8] as well as *in vitro*.[9, 10] In our studies *in vivo*, depolarizations developed either before or concurrently with the time of increase in the temperature of the brown fat. Similarly, during electrical stimulation of the nerves innervating the fat pad, the depolarization of the fat cells consistently occurred prior to any detectable temperature change. Moreover, repolarization following completion either of norepinephrine administration or nerve stimulation took place while the temperature of the tissue was still elevated. These temporal relationships thus indicate that the observed depolarizations do not result from thermal elevation of the brown fat induced by norepinephrine (whether this be injected or released from nerve endings).

The calorigenic effect of norepinephrine on brown fat evidently derives from

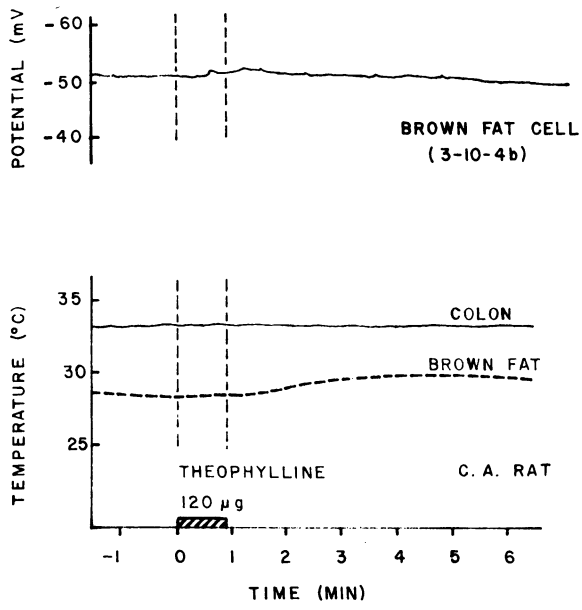

FIG. 6.—Effect of injected theophylline (325 µmoles/kg body wt) on the intracellular potential of a brown fat cell and the temperatures of the colon and fat pad.

stimulation of lipolysis via the membrane-bound[14] adenylate cyclase-cyclic 3′,5′-AMP system (Fig. 7); this effect is apparently a β-adrenergic response.[15, 16] Since injection of the β-adrenergic antagonist, propranolol, substantially diminished the magnitude of the norepinephrine-induced membrane depolarization as well as abolished the temperature increase, it would appear that the depolarization is at least partially associated with an early step(s) in the norepinephrine stimulation of brown fat thermogenesis. The fact that administration of theophylline was followed by an elevation of the temperature of the brown fat but no significant membrane depolarization suggests that the depolarizations observed with norepinephrine occur prior to the formation of cyclic AMP. That is, since theophylline effectively prolongs the life of existing cyclic AMP (by inhibiting its enzymatic degradation by phosphodiesterase), it effects a calorigenic response from brown fat qualitatively similar to that induced by norepinephrine. Moreover, the biochemical events resulting in stimulation of thermogenesis by these two agents presumably differ only in those initial steps leading to the

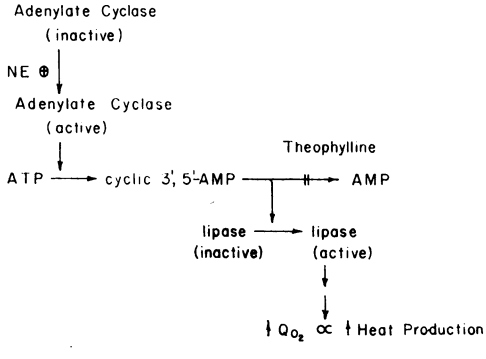

FIG. 7.—Schema of NE effect on brown fat metabolism.

formation of cyclic AMP. Thus, the observations that (1) norepinephrine depolarizes the membrane of the brown fat cell, (2) this response is inhibited by a β-adrenergic blocker, and (3) theophylline does not depolarize the membrane indicate that the NE-induced depolarization is probably associated, in part, with events prior to the formation of cyclic AMP (i.e., the sequence terminating in activation of adenylate cyclase). The fact that this depolarization is also antagonized by the α-adrenergic blocker suggests that the membrane phenomenon may be partially associated with biochemical events other than those described in Figure 7. However, at the present time the pathway effecting an α-adrenergic response in brown fat cells is still undefined.

