Purinergic Receptor Stimulation Increases Membrane Trafficking in Brown Adipocytes

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ABSTRACT Stimulation of brown adipocytes by their sympathetic innervation plays a major role in body energy homeostasis by regulating the energy-wasting activity of the tissue. The norepinephrine released by sympathetic activity acts on adrenergic receptors to activate a variety of metabolic and membrane responses. Since sympathetic stimulation may also release vesicular ATP, we tested brown fat cells for ATP responses. We find that micromolar concentrations of extracellular ATP initiates profound changes in the membrane trafficking of brown adipocytes. ATP elicited substantial increases in total cell membrane capacitance, averaging ~30% over basal levels and occurring on a time scale of seconds to minutes. The membrane capacitance increase showed an agonist sensitivity of 2-methylthio-ATP > ATP > ADP >> adenosine, consistent with mediation by a P2y type purinergic receptor. Membrane capacitance increases were not seen when cytosolic calcium was increased by adrenergic stimulation, and capacitance responses to ATP were similar in the presence and absence of extracellular calcium. These results indicate that increases in cytosolic calcium alone do not mediate the membrane response to ATP. Photometric assessment of surface-accessible membrane using the dye FM1-43 showed that ATP caused an approximate doubling of the amount of membrane actively trafficking with the cell surface. The discrepancy in the magnitudes of the capacitance and fluorescence changes suggests that ATP both activates exocytosis and alters other aspects of membrane handling. These findings suggest that secretion, mobilization of membrane transporters, and/or surface membrane expression of receptors may be regulated in brown adipocytes by P2y purinergic receptor activity.

KEY WORDS: exocytosis • ATP • P2 purinergic receptor • FM1-43

INTRODUCTION

The major role of adipocytes is to store and mobilize body energy reserves. Until recently it was thought that adipocytes were essentially passive deposition sites in this process, effecting the net uptake or release of metabolic energy in response to hormonal commands generated elsewhere. However, a rapidly growing body of evidence indicates that fat cells play an active role in regulating body nutritional energy homeostasis and body weight (Flier, 1995). The regulatory mechanisms involved are complex and incompletely understood but are known to be mediated by extensive cross-talk between fat and a number of other tissues. Adipocyte proliferation, differentiation, and energy handling are modulated by a wide variety of neuronal and circulating effectors, and adipocytes in turn secrete a number of products that act as autocrine, paracrine, or endocrine factors to affect fat cell properties and energy homeostasis (Himms-Hagen 1989; Ailhaud et al., 1992; Spiegelman and Hotamisligil, 1993). Awareness of these interactions has fueled growing interest in the receptor responses of fat cells.

Two classes of adipocyte are involved in maintaining nutritional energy homeostasis. White adipocytes subserve the classic fat cell role of storing energy in times of plenty and releasing the energy stores in times of need. Brown adipocytes on the other hand can actively waste energy through their capacity to rapidly convert metabolic energy to heat (Nicholls and Locke, 1984). Sympathetic adrenergic neuronal activity stimulates thermogenesis in brown fat by activating a hormone-sensitive lipase and activating a unique uncoupling protein in the inner mitochondrial membrane. The lipase action mobilizes fatty acids from intracellular lipid droplets to provide substrate for electron transport and the uncoupling protein acts to shunt the mitochondrial proton gradient. Activated brown fat cells are capable of prodigious rates of energy usage, up to 60 times that of a liver cell (Nicholls, 1974). The energy wasting activity of brown fat is used to burn off excess food energy, as well as to generate heat during cold stress and with arousal from hibernation. This action of brown fat is essential for normal weight regulation, as evidenced by the observations that compromised brown fat function is a feature of most animal models of obesity (Johnson et al., 1991) and that selective genetic knock-
out of brown fat thermogenic capacity causes obesity in mouse models (Lowell et al., 1993).

The immediate brown adipocyte responses to adrenergic stimulation include a number of membrane effects in addition to the cytoplasmic energy-wasting response. α-adrenergic stimulation increases cytosolic pH (Horwitz and Hamilton, 1993) through activation of Na/H antiporters and effects on other transporters (Giovannini et al., 1988; Lee et al., 1994). α-adrenergic activation also results in increases in cytosolic calcium levels, both through release from intracellular stores and influx across the plasma membrane (Wilcke and Nedergaard, 1989; Lee et al., 1993). The increase in intracellular calcium can activate two calcium-sensitive currents present in brown fat cells, a hyperpolarizing potassium current (Lucero and Pappone, 1990), and a depolarizing chloride current (Pappone and Lee, 1995). β-adrenergic stimulation, possibly as a consequence of changes in cytosolic redox state (Koivisto et al., 1993; Koivisto and Nedergaard, 1995), activates a depolarizing nonselective cation conductance (Lucero and Pappone, 1990). The amount of these conductances present in brown adipocytes is highly variable from cell to cell, and may be regulated in response to changes in stimulation levels or other factors in the local environment (Pappone and Lee, 1995). The variability in membrane conductance properties gives rise to diverse membrane potential responses to adrenergic stimulation (Girardier and Schneider-Picard, 1983; Horwitz and Hamilton, 1984; Schneider-Picard et al., 1985; Lucero and Pappone, 1990; Pappone and Lee, 1996). The functions of these many membrane responses to adrenergic activation are not yet fully understood, but they do not seem to be directly involved in generating thermogenic responses (Nedergaard and Lindberg, 1982; Nicholls and Locke, 1984; Pappone and Lucero, 1992). The amount of brown adipose tissue and its energy-utilizing capacity are highly regulated and modulated by adrenergic and other stimuli, and it is thought that the membrane responses to adrenergic agents play a role in the regulation of cell growth and gene expression (Himms-Hagen, 1989).

