PKA-independent cAMP stimulation of white adipocyte exocytosis and adipokine secretion: modulations by Ca^{2+} and ATP

Ali M. Komai[1], Cecilia Brännmark[2], Saliha Musovic[1], Charlotta S. Olofsson[1]

[1]Department of Neuroscience and Physiology, Metabolic Physiology Section, University of Gothenburg, Medicinaregatan 11, SE41390 Göteborg, Sweden

[2]Discovery Sciences, AstraZenecaR&D, Pepparedsleden 1, SE43153 Mölndal, Sweden

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Corresponding author:

Dr Charlotta Olofsson

University of Gothenburg, The Sahlgrenska Academy

Dept. Neuroscience and Physiology, Section of Metabolic Physiology

Postal adress: Box 432

SE-405 30 Göteborg

Sweden

E-mail: charlotta.olofsson@physiol.gu.se

Key points summary

- The molecular and cellular mechanisms involved in short-term regulation of white adipocyte adipokine release remain elusive.
- Here we have examined effects of intracellular cAMP, Ca^{2+} and ATP on exocytosis and adipokine secretion by a combination of membrane capacitance patch-clamp recordings and biochemical measurements of secreted adipokines.
- Our findings show that white adipocyte exocytosis is stimulated by cAMP/Epac (exchange proteins activated by cAMP)-dependent but Ca^{2+}- and PKA-independent mechanisms and can largely be correlated to release of adiponectin vesicles residing in a readily releasable vesicle pool.
- A combination of Ca^{2+} and ATP augments exocytosis/adiponectin secretion via a direct action on the release process and by recruitment of new releasable vesicles.
- Our results elucidate several previously unknown cellular mechanisms involved in regulation of white adipocyte exocytosis/secretion. The well-established disturbances of adipokine secretion in obese individuals highlights the significance of understanding regulatory mechanisms and intracellular mediators involved in white adipocyte adipokine secretion.

148 words

Abstract

We examined the effects of cAMP, Ca^{2+} and ATP on exocytosis and adipokine release in white adipocytes by a combination of membrane capacitance patch-clamp recordings and biochemical measurements of adipokine secretion. 3T3-L1 adipocyte exocytosis proceeded even in the complete absence of intracellular Ca^{2+} ($[Ca^{2+}]_i$; buffered with BAPTA) provided cAMP (0.1 mM) was included in the intracellular (pipette-filling) solution. Exocytosis typically plateaued within ~10 minutes, likely signifying depletion of a releasable vesicle pool. Inclusion of 3 mM ATP in combination with elevation of $[Ca^{2+}]_i$ to ≥700 nM augmented the rate of cAMP-evoked exocytosis ~2-fold and exocytosis proceeded for longer periods (≥20 min) than with cAMP alone. Exocytosis was stimulated to a similar extent upon substitution of cAMP by the Epac agonist 8-Br-2'-O-Me-cAMP (1 mM included in the pipette solution). Inhibition of PKA by addition of Rp-cAMPS (0.5 mM) to the cAMP-containing pipette solution was without effect. A combination of the adenylate cyclase activator forskolin (10 μM) and the phosphodiesterase inhibitor IBMX (200 μM; forsk/IBMX) augmented adiponectin secretion measured over 30 min 3-fold and 2-fold in 3T3-L1 and human subcutaneous adipocytes respectively. This effect was unaltered by pre-loading of cells with the Ca^{2+} chelator BAPTA-AM and 2-fold amplified upon inclusion of the Ca^{2+} ionophore ionomycin (1μM) in the extracellular solution. Adiponectin release was also stimulated by the membrane permeable Epac agonist 8-Br-2'-O-Me-cAMP-AM but unaffected by inclusion of the membrane-permeable PKA inhibitor Rp-8-Br-cAMPS (200 μM). The adipokines leptin, resistin and apelin were present in low amounts in the incubation medium (1-6% of measured adiponectin). Adipsin was secreted in substantial quantities (50% of adiponectin concentration) but release of this adipokine was unaffected by forsk/IBMX. We propose that white adipocyte exocytosis is stimulated by cAMP/Epac-dependent but Ca^{2+}- and PKA-independent release of vesicles residing in a readily releasable pool and that the release is augmented by a combination of Ca^{2+} and ATP. We further suggest that secreted vesicles chiefly contain adiponectin.

Abbreviations

ΔC_{tot}, total capacitance increase; C_m, membrane capacitance; $\Delta C/\Delta t$, exocytotic rate; Glut4, glucose transporter type 4; PKA, Protein kinase A; EC, extracellular solution; Rp-cAMPS, (*R*)-Adenosine, cyclic 3',5'-(hydrogenphosphorothioate) triethylammonium; Rp-8-Br-cAMPS, 8-Bromoadenosine-3',5'-cyclic monophosphorothioate, Rp-isomer; IBMX, 3-Isobutyl-1-methylxanthine; Me-cAMP, 8-Bromo- 2'- O- methyladenosine- 3', 5'- cyclic monophosphate; IBMX, 3-Isobutyl-1-methylxanthine; Epac, exchange proteins activated by cAMP.

Introduction

White adipose tissue, traditionally regarded as a tissue primarily involved in lipid storage, has in recent years been increasingly recognised to be an endocrine organ that expresses and secretes a variety of bioactive molecules with auto- para- and endocrine functions. Secreted products, collectively known as *adipokines*, are known to play important roles in energy homeostasis as well as in inflammatory responses (Trujillo & Scherer, 2006; Maury & Brichard, 2010). With increasing weight, adipose tissue disturbances arise resulting in dysregulated adipokine secretion. Although the secretion of almost every known adipokine is disturbed in obesity (Maury & Brichard, 2010), the molecular and cellular mechanisms involved in short-term regulation of adipokine release remain poorly characterised. Given the severe medical consequences of obesity, including type-2 diabetes, hypertension and cardiovascular disease (Hevener & Febbraio, 2009), the significance of understanding regulatory mechanisms and intracellular mediators involved in white adipocyte adipokine secretion is evident.

Adiponectin is an adipokine exclusively secreted from mature adipocytes, with insulin-sensitising, fat-burning, anti-inflammatory and anti-oxidant properties. Plasma adiponectin levels are decreased in obesity, due to disturbances of both production and secretion of the adipokine (Hoffstedt *et al.*, 2004; Maury & Brichard, 2010; Kovacova *et al.*, 2012). Elevated adiponectin levels have been shown to have a protective role in the development of type-2 diabetes (Spranger *et al.*, 2003). Most investigations aimed at elucidating regulatory mechanisms involved in adipokine secretion are conducted as long-term (several hours or days) studies. Shorter-term (≤4 hours) regulation has been investigated in a few studies and release of adiponectin was found to be triggered by

insulin (Bogan & Lodish, 1999; Cong *et al.*, 2007; Szkudelski, 2007; Xie *et al.*, 2008), to involve phosphoinositide 3-kinase signalling (Cong *et al.*, 2007) and to depend on an elevation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$; Bogan & Lodish, 1999). Adiponectin has been suggested to be targeted to a regulatory secretory pathway (Bogan & Lodish, 1999; Xie *et al.*, 2008) but the regulation of its secretion is inadequately investigated.

The term "stimulus-secretion coupling" is used to describe events occurring in neuroendocrine cells when exposed to its immediate stimulus leading to release of its secretory product (Kits & Mansvelder, 2000). In most neuroendocrine cells, an elevation of $[Ca^{2+}]_i$ is the trigger for secretion of hormone or neuropeptide containing vesicles via regulated exocytosis (Burgoyne & Morgan, 2003). In addition to the triggering signal, the Ca^{2+}-dependent secretion is modulated by several intracellular mediators such as cAMP and ATP and requires the interaction of numerous proteins involved in secretory vesicle exocytosis (reviewed in Burgoyne & Morgan, 2003; Seino & Shibasaki, 2005). However, both the cellular mechanisms and the mediators involved in white adipocyte stimulus-secretion coupling remain unknown.

Here we have used *in vitro* differentiated 3T3-L1 cells, an extensively characterized model of white adipocytes, as well as *ex vivo* primary human subcutaneous adipocytes. We report, for the first time, how white adipocyte exocytosis is affected by the intracellular mediators Ca^{2+}, cAMP and ATP. Exocytosis, monitored as increases in membrane capacitance (Lindau & Neher, 1988), was compared to measurements of short-term (30 min) secretion of the adipokines adiponectin, leptin, resistin, apelin and adipsin. Our study is, to our best knowledge, the first where adipokine secretion is investigated using this methodology. We show that although exocytosis in white adipocytes shares some characteristics with archetypal endocrine cell types, there are important differences. Thus, white adipocyte exocytosis is stimulated by cAMP whilst Ca^{2+} and ATP exert modulatory effects. cAMP triggers exocytosis also in the complete absence of intracellular Ca^{2+} and stimulation occurs mainly via a PKA-independent pathway involving Epac (exchange proteins activated by cAMP). We further demonstrate that a large fraction of the vesicles may be released in an ATP-independent fashion. Our results show that exocytosis measured as increase in

membrane capacitance can to a large extent be functionally correlated with release of significant quantities of adiponectin.

Methods

3T3-L1 cell culture

3T3-L1 cells were maintained as subconfluent cultures in DMEM (high-glucose, 4500 mg/liter; Gibco) containing 10% foetal bovine serum (FBS Gold; PAA laboratories) and 1% Pen Strep (Gibco; medium 1). Differentiation was carried out according to established procedures (Kohn *et al.*, 1996). Briefly, cells were grown to confluence (day 0) and thereafter incubated in medium 1 supplemented with 1 μM dexamethasone, 850 nM insulin, and 0.5 mM 3-isobutyl-1-methylxanthine. After 48 hours (day 2) the medium was changed to medium 1 supplemented with insulin only. After an additional 48 hours (day 4) the medium was replaced, now by medium 1 alone. The medium was thereafter freshly replaced every two days. Experimental studies were performed in mature 3T3-L1 adipocytes (determined by occurrence of large lipid droplets; see Fig. 1A) between day 8 and 10 from start of differentiation.

