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Differential Regulation of Ca^{2+} Signaling and Membrane Trafficking by Multiple P2 Receptors in Brown Adipocytes

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Abstract. Extracellular ATP triggers changes in intracellular Ca^{2+}, ion channel function, and membrane trafficking in adipocytes. The aim of the present study was to determine which P2 receptors might mediate the Ca^{2+} signaling and membrane trafficking responses to ATP in brown fat cells. RT-PCR was used to determine which P2 receptors are expressed in brown fat cells. Responses to nucleotide agonists and antagonists were characterized using fura-2 fluorescence imaging of Ca^{2+} responses, and FM 1-43 fluorescence imaging and membrane capacitance measurements to assess membrane trafficking. The pharmacology of the Ca^{2+} responses fits the properties of the P2Y receptors for which mRNA is expressed, but the agonist and antagonist sensitivity of the membrane-trafficking response was not consistent with any P2 receptor described to date. Brown adipocytes expressed mRNA for $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ metabotropic receptors and $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, and $P2X_7$ ionotropic receptors. The agonists ATP, ADP, UTP, UDP and 2′, 3′-(benzoylbenzoyl) ATP (BzATP) increased intracellular Ca^{2+}, while 100 μM suramin, pyridoxal-phosphate-6-azophenyl-2′ 4′-disulfonic acid (PPADS), or Reactive Blue 2 partially blocked Ca^{2+} responses. ATP, but not ADP, UTP, UDP or BzATP activated membrane trafficking. The membrane response could be blocked completely with 1 μM PPADS but not by the antagonist MRS2179. We conclude that multiple P2 receptors mediate the ATP responses of brown fat cells, and that membrane trafficking is regulated by a P2 receptor showing unusual properties.

Key words: P2 Receptors — ATP — Brown Fat — P2Y Receptor Expression — P2X Receptor Expression — Membrane Trafficking — FM 1-43 — Calcium Signaling

Introduction

Release of nucleotides from cells and subsequent activation of P2 nucleotide receptors form a ubiquitous and influential intercellular communication system (Abbracchio & Burnstock, 1998). Two classes of receptors mediate these responses; P2X receptors form Ca^{2+}-permeable cation channels in the plasma membrane and P2Y receptors are G protein-coupled receptors linked to enzyme activation. P2 receptors act through diverse intracellular signaling systems known to be important in adipocyte physiology by affecting cAMP, lipid, and intracellular Ca^{2+} signals (Ralevic & Burnstock, 1998). P2 receptors can also stimulate the secretion of cytokines, thus generating additional intercellular communication (Abbracchio & Burnstock, 1998). These actions can contribute to the cells' responsiveness to other regulators by setting the overall "signaling tone" of the cells (Ostrom, Gregorian & Insel, 2000).

Communication between adipocytes and between adipocytes and other cell types in adipose tissue influences the proliferation and differentiation of fat cells and their responsiveness to hormones (Schling & Loffler, 2002). Extracellular nucleotides acting at P2 receptors are likely to play a role in mediating these effects. Reported effects of extracellular ATP in brown and white adipocytes include increases in intracellular free calcium (Ca_i) (Kelly, Deeney & Corkey, 1989; Lee & Pappone, 1997a; Omatsu-Kanbe & Kitasato, 1997; Omatsu-Kanbe & Matsuura, 1999), lipogenesis (Schodel et al., 2004), and membrane turnover (Pappone & Lee, 1996; Lee & Pappone, 1997b), and modulation of membrane

Abbreviations: BzATP, (benzoylbenzoyl) ATP; αβMeATP, α,β,-methylene-ATP; 2MeSATP, 2-methylthio-ATP; PPADS, pyridoxal-phosphate-6-azophenyl-2′ 4′-disulfonic acid

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conductances (Lee & Pappone, 1997b; Wilson et al., 1999). Extracellular nucleotides also increase Ca_i in preadipocytes (Schmidt & Loffler, 1998), and promote their proliferation in culture (Wilson et al., 1999) and aromatase activity (Schmidt & Loffler, 1998).

Nine different P2Y receptors and seven P2X receptors have been discovered in mammals so far (Ralevic & Burnstock, 1998; Communi et al., 2001; Hollopeter et al., 2001; Abbracchio et al., 2003; Inbe et al., 2004). The P2 receptors involved in nucleotide signaling in adipocytes are not known, though there is evidence that multiple P2 receptors mediate the physiological responses. The peak and plateau phases of the ATP-induced Ca_i signals in brown fat cells are differentially sensitive to the P2 receptor blocker PPADS, suggesting that different receptors might be involved in generating the early and late phases of the Ca_i responses (Omatsu-Kanbe, Isono & Matsuura, 2002). UTP is also effective in eliciting cytosolic Ca^{2+} increases in these cells (Omatsu-Kanbe & Matsuura, 1999; Leaver & Pappone, 2002), but UTP does not activate membrane trafficking or modulate voltage-gated K channels (Pappone & Lee, 1996; Wilson & Pappone, 1999), also indicating that brown adipocytes express multiple P2 receptors. Neither white nor brown fat are generally included in expression screens for cloned P2 receptors, and the two studies that have examined P2 receptor expression in fat used RT-PCR of mRNA from all the cells in adipose tissue (Moore et al., 2001; Omatsu-Kanbe et al., 2002). Mature adipocytes constitute only ~40% of the cells in fat (Bukowiecki et al., 1982; Trayhurn & Beattie, 2001), so these results may overestimate the number and types of P2 receptors expressed. In the present study, we used RT-PCR of mRNA from pure adipocyte preparations to determine which P2 receptors could be present in mature brown fat cells in vivo. We then investigated the pharmacology of the Ca_i and membrane trafficking responses to determine which P2 receptors might mediate these responses. The results indicate that Ca_i responses to extracellular nucleotides in brown fat cells are mediated by $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ receptors. In addition, brown fat cells express mRNA for six of the seven known P2X receptors. However, the pharmacology of the membrane trafficking response is not consistent with the reported properties of any of these P2 receptors.

Materials and Methods

Cell Isolation and Culture

Brown fat cells were isolated from the interscapular fat pads of 1–8 day old rats. For RT-PCR experiments mature, floating adipocytes were isolated by collagenase digestion (3 mg/ml in Krebs–Ringer bicarbonate buffer, 3% BSA for 15 min). The pups for these experiments were sacrificed immediately following their removal from the dam to maximize the fat content of the cells and increase the yield of floating fat cells. The digest was expressed through silk filter cloth (Precision Cells, Farmington, NY). Retained tissue was incubated an additional 30 min in 1 mg/ml collagenase, then expressed through silk. Floating cells were harvested by centrifugation and used for the mRNA isolation.

