ATP Can Stimulate Exocytosis in Rat Brown Adipocytes without Apparent Increases in Cytosolic Ca^{2+} or G Protein Activation

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ABSTRACT Extracellular ATP activates large increases in cell surface area and membrane turnover in rat brown adipocytes (Pappone, P. A., and Lee, S. C. 1996. *J. Gen. Physiol.* 108:393–404). We used whole-cell patch clamp membrane capacitance measurements of membrane surface area concurrently with fura-2 ratio imaging of intracellular calcium to test whether these purinergic membrane responses are triggered by cytosolic calcium increases or G protein activation. Increasing cytosolic calcium with adrenergic stimulation, calcium ionophore, or calcium-containing pipette solutions did not cause exocytosis. Extracellular ATP increased membrane capacitance in the absence of extracellular calcium with internal calcium strongly buffered to near resting levels. Purinergic stimulation still activated exocytosis and endocytosis in the complete absence of intracellular and extracellular free calcium, but endocytosis predominated. Modulators of G protein function neither triggered nor inhibited the initial ATP-elicited capacitance changes, but GTPγS or cytosolic nucleotide depletion did reduce the cells' capacity to mount multiple purinergic responses. These results suggest that calcium modulates purinergically-stimulated membrane trafficking in brown adipocytes, but that ATP responses are initiated by some other signal that remains to be identified.

INTRODUCTION

Brown adipose tissue generates heat in response to norepinephrine released by sympathetic nerves (Nicholls and Locke, 1984). Most sympathetic nerves are thought to release ATP (Westfall et al., 1990), and extracellular ATP also evokes a number of responses in brown and white adipocytes (Pappone and Lee, 1996; Lee and Pappone 1997a, b). Micromolar concentrations of ATP increase intracellular calcium ($[Ca^{2+}]_i$), alter membrane conductance, and activate exocytosis in adipocytes. The membrane trafficking response is substantial, and can result in as much as twofold increases in cell membrane surface area in brown adipocytes (Pappone and Lee, 1996). The role of purinergic activation in adipocyte physiology is not known, but the membrane mobilization is likely involved in secretion and/or the redistribution of receptors, transporters, or other membrane components.

Regulated secretion in neurons and endocrine and exocrine cells is typically triggered by a rise in $[Ca^{2+}]_i$ (Burgoyne and Morgan, 1995; Augustine et al., 1996), and secretory responses to purinergic stimulation mediated by $[Ca^{2+}]_i$ increases have been reported (Blachier and Malaisse, 1988; Kim and Westhead, 1989; Li et al., 1991). However, our previous work suggested that intracellular calcium alone is not sufficient to activate exocytosis in adipocytes (Pappone and Lee, 1996). α-Adrenergic stimulation of brown adipocytes also increases $[Ca^{2+}]_i$ levels (Wilcke and Nedergaard, 1989; Lee et al., 1993), but fails to increase cell surface area (Pappone and Lee, 1996). The lack of a membrane response to adrenergic stimulation was not due to inhibition of exocytosis, since normal increases in cell surface area were still elicited by ATP in the presence of norepinephrine. While these experiments suggested that calcium is not solely responsible for activating the purinergic membrane trafficking response, they were all performed in intact, perforated patch clamped cells (Pappone and Lee, 1996) in which $[Ca^{2+}]_i$ levels were not measured or controlled. Thus, the possibility remained that ATP generates a signal that acts in concert with elevated cytosolic calcium to increase membrane trafficking.

The experiments presented here had two aims. The first was to ascertain what role intracellular calcium levels play in the purinergic activation of exocytosis in adipocytes. We used whole-cell patch clamp techniques to measure membrane capacitance and control cytosolic calcium levels together with simultaneous fura-2 photometry to directly assess $[Ca^{2+}]_i$. Second, we tested whether G protein activation is involved in triggering exocytosis. The pharmacology of the membrane trafficking response in brown adipocytes is consistent with mediation by a P2Y purinergic receptor (Pappone and Lee, 1996; Burnstock, 1997). The P2Y purinergic receptors couple to G proteins to activate phospholipases (Harden et al., 1995), consistent with the action of ATP releasing intracellular calcium stores in brown adipocytes (Lee and Pappone, 1997a). Surprisingly, we find that the initiation of ATP-activated exocytosis does not require cytosolic calcium increases and is insensitive to G protein manipulations, although both modulated the re-

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Abbreviations used: MOPS, 3-(*N*-morpholino)propanesulfonic acid; BAPTA, 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, potassium; FM 1-43, *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino) styryl) pyridinium dibromide; fura-2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, potassium; GDPβS, guanosine-5′-*O*-(2-thiodiphosphate) lithium; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate) lithium; XTP, xanthosine 5′-triphosphate, sodium.

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sponse. These results suggest that the signaling system used by brown adipocytes to activate membrane trafficking differs from those described previously for most P2Y purinergic and exocytotic responses.

EXPERIMENTAL PROCEDURES

Cell isolation and culture

Brown fat cells were isolated by collagenase digestion from the interscapular fat pads of 1–8-day-old neonate rats and cultured as described previously (Pappone and Lee, 1996; Lucero and Pappone, 1989, 1990). Experiments were performed on cells maintained 1–7 days in culture. Fat cells grow in size with time in culture, and the cells used in these experiments varied from 15 to 150 pF in size. There were no apparent differences in the ATP sensitivity or responses of these cells with time in culture.

Electrophysiology

Cell membrane currents were measured using standard whole-cell patch clamp techniques (Pappone and Lee, 1995, 1996; Lucero and Pappone, 1989). Cell membrane capacitance and conductance were determined from the integral of the charging current and the steady-state currents elicited by small voltage steps from the holding potential of −60 mV (Pappone and Lee, 1996). Pipette capacitance was nulled at the amplifier, but no other on-line series resistance or capacitance compensation was used. Access resistance was determined from the time constant of the charging current and was used to correct for series resistance errors. Typical pipette resistances were 2–5 MΩ (median 3.2 MΩ) and access resistances were 5–35 MΩ (median 12 MΩ). Total charge and corrected voltage were used to calculate cell membrane capacitance; leak current and corrected voltage were used to calculate cell membrane conductance. This method gives consistent values for membrane capacitance even when membrane conductance is very high and cell input impedance is comparable to the access resistance (Pappone and Lee, 1996). Average initial cell capacitance was 33.6 ± 1.1 pF (mean ± SE, median 29.3 pF) and average peak ATP-stimulated conductance was 3.9 ± 0.3 nS (median 3.0 nS). Only ~5% of the cells had maximum membrane conductances in excess of 10 nS, so the actual applied series resistance corrections at peak current were small in most cases (average ~6%, median ~4%).

Solutions

The extracellular solution was nominally Ca^{2+}-free Krebs-Ringer saline (Sigma K4002) containing (in mM) 120 NaCl, 4.5 KCl, 0.5 $MgCl_2$, 10 glucose, 0.7 Na_2HPO_4, 1.5 NaH_2PO_4, 24 $NaHCO_3$, bubbled with 95% O_2/5% CO_2, with or without 2 mM $CaCl_2$ added. Experiments were performed at room temperature (22–24°C) with continuous solution flow.