Isolation of human adipocytes

Human adipocytes were isolated from subcutaneous adipose tissue biopsies taken during skin reduction surgery under general anesthesia at the Sahlgrenska University Hospital, Gothenburg, Sweden. All subjects gave their informed consent. The procedures were approved by the Regional Ethical Review Board, University of Gothenburg and were carried out in compliance with the Declaration of Helsinki. The subjects were women with a mean age of 35.3 (range 35-36) years and mean BMI of 24.9 (range 23.6-26.7). Subjects had lost weight by gastric bypass or reduced caloric intake, mean weight reduction 35 kg (range 18-57 kg). The biopsies were transported in a heated container prior to cell isolation. Adipocyte isolation was performed as described (Stralfors & Honnor, 1989), with small modifications. Briefly, adipose tissue was minced and degraded using collagenase type 1 (Worington, USA) and filtered through a nylon mesh. Adipocytes were washed by flotation in a Krebs-Ringer solution containing (in mM) 120 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 20 HEPES

(pH 7.4). The solution was supplemented with 1% (w⁄v) fatty acid-free bovine serum albumin, 100 nM phenylisopropyladenosine, 0.5 UmL-1 adenosine deaminase with 2 mM glucose. Cells were subsequently incubated over night (37ºC and 5% CO_2) in the same solution mixed with an equal volume of DMEM containing 7% (w⁄v) albumin, 200 nM adenosine, 20 mm HEPES, 50 UmL-1 penicillin, 50 lgmL-1 streptomycin (pH 7.4).

Electrophysiology and $[Ca^{2+}]_i$ imaging

3T3-L1 adipocytes, grown in Petri culture dishes (Nunc or MatTek for plastic and glass respectively), were during the recordings continuously superfused with an extracellular solution containing (in mM): 140 NaCl, 3.6 KCl, 2 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgSO_4$, 5 HEPES (pH 7.4 with NaOH), 2.6 $CaCl_2$ supplemented with 5 mM glucose (EC).

Exocytosis, measured as increase in membrane capacitance (Lindau & Neher, 1988), was studied with the whole-cell configuration of the patch-clamp technique using an EPC-9 patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany) and PatchMaster software. Patch pipettes were prepared from borosilicate glass capillaries coated with Sylgard® (Dow Corning) and heat-polished prior to use. The pipette resistance ranged between 3 and 5 MΩ when filled with pipette-filling solution. Access and seal resistances during the recordings were typically <15 MΩ and >1 GΩ respectively. Cells were clamped at -70 mV. The pipette filling solutions consisted of (in mM): 125 potassium glutamate, 10 KCl, 10 NaCl, 1 $MgCl_2$, 5 HEPES (pH 7.15 with KOH). The solutions were prepared containing different Ca^{2+}-EGTA or -BAPTA mixtures to achieve varying $[Ca^{2+}]_i$ and were supplemented with Mg-ATP and/or cAMP, Rp-cAMPS (Tocris) or 8-Br-2'-O-Me-cAMP (BIOLOG Life Science Institute, GmbH, Germany) as specified.

Alterations of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were recorded with dual-wavelength ratio imaging as previously described (Astrom-Olsson *et al.*, 2012). Briefly, 3T3-L1 adipocytes were loaded for 1.5 hours at room temperature with 2 μM Fura-2 AM together with 0.02% Pluronic F-127 (wt/vol, Life Technologies). A Lambda DG-4 illumination system was employed (Sutter Instrument Company, USA) together with a Nikon Diaphot 300 inverted microscope (Nikon, Japan). Image capturing was performed using a QuantEM 512SC CCD camera (Photometrics, USA) and Metafluor

software (Meta imaging series 7.5, Molecular Devices, USA). The excitation wavelengths were 340 and 380 nm and emitted light was collected at 510 nm. The $[Ca^{2+}]_i$ was determined using equation (5) of Grynkiewicz *et al.*, 1985) and a Kd of 224 nM. Measurements were carried out at 32˚C.

Adipokine secretion in 3T3-L1 adipocytes

3T3-L1 adipocytes were grown and differentiated on 6- or 12-well plates (Sarstedt). Cells were washed and pre-incubated for 30 minutes in EC without glucose, in the presence or absence of BAPTA-AM (Life Technologies) or Rp-8-Br-cAMPS (BIOLOG Life Science Institute, GmbH, Germany) as indicated. Subsequently, EC supplemented with test substances as specified was added and cells were incubated on gentle shaking for 30 min. Incubations were carried out at 32˚C. At the end of the incubation the EC was removed and centrifuged (2000 rpm, 5 min) to remove non-adherent cells. Samples were aliquoted and stored at -80˚C. Secreted adiponectin, leptin and resistin were measured using mouse ELISA DuoSets for the respective adipokines (R&D Systems Europe, UK). Adipsin and apelin were analysed using pre-coated ELISA plates (Antibodies-Online GmbH). Secreted adipokine was expressed in relation to total protein content.

For measurements of protein content, the adherent cells in the wells were washed with PBS and then lysed using a PBS-solution containing protease inhibitor (1 tablet/10 ml; Complete Mini, Roche, Germany) and SDS (2 %). Cells were removed by scraping and the cell mixture was stored at -80˚C. To ensure complete cell lysis the thawed cell mixture was ultra sonicated before protein measurements were performed using Pierce BCA protein kit (Thermo scientific, USA) according to instructions. Samples were centrifuged and the lipid layer removed.

Adiponectin release in human adipocytes

Human adipocytes (200 µl packed cells/ml) were washed and pre-incubated in EC for 30 min at 32˚C. Subsequently, test substances were added as indicated and adipocytes were incubated on gentle shaking for 30 min at 32˚C. The incubation was terminated by separation of the cells from the media by centrifugation through diisonoyl phtalate (Sigma-Aldrich, USA) and instant freezing on dry ice. Tubes were cut through the

diisononyl phtalate layer at two points to separate the cells from the media. Samples were stored at -80˚C. Secreted adiponectin was measured as described above and compared to total protein content.

Measurements of cAMP content

Cyclic AMP XP® Assay Kit #4339 (Cell Signaling) was used to determine intracellular levels of cAMP. Measurements were carried out according to manufacturer's instructions.

Data analysis

In all infusion experiments the rate of capacitance increase ($\Delta C/\Delta t$) was measured at two or more different time points applying linear fits as illustrated in Fig. 1B. The total capacitance increase at the end of the recording (ΔC_{tot}), reflecting the number of vesicles released, as well as the duration of the exocytotic response were also determined as illustrated in Fig. 1B. As specified in Results, ΔC_{tot} corresponds to a plateau value (when applicable) or the magnitude of the capacitance increase observed 10 min after establishment of the whole-cell configuration. The statistical significance of variance between two means was calculated using Origin Pro (OriginLab Corporation, USA) and Student's t-test, paired or unpaired as appropriate. When more than one group is compared to the same control group one-way ANOVA was applied. All data are presented as mean values ± S.E.M. for the designated number of experiments. The free [Ca^{2+}] of the intracellular solutions was calculated using MAXC DOWNLOADS (http://www.stanford.edu/~cpatton/maxc.html).

Results

Ca^{2+} dependence of exocytosis in 3T3-L1 adipocytes

The role of Ca^{2+} in white adipocyte exocytosis was investigated in 3T3-L1 adipocytes voltage-clamped at -70 mV and infused with solutions containing different [Ca^{2+}] as well as 3 mM ATP and 0.1 mM cAMP. Infusion of cells with 9 mM $CaCl_2$ together with 10 mM of the Ca^{2+} chelator EGTA, corresponding to a free [Ca^{2+}] of ~1.5 μM,

triggered exocytosis and the exocytotic rate ($\Delta C/\Delta t$) measured during the second minute from start of infusion averaged 26±2.2 fF/s (Fig. 2A, C). This rate is similar to what has been measured in pancreatic beta-cells using the same intracellular solution (Proks *et al.*, 1996; Barg *et al.*, 2002; Olofsson *et al.*, 2009). Exocytosis continued for >10 min (measured in 8 recordings with a sustained seal).

Exocytosis was still triggered using pipette solutions containing lower [Ca^{2+}] of either 8 or 6 mM $CaCl_2$ (10 mM EGTA), corresponding to free Ca^{2+} concentrations of ~700 nM and ~250 nM respectively (3 mM ATP and 0.1 mM cAMP still included). As can be seen in Fig. 2A and C, exocytosis at 700 nM free Ca^{2+} proceeded at a rate corresponding to ~75% of that observed using 1.5 μM Ca^{2+} (P=0.07). Again, exocytosis continued for >10 minutes (measured in 10 recordings). Infusion of cells with the solution containing 250 nM free Ca^{2+} stimulated exocytosis at a rate equal to ~50% of that achieved with the highest Ca^{2+} solutions. In 8 cells infused with 250 nM free Ca^{2+} the capacitance increase plateaued within ~6 min, possibly signifying the depletion of a releasable vesicle pool (Burgoyne & Morgan, 2003).

We next investigated if exocytosis could still be triggered in the absolute absence of intracellular Ca^{2+}. To ascertain complete Ca^{2+}buffering, the fast Ca^{2+} chelator BAPTA was used (0 $CaCl_2$ and 10 mM BAPTA; cAMP and ATP included). Exocytosis was still triggered by cAMP under Ca^{2+}-free conditions, at a rate similar to that seen at 250 nM free Ca^{2+} (Fig. 2B and C) and the capacitance increase again plateaued (at ~8 min). Values of total capacitance increase ($\Delta C_{tot,}$) as well as rates at exocytotic plateaus or t=10 min using the different solutions are summarised in panel 2D.

Our results demonstrate that 3T3-L1 adipocyte exocytosis may proceed for long periods in the presence of Ca^{2+}, cAMP and ATP, possibly indicating that recruitment of vesicles for release is a Ca^{2+}-dependent process.

The role of intracellular ATP in 3T3-L1 adipocyte exocytosis

To explore the involvement of ATP in adipocyte exocytosis, cells were infused with solutions containing or lacking the nucleotide, in the presence of 0 or 1.5 μM Ca^{2+} (Fig. 3A-D). At 1.5 μM Ca^{2+}, 3 mM ATP and 0.1 mM cAMP exocytosis was again triggered at a rate comparable to that seen in Fig. 2. Exclusion of ATP decreased the rate of capacitance increase ~60% (P<0.01 vs. ATP included). Interestingly, the

capacitance continued to increase for ≥10 min and did not plateau even in experiments lasting up to 20 minutes, regardless of the absence or presence of ATP. Our results indicate that ATP is not required to sustain exocytosis/vesicle replenishment in 3T3-L1 adipocytes.