For imaging and electrophysiology experiments the rat pups were isolated from their dam for ~12 hours prior to sacrifice. The cold stress causes the pups to activate their brown adipose tissue, thus depleting the brown adipocytes of lipid. This increases adipocyte density and results in large numbers of mature brown adipocytes sinking following isolation. The tissue was digested with collagenase and the sinking cells cultured as described previously (Lucero & Pappone, 1989; 1990; Pappone & Lee, 1996). Experiments were performed on mature adipocytes containing multiple lipid droplets maintained 1–7 days in culture. The percentage of cells showing Ca_i and membrane trafficking responses to nucleotides was independent of culture age.

Imaging

Intracellular free $[Ca^{2+}]$ was estimated by ratio fura-2 digital imaging of 1–20 morphologically-identified brown fat cells attached to a glass coverslip as described previously (Lee, Nuccitelli & Pappone, 1993; Lee & Pappone, 1997a). During the Ca_i imaging experiments, cells were continuously superfused with modified Krebs solution containing (in mM) 120 NaCl, 4.6 KC1,0.5 $MgCl_2$, 0.7 Na_2HPO_4,1.5 Na_2HPO_4, 10 glucose, supplemented with 24 mM $NaHCO_3$ and 2 mM $CaCl_2$, gassed with 5% CO_2 in either O_2 or air. Experiments were performed at room temperature.

Changes in surface-accessible cell membrane were estimated using FM 1-43 fluorescence as described previously (Betz & Bewick, 1993; Pappone & Lee, 1996; Smith & Betz, 1996). Cells were exposed to 1–4 μM FM 1-43 and cell-associated fluorescence due to excitation at 480 nm was assessed with a 510 nm long-pass emission filter. The Krebs solution was not gassed in these experiments because gassing degraded the FM 1-43 fluorescence within 5–10 min. The amphipathic FM 1-43 rapidly partitions into bath-accessible cell membrane. The dye's fluorescence is greatly increased in the membrane environment, so increases in the amount of membrane in contact with the FM 1-43 solution increases the measured fluorescence signal.

Electrophysiology

Cell membrane capacitance (C_m) and conductance were measured using standard whole-cell path-clamp techniques as described previously (Pappone & Lee, 1996). C_m was determined from the integral of the charging transient during 10 mV depolarizing voltage steps applied every 5–10 s from the −60 mV holding potential, corrected for the measured series resistance. The pipet solution contained (in mM) 100 K-aspartate, 35 KCl, 10 K_2EGTA, 1 $CaCl_2$, 1 MgATP and 10 MOPS, pH 7.2 and calculated 18 nM free calcium. The external solution and other conditions were the same as for the imaging experiments.

ADP Solutions

ADP solutions for some experiments were treated with hexokinase to remove contaminating ATP. On the day of the experiment, a stock solution containing 2.5 mM ADP in Krebs solution with 25 mM D-glucose and 10 U/ml hexokinase was incubated for 30 min at room temperature. The solution was then diluted with Krebs solution for use in imaging experiments. Light output from a

Table 1. RT-PCR Primers

Receptor	Sequence	bp
$P2Y_1$	Forward 5′ GTGGCGTGGTGTACCCTCTCAAGT 3′ Reverse 5′ AGCCGTGCCCTCAAATTCATCGTT 3′	411
$P2Y_2$	Forward 5′ CAAGCCCGCCACAGAGCCTACAC 3′ Reverse 5′CCCCAACCCCTCGATTCTACTTCT 3′	529
$P2Y_4$[a]	Forward 5′ CACCGATACCTGGGTATCTGCCAC 3′ Reverse 5′ CAGACAGCAAAGACAGTCAGCACC 3′	340
$P2Y_6$	Forward 5’ CCGGCGGCAACCACACGAT 3′ Reverse 5′ ACAAGAACGGTCAGGCGGGAAGA 3′	414
$P2Y_{11}$	Forward 5′ CCCCGCTGGCCGCCTACCTCTATC 3′ Reverse 5′ CACGCAGCTTCTCGGCCACAGTCA 3′	488
$P2Y_{12}$[b]	Forward 5′ CAGGTTCTCTTCCCATTGCT 3′ Reverse 5′ CAGCAATGATGATGAAAACC 3′	657
$P2Y_{13}$[c]	Forward 5′ TGTGTCGTTTTTCTTCGGTG 3′ Reverse 5′ CTGCCAAAAAGAGAGTTG 3′	575
$P2X_1$[d]	Forward 5′ TGGGTGGGTGTTTGTCTATG 3′ Reverse 5′ TGAAGTTGAAGCCTGGAGAC 3′	750[e]
$P2X_2$[d]	Forward 5′ TCCATCATCACCAAAGTCAA 3′ Reverse 5′ TTGGGGTAGTGGATGCTGTT 3′	392[e]
$P2X_3$[d]	Forward 5′ TTGAGGGTAGGGGATGTGGT 3′ Reverse 5′ GCTGATAATGGTGGGGATGA 3′	327[e]
$P2X_4$[d]	Forward 5′ GCTTATACAGTGTGAGCGTATGGAGGTAGGAAG 3′ Reverse 5′ TTTAATGTTTGGTCCTAGGTCATTTCAACACAG 3′	250[e]
$P2X_5$[d]	Forward 5′ TGAAGGGTGGTGTGATAGGA 3′ Reverse 5′ GTTGATGACTGTGGGGATGA 3′	269[e]
$P2X_6$[d]	Forward 5′ CTGTGGGATGTGGCTGACTT 3′ Reverse 5′ TCAAAGTCCCCTCCAGTCAT 3′	485[e]
$P2X_7$[d]	Forward 5′ AGGGAGGAATC ATGGGC ATT 3′ Reverse 5′ CCTCCAGTGCCAAAAACCAG 3′	235

[a](Webb et al., 1998).
[b](Jin et al., 2001).
[c](Communi et al., 2001).
[d](Luo et al., 1999).
[e]In the original publication by Luo et al. (1999) there were incorrect sequences and expected lengths, which have been corrected here (X. Luo, personal communication).

luciferin-luciferase solution was used to assay the ATP concentration of the solutions as in Taylor et al. (1998). ADP solutions contained ~1% ATP without hexokinase treatment, and contained < 1 nM (~0.001%) ATP after exposure to hexokinase.