The typical pipette solution for whole-cell recording contained (in mM) 100 potassium aspartate, 35 KCl, 10 K_2EGTA, 1 $CaCl_2$, 1 MgATP, and 10 MOPS, pH 7.2 and calculated 18 nM free calcium. Measurement of intracellular $[Ca^{2+}]$ in cells preloaded with fura-2 indicated there was no stimulation of calcium responses by the pipette ATP if there was no back-pressure on the pipette solution during seal formation. Solutions of different calcium buffering capacity were made by varying the concentrations of EGTA (0–75 mM), BAPTA (1.9–10 mM), and added calcium. Free $[Ca^{2+}]$ of the solutions was calculated using the MaxChelator program (Bers et al., 1994). Potassium aspartate concentrations were varied as needed to maintain solution osmolarity of 270–300 mOsM and Cl^- concentrations near 35 mM.

Photometry

Intracellular free $[Ca^{2+}]$ was estimated by ratio fura-2 photometry. Fluorescence intensity at 510 nm due to alternating excitation at 340 and 380 nm was recorded from a single cell in an iris-defined spot using hardware and software from IonOptix Corp. (Milton, MA) (Lee and Pappone, 1997a). Fura-2 (free acid, 0.1–0.5 mM) was introduced through the patch pipette. The intracellular fura-2 concentration reached steady-state within 20 min of entering whole-cell configuration, indicating that this period should be sufficient for the similarly sized calcium buffers to equilibrate. Since individual cells were monitored before the introduction of dye, we had an accurate measure of 340 and 380 nm excited autofluorescence for each cell, which was subtracted from all subsequent data. The fura-2 signal was calibrated using high K^+ solutions with defined Ca^{2+} concentrations loaded in 20-μm pathlength microcuvettes (VitroCom, Mountain Lakes, NJ).

The dye FM 1-43 was used to measure total surface membrane and membrane trafficking (Betz et al., 1996), as described previously (Pappone and Lee, 1996). FM 1-43 in the bath quickly partitions into all surface-accessible cell membranes. Since the dye is much more fluorescent in a hydrophobic environment than in the aqueous phase, its fluorescence reflects the amount of cell membrane communicating with the bath solution (Betz et al., 1996). Cell-associated FM 1-43 fluorescence was monitored at 485 nm excitation, 530 nm emission with the same IonOptix hardware and software.

Reagents

GTPγS and GDPβS were obtained from both Sigma Chemical Co. (St. Louis, MO) and Research Biochemicals International (Natick, MA). EGTA was from Fluka Chemical Corp. (Ronkonkoma, NY). Fura-2, FM 1-43, BAPTA and 5,5′-dibromo-BAPTA were from Molecular Probes (Eugene, OR). Fura-2 acetoxymethyl ester was from Calbiochem. All other reagents were from Sigma.

RESULTS

Responses to ATP are maintained in whole-cell recordings

Extracellular ATP activates Ca^{2+}-dependent and Ca^{2+}-independent conductances and elicits an average ~30% increase in cell electrical capacitance in intact brown adipocytes voltage clamped in the perforated-patch configuration (Pappone and Lee, 1996). Fig. 1 shows that similar responses can be seen in cells patch clamped for long periods in the whole cell configuration. In this cell ATP stimulated a doubling of cell membrane capacitance (C_m) and an approximately fourfold, transient increase in membrane conductance after >10 min in the whole-cell configuration. The capacitance increases in whole-cell experiments with $[Ca^{2+}]_i$ buffered by 10 mM EGTA closely resembled those seen in intact cells in magnitude and time course. The average capacitance change in response to initial ATP exposures was a 31 ± 3% (mean ± SE; $n = 47$) increase, compared to an average 33 ± 3% ($n = 50$) increase in intact cells (Pappone and Lee, 1996). Endocytosis following washout of ATP was also similar in whole-cell patch clamped cells. In intact cells C_m typically requires 5–30 min to return to prestimulation levels following brief exposure to ATP, and often remains elevated following repeated or sustained stimulation (Pappone and Lee, 1996). The capacitance responses in the cell in Fig. 1 show a similar pattern.

Cell conductance increases were somewhat smaller in whole-cell recordings than in intact cells, probably because

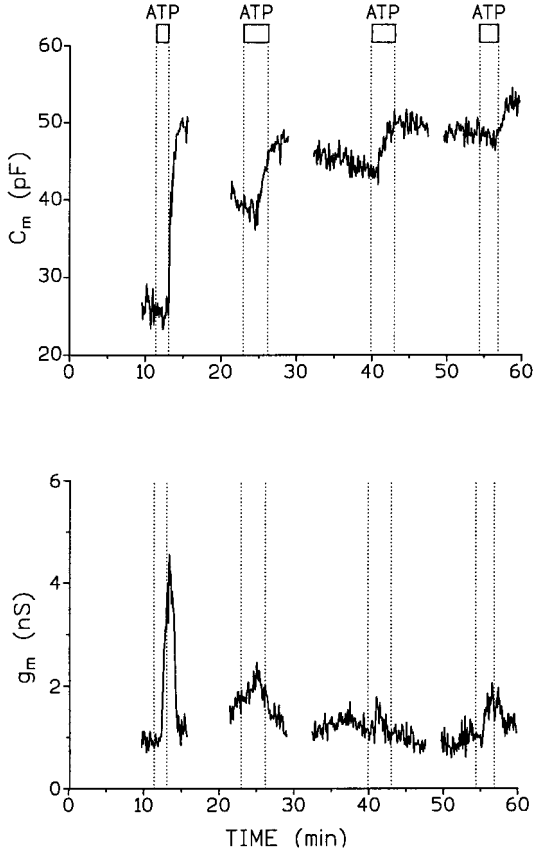

FIGURE 1 Extracellular ATP increases membrane capacitance and conductance in whole-cell patch clamped cells. Membrane capacitance (C_m) and conductance (g_m) were measured from the current transients recorded during 10-mV voltage steps applied every 6 s. During the times indicated by the bars and dotted lines, the cell was perfused with solution containing 5 μM ATP. No correction was made for time delays in the perfusion system (estimated to be 15–20 s). The pipette solution contained 10 mM EGTA and 1 mM Ca^{2+}, corresponding to a calculated free calcium concentration of 18 nM. There was no added nucleotide in the pipette solution. Zero time in this and all subsequent figures was the moment of membrane patch rupture during the formation of whole cell patch clamp configuration.