We next investigated how exclusion of intracellular ATP affected Ca^{2+}-independent exocytosis (using 10 mM BAPTA as a Ca^{2+} chelator; Fig. 3C-D). Under these conditions, exocytosis still occurred when the intracellular solution contained 3 mM ATP and 0.1 mM cAMP; $\Delta C/\Delta t$ measured at 2 min averaged 12±3 fF/s, comparable to that in Fig. 2. Exclusion of ATP from the pipette solution did not affect exocytotic rate measured at any time point. The total capacitance increase measured at the plateaus averaged 4.4±1.1 pF in the presence of ATP and 3.8±0.7 pF in the absence of the nucleotide (measured at ~9 and ~7 min respectively; P=0.7).

The involvement of cAMP in 3T3-L1 adipocyte exocytosis

We next tested the effects of Ca^{2+} and ATP in the absence of cAMP. As shown in Fig. 4A-B, exocytosis was completely abolished upon exclusion of cAMP, regardless of the presence or absence of Ca^{2+} (1.5 μM). Likewise, no effects of ATP were detected in the absence of cAMP. These data underscore the central role of cAMP in adipocyte exocytosis.

Exocytosis regulated by cAMP may occur via pathways involving PKA- or the cAMP receptor Epac (Seino & Shibasaki, 2005). To determine the involvement of PKA and Epac in 3T3-L1 adipocyte exocytosis, we again infused cells with the solution containing 0.1 mM cAMP (no Ca^{2+}, 3 mM ATP) supplemented or not with 0.5 mM of the PKA-inhibtor Rp-cAMPS (Fig. 4C and D). In accordance with results in Fig. 3, cAMP stimulated exocytosis. Inclusion of the inhibitor was without effect at any measured time point. It was verified that exocytosis was not induced by Rp-cAMPS alone (n=4; $\Delta C/\Delta t$ at 2 min = 1.2±0.6 fF/s).

To further investigate the contribution of PKA-independent signalling, we infused cells with a pipette solution lacking Ca^{2+} and cAMP supplemented with 8-Br-2'-O-Me-cAMP, a potent and specific activator of Epac (Herfindal *et al.*, 2013). Agonist concentrations were chosen based on the binding affinity for Epac (5 times lower than the more commonly used but less specific 8pCPT-2Me-cAMP; information from

BIOLOG). As shown in Fig. 4C and D, 1 mM 8-Br-2'-O-Me-cAMP stimulated exocytosis as potently as regular cAMP. Exocytosis was still triggered by 0.5 mM of the Epac agonist, at a rate amounting to 50% of that achieved using the higher concentration (n=9; not shown). Taken together those results suggest that cAMP-stimulation of 3T3-L1 adipocyte exocytosis occurs predominantly via PKA-independent pathways involving Epac.

Endocytosis vs. exocytosis

Recordings of cell membrane capacitance measure the balance between exo- and endocytosis. The fact that exocytosis is abolished using a pipette solution lacking cAMP while no apparent endocytosis is observed (see Fig. 4A, B) reinforces the proposal that measured alterations of membrane capacitance chiefly reflect exocytosis. Endocytosis has in several endocrine cell types been shown to be Ca^{2+} dependent (Eliasson *et al.*, 1996; Mansvelder & Kits, 1998). It is therefore noteworthy that no endocytosis was detected in cells infused with a solution containing 1.5 μM free Ca^{2+} (cAMP absent) even in recordings lasting up to 20 minutes (Fig. 4B).

To explore the role of Ca^{2+} in adipocyte endocytosis further, we infused cells with a solution containing an unphysiologically high free [Ca^{2+}] >10 μM. A reduction of $\Delta C/\Delta t$ by ~40% at t=2 min (15±2.4 fF/s *cf.* 26±2.2 fF/s at 1.5 μM free Ca^{2+} in Fig.2; P=0.01) and by ~70% at t=10 min (2.9±0.6 fF/s *cf.* 9.0±2.2 fF/s using 1.5 μM Ca^{2+}) was evident, possibly resulting from exocytosis being masked by contaminant endocytosis occurring at a higher speed at high $[Ca^{2+}]_{i}$. Interestingly, endocytosis was in some experiments apparent as "bursts" of membrane retrieval resulting in a partly wave-like capacitance trace; this was never seen in experiments using a solution containing a lower [Ca^{2+}]. In other experiments net endocytosis was evident as negative rates at later time-points (examples of endocytosis depicted in Fig. 4E). Our results with >10 μM free Ca^{2+} are in agreement with a study in pancreatic beta-cells where endocytosis was first detectable upon infusion of [Ca^{2+}] >10 μM (Eliasson *et al.*, 1996). Collectively, these findings make it justifiable to conclude that endocytosis interferes minimally with exocytosis triggered by the pipette solutions used in our study. It should be noted that endocytosis triggered by high [Ca^{2+}] would, if anything, result in an underestimation of the Ca^{2+}-dependence of exocytosis in our cells.

Exocytosis depends on cell size

Compared to most other cell types, white adipocytes are exceptionally large and the variation in cell size is immense. Adipocyte expansion is strongly associated with insulin resistance and type-2 diabetes (Jernas *et al.*, 2006) and secretion of several adipokines has been suggested to depend on adipocyte cell size and to correlate with cell surface area (Skurk *et al.*, 2007).

We plotted the initial cell capacitance (C_m value at the start of the recording before exocytosis was triggered) for all cells included in this study. The distribution of cell size (expressed as membrane capacitance) in a total of 155 experiments is shown in Fig. 5A. Observed cell capacitance ranged between 16 pF and 129 pF. The average cell size amounted to 43±1 pF and the median was 39 pF. The 8-fold variation of cell capacitance translates into a ~3-fold variation of cell diameter, thus significantly smaller than the 20-fold variation reported in human adipocytes (Jernas *et al.*, 2006), likely in part reflecting a selection bias (avoiding the smallest and largest cells for electrophysiology). In order to investigate the importance of cell size in our study, we plotted $\Delta C/\Delta t$ at t=2 min and ΔC_{tot} values as a function of C_m. As can be seen in Fig. 5B-E, there were positive correlations between cell size and $\Delta C/\Delta t$ and ΔC_{tot} both at 0 and 1.5 μM $[Ca^{2+}]_i$ (cAMP and ATP included). Thus, exocytosis is in part a function of cell surface area. Reanalysis of $\Delta C/\Delta t$ and ΔC_{tot} values in Figs. 2 and 3 in relation to initial cell size did not significantly affect the outcome of the experiments described.

It is interesting that adipocytes that are 10 times bigger than archetypal endocrine cells like pancreatic β-cells and melanotrophs, secrete at a similar rate (~25 fF/s; Thomas *et al.*, 1990; Proks *et al.*, 1996; Barg *et al.*, 2002; Olofsson *et al.*, 2009). However, it is worth remembering that the cytosol of white adipocytes comprises only 5% of the total cell volume while the remaining 95% of the cell interior is filled with lipids (Trujillo & Scherer, 2006). Thus, the large size of the cells is more likely related to their lipid storage capacity than to their secretory functions.

The role of Ca^{2+} and cAMP in white adipocyte adiponectin secretion

Adiponectin is released from mature white adipocytes and several studies have shown that also 3T3-L1 adipocytes secrete this adipokine (Bogan & Lodish, 1999; Blumer *et*

al., 2008). To explore a possible association between our capacitance data and adiponectin secretion, we investigated how experimental alterations of intracellular Ca^{2+} and cAMP levels affected release of this adipokine. To compare the secretion results with the capacitance measurements, experiments were carried out at 32ºC and secretion was measured in 30 min incubations. We argued that this would be sufficiently long to allow secretion of measurable adiponectin levels, yet short enough to permit comparison with the capacitance recordings. It should be noted that previous secretion studies have used very long (several hours or days) incubation times.

In order to investigate effects of elevated cAMP, 3T3-L1 adipocytes were incubated with 10 μM forskolin and 200 μM IBMX (forsk/IBMX). This combination elevated cytoplasmic cAMP levels >6-fold in our experiments and the elevation was unaffected by Ca^{2+} buffering (Fig. 6A). As shown in Fig. 7A, forsk/IBMX stimulated adiponectin secretion >3-fold compared to control. Forskolin has, in addition to elevating cAMP, been shown to increase $[Ca^{2+}]_i$ in pancreatic islet cells (Gao *et al.*, 2002) and brown adipocytes (Nakagaki *et al.*, 2005). Ratiometric recordings of cytosolic $[Ca^{2+}]$ showed that forsk/IBMX elevated 3T3-L1 adipocyte $[Ca^{2+}]_i$, from a basal 92±3 nM to an average peak value of 140±4 nM (Fig. 6B; 144 analysed cells in five separate experiments). Thus, to study the role of intracellular Ca^{2+} in forsk/IBMX stimulated secretion, 3T3-L1 adipocytes were pre-incubated with membrane permeable BAPTA-AM (50 μM; 30 minutes). The Ca^{2+} buffering effect was verified in pre-treated cells stimulated with extracellular ATP. In four separate experiments, preloading with BAPTA abolished the $[Ca^{2+}]_i$-elevating effect of 100 μM ATP (not shown). Forsk/IBMX elevated secretion of adiponectin to the same extent in BAPTA-treated cells (Fig. 7A).

To further study the role of Ca^{2+} in cAMP-stimulated adiponectin secretion, 3T3-L1 adipocytes were exposed to the Ca^{2+} ionophore ionomycin (Fig. 7B). Forsk/IBMX again stimulated adiponectin secretion >3-fold, an effect that was augmented 2-fold in the presence of 1 μM ionomycin.

To verify the physiological importance of our findings, primary human subcutaneous adipocytes were incubated for 30 minutes together with forsk/IBMX. Forskolin was included at 0.5 or 10 μM. As shown in Fig. 7C, both concentrations of

forskolin (combined with 200 μM IBMX) stimulated adiponectin release also in human adipocytes.

The presence of basal adiponectin secretion under control conditions is evident in both 3T3-L1 and human adipocytes. Every hormone released by regulated secretion also has a basal background release, controlled by in much unknown mechanisms. In our adiponectin secretion experiments basal release can be envisaged to partly arise from the intracellular presence of some cAMP also in unstimulated cells. Moreover, plasma adiponectin levels are continuously high and a notable basal secretion of this adipokine is thus expected.