It is likely that sympathetic neuronal stimulation of brown fat results in exposure of the cells to extracellular ATP and the products of its breakdown by ectoenzymes. ATP is colocalized with norepinephrine in sympathetic adrenergic nerve terminals in many systems (Westfall et al., 1990). In addition, receptors for extracellular ATP are present in many tissues, and responses to extracellular adenosine have been found in brown fat cells (Szillat and Bukowiecki, 1983; Schimmel and McCarthy, 1984; Schimmel et al., 1987). We therefore tested whether brown fat cells have membrane responses to purine nucleotides. We find that exposure of brown fat cells to micromolar concentrations of extracellular ATP raises intracellular calcium levels, activates membrane ion conductances, and increases cell membrane surface area. The size of the surface area increase indicates that it may reflect a substantial secretory event, suggesting that purinergic stimulation may regulate vesicle-mediated secretion by adipocytes. Alternatively, the localization of membrane transport proteins or receptors may be under purinergic control.

MATERIALS AND METHODS

Cells

Brown fat cells were isolated by collagenase digestion of interscapular fat pads from 1–5-d-old rats as described previously (Lucero and Pappone, 1989). The rat pups were fasted for ~12 h and cold-anaesthetized at 5°C for ~1 h before killing by decapitation. These procedures were approved by the University of California (Davis) animal care committee. The food-deprivation and cold exposure mobilize fat from the cells and increase their density, so that a significant proportion of the adipocytes sink and adhere to the substrate when plated. The cells were plated on collagen-coated coverslips and incubated at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf/horse serum, 0.2 U/ml insulin, 100 μg/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. Most of the experiments were performed on cells maintained 2–10 d in culture, but some experiments used cells immediately (0–1 d) after the isolation procedure. There were no differences apparent in the responses of cells with culture age. All cells used in these experiments contained multiple lipid droplets, identifying them as brown fat cells.

Electrophysiology

Whole-cell membrane currents were measured in cultured brown fat cells using our standard perforated patch voltage clamp methods (Lucero and Pappone, 1990). Thick-walled borosilicate glass was used to manufacture pipets with resistances of 1–4 MΩ. Membrane currents were recorded, filtered (2–3 kHz), and pipet capacitance was nulling used either an EPC-7 (List Electronic, Darmstadt, Germany) or Dagan 3900 (Dagan Corp., Minneapolis, MN) patch clamp amplifier, connected to a computer via a Basic23 interface (INDEC Systems, Sunnyvale, CA). Pulse protocols were delivered and data collected and analyzed using software developed by R.S. Lewis, Stanford University (Stanford, CA).

Unless noted otherwise, the cells were continuously perfused with Krebs' solution consisting of (in mM) 120 NaCl, 4.5 KCl, 2 CaCl₂, 0.5 MgCl₂, 25 NaHCO₃, 0.7 Na,HPO₄, 1.3 Na,H₂PO₄, 10 glucose, pH 7.4, equilibrated with 95% O₂/5% CO₂. 0 Ca/0 Mg Krebs' was identical to normal Krebs' except that calcium and magnesium salts were omitted without substitution. The pipet solution contained (in mM) 115 K aspartate, 25 KCl, 10 NaCl, 10 MOPS, KOH to pH 7.2, 280 mosM, 400 μg/ml Phlorphonic F-127 and 250 μg/ml nystatin (Fluka Chemical Corp., Ronkonkoma, NY). All voltage clamp measurements were made at room temperature (22–25°C).

Cell electrical capacitance was determined from the analysis of membrane currents measured during 10-mV depolarizing voltage steps from the holding potential of ~60 mV. Voltage steps were of sufficient duration (5–25 ms) for the current level to reach a steady state. Brown fat cells have no voltage-gated currents in this potential range (Lucero & Pappone, 1989), and activation of the calcium-activated K and Cl conductances that can be present is voltage independent (Lucero & Pappone, 1990;
was determined by adjustment of the clamp amplifier compensation computer-fit to the capacity current transient and the relationship \( \tau = R_A C_M \), as diagrammed in Fig. 1. Capacity current transients were fit well by a single exponential function both before and after exposure to ATP (see Fig. 3 A). Alternatively, \( R_A \) was determined by adjustment of the clamp amplifier compensation circuitry. The two methods gave similar values of \( R_A \). The resulting \( R_A \) values were used to correct for access resistance errors in the subsequent analysis. \( R_A \) values were typically 10-50 MΩ and generally changed by <10% in the course of the experiments.

**Figure 1.** Diagram illustrating our assumed equivalent circuit (A) and our methods for determining membrane capacitance and resistance from the currents during voltage steps (B). \( R_A \) is the access resistance, \( C_M \) the membrane capacitance, \( R_M \) the membrane resistance, \( E_{com} \) the voltage clamp command potential, and \( E_M \) is the cell membrane potential. \( \tau \) was determined from the computed fit of a single exponential function to the current record, which was then used to correct the current through the membrane resistance, \( I_{ss} \), for the finite exponential rise of the voltage step. The difference between the total current and \( I_R \) was integrated to give the capacitative charge movement, \( \Delta Q \). The steady-state current, \( I_{ss} \), was used to determine membrane conductance.

Measurements of \( C_M \) and membrane conductance (\( =1/R_M \)) with time were made by applying voltage steps at 5-10 s intervals. Post-experimental analysis of the membrane currents as outlined above gave single values for \( C_M \) and \( R_M \) from each voltage step, which were then used to generate time course data like that shown in Fig. 2. Fig. 2 shows \( C_M \) and conductance measured from voltage steps applied every 6 s in a cell exposed to the \( \alpha \)-adrenergic agonist phenylephrine. The thin lines show the values of \( C_M \) and \( 1/R_M \) without correction for access resistance, and the thick lines show data corrected as above for the measured 34 MΩ access resistance. Phenylephrine increases cytosolic calcium levels, activating both hyperpolarizing K (Lucero & Pappone, 1990) and depolarizing Cl (Pappone & Lee, 1995) conductances, producing an ~15-fold increase in membrane conductance in this cell. The initial large conductance increase in this cell was produced by activation of net outward current, while the later conductance was due to a net inward current increase. The corrected data shows that the \( C_M \) measured by these methods is stable, even in the face of the large increases in membrane conductance. All data presented in RESULTS has been corrected for \( R_A \) as outlined above.