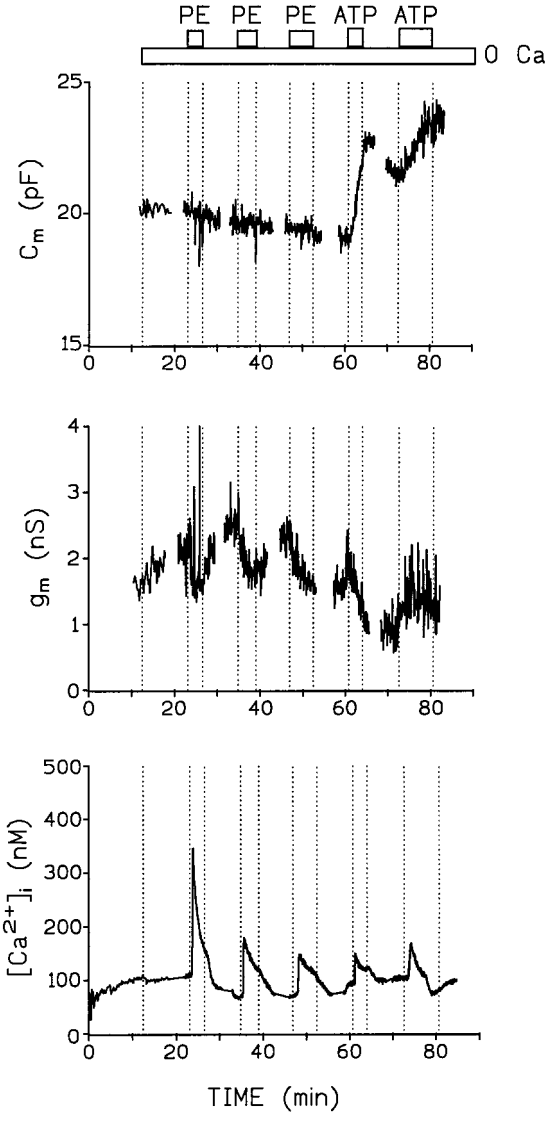

FIGURE 2 α-Adrenergic stimulation increases $[Ca^{2+}]_i$ without altering membrane capacitance. C_m, g_m, and intracellular $[Ca^{2+}]$ were measured simultaneously in a whole-cell patch clamped cell with internal solution buffered to 18 nM Ca^{2+} with 10 mM EGTA, and supplemented with 1 mM MgATP and 0.1 mM fura-2. Where indicated, the perfusing solution contained nominally zero calcium and/or 20 μM phenylephrine (PE) or 10 μM ATP.

activation of Ca^{2+}-dependent conductances is diminished by the added intracellular Ca^{2+} buffers. However, pipette solutions containing Ca^{2+} and ATP with EGTA as the sole Ca^{2+} buffer did not control cytosolic Ca^{2+} well. Resting Ca^{2+} levels reported by the introduced fura-2 were often significantly higher than the calculated free Ca^{2+} predicted in such solutions, as shown, for example, in Fig. 2. In contrast, cells containing similar EGTA-buffered solutions without Ca^{2+} and/or ATP, or with 5 mM BAPTA added, consistently showed Ca^{2+} levels within 20 nM of the calculated value (see Figs. 5, 7, and 8). Free Ca^{2+} levels reported by fura-2 in microcuvette measurements of the EGTA pipette solutions were not affected by the addition of ATP (not shown). These results suggest that cytosolic Ca^{2+} levels are actually higher than expected in cells dialyzed with EGTA-buffered solutions containing ATP. In addition, cells with this internal solution were able to produce many more agonist-elicited increases in cytosolic Ca^{2+} in zero Ca^{2+} bath solution than were intact cells (Lee and Pappone, 1997a) or cells buffered with BAPTA (see below), suggesting that Ca^{2+} stores are able to load when ATP and Ca^{2+} are present and EGTA is the sole Ca^{2+} buffer in the pipette solution.

In these experiments cells were maintained in whole cell configuration for 5–35 min (median time 20 min) before exposure to ATP. In Fig. 1 and all subsequent figures, time = 0 indicates the point at which whole-cell recording was initiated. The duration of internal dialysis prior to stimulation with ATP did not affect the frequency or the magnitude of the initial capacitance responses. Furthermore,

it was possible to elicit many ATP responses in a single cell, as seen in Fig. 1. In all, 95% of 207 cells stimulated with 1–10 μM extracellular ATP in whole-cell recordings responded with changes in membrane conductance and capacitance, indicating that the signaling and effector systems for the membrane responses to ATP are not readily washed out of the cell.

Increases in $[Ca^{2+}]_i$ do not correlate with increases in membrane capacitance

In intact brown adipocytes, both α-adrenergic (Lee et al., 1993) and purinergic (Lee and Pappone, 1997a) stimulation can increase $[Ca^{2+}]_i$ levels through release of Ca^{2+} from intracellular stores and influx of Ca^{2+} across the plasma membrane. Only purinergic stimulation increases membrane capacitance (Pappone and Lee, 1996), suggesting that increased $[Ca^{2+}]_i$ is not sufficient to trigger an increase in C_m. However, calcium responses in adipocytes are highly variable, so we tested this contention more rigorously by simultaneously monitoring C_m and intracellular $[Ca^{2+}]$ in the same cell, as shown in Fig. 2. Three exposures to the α-adrenergic agonist phenylephrine (PE) in zero calcium solution increased cytosolic $[Ca^{2+}]$ but did not change C_m. Two subsequent exposures to ATP significantly increased capacitance, although the calcium transients were smaller. Similar results were seen in four other cells. Thus, the signaling pathways that release intracellular calcium stores remain active in whole-cell patch clamped brown fat cells, but adrenergically activated release of stored calcium is not sufficient to increase C_m.

The possibility remained that adrenergic stimulation inhibits calcium-activated exocytosis, but that the inhibition could be overcome by purinergic stimulation. However, increasing $[Ca^{2+}]_i$ directly by exposing cells to a Ca^{2+} ionophore or pipette solutions containing high calcium also failed to activate increases in C_m. Fig. 3 shows that exposure to 4-bromo-A23187 raised intracellular $[Ca^{2+}]$ to micromolar levels without changing cell capacitance. Subsequent exposure of the cell to ATP in zero calcium solution resulted in a normal increase in capacitance without measurable increase in cell calcium. Ionophore exposure failed to increase C_m in nine cells under similar conditions even though $[Ca^{2+}]$ increased on average to ~900 nM. Four of five cells subsequently responded with increases in C_m when exposed to ATP.

Raising intracellular $[Ca^{2+}]$ with a pipette solution containing high levels of free Ca^{2+} also failed to activate exocytosis, as shown in Fig. 4. Eight similar experiments with pipette $[Ca^{2+}]$ as high as 100 μM all failed to increase capacitance. As is seen in Fig. 4, ATP stimulation in cells with sustained high internal calcium could result in net endocytosis. Clearly, elevation of cytosolic calcium does not itself trigger exocytosis. However, high calcium levels do modify membrane responses to ATP.