RT-PCR

Total RNA was extracted from floating brown fat cells by adding to the cell suspension twice its volume of Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA), followed by two chloroform extractions and isopropanol precipitation. Total RNA yield ranged 100–400 μg/ml as inferred by UV spectroscopy, however, subsequent mRNA isolates and cDNA products were not quantified. Messenger RNA was isolated from the total RNA using the PolyA Tract mRNA Isolation System IV (Promega, Madison, WI), following the manufacturer's instructions. First-strand synthesis was performed using the RetroScript kit (Ambion, Austin, Texas) and random decamer priming as per the manufacturer's instructions. Primers were designed to rat sequence where available. Alternatively, previously published primers were used (Table 1). PCR reactions were conducted in PCR Gold Buffer (Applied Biosystems, Foster City, CA), and included cDNA (6% of the total reaction volume), 0.5 mM dNTP mix, 3.5 mM $MgCl_2$, 0.2 μM of each primer, and 0.04 units/μl of AmpliTaq Gold (Applied Biosystems). $P2Y_4$ and P2X primers were used with 1.5 mM $MgCl_2$. Cycling temperatures for P2Y (except $P2Y_4$) primer PCR were 95°C for 10 min followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min. P2Y4 reactions were cycled at 95°C for 10 min followed by 35 cycles of 95°C for 1 min, 65°C for 30 s, and 72°C for 1 min. P2X cycling temperatures were 95°C for 10 min followed by 35 cycles of 95°C for 45 s, 53°C for 45 s, and 72°C for 1 min. All PCR cycles included a final extension at 72°C for 10 min. Products were visualized on 1.8% agarose stained with ethidium bromide. PCR amplicons were either excised from the gel using QIAquick Gel Extraction Kit (Qiagen, Maryland USA), or concentrated using QIAquick PCR purification kit (Qiagen) for sequencing. If only faint amplification was seen in the original RT-PCR, the gel extract products were then used for additional rounds of PCR using the same protocols as above. Sequencing was performed at the UC Davis Division of Biological Sciences DNA Sequencing Facility using Big Dye Terminators v. 3.1 cycle sequencing (Applied Biosystems). All sequences were BLAST searched to confirm that the corresponding P2 receptor sequence had been amplified. The efficacy of primers that did not produce PCR products from brown fat cell mRNA was confirmed by performing RT-PCR and sequencing using rat spleen or rat brain mRNA (BD Biosciences Clonetech, Palo Alto, CA). All primers used produced the appropriate product from rat mRNA except the primer designed for the $P2Y_{11}$ receptor.

The $P2Y_{11}$ receptor primer was based on the human sequence because the $P2Y_{11}$ gene has not been shown in rat.

Reagents

PPADS (Pyridoxal-phosphate-6-azophenyl-2′ 4′-disulfonic acid tetrasodium) was obtained from RBI (Natick, MA). EGTA was from Fluka Chemical (Ronkonkoma, NY). Fura-2/AM was from Calbiochem (San Diego, CA) or Molecular Probes (Eugene, OR). FM 1-43 was from Molecular Probes. Pluronic F-127 is a product of BASF (Mount Olive, NJ). All other reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted.

Data Analysis

Data are presented as means ± SEM. Statistics were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Further analysis of Ca_i measurements was performed using Igor Pro (Wavemetrics, Lake Oswego, OR) analysis software.

Results

Brown Adipocytes Express Message for Many P2 Receptors

Previous RT-PCR of cultures from brown adipose tissue found message for several P2Y and P2X receptors (Omatsu-Kanbe et al., 2002), but because the cultures contained other cell types in addition to adipocytes, expression could not be unequivocally attributed to the fat cells. We used RT-PCR of mRNA from acutely isolated mature brown adipocytes to determine which P2 receptors brown fat cells expressed in vivo. By using RNA from floating cells we were able to exclude other cell types present in brown adipose tissue from this analysis. Figure 1 shows the expression pattern for P2 receptors found using mRNA solely from mature brown adipocytes. Message of the correct size and sequence was present for nine different P2 receptors: $P2Y_2$, $P2Y_6$, $P2Y_{12}$, $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, and $P2X_7$. Sequence data confirmed that the products amplified were the rat P2 receptors targeted. We did not test for the presence of the $P2Y_{14}$ receptor, which is specific for UDP-glucose and is not activated by ATP or UTP (Abbracchio et al., 2003). Nor did we test for the $P2Y_{15}$ receptor, which is activated by AMP and adenosine (Inbe et al., 2004)—agonists that do not activate Ca_i or membrane trafficking responses in brown fat cells (Pappone & Lee, 1996).

Ca^{2+} -Mobilizing Responses Are Consistent with the Expression of $P2Y_2$, $P2Y_6$ and $P2Y_{12}$ Receptors

Brown adipocytes respond with Ca_i increases to a number of different nucleotide agonists, including the endogenous ligands ATP, ADP, UTP, UDP, and the drug BzATP. The Ca_i responses of individual cells to each agonist varied, as shown in Fig. 2*A* for ATP, UTP, and BzATP. Ca^{2+}-mobilizing agonists were also effective in raising Ca_i in zero Ca^{2+} solutions, as shown in Fig. 2*B*, indicating that Ca_i responses are mediated by P2Y receptors activating Ca^{2+} release from internal stores, not by Ca^{2+} influx through P2X receptor channels.

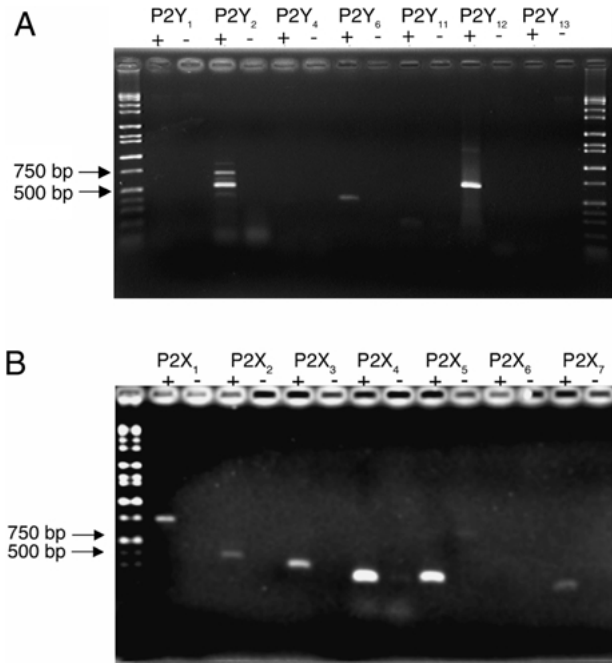


Fig. 1. RT-PCR of mRNA from mature, floating brown fat cells. (*A*) Brown adipocytes express mRNA for $P2Y_2$, $P2Y_6$ and $P2Y_{12}$ receptors. The strongest band from the $P2Y_2$ reaction was sequenced and found to correspond to the predicted sequence. The faint amplification in the $P2Y_{11}$ reaction was sequenced and found to be random amplification, not matching any rat sequences. (*B*) Brown adipocytes express mRNA for $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, and $P2X_7$ receptors. Results are shown for reaction mixtures run in parallel with (+) and without (−) reverse transcriptase.