**Fluorimetry**

Cell surface-accessible membrane was assessed using the dye FM1-43 (N-[3-triethylammoniumpropyl]-4-[4-(dibutylamino)styryl]pyridinium dibromide)\(^1\) (Betz et al., 1992; Betz and Bewick, 1993). Total fluorescence from a single cell was assessed using epifluorescence on a Nikon Diaphot with a 40× objective. Excitation and emission separation was achieved using a standard fluorescein filter set (480-nm excitation filter, 530-nm emission filter). Brown adipocytes show considerable but constant autofluorescence under these conditions (not shown). Dye-specific cellular fluorescence was greater than twice the autofluorescence at the 2 μM concentration of FM1-43 that we used. Excitation was controlled and fluorescence was quantified with a photon counting system and data collection and analysis software from IonOptix Corp. (Milton, MA). Data points were acquired at a rate of 10 s/pt with exposure times of 0.1-0.2 s/pt. FM1-43 fluorescence exhibited no photobleaching under this paradigm.

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\(^1\)Abbreviations used in this paper: FM1-43, N-[3-triethylammoniumpropyl]-4-[4-(dibutylamino)styryl]pyridinium dibromide; NE, norepinephrine.
PHENYLEPHRINE

LI_v BID, 80-60-40-

20-0

5

30-

15

20

0

10

25

Time (min)

PHENYLEPHRINE

Conductance (nS)

30

20

10

5

10

25

Time (min)

FIGURE 2. Time course of capacitance and conductance changes during α-adrenergic stimulation. Cell membrane capacitance and conductance were measured from the currents during voltage steps applied every 6 s. 20 μM phenylephrine was present during the times shown by the bar. Thin traces show the values calculated without correcting for the access resistance. Thick traces show the values corrected for the measured access resistance of 34 MΩ. The x-axis shows time from the initiation of recording after access resistance had stabilized. The initial conductance increase initiated by phenylephrine corresponded to activation of net outward currents, while the conductance increase after ~14.5 min corresponded to increased inward current.

RESULTS

We find that extracellular ATP, acting through purinergic membrane receptors, has a number of effects on the physiology of brown adipocytes (Pappone and Lee, 1994; Pappone and Lee, manuscript in preparation). ATP increases cytosolic calcium levels, both through release of calcium from cytoplasmic stores and the activation of calcium influx. Adipocyte membrane conductance is increased, both through subsequent activation of calcium-sensitive K (Lucero and Pappone, 1990) and Cl (Pappone and Lee, 1995) conductances and through induction of a nonselective cation conductance. Probably as a result of these effects on cellular ion fluxes, ATP also produces a modest increase in cell metabolic rate. Here we present results examining a novel action of extracellular ATP on adipocytes: the mobilization of substantial exocytosis of intracellular membrane. We find that ATP, acting through P2Y purinergic receptors, dramatically increases membrane trafficking in brown adipocytes.

ATP Increases Adipocyte Membrane Capacitance

Exposure of perforated patch-clamped brown fat cells to extracellular ATP caused a dramatic increase in cell membrane electrical capacitance, reflecting a large increase in cell surface membrane area. Fig. 3 A shows the current transients measured in an intact cell in response to a 10-mV step depolarization before and ~7 min after exposure to ATP. In both records the capacity current transient is fit well by a single exponential function, as expected for these spherical cells. Direct integration of the current transient, corrected for errors due to the access resistance as discussed in MATERIALS AND METHODS, indicates that the cell membrane capacitance, $C_M$, increased by >70%, from an initial value of 27 to 47 pF, while the access resistance did not change. There was no consistent change in access resistances after ATP exposure in these experiments, but the membrane conductance usually increased substantially as in Fig. 3 B, due to activation of the calcium-sensitive and/or nonspecific cation conductances. ATP-induced increases in membrane capacitance were present in 80% of the >140 perforated patch-clamped cells with intact cytoplasm used for these studies. These responses to ATP likely reflect direct actions of ATP on receptors in brown fat cell membranes rather than in associated sympathetic synaptosomes, since ~85% of our experiments were performed on cells maintained in culture for two or more days that are unlikely to retain any remnants of innervation. We observed no differences in cell responses with time in culture.

There was no direct correspondence between membrane conductance increases and capacitance increases in these experiments as a whole. Cells responded to

Chemicals

ATP, GTP, UTP, adenosine, α,β-methylene ATP, ADP, norepinephrine, insulin and Reactive Blue 2 (Cibacron Blue 3GA) were obtained from Sigma Chemical Co. (St. Louis, MO). 2-methylthio ATP was from Research Biochemicals International. Pluronic F-127 and FM1-43 were from Molecular Probes Inc. (Eugene, OR).

Statistical Analysis

Means are presented ±SEM. Statistical significance was determined using Student's t test.
FIGURE 3. (A) Capacity current transients recorded from a perforated patch voltage-clamped brown fat cell before (CONTROL) and after (ATP) a brief exposure to ATP. Currents recorded during 10-mV depolarizations from the holding potential of -60 mV. Superimposed on each current trace is a single exponential function fitted to the data (dotted line). In the currents recorded before and after ATP, the data and the fitted functions superimpose almost exactly at all times after the first few ms of the voltage step. Membrane capacitances, $C_M$, determined from the integral of the current transient were 27 pF for the control and 46 pF after ATP. Cell membrane resistance, $R_M$, determined from the steady-state current level was 1.1 GΩ in the control and 0.9 GΩ after ATP. (B) Time course of membrane responses to a brief exposure to extracellular ATP in the same cell. 100 μl of 100 μM ATP was added to the 200 μl bath with continuous perfusion at ~2 ml/min of agonist-free solution. Membrane capacitance and conductance were determined from the currents during 10-mV steps from the holding potential, applied every 6 s. The asterisks mark the times at which the current traces in A were recorded. All records in this and subsequent figures are corrected for the measured access resistance.