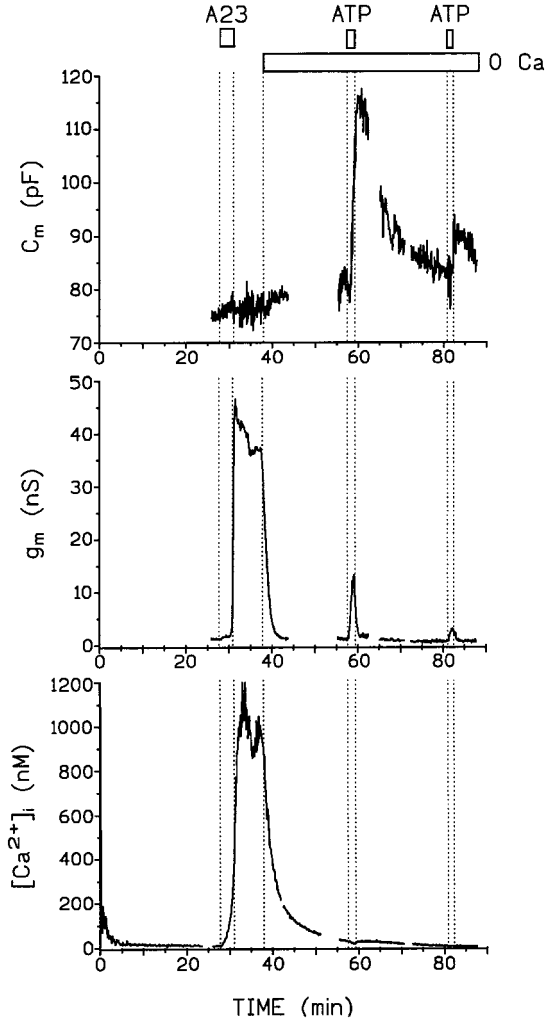

FIGURE 3 Calcium ionophore raises $[Ca^{2+}]_i$ without affecting membrane capacitance. The cell was exposed to 5 μM of the nonfluorescent ionophore 4-bromo-A23187 (A23) in bath solution containing 2 mM Ca^{2+}, increasing g_m and intracellular $[Ca^{2+}]$, but not C_m. The cell was then exposed to 10 μM ATP in nominally zero Ca^{2+} bath solution, which resulted in ~40% increase in C_m. The 0 Ca^{2+} pipette solution contained (in mM) 1.9 BAPTA and 3.8 EGTA, supplemented with 0.9 MgATP, 0.2 fura-2, and 0.2 GTPγS.

Removing calcium alters ATP responses

To determine whether any calcium was required for ATP-stimulated exocytosis, we performed experiments with pipette solutions containing BAPTA and/or high concentrations of EGTA that more effectively control $[Ca^{2+}]$ levels (Adler et al., 1991; Roberts, 1993). The initial ATP-stimulated capacitance increases averaged 21 $\pm$ 4% (n = 16) in cells buffered with 5–10 mM BAPTA, 30–75 mM EGTA, or a combination of BAPTA and EGTA to fix free calcium at low to normal levels (~20–120 nM). This capacitance increase is smaller but not statistically different from the average 31% increase seen in cells buffered with 10 mM intracellular EGTA (see above). Thus, adipocytes can still respond to ATP with normal capacitance increases with

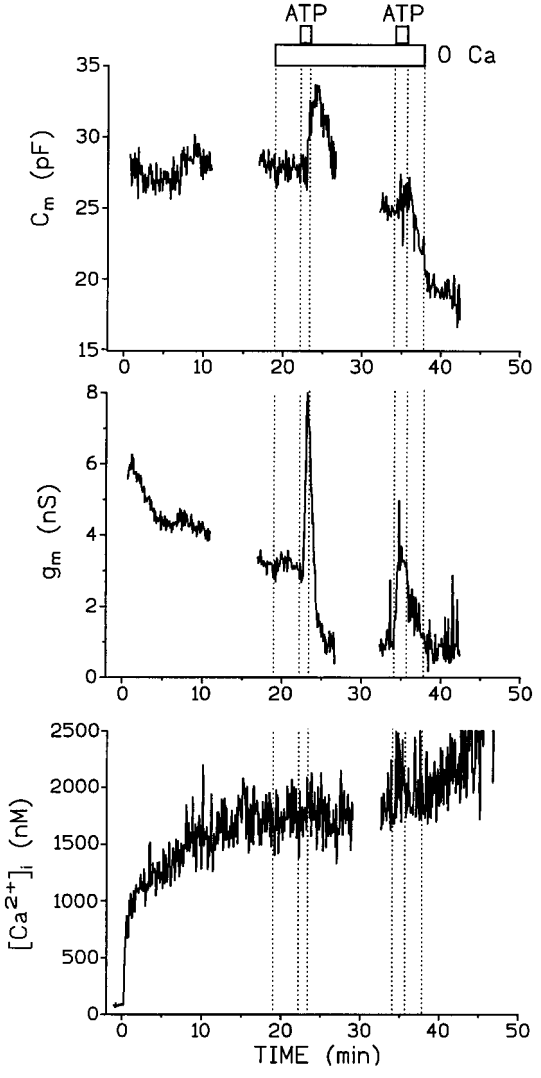

FIGURE 4 High-calcium pipette solution does not increase membrane capacitance. The cell was preloaded for 12 min with 2.5 μM fura-2 acetoxymethyl ester at room temperature. The pipette solution was buffered with 10 mM dibromo-BAPTA to contain ~1.8 μM free Ca^{2+}, with (in mM) 1 Mg^{2+}, 1.4 ATP, 0.2 GTP, and 0.2 fura-2. Where indicated, the cell was exposed to nominally 0 Ca^{2+} solution and/or 5 μM ATP.

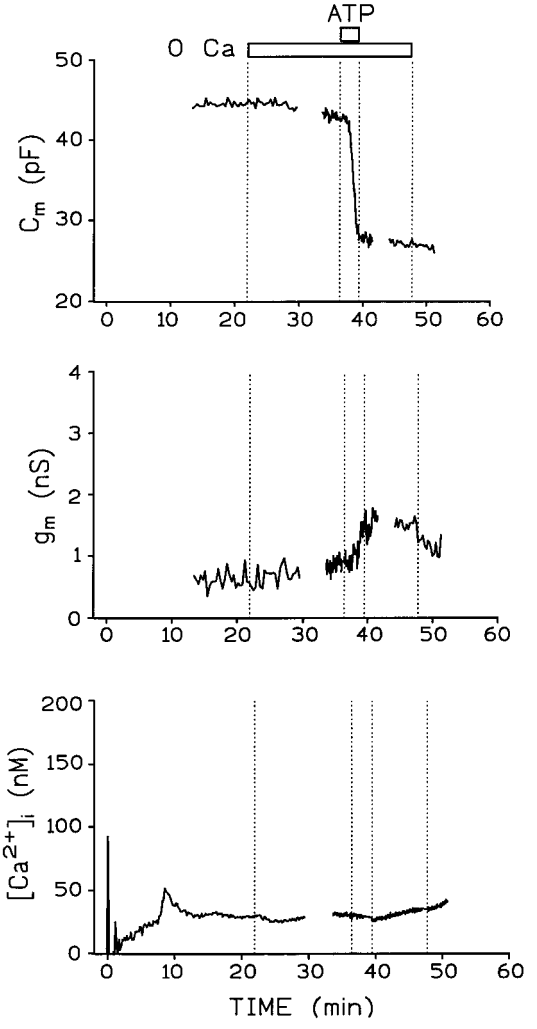

FIGURE 5 $[Ca^{2+}]_i$ buffering alters the C_m responses to ATP. Under conditions of maintained low calcium this cell showed only a sharp capacitance decrease in response to 5 μM ATP. The pipette solution was buffered with 30 mM EGTA and supplemented with 1 mM MgATP and 0.1 mM fura-2. Where indicated, the bath was nominally Ca^{2+} free.

strong calcium buffering when free $[Ca^{2+}]$ is set near normal basal levels.