Not all cells responded to all agonists. The pattern of agonist sensitivities seen for individual cells in these experiments is shown in Table 2. The pattern of Ca_i responses to ATP, ADP, UTP and UDP is consistent with the variable expression of three different P2Y receptors. $P2Y_{12}$ receptors are responsive to ATP and ADP but not UTP or UDP (Simon et al., 2002), $P2Y_2$ receptors are responsive to UTP but not UDP (von Kuegelgen, 2000), and $P2Y_6$ receptors respond to UDP, UTP and ATP (von Kuegelgen, 2000). These agonist sensitivities are summarized in Table 3.

Almost all brown fat cells that showed Ca_i increases with any nucleotide responded with Ca_i increases when stimulated with 10 μM ATP or ADP, and all cells responding to ADP also responded to ATP. Ca_i responses to the ADP solution were not due to contaminating ATP because they were elicited with the same efficacy by hexokinase-treated ADP solutions (18 cells). A significantly lower fraction of cells responded to UTP, and 18% of cells exposed to both

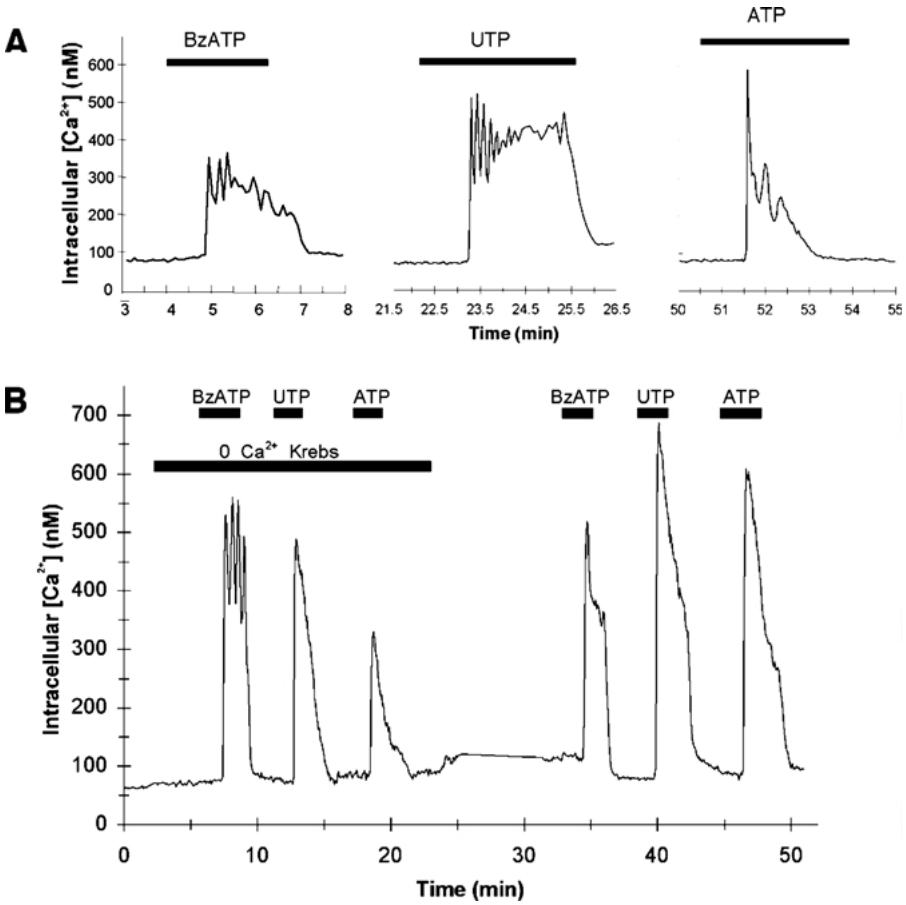


Fig. 2. (*A*) Ca_i responses to 100 μM BzATP, 10 μM UTP and 10 μM ATP in the same cell showing the different form of the responses to each agonist. Agonists were applied for the times indicated by the bars. (*B*) BzATP, UTP and ATP release Ca^{2+} from intracellular stores. 50 μM BzATP, 20 μM UTP, and 10 μM ATP each increased Ca_i in nominally zero Ca^{2+} solution. The declining magnitude of the responses in 0 Ca^{2+} probably reflects loss of cellular Ca^{2+} to the bath. When the cells were returned to normal (2 mM) extracellular Ca^{2+}, the peak responses to UTP and ATP increased. Data from a single cell. The time axes in this and subsequent figures show time from the start of the experiment.

Table 2. Agonist Ca_i Responses

Agonist	% Cells[a] responding	*n*	Responsiveness comparison ADP	UTP	BzATP	UDP
ATP	99%	273	—	***	***	***
ADP	97%	91		***	***	***
UTP	82%	246			***	***
BzATP	68%	174				**
UDP	41%	27				

Data are for cells exposed to maximally effective concentrations (10–100 μM) of agonist.

[a]*% Cells responding* is the number of tested cells (*n*) showing a measurable increase in intracellular Ca^{2+} in response to agonist using fura-2 imaging. *% Cells responding* were compared between agonists using 2 by 2 contingency tables and two-sided Fisher's exact test.

***% *Cells responding* is significantly different with $p \leq 0.001$ for the two agonists.

** *% Cells responding* is significantly different with $p \leq 0.01$ for the two agonists.

— *% Cells responding* is not significantly different ($p > 0.05$) for the two agonists.

Table 3. P2Y Receptor nucleotide sensitivity reported in other cells

	$P2Y_2$*	$P2Y_6$*	$P2Y_{12}$**
ATP	+	+	+
ADP	–	+	+
UTP	+	+	–
UDP	–	+	–

*von Kuegelgen, 2000.