The involvement of PKA-independent mechanisms in cAMP-stimulation of adiponectin secretion

To elucidate the cAMP signalling pathway, we investigated the ability of forsk/IBMX to stimulate adiponectin secretion in cells treated with the membrane-permeable protein kinase inhibitor Rp-8-Br-cAMPS (present during 30 min pre-incubation and throughout 30 min stimulations). Forsk/IBMX stimulated adiponectin secretion >4-fold in this series of experiments. As shown in Fig. 7D, the stimulation was unaffected by inclusion of 200 μM Rp-8-Br-cAMPS. It was verified that Rp-8-Br-cAMPS alone was without effect on adiponectin release. The results using Rp-8-Br-cAMPS indicates that cAMP stimulates adiponectin secretion via a PKA-independent pathways. It is therefore of interest that the effect of forsk/IBMX was mimicked by the membrane-permeable Epac agonist 8-Br-2'-O-Me-cAMP-AM (20 μM; Fig. 7E). In fact, the Epac agonist was only a marginally weaker stimulus than forsk/IBMX and this difference did not attain statistical significance. A higher concentration of the agonist (60 μM) had similar effects (investigated concentrations chosen based on Chepurny *et al.*, 2010 and discussions with BIOLOG).

The experiment was replicated using primary human subcutaneous adipocytes (Fig. 7F). When included at a concentration of 20 μM, 8-Br-2'-O-Me-cAMP-AM stimulated adiponectin release ~2-fold above control, similar to the stimulation produced by 10 μM forskolin in the presence of IBMX (*cf.* Fig. 7C).

Secretion of additional adipokines

White adipocytes secrete several known adipokines in addition to adiponectin whose release may contribute to observed membrane capacitance increases. Leptin and resistin are, together with adiponectin, classified as adipocyte-derived peptide hormones and have been suggested to be released via regulated exocytosis (Bogan & Lodish, 1999; Bradley & Cheatham, 1999; Roh *et al.*, 2000; Zhong *et al.*, 2002; Xie *et al.*, 2008; Ye *et al.*, 2010). We thus investigated the secretion of those adipokines in 30 min incubations. Resistin is secreted from rodent and 3T3-L1 adipocytes (Zhong *et al.*, 2002; Ye *et al.*, 2010) although in humans this adipokine is mainly secreted from macrophages as well as from immature adipocytes (Janke *et al.*, 2002; Lazar, 2007). Resistin was present in the medium from cells stimulated with forsk/IBMX but amounted to only 6% of measured adiponectin (2.0±0.3 ng/ml resistin vs. 34±3 ng/ml adiponectin; analysed in 22 samples). The ratio between adiponectin and resistin remained constant upon inclusion of ionomycin (3.0±0.5 ng/ml resistin vs. 50±5 ng/ml adiponectin). The 3T3-L1 adipocytes used in this study are known to express/secrete very low levels of leptin (MacDougald *et al.*, 1995; Norman *et al.*, 2003); this was confirmed by measurements showing that this adipokine is secreted in small amounts in our cells (secreted leptin amounted to 2.8±0.3 ng/ml compared to 114±0.1 ng/ml adiponectin; analysed in 12 samples exposed to forsk/IBMX). We further investigated the content of apelin and adipsin in the same samples, two additional adipokines suggested to be abundantly released from 3T3-L1 adipocytes (Kitagawa *et al.*, 1989; Than *et al.*, 2012). Apelin secretion was very low (4.5±0.5 ng/ml) and thus amounted to 2% of secreted adiponectin. Adipsin was released in slightly larger amounts but the secretion was unaffected by stimulation with forsk/IBMX (13±0.2 ng/ml in control cells compared to 18±0.4 ng/ml apelin in cells exposed to forsk/IBMX (P=0.3; compare with a 4-fold elevation of adiponectin release in this series from 27±3 ng/ml under control conditions to 114±0.1 ng/ml when incubated with forsk/IBMX).

Discussion

Here we combine electrophysiological recordings of membrane capacitance (exocytosis) with measurements of released adiponectin in white adipocytes with the aim to elucidate the mechanisms involved in short-term regulation of white adipocyte exocytosis. Neuroendocrine cells typically contain thousands of hormone or neurotransmitter containing vesicles that need to undergo Ca^{2+}-, ATP-, and cAMP-dependent priming steps in order to attain release competence. Primed vesicles may be directly secreted upon stimulation, usually by Ca^{2+}, and once depleted the readily releasable vesicle pool must again be refilled by priming of new vesicles (Burgoyne & Morgan, 2003; Rorsman & Renstrom, 2003; Alvarez & Marengo, 2011). Our results show that although white adipocytes share some features of regulation of exocytosis with other endocrine cell types there are some fundamental differences. The key findings in our study as well as certain aspects of particular interest are discussed below.

Regulation of white adipocyte exocytosis.

We here show that 3T3-L1 adipocyte exocytosis is triggered by cAMP but that this effect is influenced by both Ca^{2+}-dependent and -independent processes. We propose that white adipocytes release adiponectin-containing vesicles according to the model summarised in Fig. 8A. Our model postulates that cAMP stimulates exocytosis of a readily releasable pool of adiponectin-containing vesicles in a Ca^{2+}- and ATP-independent manner. A combination of Ca^{2+} ($\geq$700 nM) and ATP amplifies cAMP-triggered exocytosis via potentiation of vesicle release. Ca^{2+} is further necessary for vesicle replenishment. We base our model on the following findings: First, cAMP stimulates exocytosis (monitored as an increase in cell capacitance) at $[Ca^{2+}] \leq$250 nM whilst adiponectin secretion (detected biochemically) is stimulated by forsk/IBMX under intracellular Ca^{2+}-free conditions (*cf.* Figs. 2B and 7A). Second, the rate of capacitance increase evoked by cAMP in the absence of ATP is equal at early time-points (t$\leq$6 min) regardless of the absence or presence of intracellular Ca^{2+}. However, plateaus of capacitance increase are not seen when exocytosis is triggered in the presence of $[Ca^{2+}] \geq$700 nM (*cf.* Figs. 2A and 3A), indicating a role for Ca^{2+} in recruitment of new vesicles for release. Third, the combination of $[Ca^{2+}] \geq$700 nM and

ATP augments cAMP-triggered capacitance increase at early time points (t=2-8 min; see Fig. 3B and schematic Fig. 8B) akin to a strong potentiation of adiponectin secretion in cells incubated with ionomycin (*cf.* Figs. 2A and 7B). Further, the exocytotic rate in the presence of high Ca^{2+} measured at times ≥10 min equals that observed in the absence of ATP (Fig. 3B) indicating that ATP exert its effect on exocytosis at a distal step equivalent to augmentation of cAMP-stimulated vesicle release. Fourth, the potentiation of exocytosis by ATP is clearly Ca^{2+}-dependent since ATP is without effect on exocytosis in the absence of Ca^{2+} (Fig. 3C-D). The finding that a selective Epac agonist stimulates exocytosis and adiponectin secretion to the same extent as cAMP further indicates that the cAMP stimulation occurs largely via PKA-independent signalling mediated by the cAMP-sensing protein Epac.

The ultrastructural characteristics of adipokine-containing vesicles remain poorly defined. However, based on the size of known peptide hormone vesicles (Parsons *et al.*, 1995; Olofsson *et al.*, 2002) and the abundant presence of large (200-500 nm), electron-dense vesicle structures in close proximity to the white adipocyte plasma membrane (Ramm *et al.*, 2000) secretion of adipokines can be envisaged to significantly contribute to membrane capacitance and account for capacitance increases amounting to several pico-Farad (Fig. 8B).

We acknowledge that fusion of vesicles containing cargo other than adiponectin may contribute to the capacitance increases illustrated in Fig. 8B. Increases in adipocyte membrane capacitance have previously been deduced to fusion of Glut4 vesicles with the plasma membrane (Chowdhury *et al.*, 2002). However, the small diameter of those vesicles (50-70 nm; reviewed in Stockli *et al.*, 2011) together with the reported maximal fusion rate (0.15 vesicles/min/μm^2; Stenkula *et al.*, 2010) suggest that Glut4 vesicle fusion only marginally (<5%) contributes to the observed increases in membrane capacitance. Our measurements indicate that the adipokines leptin, resistin and apelin are secreted at low levels compared to adiponectin (1-6%). Adipsin is released at significant levels but secretion of this adipokine was not further stimulated by forsk/IBMX indicating that it is not released from the same vesicle population. Thus, even if we cannot exclude that release of additional adipokines contributes to the capacitance increases (if they are at all released by regulated exocytosis of a distinct vesicles population), quantitative considerations argue that the increases in cell

capacitance we observe principally reflect adiponectin release. This conclusion is reinforced by the excellent correlation between measurements of cell capacitance and adiponectin secretion data.

Roles of cAMP, Ca^{2+} and ATP in control of exocytosis in white adipocytes and other endocrine cell types

Both the cAMP dependence as well as its Ca^{2+} independence distinguishes exocytosis in white adipocytes from that in archetypal endocrine cell types. An interplay between Ca^{2+} and cAMP is usually involved in non-neuronal cell type exocytosis but Ca^{2+} is typically the trigger of secretion while cAMP acts as an enhancer (Szaszak *et al.*, 2008). In pancreatic beta-cells exocytosis can be triggered by Ca^{2+} alone, albeit at a rate lower than observed in the presence of cAMP (Renstrom *et al.*, 1997; Eliasson *et al.*, 2003). However, cAMP alone is unable to stimulate beta-cell exocytosis in the absence of Ca^{2+} (Renstrom *et al.*, 1997). By contrast, secretion in some exocrine cell types have been shown to be stimulated by cAMP while Ca^{2+} has augmenting effects (Szaszak *et al.*, 2008).

The involvement of cAMP in white adipocyte adiponectin secretion has been investigated previously. With few exceptions, these studies investigate long-term (several hours or days) effects of alterations of cAMP and report that an elevation of the nucleotide significantly *decreases* adiponectin release (Delporte *et al.*, 2002; Fasshauer *et al.*, 2003; Cong *et al.*, 2007; Fu *et al.*, 2007). Short-term ($\leq$3 h) effects of cAMP elevation were in addition investigated in a couple of the studies and either show no alteration (Delporte *et al.*, 2002) or stimulation (Juan *et al.*, 2007) of adiponectin secretion. Our own data demonstrate, both in 3T3-L1 cells and human subcutaneous adipocytes, that the acute response to cAMP is stimulation of adiponectin secretion. The finding that the stimulatory effect of cAMP is chiefly via a PKA-independent pathway further distinguishes adipocytes from other endocrine cell types. In pancreatic beta-cells only approx. 15% of the cAMP effect on vesicle replenishment is due to PKA-independent signalling (Renstrom *et al.*, 1997). It is interesting to hypothesise that the usage of a PKA-independent pathway for control of adiponectin release is a way to differentiate regulation of secretion and lipolysis since the latter is known to be regulated mainly via PKA-dependent mechanisms (Carmen & Victor, 2006).