Complete removal of intracellular and extracellular calcium altered but did not prevent membrane responses to ATP. Both in intact cells (Pappone and Lee, 1996) and in whole cell experiments with some calcium present (see Fig. 9 below) 80–85% of cells respond to ATP with a capacitance increase. However, with intracellular and extracellular calcium levels held near zero, net capacitance decreases became common. In Fig. 5, ATP elicited a sharp decrease (~37%) in capacitance with no change in measured $[Ca^{2+}]$. Net endocytosis in response to a single ATP exposure was seen in 22 of 36 cells under strictly zero calcium conditions.

Even when capacitance measurements showed a net decrease in cell surface area, both exocytosis and endocytosis were activated by ATP. The fluorescence of the amphipathic membrane dye FM 1-43 is proportional to the total amount of bath-accessible cell membrane (Betz et al., 1996). In intact brown fat cells FM 1-43 fluorescence increased by ~100% with ATP stimulation, while simultaneously measured C_m increased only ~30%, indicating that both endocytosis and exocytosis are activated (Pappone and Lee, 1996). Figure 6 shows simultaneous capacitance and FM 1-43 fluorescence measurements from two whole-cell experiments with different intracellular Ca^{2+} buffering. When the pipette solution contained 10 mM EGTA/1 mM Ca^{2+} the membrane capacitance increased with ATP stimulation (Fig. 6 *A*), while when the pipette solution contained 10 mM BAPTA with no added Ca^{2+} the capacitance decreased (Fig. 6 *B*). Despite the different C_m responses, both cells showed similar increases in FM 1-43 fluorescence. Thus new membrane is mobilized to the cell surface whether ATP triggers net endocytosis or exocytosis. Equiv-

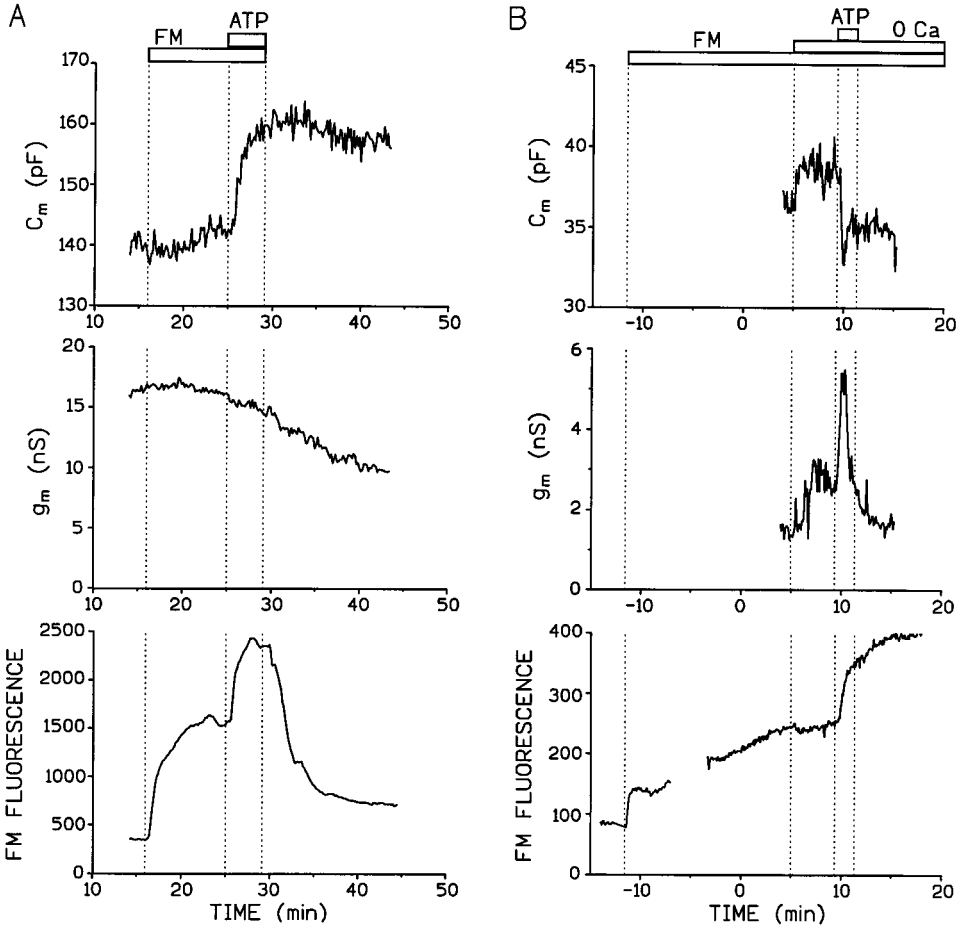

FIGURE 6 Membrane trafficking measured by simultaneous assessment of FM 1-43 fluorescence and membrane capacitance. (*A*) C_m and fluorescence responses to 0.1 μM ATP with $[Ca^{2+}]_i$ buffered to a calculated concentration of 18 nM by 10 mM EGTA. The cell was exposed to 2 μM FM 1-43 with or without 0.1 μM ATP in normal calcium-containing solution during the times indicated. The initial increase in fluorescence with wash-in of the dye reflects staining of surface-accessible membranes. Addition of ATP causes simultaneous increases in fluorescence and capacitance. (*B*) Results from a similar experiment in 0 Ca^{2+} bath solution with cytosolic Ca^{2+} buffered with 10 mM BAPTA and no added Ca^{2+}. 10 μM ATP causes an increase in fluorescence of similar magnitude, but it is accompanied by a simultaneous decrease in C_m. FM 1-43 fluorescence is given in photon counts/ms.

alent results were obtained in three other cells under the conditions of Fig. 6 *A* and in one additional cell under the conditions of Fig. 6 *B*.

Zero calcium also affected other features of the membrane response to ATP. In some cells ATP initiated a slow capacitance decrease that continued after ATP was removed. In other cells (Fig. 7), C_m increased normally in the presence of ATP, but rapidly decreased when ATP was removed and could even undershoot its prestimulation value. Cells also were generally less likely to show responses to multiple applications of ATP in the complete absence of Ca^{2+}, suggesting that some Ca^{2+} is necessary for repriming of the membrane responses. Thus, keeping intracellular Ca^{2+} levels very low seems to promote endocytosis over exocytosis and decrease the steady-state surface area of the cells.

Extracellular Ca^{2+} can support net exocytosis in response to ATP even when intracellular Ca^{2+} is strongly buffered to very low levels. Fig. 8 shows an experiment with internal Ca^{2+} buffered by both BAPTA and EGTA and no added calcium inside the cell. In the absence of extracellular Ca^{2+}, the immediate effect of extracellular ATP was a small net decrease in capacitance (~6%). However, the decline in C_m reversed when 2 mM Ca^{2+} was introduced to the bath solution, and a net increase in capacitance (~7%) resulted. The introduction of extracellular Ca^{2+} also caused an increase in membrane conductance. Both these changes in cell response occurred with no detectable change in intracellular $[Ca^{2+}]$. Similar results—i.e., an initial decrease in capacitance with ATP stimulation in the absence of extracellular Ca^{2+} followed by an increase in C_m when Ca^{2+} was added in the continued presence of ATP—were observed in 11 of 11 cells. These data suggest that some Ca^{2+} is necessary to sustain a net capacitance increase in response to ATP, and that the Ca^{2+} can come from the extracellular solution.