ATP with both capacitance and conductance changes within a few seconds, but the membrane capacitance changes were generally slower in both onset and recovery than the conductance changes, as shown in Fig. 3 B. The lack of temporal correspondence indicates that the conductance and capacitance increases are not tightly linked. The magnitude of the capacitance increases in response to ATP averaged +33 ± 3% ($n = 50$) in perforated patch-clamped cells bathed with normal Krebs’ solution. Both the size of the capacitance responses and their time courses were highly variable from cell to cell. The amplitude of the $C_M$ increases ranged from no measurable change to as much as a 97% increase. Most of our experiments do not show maximal capacitance responses to ATP, since we limited the duration of cells’ exposure to ATP in order to minimize activation of the cation conductance. Repeated or sustained exposure to ATP irreversibly activated such large, noisy currents that the recordings became unreliable. To prevent this, we usually removed the ATP when the capacitance was still rising. Some cells returned to their basal capacitance levels within a few minutes, while others showed little or no decline after washout of the ATP. Micromolar extracellular ATP was sufficient to elicit membrane conductance and capacitance responses, as illustrated in Fig. 4. In spite of
the profound changes in cell membrane electrical properties induced by ATP, there was no apparent change in cell appearance after ATP stimulation.

Membrane conductance responses to ATP and norepinephrine (NE) seemed independent and additive, as shown in Fig. 5. NE alone stimulated a small capacitance increase in the cell of Fig. 5 and in 8 out of 10 cells in which $C_M$ was continuously monitored, but the average increase with NE was only $6 \pm 4\%$ ($n = 8$). In 15 additional cells in which $C_M$ was measured before and after, but not during, exposure to agonist, NE produced a $4 \pm 1\%$ increase in $C_M$, while ATP responses were four times larger, averaging a $17 \pm 5\%$ $C_M$ increase. All of these cells showed substantial conductance increases in response to NE, reflecting cytosolic calcium increases and activation of calcium-sensitive conductances. These experiments indicate that ATP-induced membrane capacitance changes are regulated independently from adrenergic signalling systems.

Since adrenergic receptor activation releases calcium from intracellular stores as effectively as does purinergic stimulation (Lee et al., 1993; Pappone and Lee, manuscript in preparation), the lack of capacitance response to NE indicates that cytosolic calcium increases alone are insufficient to trigger capacitance increases. Fig. 5 shows that adrenergic stimulation does not suppress capacitance responses to ATP. In addition, the time course of ATP-induced capacitance increases and their magnitude were similar in the presence and absence of extracellular calcium, as shown in Figs. 6 and
indicating that membrane calcium influx is not required to develop a capacitance increase. Although the time course of $C_M$ recovery back to basal levels in Fig. 6 is more rapid in the absence of calcium than in its presence, this difference in time course was not seen consistently in zero calcium experiments. It was possible to elicit capacitance responses to ATP after prolonged periods (20–30 min) in divalent cation free solution, even after multiple stimulations with purinergic and/or adrenergic agonists, which would be expected to deplete intracellular stores of releasable calcium (Lee et al., 1993). Thus, it seems that the capacitance increases are not mediated by increases in intracellular calcium alone. However, significant intracellular calcium may be present in all these experiments, so it remains possible that cytosolic calcium plays a permissive or highly localized role in the membrane capacitance response.

**ATP Acts through a $P_{2Y}$ Purinergic Receptor**

The pharmacology of the capacitance responses indicates regulation through a $P_{2Y}$ purinergic receptor system (Burnstock, 1990). Both 2-methylthio-ATP (2-MeSATP) and ADP were highly effective in generating capacitance increases, as shown in Fig. 7. The minimal concentration of these agonists necessary to elicit a measurable response gave a potency order of 2-MeSATP $\geq$ ATP $>$ ADP. Prior exposure of the cells to 10–100 $\mu$M of the $P_{2Y}$ antagonist Reactive Blue 2 reversibly blocked the response to 1–5 $\mu$M ATP. The threshold concentration of ATP necessary to elicit a discernible membrane response was in the range of 0.1–0.5 $\mu$M in normal Krebs' solution and was similar in solutions with no calcium added or with both calcium and magnesium ions omitted, arguing against involvement of an ATP$^4$-sensitive $P_{2Y}$ receptor. Membrane capacitance responses were not seen with exposure to adenosine (100 $\mu$M, $n = 2$), $\alpha$-methylene ATP (0.1–1 mM, $n = 3$), GTP (0.5 mM, $n = 4$), or UTP (0.25–0.5 mM, $n = 3$). Insulin (100–200 nM), which can mobilize glucose transporters in brown fat cells (Slot et al., 1991), did not measurably affect membrane capacitance in 11 of 11 cells, even when insulin-deprived for 4 ($n = 3$) or 24 ($n = 4$) hours preceding exposure to hormone.