**Simon et al, 2002.

ATP and UTP responded to ATP but not UTP. The finding that not all ATP-sensitive receptors in fat cells can be activated by UTP indicates that adipocytes express a receptor that is sensitive to purines but not pyrimidines. These results are consistent with expression of $P2Y_{12}$ receptors activated by ATP and ADP but not UTP. Brown fat cells also responded to UDP with Ca_i increases, but UDP responses were significantly less frequent (41% of cells) than responses to any of the other agonists tested. All UDP-sensitive cells also responded to ATP and to UTP, consistent with mediation of the UDP Ca_i response by $P2Y_6$ receptors. A significantly greater fraction of cells responded to ATP and UTP than to UDP. This indicates the presence of an additional receptor activated by the triphosphate nucleotides but not UDP, consistent with the expression of $P2Y_2$ receptors. Thus the Ca_i responses of cultured brown adipocytes to nucleotides are consistent with differential

expression of all three P2Y receptor messages seen in the RT-PCR results from acutely isolated cells, suggesting that the P2Y receptor expression of cultured cells is qualitatively the same as in vivo.

The actions of P2 receptor antagonists were also consistent with the expression of three different P2Y receptors in brown fat cells. Ca_i responses to BzATP, UTP and ATP are sensitive to block by the nonselective P2 receptor antagonists RB2, suramin, and PPADS. However, the extent to which each agonist response was inhibited differed among these antagonists. All three antagonists (100 μM) blocked BzATP responses by >80%. UTP responses were substantially inhibited by RB2 and suramin, but reduced by only 35% by PPADS. ATP responses were blocked by RB2, but only ~50% inhibited by 100 μM suramin or PPADS. These disparate antagonist effects on the different agonist responses are consistent with the presence of three functional P2Y receptors. However, because of the nonselective actions of the antagonists these data could not be used to distinguish receptor types.

Membrane Trafficking Is Activated by ATP but not by Other Nucleotides

We have previously shown that extracellular ATP activates membrane trafficking in brown fat cells. The membrane response can be monitored by measuring electrical capacitance (C_m) in voltage-clamp experiments or measuring FM1-43 fluorescence in imaging experiments (Pappone & Lee, 1996). C_m, which is proportional to plasma membrane surface area, increases by an average of 33% in response to concentrations of ATP ≥2 μM, and imaging and voltage-clamp studies suggest that activation of both exocytosis and endocytosis are involved in the response. In those studies, 2MeSATP, ATP, and ADP activated membrane trafficking, while UTP, GTP, αβMeATP and adenosine did not. 2MeSATP and ATP were roughly equipotent, but much higher concentrations of ADP were required to elicit responses.

In the present experiments we tested if the membrane-trafficking responses to solutions containing high concentrations of ADP seen in previous experiments could be explained by contaminating ATP in the ADP solutions. To do this we compared the effects of ADP solutions that had or had not been pretreated with hexokinase to degrade any ATP present. Application of untreated 100 μM ADP solution ("ADP") activated FM1-43 fluorescence increases in 5/18 cells that subsequently showed robust membrane-trafficking responses to 10 μM ATP, as shown in the example in Fig. 3. However, previous exposure to 100 μM ADP solution that had been pretreated with hexokinase (ADP in Fig. 3) failed to elicit membrane responses from any of these cells, even though 10 μM enzyme-treated ADP consistently

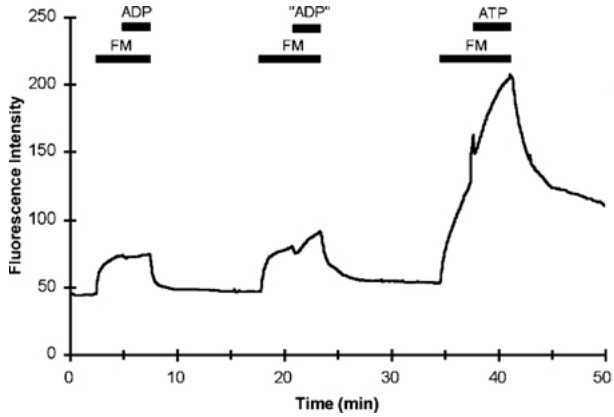


Fig. 3. ADP does not activate membrane trafficking. The signal shows fluorescence measured from a single brown fat cell. 1 μM FM 1-43 was washed in and out of the chamber at the times indicated by the bars. After the fluorescence signal reached a steady rate of change, agonist was washed into the chamber. Agonists were 100 μM ADP that was treated with hexokinase to remove contaminating ATP (*ADP*), 100 μM ADP that was not treated with hexokinase and so contained ~1% ATP ("*ADP* "), or 10 μM ATP (*ATP*). Only the solutions containing ATP increased the rate of rise of the fluorescence, indicating that ADP is not an agonist for the membrane-trafficking response. The small dips in fluorescence occurring initially with agonist applications are artifacts of the solution change. The incomplete washout following the second exposure to FM 1-43 and the increase in fluorescence when FM1-43 solution is applied for a third time reflect the increase in surface membrane trafficking and membrane surface area activated by the previous exposure to ATP in the "ADP " solution.

elicited Ca_i responses (*see* above). These data demonstrate that ADP does not activate membrane-trafficking and that the membrane trafficking activated by ADP solutions in our previous experiments (Pappone & Lee, 1996) was due to contaminating ATP.

We further characterized the agonist sensitivity of the membrane-trafficking response by examining the effects of BzATP and UDP. We found that 200–250 μM BzATP had no effect on C_m (5 ± 1% increase in C_m, $n = 3$) in cells which subsequently showed an average 40 ± 9% increase in C_m when stimulated with 10 μM ATP. UDP was also ineffective in activating membrane trafficking. FM1-43 fluorescence did not increase (average change of 0 ± 4%, $n = 9$) when cells were exposed to 100 μM UDP, while the same cells showed an average 68 ± 13% increase in fluorescence when stimulated with 10 μM ATP.

Low Concentrations of PPADS Block Membrane Trafficking

The membrane-trafficking response is much more sensitive to block by the antagonist PPADS than are the nucleotide-induced Ca_i responses. Fig. 4 shows that just 1 μM PPADS was sufficient to completely inhibit C_m changes in response to ATP in patch-clamped cells ($n = 6$) or fluorescence increases in

experiments with FM1-43 (n = 45 cells). This contrasts with the much smaller effect of PPADS on ATP-elicited Ca_i responses in cells that were insensitive to other nucleotides (i.e., having a Ca_i response profile like the membrane-trafficking response). Most cells (8/11) that showed Ca_i responses to ATP, but not UTP or BzATP in the absence of PPADS, had similar initial Ca_i increases when stimulated with ATP in the presence of 1 μM PPADS (*not shown*). This suggests that the UTP-insensitive (presumably $P2Y_{12}$) receptors mediating Ca_i increases are distinct from the receptors mediating membrane trafficking.