The effects of calcium inside and outside the cell on C_m responses to a first exposure to ATP are summarized in Fig. 9. When Ca^{2+} was eliminated from both the bath and pipette solutions, 83% of cells showed a significant net endocytosis in response to extracellular ATP (Fig. 9 *A*) When intracellular Ca^{2+} was available (low calcium in) and/or mM extracellular Ca^{2+} was present (2 calcium out), net exocytosis was strongly favored (81% of cells; Fig. 9 *A*). The effect of 0 Ca^{2+} is apparent in the average net capacitance decrease seen in all responding cells (Fig. 9 *B*). Analysis of variance ($P < 0.0001$) and post-hoc pairwise comparisons (Tukey test; $P < 0.001$) indicate that the Ca^{2+}-free net capacitance change is significantly different from the other conditions, but the differences between the C_m changes with Ca^{2+} present are not statistically significant. Overall, these data suggest that some Ca^{2+} is necessary for a net C_m increase, and that this Ca^{2+} can come from

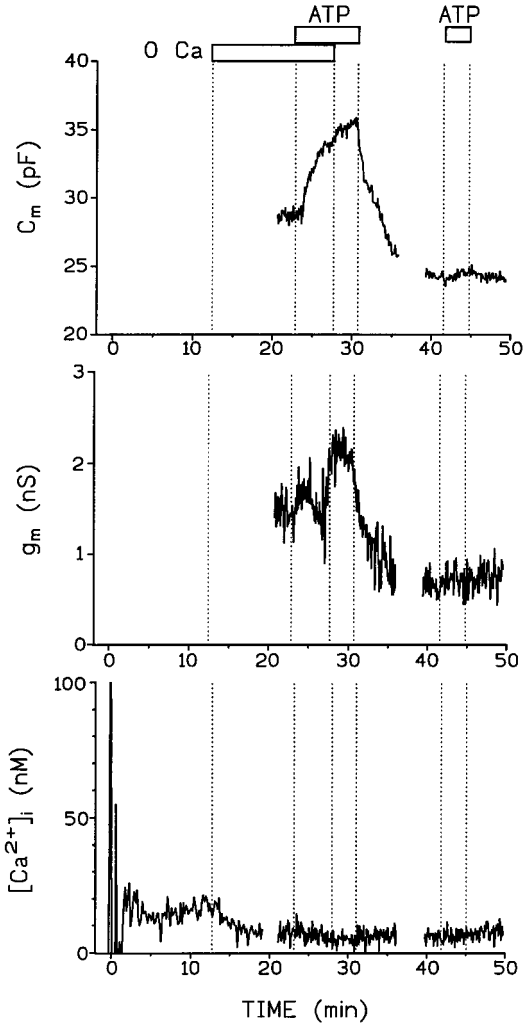

FIGURE 7 Removing both extracellular and intracellular calcium alters the response to ATP. ATP stimulation caused an initial capacitance increase in this cell with cytosolic $[Ca^{2+}]$ strongly buffered to very low levels, but C_m declined rapidly and undershot its initial value when ATP was washed out. The pipette solution was buffered with 5 mM BAPTA and 15 mM EGTA to be Ca^{2+}-free and was supplemented with 0.2 mM ATP and 0.2 mM fura-2. Where indicated, the cell was exposed to 10 μM ATP and/or 0 Ca^{2+} bath solution with 0.2 mM EGTA.

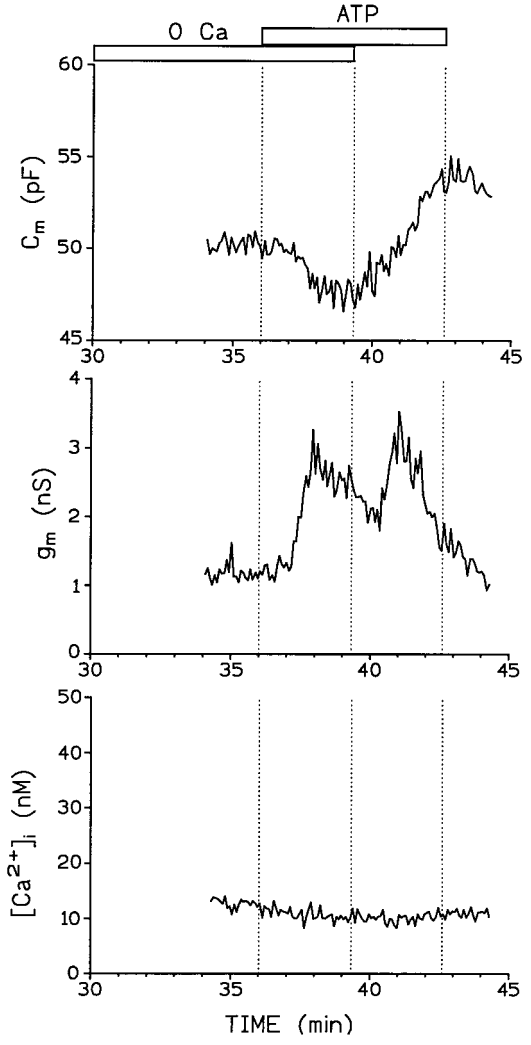

FIGURE 8 Extracellular calcium can support a capacitance increase. Under Ca^{2+}-free conditions, 10 μM ATP initiated a small capacitance decrease. When 2 mM Ca^{2+} was introduced into the perfusing solution in the continued presence of ATP, membrane capacitance increased. The Ca^{2+}-free pipette solution contained 5 mM BAPTA, 15 mM EGTA, 1 mM ATP, and 0.5 mM fura-2. Where indicated, the bath was 0 Ca^{2+} supplemented with 0.2 mM EGTA.

either extracellular or intracellular sources. However, endocytosis is also stimulated by ATP, and this remains robust even in near-zero calcium conditions.

Initial ATP responses are insensitive to modulations of G protein function

Exocytosis is initiated in some systems by G protein activation, but G protein activation may not be directly involved in triggering adipocyte membrane responses to ATP. Including nonspecific activators of G proteins (GTPγS, xanthosine triphosphate (XTP), or AlF_4^-) in the pipette solution did not initiate exocytosis in brown fat cells. As shown in Fig. 10, neither these nor other modulators of G protein function affected the frequency or the magnitude of initial capacitance responses to ATP. In addition, most cells (94% of 34 cells, with or without calcium present) showed normal responses to extracellular ATP with no ATP, GTP, or other nucleotides in the pipette, even after >30 min of dialysis (n = 8; e.g., Fig. 1). Although negative results like these cannot be definitive, they suggest that the final phase of ATP-stimulated exocytosis does not require G protein activation or other nucleotide-requiring reactions.