**ATP May Increase Both Exocytosis and Endocytosis**

Cell membrane components cycle rapidly between the cell surface and intracellular structures, with some cells...
completely turning over their surface membrane in <30 min. Our capacitance measurements reflect only the size of the surface membrane, so the ATP-induced increase in membrane capacitance we measure could equally well be due to an increase in exocytosis or a decrease in endocytosis. To differentiate between these possibilities, we examined the effects of ATP on FM1-43 fluorescence. FM1-43 is a membrane-impermeant, amphipathic fluorophore that shows large increases in fluorescence when it is in a membrane rather than an aqueous environment (Betz et al., 1992; Betz and Bewick, 1993). The fluorescence measured with FM1-43 in the solution bathing the cell then reflects the amount of cell membrane accessible to the dye on the cell surface, plus the amount of intracellular membrane rapidly exchanging with the cell surface membrane. The magnitude of the FM1-43 fluorescence appears to be a linear function of the amount of stained membrane, and its fluorescence does not seem to be modified by internalization (Smith and Betz, 1996). Addition of ATP caused an increase in FM1-43 fluorescence in 17 out of 22 experiments, suggesting that the dye had access to new membrane after the stimulation with ATP, as shown in Fig. 8. The newly stained membrane could come from fusion of vesicles with the surface membrane or endosomal structures. Alternatively, changes in endosomal properties could alter the dye’s fluorescence properties. Since both cell membrane surface area and FM1-43 fluorescence increase with ATP stimulation, it is most likely that ATP induces the exocytosis of intracellular membrane that previously was not exchanging with the surface membrane.

Adrenergic stimulation of brown fat cells had no effect on FM1-43 fluorescence, as shown in Fig. 9. In five such experiments, perfusion with μM NE produced no discernible change in cell fluorescence. Subsequent exposure of the cells to ATP more than doubled the fluorescence signal in all five of these cells.

In addition to increasing exocytosis, ATP seems to affect other aspects of membrane trafficking in brown fat cells. Simultaneously measured C_M and FM1-43 fluorescence show equivalent increases during pure exocytosis in chromaffin cells (Smith and Betz, 1996). However, in brown fat cells, FM1-43 responses to ATP were on average larger than capacitance increases. ATP induced an average FM1-43 fluorescence increase of 105 ± 31% (n = 17) compared to the average 33% capacitance increase. This difference was also seen in experiments in which FM1-43 fluorescence was measured concurrently with membrane capacitance in the same cell, as shown in Fig. 10. In this cell, membrane capacitance increased ~65% in the first few minutes after ATP, while the fluorescence increased ~150% during the same period. In six such experiments, the dye signal always reported a significantly greater increase with ATP stimulation (average 113 ± 8%) than did the capacitance measurement (average 27 ± 8%). If, as in chromaffin cells, FM1-43's fluorescent properties are not altered with endocytosis, this difference in the two measurements indicates that access of the dye to intracellular membranes as well as exocytosis are increased by ATP stimulation. In addition, dye is taken up into structures that do not

![Figure 8](image8.png)

**Figure 8.** Effects of ATP on the surface accessible membrane area measured using FM1-43 fluorescence. Total cell fluorescence was measured from a single brown fat cell during 200-ms excitations every 10 s. 2 μM FM1-43 was present during the times shown by the bars. Cell fluorescence was stable in the presence of FM1-43 alone. Addition of 2 μM ATP in the presence of FM1-43 caused an ~75% increase in fluorescence. Cell fluorescence after washout of ATP and dye decreased to a level somewhat higher than the pre-stimulation value.

![Figure 9](image9.png)

**Figure 9.** Adrenergic stimulation does not increase surface-accessible membrane measured by FM1-43 fluorescence. Shown is total cell fluorescence measured from a single brown fat cell as in Fig. 8. The cell was perfused with 1 μM norepinephrine (NE) or 5 μM ATP during the times shown by the bars.
exchange rapidly with the surface membrane, since the fluorescence level often did not return to baseline levels upon washout of the dye. This uptake is presumably responsible for the slow continuous increase in fluorescence seen in Fig. 10. The residual intracellular fluorescence was absent from the fat droplets, but otherwise was not apparently localized and could not be washed out even when cells were exposed to ATP, NE, insulin, or zero calcium solution to stimulate membrane turnover.

**DISCUSSION**

We find that exposure of brown adipocytes to 10 μM extracellular ATP evokes exocytosis of intracellular membrane, resulting in as much as twofold increases in cell membrane capacitance. Even greater increases are seen in the amount of surface-accessible membrane assayed by FM1-43 fluorescence. These changes in membrane trafficking are mediated by a P2Y purinergic receptor, which can increase cytosolic calcium levels. However, the exocytosis elicited by ATP in brown fat does not seem to be mediated by cytosolic calcium alone, since adrenergic stimulation mobilizes calcium without affecting membrane trafficking.

**Possible Sources of ATP**

There are several possible physiological sources of extracellular ATP that may act on brown adipocyte purinergic receptors. We initially tested for ATP responses because brown adipose tissue has extensive sympathetic innervation (Nechad, 1986) and vesicular ATP is usually present in sympathetic nerve terminals (Westfall et al., 1990). We find that ~90% of brown adipocytes tested showed conductance and/or capacitance responses to ATP, a proportion similar to that showing adrenergic responses (Pappone and Lee, 1995). Thus, it is likely that sympathetic activation of thermogenic responses in brown adipose tissue normally has both adrenergic and purinergic components. Previous experiments in vitro examining activity-dependent changes in brown fat cell number and properties using purely adrenergic stimulation to model sympathetic activity may need to be reevaluated in light of these results. ATP is proposed to participate in the regulation of cell proliferation and differentiation in many tissues (Rathbone et al., 1992) and in adipocytes these processes are known to be regulated by autocrine and/or paracrine factors (Butterwith, 1994), which may well include extracellular ATP. ATP may be released in addition by other cell types present in brown adipose tissue. Mast cells and endothelial cells are prevalent in brown adipose tissue (Nechad, 1986) and are also potential sources of exogenous ATP (Dubyak, 1991; Osipchuk and Cahalan, 1992). ATP may be released by these cells in response to inadequate blood flow and mediate vasodilation and/or angiogenesis as has been reported in other tissues (Motte et al., 1995). In addition, ATP may be released from fat cells themselves. Many other cell types are known to contain transport proteins such as the multidrug resistance protein, the cystic fibrosis transmembrane conductance regulator, and the sulfonyleurea receptor protein that may transport ATP into the extracellular space (Al-Awqati, 1995).