$P2Y_1$ receptors resemble the membrane-trafficking receptor in that they can be very sensitive to block by PPADS in some systems and are activated by ATP and 2MeSATP, but not uracil nucleotides. ADP can activate $P2Y_1$ receptors, but the relative sensitivity to ATP and ADP differs depending on the expression system (Vöhringer, Schäfer & Reiser, 2000). Although we did not find message for $P2Y_1$ receptors in RNA from acutely isolated fat cells, $P2Y_1$ receptor message was present in mixed cultures of cells from brown adipose tissue (Omatsu-Kanbe et al., 2002). To determine if membrane trafficking in our cultured fat cells was regulated by a $P2Y_1$ receptor that was unusually insensitive to ADP, we tested the effects of the $P2Y_1$ receptor blocker MRS2179 (Boyer et al., 1998). Stimulation of membrane trafficking by ATP was not prevented by 30 μM MRS2179, as can be seen in the FM1-43 fluorescence results in Fig. 4*B*. Exposure of cells to ATP in the presence of MRS2179 resulted in fluorescence increases (indicating increased membrane turnover) in 17 out of 21 cells tested, the same response frequency (80%) as was seen in previous work in the absence of blocker (Pappone & Lee, 1996). Only one of the tested cells responded to ATP in the absence, but not the presence of MRS2179. Because $P2Y_1$ receptors are blocked by nanomolar concentrations of MRS2179 (Boyer et al., 1998), these results indicate that the membrane-trafficking response is not mediated by $P2Y_1$ receptors.

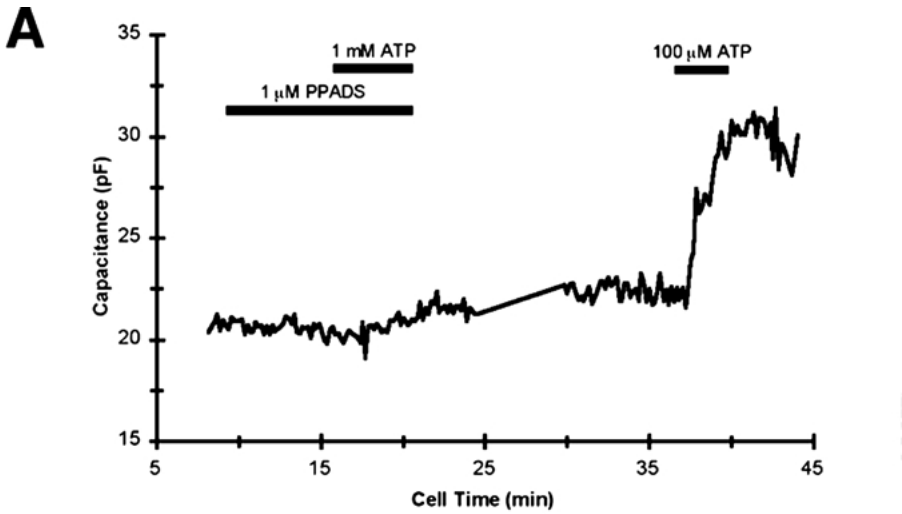
The same PPADS-sensitive P2 receptor that regulates membrane trafficking in brown fat cells may also affect adipocyte sensitivity to ATP and other nucleotides. In agreement with a previous study (Omatsu-Kanbe et al., 2002), we find that the plateau phase of Ca_i responses to ATP can be augmented by low concentrations (1–5 μM) of PPADS. Figure 5 shows the Ca_i responses recorded in a cell in the absence and presence of 1 μM PPADS. The responses in the absence of PPADS have been duplicated and superimposed (*black line*) on the responses to the same stimulus regime in the presence of PPADS. Comparison of the records with and without PPADS shows that the Ca_i response to ATP decayed more slowly when PPADS was present. Cells exposed to ATP, but not other agonists, were usually less responsive to P2 agonists applied soon after the ATP stimulus. However, low concentrations of PPADS prevented this apparent ATP-induced desensitization of the cells to nucleotide agonists. In Fig. 5 BzATP applied shortly after washout of ATP activated a robust Ca_i response in the presence of 1 μM PPADS, but elicited no Ca_i response when the antagonist was absent from the solution, even though PPADS is a highly effective blocker of BzATP responses at higher concentrations. In addition, UTP elicited a higher amplitude Ca_i response in the presence of 1 μM PPADS than in its absence.