The role of Ca^{2+} in adiponectin secretion has previously not attracted much attention. It has been reported that acute exposure of 3T3-L1 adipocytes to the Ca^{2+} ionophore A23187 results in a negligible elevation of adiponectin secretion at early time points but that there is a significant stimulation at times longer than 90 min (Bogan & Lodish, 1999). This effect is in agreement with our observation that Ca^{2+} acts at early steps in the secretory pathway including replenishment of the release-competent pool of vesicles. The effects of Ca^{2+} described here occur within much shorter time frames, in the capacitance measurements during the first minutes of exocytosis (provided ATP is present). We attribute those differences to the concurrent elevation of cAMP in our study making more adiponectin containing vesicles available for Ca^{2+}-dependent potentiation of release.

Our finding of an involvement of ATP in adipocyte exocytosis is in agreement with a study showing that a decrease of intracellular ATP levels inhibits rat adipocyte adiponectin release stimulated over 2 h (Szkudelski *et al.*, 2011). As in other known secreting cell types, ATP may be required for phosphorylation of exocytotic proteins (Burgoyne & Morgan, 2003).

Pathophysiological significance and the way forward

In view of the important roles of cAMP and Ca^{2+} in control of short-term adiponectin secretion, it seems reasonable to postulate adrenergic signalling as a physiological stimulus for secretion of the adipokine. Adrenergic signalling is well known to be involved in regulation of adipocyte metabolic function and $\alpha_{1,2}$ as well as $\beta_{1,2,3}$ adrenergic receptor subtypes have been identified in white adipocytes (Lafontan *et al.*, 1997). Catecholamine stimulation elevates intracellular levels of Ca^{2+} and cAMP via α_1 and $\beta_{1,2,3}$ receptors respectively. In contrast, activation of $\alpha 2$ receptors leads to decreased production of both mediators. Thus, a dynamic functional balance between different adrenergic receptor subtypes in the adipocyte can be envisaged to influences adiponectin secretion. In agreement with the above suggestion, own results show that incubation of primary mouse subcutaneous adipocytes during 30 min in the presence of adrenaline or the β_3-adrenergic agonist CL316243 elevates adiponectin release (Komai, Musovic, Olofsson, unpublished). Importantly, adrenergic signalling is dysfunctional in several models of obesity and an increase in the $\alpha 2$ to β receptor ratio has been

described in adipocytes from obese individuals (Valet *et al.*, 2000; van Baak, 2001). According to our model of secretion (Fig. 8A), such a switch would disrupt stimulus-secretion coupling with consequent diminished adiponectin release. Previous studies consistently report that long-term (several hours or days) adrenergic stimulation results in reduced adiponectin expression/secretion (Delporte *et al.*, 2002; Fasshauer *et al.*, 2003; Cong *et al.*, 2007; Fu *et al.*, 2007). It is conceivable that the long-term effects on release are in part due to adipocyte exhaustion (adiponectin vesicle depletion), analogous to the situation in pancreatic beta-cells exposed to prolonged stimulation of insulin secretion (Robertson *et al.*, 2003). Further, an increase in adipocyte intracellular cAMP has been shown to lead to ATP depletion (Gauthier *et al.*, 2008) which would, consistent with our model of secretion, lessen adiponectin release.

The findings that elevated adiponectin levels reduce the risk of developing type-2 diabetes (Spranger *et al.*, 2003) taken together with its ability to promote adipocyte differentiation (Fu *et al.*, 2005) suggests adiponectin as a promising pharmacological target. Production of recombinant adiponectin has proven to be problematic and the prospect of an effective strategy for therapeutic administration is uncertain. Thus, approaches are currently aimed at increasing levels of endogenous adiponectin. The accomplishment of this is undoubtedly reliant on understanding the mechanisms controlling adiponectin secretion. Most neuroendocrine cells contain a single population of peptide hormone containing vesicles (although they also contain smaller synaptic-like microvesicles; Kasai *et al.*, 2012). In white adipocytes, several adipokines as well as Glut4 may be compartmentalised into distinct types of vesicles (Barr *et al.*, 1997; Bogan & Lodish, 1999; Bradley & Cheatham, 1999; Roh *et al.*, 2000; Xie *et al.*, 2008; Ye *et al.*, 2010). Insulin stimulates secretion of both leptin (Barr *et al.*, 1997) and adiponectin (Blumer *et al.*, 2008) as well as translocation of Glut 4 containing vesicles from an intracellular location to the plasma membrane (Liu *et al.*, 2003; Huang *et al.*, 2005). It is apparent that a complex control of exocytotic pathways exists in the adipocyte. Considering the well-established disturbances of adipokine secretion in obese individuals (Maury & Brichard, 2010), it is remarkable how little is known about the molecular and cellular regulation of adipokine secretion in the shorter term. Future cell physiological studies of the type we report here may help to resolve the underlying

cellular mechanisms and ultimately pave the way for pharmacological correction of these defects.

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Competing interests

None of the authors have any conflicts of interests.

Author contributions

Conception and design of the experiments: A.M.K. and C.S.O. Data collection, analysis and interpretation of data A.M.K., C.B., S. M. and C.S.O. Drafting and revising of the manuscript A.M.K., C.B., S. M. and C.S.O. All authors have read and approved of the final version of the manuscript. All experiments were carried out at the Department of Neuroscience and Physiology, Gothenburg University with the exception of isolation and incubations of human adipocytes that were performed at AstraZenecaR&D, Mölndal.

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Figures and legends

Fig. 1 Example of differentiated 3T3-L1 adipocytes and analyses performance. A 3T3-L1 adipocytes 9 days after start of differentiation. Note the accumulation of large lipid droplets. Patch pipette attached to a 3T3-L1 adipocyte representative of cells chosen for experiments. Scale bar = 50 μm **B** Example of a typical capacitance recording with ΔC_m (delta membrane capacitance) plotted against time. Analyses were carried out as illustrated in the figure. $\Delta C/\Delta t$ was measured at different time points as indicated by linear fits (black lines) superimposed on the capacitance trace (grey trace). ΔC_{tot} was measured as the difference between the start and end values of membrane capacitance, either where a plateau was obtained or at t=10 min as indicated.

Fig. 2 The exocytotic response is affected by alterations of $[Ca^{2+}]_i$. A, B Typical recordings of ΔC_m after establishment of the whole-cell configuration. Cells were infused with intracellular solutions containing 3 mM ATP and 0.1 mM cAMP together with 1.5 μM or 700 nM free Ca^{2+} (**A**) or 250 nM or 0 free Ca^{2+} (**B**). **C** Histogram summarising the average rate of capacitance increase ($\Delta C/\Delta t$) measured at t=2 min using intracellular solutions with different [Ca^{2+}]. (**D**) Exocytotic plateau values or values at t=10 min (under conditions where plateaus were not reached) as indicated using pipette solutions containing 0 (n=8), 250 nM (n=7), 700 nM (n=10) or 1.5 μM (n=8) free Ca^{2+}. Average starting values of C_m were 38±3pF (0 Ca^{2+}), 35±4 pF (250 nM Ca^{2+}), 45±5 pF (700 nM Ca^{2+}) and 42±2 pF (1.5 μM Ca^2). Data in A-C are mean values ± S.E.M. of 8-21 recordings. **P<0.05; **P<0.01*; ****P<0.001*.

Fig. 3 Exocytosis is augmented by Ca^{2+} and ATP. A Representative traces of ΔC_m for cells infused with intracellular solutions containing 0.1 mM cAMP and 1.5 μM free Ca^{2+} with or without 3 mM ATP. **B** Average $\Delta C/\Delta t$ at indicated time points. The observation that rates of exocytosis are equal at times ≥10 min indicates continuous vesicle replenishment regardless of absence or presence of ATP. Note that analysis at early time points (30 s) show little evidence of net exocytosis after establishment of the whole cell configuration, before control of the intracellular milieu by infusion of the pipette solution. **C, D** As in A and B but cells dialysed with a pipette solution lacking Ca^{2+}. The rates of exocytosis in B and D are close to 0 at 30 sec indicating negligible

net exocytosis prior to infusion of the pipette solution. Data are mean values ± S.E.M. of: 8 (+ATP) and 7 (-ATP) recordings **(B)**, 9 (+ATP) and 13 (-ATP) **(D)** recordings. **P<0.05; **P<0.01*.

Fig. 4 3T3-L1 adipocyte exocytosis is triggered by cAMP via PKA-independent mechanisms. A Typical recordings of ΔC_m for cells dialyzed with solutions lacking cAMP in the presence or absence of 1.5 μM free Ca^{2+} and/or ATP (3 mM) as indicated. **B** Average exocytotic rates analysed at different time points for experimental series in A. **C** Representative traces of ΔC_m for cells infused with intracellular solutions containing 0.1 mM cAMP together with 3 mM ATP, in the presence or absence of Rp-cAMPS (0.5 mM) or cells infused with a solution lacking cAMP and supplemented with 1 mM 8-Br-2'-O-Me-cAMP. **D** The effect of Rp-cAMPS or 8-Br-2'-O-Me-cAMP on average $\Delta C/\Delta t$ analysed at designated time points. Data are mean values ± S.E.M. of 9 (1.5 μM Ca^{2+}), 12 (0 Ca^{2+}) and 13 (0 Ca^{2+}, 0 ATP) recordings (B), 8 (0.1 mM cAMP), 7 (0.1 mM cAMP + 0.5 mM Rp-cAMPS) and 9 (0 mM cAMP + 1 mM 8-Br-2'-O-Me-cAMP) recordings (D). **E** Two representative traces of endocytosis in cells infused with an intracellular solutions containing >10 μM free Ca^{2+} together with 0.1 mM cAMP and 3 mM ATP.1.5. For comparison, a characteristic trace recorded in a cell infused with 1.5 μM free Ca^{2+} is shown.