**Extracellular ATP Acts through a P2Y Purinergic Receptor**

Brown fat cell responses to ATP are mediated by a P2Y type purinergic receptor. The pharmacology of the membrane capacitance response, that is a potency order of 2-methylthio-ATP (2-MeSATP) ≥ ATP > ADP >> adenosine, α,β-methylene ATP, GTP, UTP, and block by Reactive Blue 2, is that of a P2Y receptor (Burnstock,
In other cell types P2Y receptors are known to mobilize intracellular calcium through G-protein-mediated generation of IP3 (Harden et al., 1995; Chen et al., 1995). Our results showing activation of calcium-sensitive membrane conductances are consistent with such an action in brown adipocytes as well. Although adenosine-sensitive A1 purinergic receptors are well known in brown and white adipocytes, we are not aware of any previous description of extracellular ATP responses in brown adipocytes. In white adipocytes ATP-induced increases in cytosolic calcium (Blackmore and Augert, 1989; Kelly et al., 1989) and modulations of glucose and pyruvate handling (Kelly et al., 1989; Cheng and Harold, 1990) have been reported. However, in neither tissue is there yet a clearly defined physiological role for ATP receptor stimulation.

**ATP Activates Exocytosis**

Purinergic stimulation results in a dramatic increase in cell membrane surface area in brown fat cells. We found an average ~30% increase in cell membrane capacitance and ~100% increase in FM1-43 fluorescence in these experiments. Membrane responses were not permitted to plateau in most of our experiments, so these values are undoubtedly less than the maximal possible responses. Large increases in membrane capacitance (10–100%) such as we see with ATP stimulation of brown fat have been associated in other cell types with the insertion of membrane transporters into the plasma membrane (Lewis and de Moura, 1982) or more commonly with hormone secretion (Neher and Marty, 1982; Fernandez et al., 1984; Tse et al., 1993). The combined results of our membrane capacitance and FM1-43 fluorescence measurements indicate that, like these processes, the surface membrane increases induced by ATP also involve fusion of intracellular membranes with the surface. Electron microscopic studies have shown numerous submembranous vesicles and plasma membrane invaginations in both brown and white adipocytes (Slavin, 1987), indicating that brown fat cells have sufficient intracellular vesicles available for a substantial exocytic response. Exocytotic responses to ATP have been reported in other cell types secondary to ATP-induced increases in cytosolic calcium. Raised intracellular calcium alone, however, does not seem sufficient to trigger the membrane response of brown fat cells, since NE raised cell calcium to similar levels without activating capacitance increases. This cannot be due to a simultaneous suppression of the membrane response by adrenergic pathways because membrane capacitance responses to ATP were unaffected by concurrent NE stimulation. In addition, membrane capacitance increases were similar in the presence and nominal absence of extracellular calcium, suggesting that high calcium levels are not required for exocytosis in these cells.

**Relation between Membrane Exocytosis and Conductances**

Although purinergic stimulation has a number of effects on membrane conductances (Pappone and Lee, manuscript in preparation), these seem to be independent of the exocytotic response. The calcium-sensitive potassium and chloride conductances can be activated by other experimental manipulations that raise calcium, such as adrenergic stimulation (Lucero and Pappone, 1990), exposure of cells to calcium ionophores (not shown), and including calcium in the pipet solution in whole cell patch clamp (Pappone and Lee, 1995), without affecting cell membrane capacitance. Thus, the activation of these conductances by ATP seems purely a consequence of its action in raising cytosolic calcium levels and does not seem to depend on ATP's actions in mobilizing intracellular membranes.

**Possible Physiological Roles of ATP Responses**

Membrane responses of brown fat cells to extracellular ATP have not been reported previously, and the role ATP-stimulated exocytosis might play in the physiology of the tissue is not known. One possibility is that ATP regulates the surface expression of a membrane protein or proteins (Bradbury and Bridges, 1994). Many studies of membrane trafficking in adipocytes have examined the insulin-activated upregulation of glucose transport, which involves significant movement of intracellular membrane containing Glut4-type glucose transporters to the surface in both white and brown adipocytes (Czech, 1995; Slot et al., 1991). These membrane events do not seem to be involved in the exocytotic response to purinergic stimulation however. First, insulin did not increase membrane capacitance or FM1-43 fluorescence in our experiments, although our cells may have lost insulin sensitivity with time in culture. Second, Glut4 mobilization is also activated by adrenergic stimulation in brown fat cells (Omatsu-Kanbe and Kita-sato, 1992), but in our experiments NE evoked minimal capacitance or fluorescence increases. Third, work on an adipocyte model cell line indicates that ATP does not mobilize glucose transporters (Robinson et al., 1992). And finally, extracellular ATP suppresses Glut4 mobilization in response to insulin in white adipocytes (Kelly et al., 1989). Thus, it is unlikely that the membrane surface changes we see in response to ATP result from the upregulation of glucose transport activity. It remains possible however that purinergic membrane mobilization mediates the surface expression of some other membrane transport protein or receptor, such as fatty acid transporters or insulin receptors.

Purinergic membrane mobilization may reflect activation of a secretory process. There is a growing catalog of adipocyte secreted peptides and proteins (reviewed in Ailhaud et al., 1992; Spiegelman et al., 1993; Spiegel-
man and Hotamisligil, 1993), which include growth factors (hepatocyte growth factor, insulin-like growth factor, vascular endothelial growth factor, tumor necrosis factor α), complement components (adipsin/factor D, factor B, factor C), and fat handling proteins (cholesterol ester transfer protein, lipoprotein lipase, apolipoprotein E). At least some adipocyte secretory products seem to be involved in regulating the growth and differentiation of adipose tissue (Lau et al., 1990; Ailhaud et al., 1992). In addition, it has been found recently that the ob gene mutation, which causes obesity in mice, codes for a peptide secreted by fat cells, termed adipin (et al., 1992). In addition, it has been found recently that the ob gene mutation, which causes obesity in mice, codes for a peptide secreted by fat cells, termed leptin, that can act centrally to suppress appetite, increase metabolism, and reduce body weight (Zhang et al., 1994; Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). While regulated secretion of fat cell products has not been reported, release of one or more or these products may be controlled by extracellular ATP levels. It is likely that in many biochemical preparations sufficient ATP could be released from only a few damaged cells to elicit the exocytotic responses we report, or there may be sufficient endogenous ATP release to trigger secretion. Fat cells are fragile, and even nanomolar concentrations of ATP are sufficient to elicit cell responses (Barnard et al., 1994). Thus it is possible that secretion that has been considered constitutive may have in fact been stimulated by ATP released from broken cells. Secretion and/or expression of many adipocyte products correlates with the metabolic and/or differentiative state of the tissue and the metabolic status of the organism, and so are altered in obesity or with development of the adipocyte phenotype. Given these phenomena, it seems likely that purinergic stimulation plays an important role in adipocyte physiology.