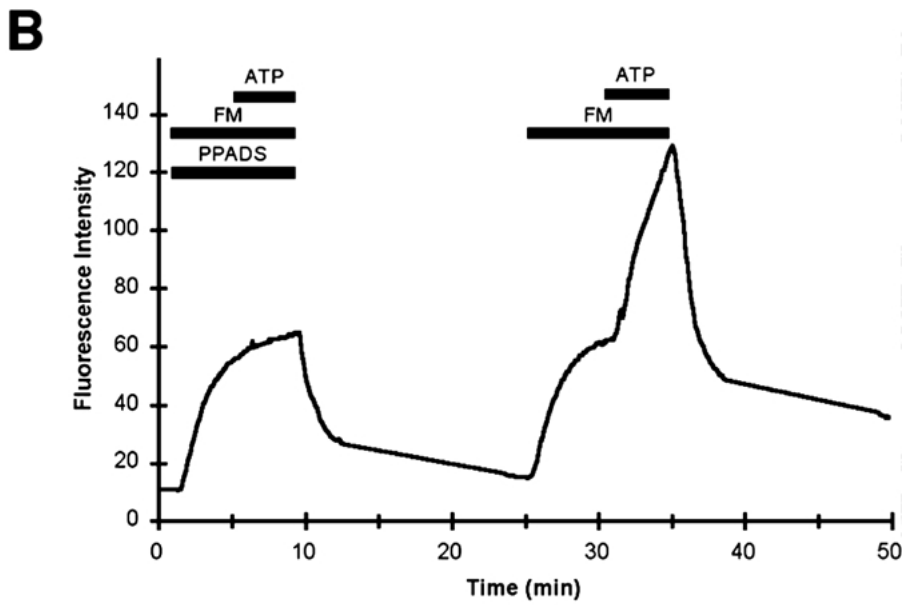
Discussion

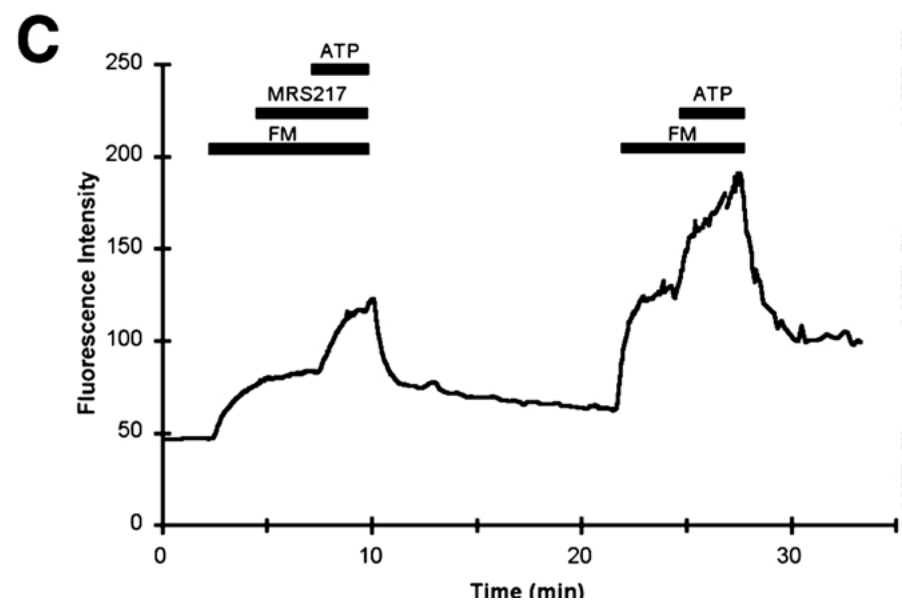
The motivation for these studies was to determine the P2 receptors present in brown adipocytes and to ascertain which receptors mediate the many responses of brown fat cells to ATP and other nucleotides. We find that acutely isolated mature brown adipocytes express mRNA for three P2Y receptors and six of the seven known P2X receptors. The agonist and antagonist profiles for nucleotide Ca_i responses reported here are fully consistent with the expected sensitivities of the P2Y receptor expression predicted by our RT-PCR results. In contrast, the agonist and antagonist sensitivities of the membrane-trafficking response to ATP do not correspond to those of any P2 receptor reported to date.

Nucleotide Ca_i Responses Are Mediated by $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ Receptors

We find that mRNA from mature adipocytes includes transcripts for $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ receptors and that the agonist profile of Ca_i responses is consistent with expression of these three P2Y receptors. The observed cell-to-cell variation in Ca_i responses to UTP and UDP is well explained by cell-to-cell variation in expressed $P2Y_2$ (UTP sensitive, UDP insensitive), $P2Y_6$ (UTP- and UDP-sensitive), and $P2Y_{12}$ (UTP- and UDP-insensitive) receptors (von Kuegelgen, 2000; Simon et al., 2002) (*see* Tables 2 and 3). These results are consistent with the conclusion from previous work that at least two P2 receptors mediate Ca_i responses in brown fat cells (Omatsu-Kanbe et al., 2002). The P2Y expression profile of isolated brown adipocytes is somewhat different than has been reported for mixed cell cultures from brown adipose tissue (Omatsu-Kanbe et al., 2002), possibly reflecting contributions of mRNA from non-adipose cells to those results. Expression of multiple P2Y receptor subtypes by individual cells has been documented in primary cells from a number of tissues (e.g., Erlinge et al., 1998; Luo et al., 1999; Ferrari et al., 2000a,b; Dockrell et al., 2001; Vartian et al., 2001; Zhu & Kimelberg, 2001)). Variable expression of these several P2Y receptors likely


A
1 mM ATP
100 μM ATP
1 μM PPADS
Capacitance (pF)
Cell Time (min)


B
ATP
FM
PPADS
ATP
FM
Fluorescence Intensity
Time (min)


C
ATP
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FM
ATP
FM
Fluorescence Intensity
Time (min)

Fig. 4. Membrane trafficking can be blocked by low concentrations of PPADS but not by the $P2Y_1$ antagonist MRS2179. (*A*) C_m responses to ATP in a whole-cell patch-clamped cell. 1 μM PPADS completely blocks the membrane-trafficking responses to ATP. (*B*) In the presence of 1 μM PPADS, addition of 10 μM ATP had no effect on FM1-43 fluorescence, indicating there was no change in the amount of cell membrane rapidly exchanging with the cell surface in this intact cell. In the absence of PPADS, ATP caused a large increase in FM1-43 fluorescence, indicating increased rates of exocytosis and/or endocytosis. (*C*) 30 μM MRS2179 failed to inhibit membrane trafficking in response to 10 μM ATP. Addition of ATP in the presence of the antagonist causes a rapid increase in fluorescence, indicating that increased membrane was exposed to the cell surface. The incomplete washout of the fluorescence following the first exposure to FM1-43 and the larger initial fluorescence signal when the dye was reapplied result from the increased dye internalization and cell surface area activated by the previous ATP application.

underlies the idiosyncratic and complex Ca_i responses of brown fat cells to ATP.

The Membrane-trafficking Response Fits no Known P2 Receptor

Stimulation of brown adipocytes with ATP evokes dramatic increases in membrane turnover that can result in as much as a doubling of membrane surface area (Pappone & Lee, 1996; Lee & Pappone, 1997b, 1999). Membrane trafficking can be activated by 2MeSATP and ATP, but not by ADP, αβMeATP, BzATP, UTP, UDP, GTP or adenosine [(Pappone & Lee, 1996), these studies]. We had previously reported that C_m increases could be evoked by high concentrations of ADP, but in the present studies we demonstrate that these responses were due to ~1% ATP present in the ADP solutions. The membrane response to ATP is blocked completely by 1 μM PPADS, and by 10–100 μM suramin (*not shown*) or Reactive Blue 2 (Pappone & Lee, 1996).

Previously we had attributed the membrane-trafficking response to activation of a P2Y receptor (Pappone & Lee, 1996; Lee & Pappone, 1999). However, none of the three P2Y receptors for which message is present in acutely isolated brown adipocytes nor the additional two receptors for which mRNA was found in mixed cell cultures (Omatsu-Kanbe et al., 2002) share the pharmacological profile of the membrane-trafficking response. $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are very sensitive to UTP and/or UDP, neither of which activates membrane responses. $P2Y_{12}$ receptors are insensitive to block by PPADS and suramin (Honda et al., 2001), both of which are effective in blocking the membrane response. Additionally, $P2Y_{12}$ receptor-mediated responses are blocked by pertussis toxin (Honda et al., 2001; Simon et al., 2002), which does not inhibit the membrane response in adipocytes (*unpublished observations*). $P2Y_1$ receptors were found in mixed brown fat cell cultures (Omatsu-Kanbe et al., 2002). $P2Y_1$ receptors are activated by ADP (Filippov, Brown & Barnard, 2000; von Kuegelgen, 2000) and blocked by MRS2179, but neither ADP nor this specific $P2Y_1$ antagonist affected membrane trafficking. In addition, 1 μM PPADS completely blocked the membrane-trafficking response to ATP, while rat $P2Y_1$ receptors are reported to be relatively insensitive to block by PPADS, with an IC_{50} of 250–430 μM (Vöhringer et al., 2000). BzATP evoked intracellular Ca^{2+} release in brown fat cells, suggesting that a $P2Y_{11}$ receptor homologue could be present in these cells (Communi, Robaye & Boeynaems, 1999). However, BzATP did not activate membrane trafficking, indicating that a $P2Y_{11}$ receptor is not involved in the membrane trafficking response.