Fig. 5 Relationship between cell size and exocytotic capacity of 3T3-L1 adipocytes. A The distribution of cell size (measured in pF) for 155 capacitance recordings included in our study. Cell sizes have been divided in 5-pF groups. **B-E** Values of $\Delta C/\Delta t$ (**B,C)** and ΔC_{tot} (**D,E**) as a function of C_m for cells dialysed with a pipette solution containing 3 mM ATP and 0. 1 mM cAMP with 1.5 μM (**B,D**) or 0 (**C,E**) free Ca^{2+}. Values for ΔC_{tot} are represented by plateau values in the absence of Ca^{2+}. In the presence of 1.5 μM free Ca^{2+}, when plateaus were not achieved, ΔC_{tot} represents the change in cell capacitance during the first 10 min of the recording.

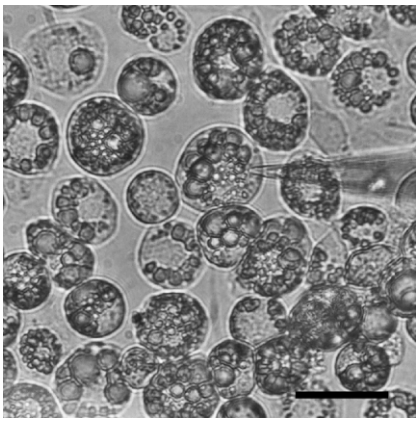
Fig. 6 A combination of forskolin and IBMX increases adipocyte cAMP levels and elevates $[Ca^{2+}]_i$. A Cytoplasmic [cAMP] in 3T3-L1 adipocytes incubated with forskolin (10 μM) + IBMX (200 μM) for 30 min in the presence or absence (pre-treatment with

50 μM BAPTA-AM) of intracellular Ca^{2+}. **B** example trace of effect of forsk/IBMX on 3T3-L1 adipocyte $[Ca^{2+}]_i$. ***P<0.01; ***P<0.001*.

Fig. 7 cAMP stimulated adiponectin secretion is Epac-dependent and potentiated by Ca^{2+}. A Adiponectin secretion in 3T3-L1 adipocytes expressed as fold increase compared to basal (5 mM glucose alone) during 30 min incubations (32°C) with forskolin (10 μM) + IBMX (200 μM) with or without BAPTA pre-treatment (50 μM). **B** As in A but cells incubated for 30 min with forsk/IBMX in the absence or presence of ionomycin (1μM). **C** Adiponectin secretion during 30 min incubation of human primary subcutaneous adipocytes with forskolin (0.5 or 10 μM) together with IBMX (200 μM). **D** Effects of the cAMP-antagonist Rp-8-Br-cAMPS (200 μM) on forsk/IBMX stimulated adiponectin secretion in 3T3-L1 adipocytes. **E** 3T3-L1 adipocyte adiponectin secretion stimulated during 30 min incubation with the Epac agonist 8-Br-2'-O-Me-cAMP-AM (20 μM). **F** Adiponectin secretion in human subcutaneous adipocytes stimulated with 8-Br-2'-O-Me-cAMP-AM (20 μM) for 30 min. Note that basal secretion (control) is somewhat lower than in C but that the fold-increase produced by the agonist is of similar magnitude as that stimulated with forsk/IBMX. Data are mean values ± S.E.M. of 14 (A), 8 (B), 10 (D) and 11 (E) experiments. Isolated adipocytes from four and three patients respectively were used in C and F. ***P<0.01; ***P<0.001*.

Fig. 8 Proposed model of regulation of white adipocyte exocytosis. A White adipocytes contain at least two functional pools of secretory vesicles whose release is differentially regulated by the intracellular messengers cAMP, Ca^{2+} and ATP. A pool corresponding to readily releasable adiponectin containing vesicles are released by an elevation of cAMP alone. Ca^{2+} and ATP augments exocytosis and Ca^{2+}-dependent mechanisms are involved in replenishment of vesicles residing in a reserve pool (see text for details). **B** Schematic of increases in membrane capacitance in relation to secretion of vesicles with regard to the intracellular presence of cAMP, Ca^{2+} and ATP.

A



B

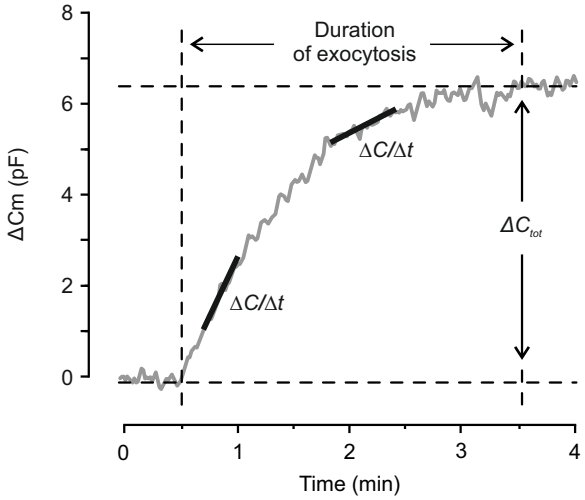
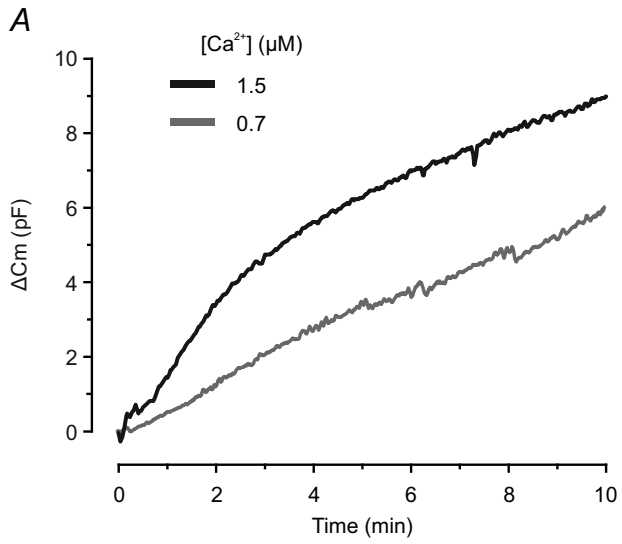
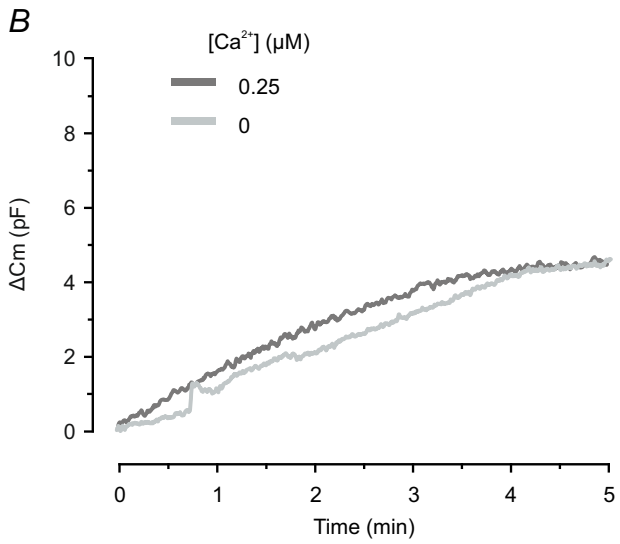
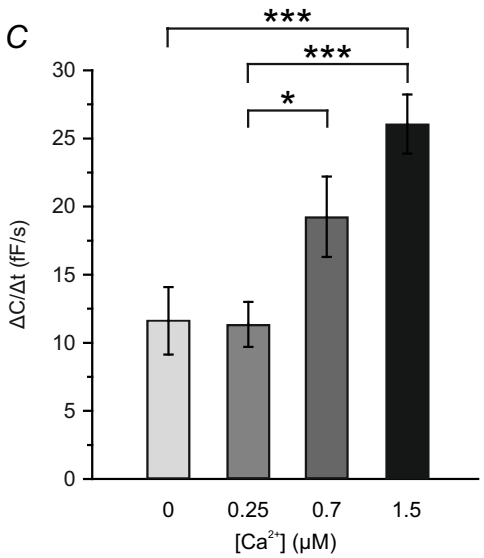

Duration
of exocytosis
ΔC/Δt
ΔC/Δt
ΔC_tot
ΔCm (pF)
Time (min)
0
2
4
6
8
0
1
2
3
4

Fig. 1







D

$[Ca^{2+}]_i$ (μM)	ΔC_{tot} (pF)	ΔC/Δt (fF/s)
0	3.7±1.1* plateau	-0.01±0.2
0.25	3.5±0.8** plateau	0.8±0.3
0.7	5.3±0.7* 10 min	7.7±1.2
1.5	9.4±1.7 10 min	9.0±2.2