With regard to the relationship between the norepinephrine-induced depolarization and stimulation of brown fat heat production, our data indicate that the two events are "causally" independent (i.e., the depolarization appears to be at least partially associated with activation of adenylate cyclase, while the ensuing elevation of brown fat thermogenesis results from the consequences of the enzyme activation rather than from any changes of electrolytes which may accompany the depolarization). On the other hand, these data do not preclude the possibility that a norepinephrine-induced alteration in the membrane permeability of the brown fat cell may play a significant role in the maintenance of an increased level of brown fat metabolism during the longer periods of physiological stimulation (i.e., cold stress). That is, previous studies[11] have suggested that the norepinephrine-stimulation of brown fat thermogenesis *in vivo* initially reflects an increase in substrate availability. However, to account for the sustained elevation of heat production of the brown fat as seen during physiological stimulation, there must be a concomitant increase in the rate of ATP turnover (to provide ADP for phosphorylation) and/or possibly an uncoupling of oxidative phosphorylation and respiration.

Since our results demonstrate that the membrane of the brown fat cell remains depolarized throughout the one- to three-minute period of electrical stimulation of the nerves innervating the tissue, it seems likely that this depolarization might also be manifested (if only periodically for each cell) throughout the longer duration of physiological stimulation of the tissue. Under such conditions, therefore, it is conceivable that if cellular changes of electrolytes do accompany the membrane depolarization, the rate of the ATP-dependent Na^+/K^+ pump may be stimulated and may thereby effect an increased availability of ADP, providing at least a portion of the increased acceptor required for maintenance of the higher respiratory rate of the brown fat. Whether such shifts of electrolytes actually occur during the norepinephrine stimulation of brown fat thermogenesis is presently under study.

* This work was supported by research grants USPHS HD-03268 and NASA NGR 05-004-035 and presented in part at the meeting of the Federation of American Societies for Experimental Biology, April 17, 1969. We wish to thank Mr. D. Zinn for his technical assistance, and Drs. B. Libet and I. Wagman for their critical review of this manuscript.

[1] Smith, R. E., *The Physiologist*, **4**, 113 (1961).
[2] Smith, R. E., and R. J. Hock, *Science*, **140**, 199 (1963).
[3] Smalley, R. L., and R. L. Dryer, *Science*, **140**, 1333 (1963).
[4] Dawkins, M. J. R., and D. Hull, *J. Physiol.*, **172**, 216 (1964).

[5] Donhoffer, Sz., F. Sardy, and Gy. Szegvári, *Nature*, **203**, 765 (1964).
[6] Hull, D., and M. M. Segall, *J. Physiol.*, **181**, 458 (1965).
[7] Smith, R. E., and B. A. Horwitz, *Physiol. Rev.*, **49**, 330 (1969).
[8] Smith, R. E., and Y. Imai, *Federation Proc.*, **28**, 721 (1969).
[9] Seydoux, J., L. Girardier, and T. Clausen, *Helv. Physiol. Pharmacol. Acta*, **26**, 251 (1968).
[10] Girardier, L., J. Seydoux, and T. Clausen, *J. Gen. Physiol.*, **52**, 925 (1968).
[11] Horwitz, B. A., P. A. Herd, and R. E. Smith, *Can. J. Physiol. Pharmacol.*, **46**, 897 (1968).
[12] Sulzer, F. G., *Versuch einer Naturgeschichte des Hamsters* (Göttingen: J. C. Dieterich, 1774).
[13] Cameron, I. L., and R. E. Smith, *J. Cell Biol.*, **23**, 89 (1964).
[14] Sutherland, E. W., in *Conferences on Cellular Dynamics*, ed. L. D. Peachey (New York: Academy of Science, 1967), p. 28.
[15] Heim, T., and D. Hull, *J. Physiol.*, **187**, 271 (1966).
[16] Beviz, A., and E. Mohme-Lundholm, *Acta Pharmacol. Toxicol. suppl.* **4**, **25**, 21 (1967).