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REFERENCES

Ailhaud, G., P. Grimaldi, and R. Negrel. 1992. Cellular and molecular aspects of adipose tissue development. Annu. Rev. Nutr. 12:207–233.

Al-Awqati, Q. 1995. Regulation of ion channels by ABC transporters that secrete ATP. Science (Wash. DC). 269:805–806.

Barnard, E.A., G. Burnstock, and T.E. Webb. 1994. G protein-coupled receptors for ATP and other nucleotides: a new receptor family. Trends Pharmacol. Sci. 15:67–70.

Betz, W.J., and G.S. Bewick. 1993. Optical monitoring of the transmitter release and synaptic vesicle recycling at the frog neuromuscular junction. J. Physiol. (Lond.). 460:287–300.

Betz, W.J., F. Mao, and G.S. Bewick. 1992. Activity-dependent fluorescent staining and destaining of living vertebrate motor nerve terminals. J. Neurosci. 12:363–375.

Blackmore, P.F., and G. Augert. 1989. Effect of hormones on cytosolic free calcium in adipocytes. Cell Calcium. 10:561–567.

Bradbury, N.A., and R.J. Bridges. 1994. Role of membrane trafficking in plasma membrane solute transport. Am. J. Physiol. 267:C1–C24.

Burnstock, G. 1990. Overview: purinergic mechanisms. In Biophysical Actions of Extracellular ATP. G.R. Dubyak and J.S. Fedan, editors. Annals of the New York Academy of Sciences. New York. 603:1–18.

Butterwith, S.C. 1994. Molecular events in adipocyte development. Pharmcol. Ther. 61:399–411.

Campfield, L.A., F.J. Smith, Y. Guisez, R. Devos, and P. Burn. 1995. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science (Wash. DC). 269:546–549.

Chen, Z.-P., A. Levy, and S.L. Lightman. 1995. Nucleotides as extracellular signalling molecules. J. Neuroendocrinol. 7:83–96.

Cheng, K., and E.G. Harold. 1990. Insulin-like effects of ATP on adipocyte pyruvate dehydrogenase and phosphorylase. Arch. Biochem. Biophys. 276:232–235.

Czech, M.P. 1995. Molecular actions of insulin on glucose transport. Annu. Rev. Nutr. 15:441–471.

Dubyak, G.R. 1991. Signal transduction by P2-purinergic receptors for extracellular ATP. Am. J. Respir. Cell Mol. Biol. 4:295–300.

Fernandez, J.M., E. Neher, and B.D. Gomperts. 1984. Capacitance measurements reveal stepwise fusion events in degranulating mast cells. Nature (Lond.). 312:453–455.

Flier, J.S. 1995. The adipocyte: storage depot or node on the energy information superhighway? Cell. 80:15–18.

Giovannini, P., J. Seydoux, and L. Girardier. 1988. Evidence for a modulating effect of Na+/H+ exchange on the metabolic response of rat brown adipose tissue. Pflug. Arch. 411:273–277.

Girardier, L., and G. Schneider-Picard. 1983. Alpha and beta-adrenergic mediation of membrane potential changes and metabolism in rat brown adipose tissue. J. Physiol. (Lond.). 353:629–641.

Halaas, J.L., K.S. Gajiwala, M. Maffei, S.L. Cohen, B.T. Chait, D. Rabinowitz, R.L. Lallone, S.I. Burley, and J.M. Friedman. 1995. Weight-reducing effects of the plasma protein encoded by the obese gene. Science (Wash. DC). 269:543–546.

Harden, T.K., J.L. Boyer, and R.A. Nicholas. 1995. P2-purinergic receptors: subtype-associated signaling responses and structure. Annu. Rev. Pharmacol. Toxicol. 35:541–579.

Himms-Hagen, J. 1989. Brown adipose tissue thermogenesis and obesity. Prog. Lipid Res. 28:67–115.

Horwitz, B.A., and J. Hamilton. 1984. Alpha-adrenergic-induced changes in hamster (Mesocricetus) brown adipocyte respiration and membrane potential. Comp. Biochem. Physiol. 78:99–104.

Horwitz, B.A., and J. Hamilton. 1993. Plasma membrane proton transport and hamster brown adipocyte thermogenesis. In Living in the Cold III. Ecological, Physiological, and Molecular Mechanisms. C. Carey, G.L. Florant, B.A. Wunder, and B.A. Horwitz, editors. Westview, Boulder, CO. 217–224.

Johnson, P.R., M.R.C. Greenwood, B.A. Horwitz, and J.S. Stern. 1991. Animal models of obesity: genetic aspects. Annu. Rev. Nutr. 11:325–353.
Kelly, K.L., J.T. Deeney, and B.E. Corkey. 1989. Cytosolic free calcium in adipocytes: distinct mechanisms of regulation and effects on insulin action. J. Biol. Chem. 264:12754-12757.

Koivistio, A., and J. Nedergaard. 1995. Modulation of calcium-activated non-selective cation channel activity by nitric oxide in rat brown adipose tissue. J. Physiol. (Lond.). 486:59-65.