Fig. 2

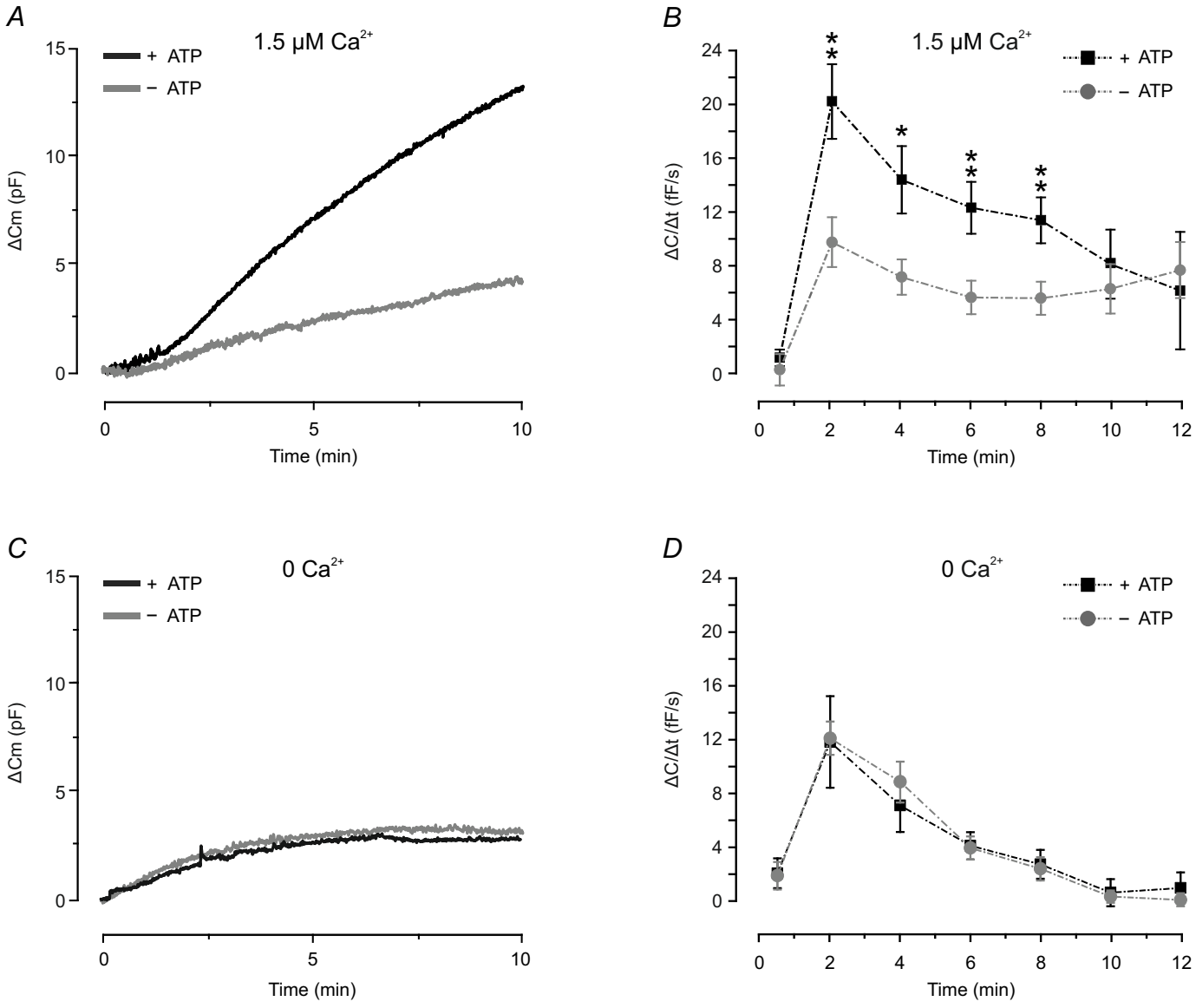

A
1.5 μM Ca2+
+ ATP
− ATP
ΔCm (pF)
Time (min)
B
1.5 μM Ca2+
+ ATP
− ATP
ΔC/Δt (fF/s)
Time (min)
C
0 Ca2+
+ ATP
− ATP
ΔCm (pF)
Time (min)
D
0 Ca2+
+ ATP
− ATP
ΔC/Δt (fF/s)
Time (min)

Fig. 3

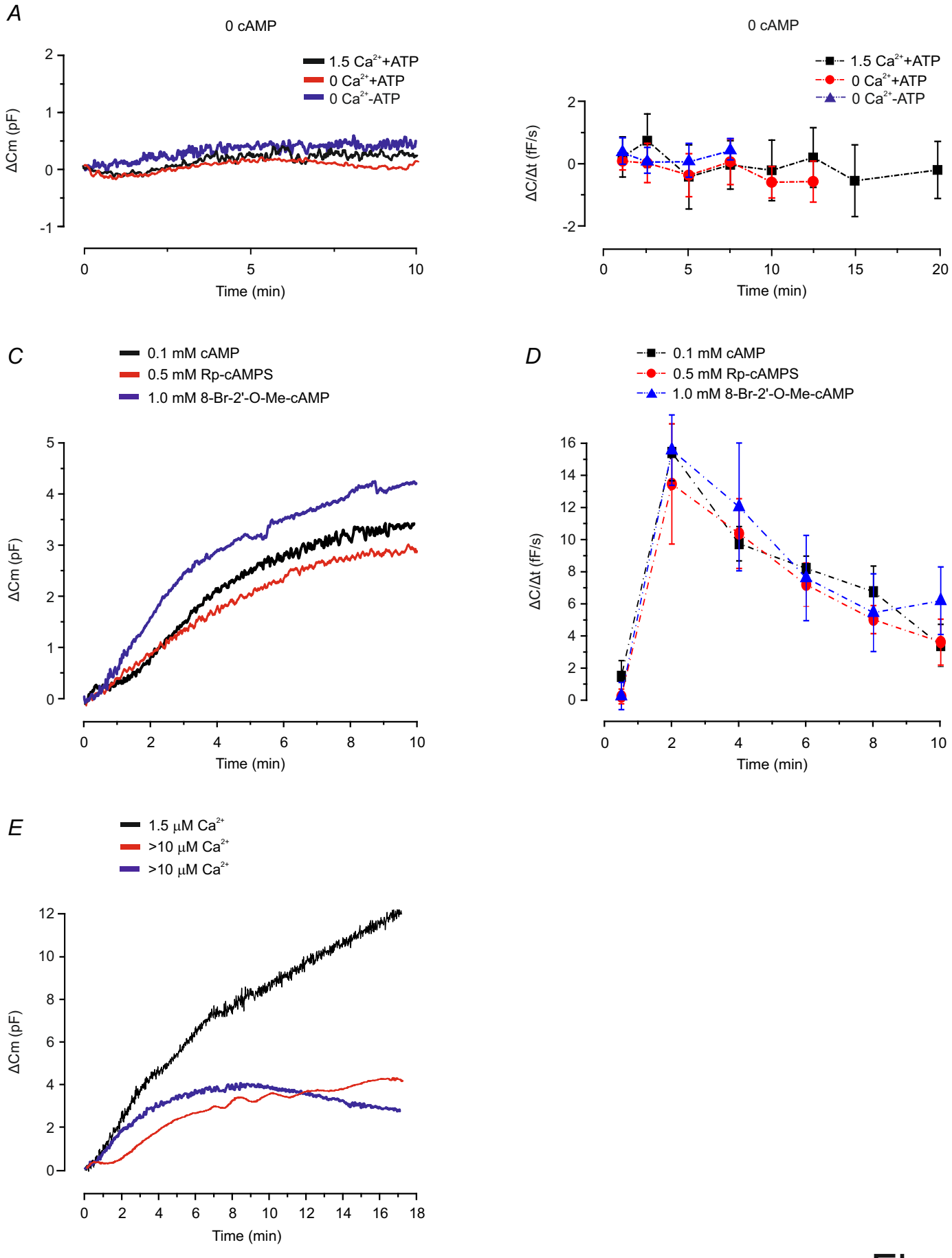

A
0 cAMP
1.5 Ca2+ATP
0 Ca2+ATP
0 Ca2+-ATP
ΔCm (pF)
Time (min)
0 cAMP
1.5 Ca2+ATP
0 Ca2+ATP
0 Ca2+-ATP
ΔC/Δt (fF/s)
Time (min)
C
0.1 mM cAMP
0.5 mM Rp-cAMPS
1.0 mM 8-Br-2'-O-Me-cAMP
ΔCm (pF)
Time (min)
D
0.1 mM cAMP
0.5 mM Rp-cAMPS
1.0 mM 8-Br-2'-O-Me-cAMP
ΔC/Δt (fF/s)
Time (min)
E
1.5 μM Ca2+
>10 μM Ca2+
>10 μM Ca2+
ΔCm (pF)
Time (min)

Fig. 4

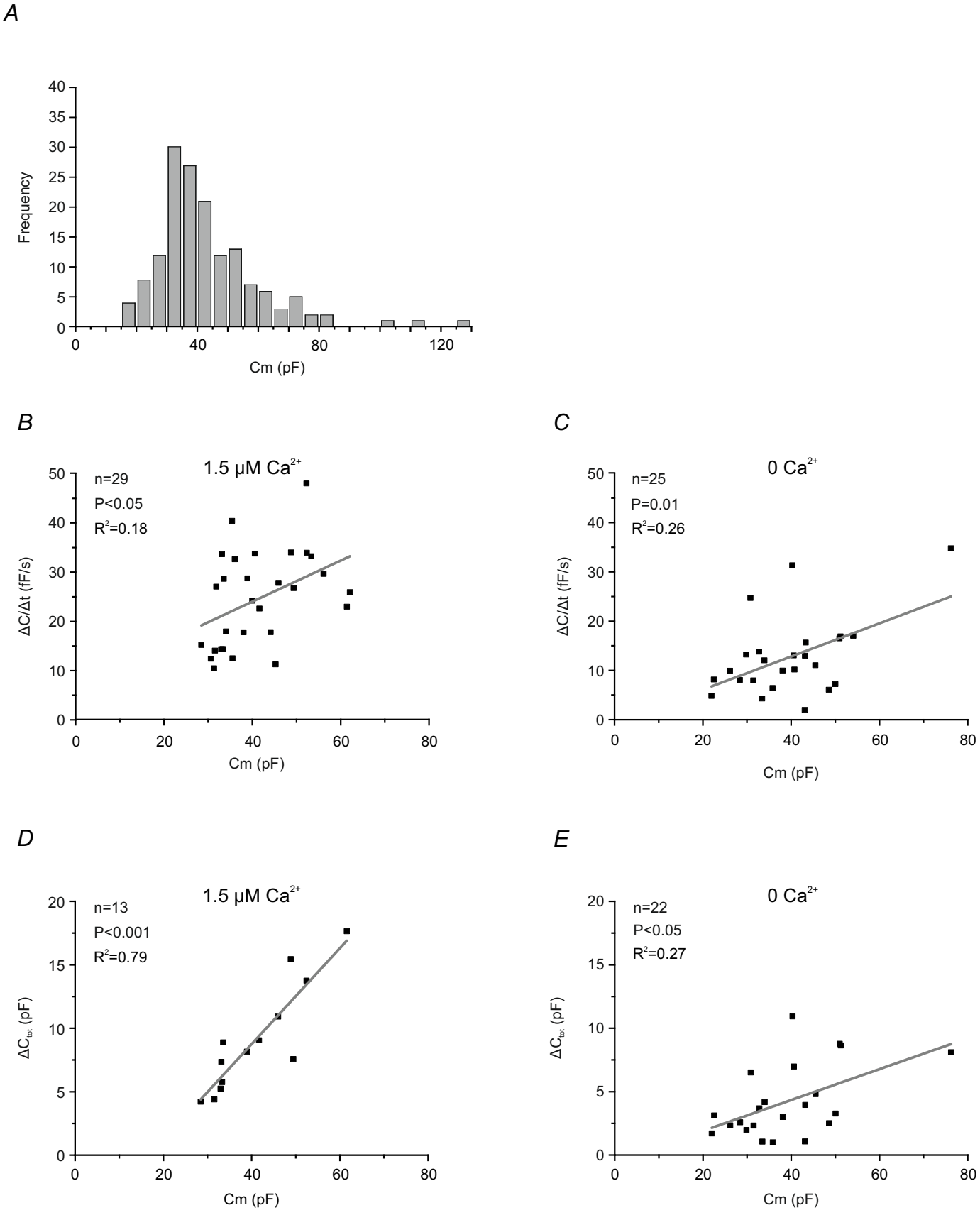
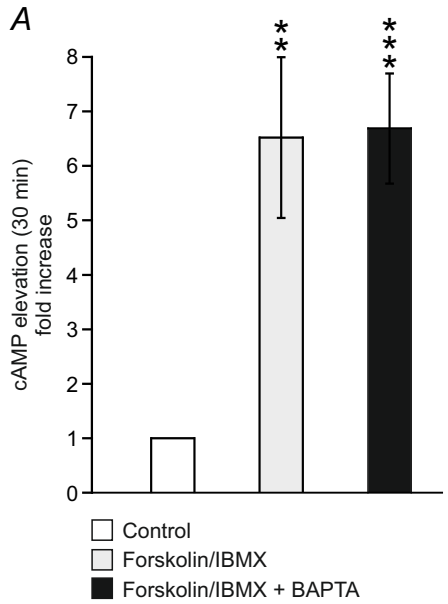