The membrane trafficking response to ATP in brown fat cells may be mediated by P2X receptors. The nucleotide sensitivity of the membrane trafficking response, i.e., activation by ATP but not ADP, UTP or UDP, is characteristic of P2X receptors. Initiation of the trafficking response in brown fat cells does not require functional G proteins (Lee & Pappone, 1999), suggesting that P2Y receptors are not involved. $P2X_7$ receptors mediate secretory or membrane-trafficking responses in other cell types (Virginio et al., 1999; Wilson et al., 2002; Gudipaty et al., 2003; Jung et al., 2004; Kochukov & Ritchie, 2004), and activation of membrane trafficking by $P2X_7$ receptors can be independent of extracellular Ca^{2+} (Kochukov & Ritchie, 2004)— a property characteristic of the brown fat membrane response (Lee & Pappone, 1999). Despite these indications of potential P2X receptor involvement, the pharmacology of the membrane-trafficking response in brown fat cells does not match that of any known homomeric P2X receptor. $P2X_1$, $P2X_2$, $P2X_5$, and $P2X_7$ receptors respond to BzATP, which does not activate membrane trafficking (North, 2002). GTP is a full agonist at the rat $P2X_5$ receptor (Wildman et al., 2002) and is completely ineffective in activating the membrane trafficking response (Pappone & Lee, 1996). $P2X_3$ receptors are very sensitive to αβMeATP (North, 2002), another agonist that is ineffective in eliciting membrane trafficking in brown adipocytes (Pappone & Lee, 1996). $P2X_4$ and $P2X_6$ receptors have reported agonist sensitivities that are similar to the membrane trafficking receptor, but they are completely insensitive to block by suramin and PPADS (North, 2002), antagonists that effectively block the membrane response in brown fat. The atypical properties of the ATP receptor activating membrane responses in brown fat could be due to mediation by a heteromeric P2X receptor (Torres, Egan & Voigt, 1999; Surprenant et al., 2000; Yoshioka, Saitoh & Nakata, 2001; Brown et al., 2002), or from effects of the expression system on receptor characteristics, as has

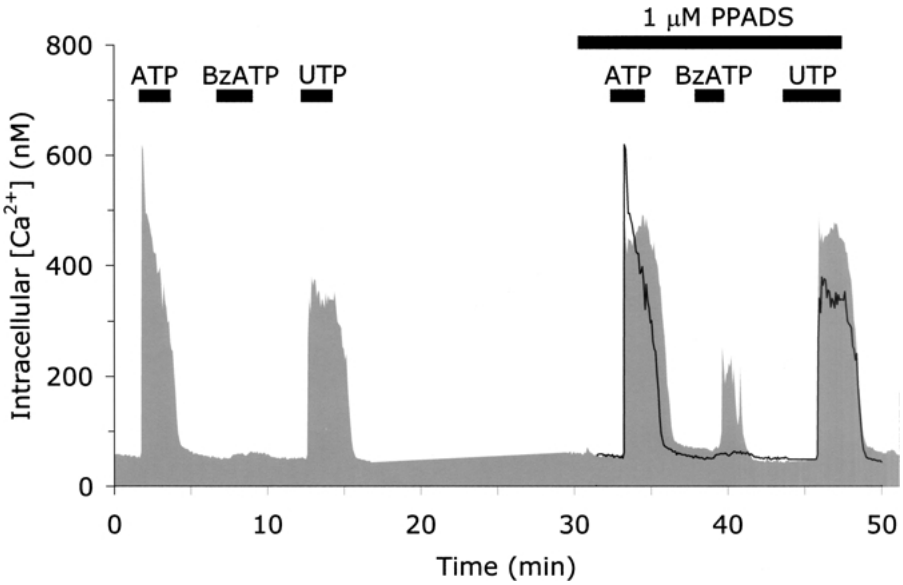

Fig. 5. Low concentrations of PPADS can augment Ca_i responses. The gray area shows that in the absence of PPADS the Ca_i response to 10 μM ATP declined rapidly, 50 μM BzATP applied shortly after ATP washout failed to elicit a Ca_i increase, and the peak Ca_i response to 20 μM UTP was only half that produced by 10 μM ATP. In the presence of 1 μM PPADS the identical paradigm of agonist exposures elicits from the cell a sustained ATP Ca_i response, a robust Ca_i response to BzATP and a UTP response of comparable amplitude to the ATP response. The responses in the absence of PPADS are duplicated, time-shifted and superimposed as a black line on the responses in the presence of PPADS.

been shown for some P2Y receptors (Simon et al., 2002). Alternatively, the brown fat receptor could represent a new P2 receptor that has not yet been described at the molecular level.

The P2 Receptor Mediating Membrane Trafficking May also Regulate Other P2 Receptors

In experiments measuring Ca_i responses to P2 agonists, treatment with ATP inhibited subsequent responses to ATP, ADP, UTP and BzATP. The pharmacology of this homologous and heterologous desensitization is consistent with it being mediated by the same receptor that increases membrane trafficking. Like the membrane-trafficking response, ATP-induced desensitization was sensitive to block by 1 μM PPADS and activated only by ATP and not the other tested nucleotides. This desensitization of P2 receptors may be a consequence of increased membrane trafficking, possibly through internalization of P2 receptors or other membrane components contributing to the Ca_i response. It has been suggested that a PPADS-sensitive ATP-activated receptor inhibits the influx of Ca^{2+} induced by emptying intracellular Ca^{2+} stores (Omatsu-Kanbe & Matsuura, 1999; Omatsu-Kanbe et al., 2002), which would have the effect of decreasing plateau Ca_i levels and reducing the Ca^{2+} available in the stores for subsequent responses. The data presented here are consistent with an ATP-induced block of Ca^{2+} influx pathways as well as with other possible desensitization mechanisms.

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