Removal of intracellular nucleotides and perturbation of G protein function did reduce the frequency of repeated membrane responses to ATP. With our usual pipette solution (10 mM EGTA/1 mM calcium/1 mM ATP), only 2 of 28 cells failed to respond to a second exposure to ATP. When the pipette solution also contained GTPγS, XTP, or GDPβS, second responses to ATP were absent or very small (4–6 cells tested under each condition). Although there

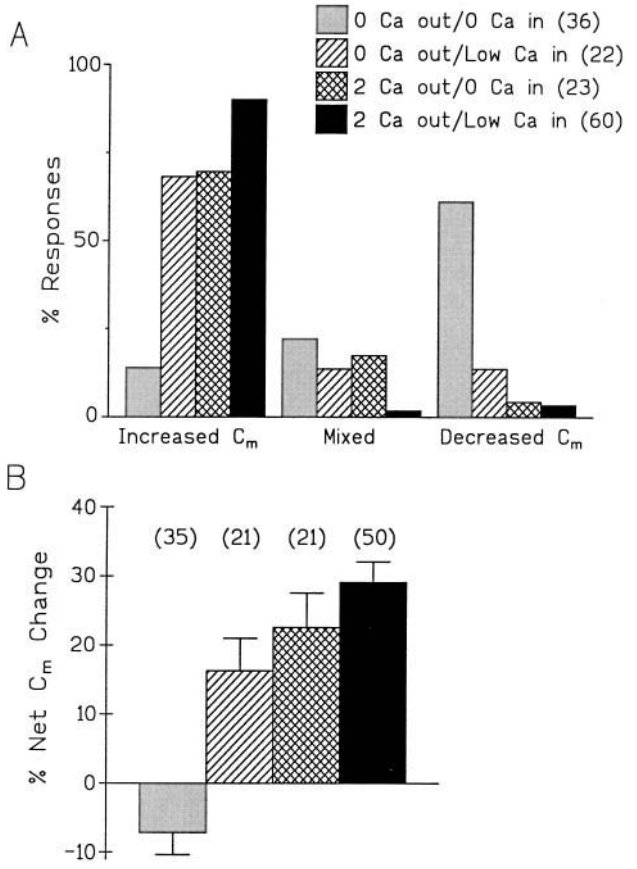

FIGURE 9 Summary of the effects of intracellular and extracellular Ca^{2+} on capacitance responses. (*A*) Percent of cells responding to a first application of extracellular ATP with a C_m increase, decrease, or mixed response. Responses were scored during or shortly after exposure to ATP. Increased C_m and decreased C_m count cells responding with monotonic capacitance changes >5% of the initial C_m. Cells that showed both a C_m increase and a C_m decrease in the presence of ATP, and cells that showed a conductance increase but C_m changed by <5%, were scored as mixed responses. Calcium conditions were as follows: 0 calcium in, no added calcium in the pipette solution, and buffered with BAPTA or high concentrations of EGTA; low calcium in, calculated pipette $[Ca^{2+}]$ of ~18–120 nM; 0 calcium out, nominally 0 Ca^{2+} Krebs-Ringer solution, sometimes supplemented with 0.1–0.2 mM EGTA; 2 calcium out, Krebs-Ringer solution with 2 mM $CaCl_2$. Number of cells under each condition is given in parentheses. (*B*) Average net percent capacitance change of responding cells in (*A*). Numbers of cells are less than in (*A*) because nonresponders and cells exposed to ATP < 60 s were not included in the calculation of net C_m change. Cells exposed to ATP for <60 s showed smaller changes. Error bars are SEM.

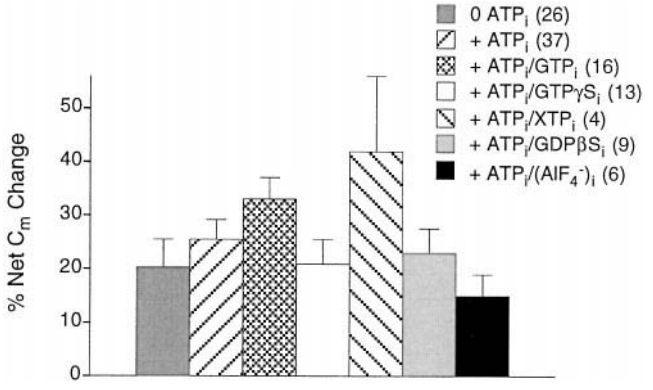

FIGURE 10 Effects of pipette solution composition on ATP responses. Shown are the average capacitance changes seen in cells showing net capacitance increases in response to 1–10 μM ATP. The pipette solutions contained no added nucleotides (0 ATP) or contained mM ATP and GTP (0.1–1.0 mM), GTPγS (0.005–1.0 mM), XTP (8.5 mM), GDPβS (0.1–1.0 mM), or AlF_4^- (0.01–1.0 mM). There are no significant differences between the groups. Error bars represent mean ± SE. Number of cells under each condition is given in parentheses.

were examples of cells that responded to ATP many times without any intracellular ATP, 3 of 10 cells without intracellular ATP failed to respond to a second ATP exposure. These results indicate that G protein activation and other nucleotide-requiring reactions may be involved in priming, maintaining, or recovery from the membrane response to ATP.

DISCUSSION

We used simultaneous fura-2 measurement of $[Ca^{2+}]_i$ and voltage clamp measurements of cell membrane capacitance to examine the role of $[Ca^{2+}]_i$ in the ATP-activated membrane trafficking response of brown adipocytes. We find that cytosolic Ca^{2+} levels modulate but do not trigger the increases in membrane trafficking evoked by extracellular ATP in brown fat cells. Increasing $[Ca^{2+}]_i$ to micromolar levels with ionophore or via the pipette solution did not change measured cell capacitance and neither prevented nor promoted ATP-stimulated capacitance changes. α-Adrenergic stimulation, which raises $[Ca^{2+}]_i$ (Wilcke and Nedergaard, 1989; Lee et al., 1993) and activates phospholipases (Nånberg and Putney, 1986; Schimmel et al., 1986) like purinergic stimulation (Harden et al., 1995; Lee and Pappone, 1997a), also does not stimulate increases in membrane trafficking (Pappone and Lee, 1996). Conversely, when measurable increases in $[Ca^{2+}]_i$ were prevented by intracellular BAPTA or high concentrations of EGTA, extracellular ATP still activated membrane trafficking. These results indicate that triggering the membrane response to ATP does not require any substantial increases in $[Ca^{2+}]_i$.

Strongly buffering $[Ca^{2+}]_i$ to very low levels did alter the nature of the membrane trafficking response to ATP. ATP evoked similar increases in membrane turnover with and without strong $[Ca^{2+}]_i$ buffering, as measured by increased FM 1-43 fluorescence. However, the net change in membrane surface area reported by capacitance measurements showed clear differences with changes in the effectiveness of $[Ca^{2+}]_i$ control. When $[Ca^{2+}]_i$ was not well buffered or was held near normal resting levels (50–100 nM), effective ATP stimulation always resulted in net exocytosis, while net endocytosis predominated when $[Ca^{2+}]_i$ was held at low levels. Membrane capacitance could decrease rapidly with ATP in cells with low $[Ca^{2+}]_i$, and could reduce C_m to below its prestimulation value. Thus, $[Ca^{2+}]_i$ influences the relative rates of ATP-stimulated exocytosis and endocytosis and the steady-state surface area to which the cell recovers following purinergic activation.