Koivistio, A., D. Siemen, and J. Nedergaard. 1993. Reversible blockade of the calcium-activated nonselective cation channel in brown fat cells by the sulfhydryl reagents mercury and thimerosal. Pflug. Arch. 425:549-551.

Lau, D.C.W., G. Shillabeer, K.-L. Wong, S.C. Tough, and J.C. Russel. 1990. Influence of paracrine factors on preadipocyte replication and differentiation. Int. J. Obs. 14(Suppl. 3):193-201.

Lee, S.C., J.S. Hamilton, T. Trammell, B.A. Horwitz, and P.A. Pappone. 1994. Adrenergic modulation of intracellular pH in isolated brown fat cells from hamster and rat. Am. J. Physiol. 267: C349-C356.

Lucero, M.T., and P.A. Pappone. 1989. Voltage-gated potassium channels in brown fat cells. J. Gen. Physiol. 93:451-472.

Lucero, M.T., and P.A. Pappone. 1990. Membrane responses to norepinephrine in cultured brown fat cells. J. Gen. Physiol. 95: 523-544.

Motte, S., D. Communi, S. Pirotton, and J.-M. Boeynaems. 1995. Involvement of multiple receptors in the actions of extracellular ATP: the example of vascular endothelial cells. Int. J. Biochem. Cell Biol. 27:1-7.

Nechad, M. 1986. Structure and development of brown adipose tissue. In Brown Adipose Tissue. P. Trayhurn and D.G. Nicholls, editors. Van Nostrand Reinhold Co. New York. 52-85.

Nicholls, D.G. 1974. Hamster brown adipose tissue mitochondria: the control of respiration and the proton electrochemical potential by possibly physiological effectors of the proton conductance of the inner membrane. Eur. J. Biochem. 49:573-583.

Nicholls, D.G., and R.M. Locke. 1984. Thermogenic mechanisms in brown fat. Physiol. Rev. 64:1-64.

Osipchuk, Y., and M. Cahalan. 1992. Cell-to-cell spread of calcium signals mediated by ATP receptors in mast cells. Nature (Lond.). 359:241-244.

Pappone, P.A., and S.C. Lee. 1994. Purinergic stimulation alters the membrane properties of brown fat cells. Biophys. J. 66:429A (Abstr.).

Pappone, P.A., and S.C. Lee. 1995. α-Adrenergic stimulation activates a calcium-sensitive chloride current in brown fat cells. J. Gen. Physiol. 106:231-258.

Pappone, P.A., and S.C. Lee. 1996. Exocytosis in adipocytes is activated by purinergic receptor stimulation. Biophys. J. 70: A85 (Abstr.).

Pellemounter, M.A., M.J. Cullen, M.B. Baker, R. Hecht, D. Winters, T. Boone, and F. Collins. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. Science (Wash. DC). 269:540-543.

Robinson, I.J., S. Pang, D.S. Harris, J. Heuser, and D.E. James. 1992. Translocation of the glucose transporter (GLUT4) to the cell surface in permeabilized 3T3-L1 adipocytes: effects of ATP, insulin and GTPγS and localization of GLUT4 to clathrin lattice. J. Cell Biol. 117:1181-1196.

Schimmel, R.J., M.E. Elliott, and V.C. Dehmel. 1987. Interactions between adenosine and α-adrenergic agonists in regulation of respiration in hamster brown adipocytes. Mol. Pharmacol. 32:26-33.

Schimmel, R.J., and L. McCarthy. 1986. Role of adenosine as an endogenous regulator of respiration in hamster brown adipocytes. Am. J. Physiol. 246:C301-C307.

Schneider-Picard, G., J.A. Coles, and L. Girardier. 1985. Alpha- and beta-adrenergic mediation of changes in metabolism and Na/K exchange in rat brown fat. J. Gen. Physiol. 86:169-188.

Slavin, B.G. 1987. The ultrastructure of the adipocyte. In Biology of the Adipocyte: Research Approaches. G.J. Hausman and R. Martin, editors. Van Nostrand Reinhold Co. New York. 52-85.

Slavt, J.W., H.J. Geuze, S. Gigengack, G.E. Lienhard, and D.E. James. 1991. Immunolocalization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. J. Cell Biol. 113:125-135.

Smith, C.B., and W.J. Betz. 1996. Simultaneous independent measurement of endocytosis and exocytosis. Nature (Lond.). 380:531-534.

Sjostrand, B.M., L. Choy, G.S. Hotamisligil, R.A. Graves, and P. Tojntonoz. 1993. Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes. J. Biol. Chem. 268:6823-6826.

Spiegelman, B.M., and G.S. Hotamisligil. 1993. Through thick and thin: wasting, obesity, and TNFα. Cell. 73:625-627.

Szillat, D., and L.J. Bukowiecki. 1983. Control of brown adipose tissue lipolysis and respiration by adenosine. Am. J. Physiol. 245: E555-E559.

Tse, A., F.W. Tse, W. Almers, and B. Hille. 1993. Rhythmic exocytosis stimulated by GnRH-induced calcium oscillation in rat gonadotropes. Science (Wash. DC). 260:82-84.

Westfall, D.P., K.O. Sedaa, K. Shinozuka, R.A. Bjur, and I.L.O. Buxton. 1990. ATP as a cotransmitter. In Biological Actions of Extracellular ATP. G.R. Dubyk and J.S. Fedan, editors. Annals of the New York Academy of Sciences. New York. 603:300-310.

Wilcke, M., and J. Nedergaard. 1989. α- and β-adrenergic regulation of intracellular Ca2+ levels in brown adipocytes. Biochem. Biophys. Res. Commun. 163:292-300.

Zhang, Y., R. Proctor, M. Maffei, M. Barone, L. Leopold, and J.M. Friedman. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature (Lond.). 372:425-432.