While our experimental manipulations clearly affected cytosolic calcium levels, $[Ca^{2+}]_i$ was not perfectly controlled. We routinely saw ATP-stimulated $[Ca^{2+}]_i$ increases in the presence of 10 mM EGTA, indicating that EGTA cannot effectively buffer the Ca^{2+} released from intracellular stores. In contrast, 5–10 mM BAPTA completely prevented measurable $[Ca^{2+}]_i$ changes in 90% of the experiments, indicating that $[Ca^{2+}]_i$ control is better with this faster buffer, as others have reported (Adler et al., 1991). Apparently even these high concentrations of BAPTA could not completely control $[Ca^{2+}]_i$ at the relevant sites, since addition of extracellular calcium could switch cells from net endocytosis to net exocytosis. Thus, it is likely that localized $[Ca^{2+}]_i$ increases went undetected in the BAPTA buffered cells. However, the Ca^{2+} buffering properties of BAPTA severely constrain the extent to which $[Ca^{2+}]_i$ can be changing. Introduced mobile buffer concentrations will decrease the distance over which $[Ca^{2+}]_i$ is increased by a point source, restricting the concentrations of Ca^{2+} seen at distant sites (Roberts, 1993; Smith et al., 1996). The distance for a 1/e reduction of $[Ca^{2+}]_i$ assuming a point source for Ca^{2+} influx is ~10 nm for 5 mM BAPTA, as calculated by Roberts (1993). We estimate that local $[Ca^{2+}]_i$ increases to 2 μM or more in a 10-nm-thick shell below the plasma membrane would be readily detected in our experiments. Release sites sensitive to these levels of $[Ca^{2+}]_i$ would have been expected to respond in experiments with calcium ionophore or high pipette calcium, which was not seen. Hence, greater increases in $[Ca^{2+}]_i$ triggering exocytosis in brown adipocytes would have to occur within well less than 10 nm of the fusion sites.

Less buffering of $[Ca^{2+}]_i$ than used in these experiments has been effective in preventing vesicle fusion in systems with Ca^{2+}-dependent exocytosis. In pituitary gonadotrophs, where exocytosis depends on release of Ca^{2+} from intracellular stores, 10 mM EGTA almost completely inhibits the agonist-stimulated capacitance increase, even though $[Ca^{2+}]_i$ is not well controlled (Tse et al., 1997). In adrenal chromaffin cells, where initiation of exocytosis depends on channel-mediated Ca^{2+} influx (Burgoyne, 1991), capacitance increases, but not Ca^{2+}-sensitive potassium channel activation, are completely blocked by 11 mM intracellular EGTA (Neher and Marty, 1982). Net exocytosis in brown adipocytes was only inhibited by the most severe restriction of Ca^{2+} availability, requiring that the Ca^{2+}-sensitive regulatory sites have high affinity for Ca^{2+} and/or very close association with the ER release mechanisms and Ca^{2+} influx sites.

G proteins are involved in most membrane trafficking, and can activate Ca^{2+}-independent exocytosis in other systems (Lindau and Gomperts, 1991; Burgoyne and Handel, 1994). However, many modulations of G protein function failed to activate or inhibit the initial membrane trafficking response of brown adipocytes to ATP. Conditions tested included pipette solutions containing no added nucleotides or containing GTP, GTPγS, XTP, GDPβS, or AlF_4^-. It can be argued that nucleotide depletion may be incomplete, even with the long exchange times used in these experiments, and that the added nucleotides were ineffective because of slow exchange rates of the G proteins involved, so these negative results are not conclusive. However, many of these manipulations did inhibit subsequent responses to ATP, indicating that the internal milieu had been altered. In addition, AlF_4^- activates the GDP-bound form of most receptor-linked and small G proteins, and so acts rapidly without requiring nucleotide exchange (Sternweis and Gilman, 1982; Gilman, 1987; Higashijima et al., 1991). Therefore it seems likely that G protein activation is not central to triggering ATP-stimulated membrane trafficking in brown adipocytes, but is probably involved in other aspects of membrane handling in these cells.

The mechanism of ATP-activated membrane trafficking in adipocytes does not correspond well to the standard actions of any of the known P2 receptors. The agonist potency for activating membrane trafficking in brown adipocytes is consistent with mediation of the response by a $P2Y_1$ receptor or by any of several P2X receptors (Pappone and Lee, 1996; Harden et al., 1995; Burnstock, 1997). P2Y receptors normally couple to heterotrimeric G proteins (Harden et al., 1995), while we find that the purinergic activation of membrane trafficking is insensitive to modification of G protein function. P2X receptors usually form ligand-gated nonselective cation channels (Suprenant et al., 1995) that often can carry Ca^{2+} (Bean, 1992; Edwards, 1994), and so could activate calcium-triggered exocytosis. However, it is difficult to reconcile the slow, sustained, calcium-independent membrane responses to ATP in adipocytes with an ionotropic effect. There is evidence indicating that some P2 receptors may act through mechanisms other than G protein activation or channel formation. $P2Y_1$ receptors expressed in *Xenopus* oocytes induce a purine-activated cation current, the activation of which is insensitive to modulators of G protein function (O'Grady et al. 1996). $P2X_7$ receptors can activate phospholipase D (El-Moatassim and Dubyak, 1992) without calcium influx through the $P2X_7$ channel (Humphreys and Dubyak, 1996). In addition, Zou et al. (1997) report that $P2X_7$ receptors can activate fatty acid-sensitive potassium channels in smooth muscle, presumably through the generation of fatty acids. These results suggest that P2 receptors may have additional modes of action that are not yet well documented. The ATP effects we report may well be mediated by such G protein-independent actions of a P2Y receptor or nonionotropic actions of a P2X receptor.

The physiological functions of ATP-activated membrane trafficking in brown adipocytes are unknown. In addition to increasing capacitance, extracellular ATP elicits increases in cytosolic calcium, alters membrane conductances, and increases basal, but not norepinephrine-stimulated, metabolic rate in brown adipocytes (Pappone and Lee, 1996; Lee and Pappone, 1997a; Omatsu-Kanbe and Kitasato, 1997). Responses in white adipocytes are similar (Lee and Pappone, 1997b). There have been only a few reports in the literature of any other adipocyte responses to extracellular

ATP. The most intriguing of these are data indicating that preexposure of white adipocytes to ATP inhibits subsequent stimulation of glucose uptake by insulin (Chang and Cuatrecasas, 1974). The insulin response is mediated through redistribution of GLUT4 glucose transporters from intracellular membranes to the cell surface (Corvera and Czech, 1996). It seems likely that the profound changes in membrane trafficking caused by ATP in adipocytes could affect such transporter mobilization or the redistribution of receptors or other membrane proteins.

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