A
Frequency
Cm (pF)
B
1.5 μM Ca2+
n=29
P<0.05
R2=0.18
ΔC/Δt (fF/s)
Cm (pF)
C
0 Ca2+
n=25
P=0.01
R2=0.26
ΔC/Δt (fF/s)
Cm (pF)
D
1.5 μM Ca2+
n=13
P<0.001
R2=0.79
ΔCtot (pF)
Cm (pF)
E
0 Ca2+
n=22
P<0.05
R2=0.27
ΔCtot (pF)
Cm (pF)

Fig. 5


A
cAMP elevation (30 min)
fold increase
0
1
2
3
4
5
6
7
8
**
***
Control
Forskolin/IBMX
Forskolin/IBMX + BAPTA

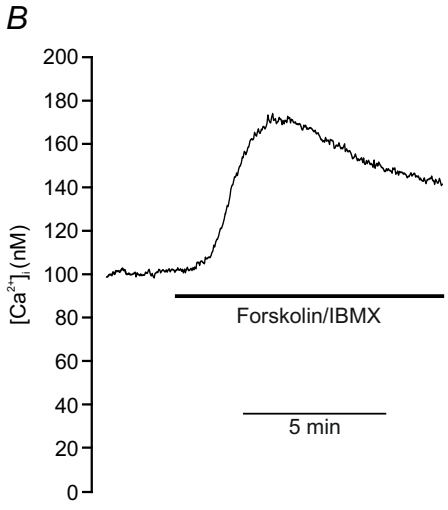

B
[Ca2+]i (nM)
0
20
40
60
80
100
120
140
160
180
200
Forskolin/IBMX
5 min

Fig. 6

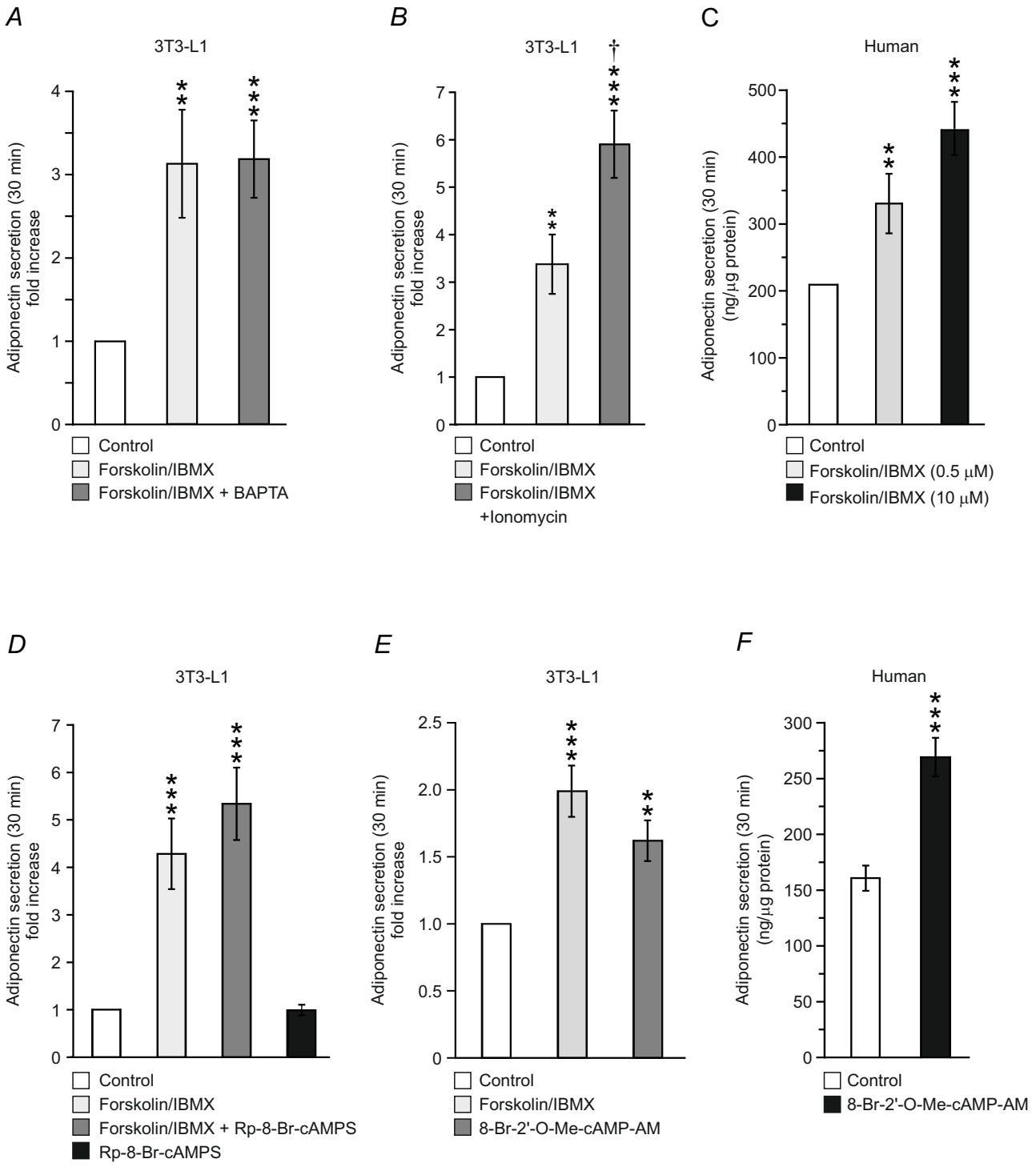
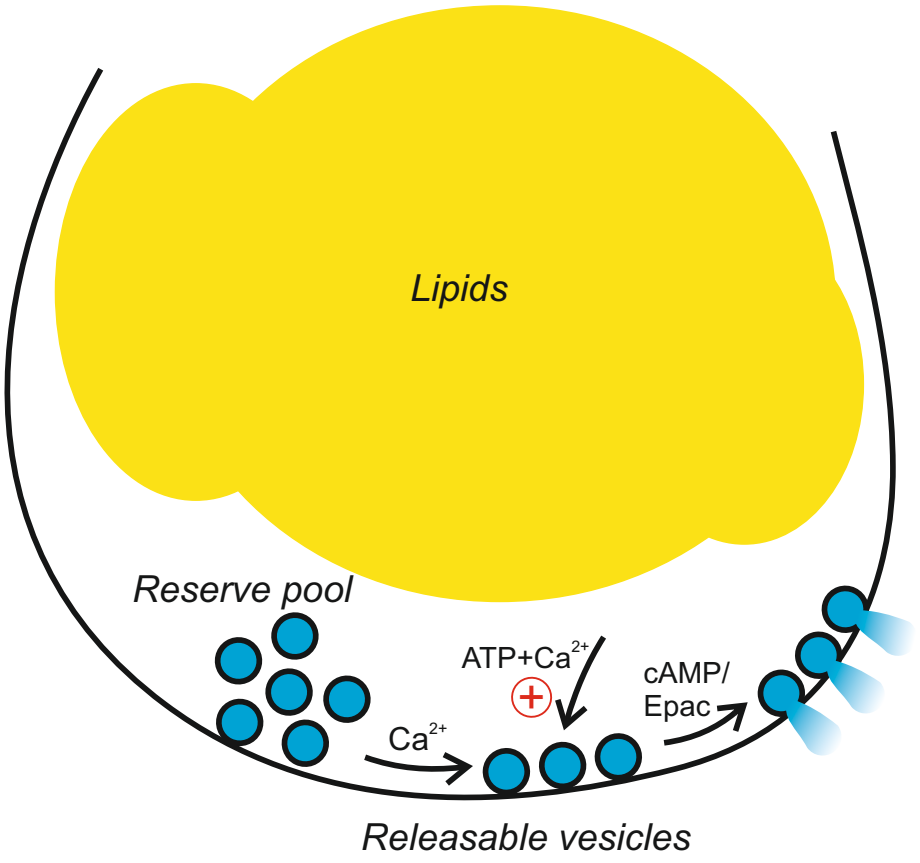

A
3T3-L1
Adiponectin secretion (30 min)
fold increase
0
1
2
3
4
**
***
Control
Forskolin/IBMX
Forskolin/IBMX + BAPTA
B
3T3-L1
Adiponectin secretion (30 min)
fold increase
0
1
2
3
4
5
6
7
**
†
***
Control
Forskolin/IBMX
Forskolin/IBMX
+Ionomycin
C
Human
Adiponectin secretion (30 min)
(ng/μg protein)
0
100
200
300
400
500
**
***
Control
Forskolin/IBMX (0.5 μM)
Forskolin/IBMX (10 μM)
D
3T3-L1
Adiponectin secretion (30 min)
fold increase
0
1
2
3
4
5
6
7
***
***
Control
Forskolin/IBMX
Forskolin/IBMX + Rp-8-Br-cAMPS
Rp-8-Br-cAMPS
E
3T3-L1
Adiponectin secretion (30 min)
fold increase
0
0.5
1.0
1.5
2.0
2.5
***
**
Control
Forskolin/IBMX
8-Br-2'-O-Me-cAMP-AM
F
Human
Adiponectin secretion (30 min)
(ng/μg protein)
0
50
100
150
200
250
300
***
Control
8-Br-2'-O-Me-cAMP-AM

Fig. 7

A


Lipids
Reserve pool
ATP+Ca2+
cAMP/
Epac
Ca2+
Releasable vesicles

B

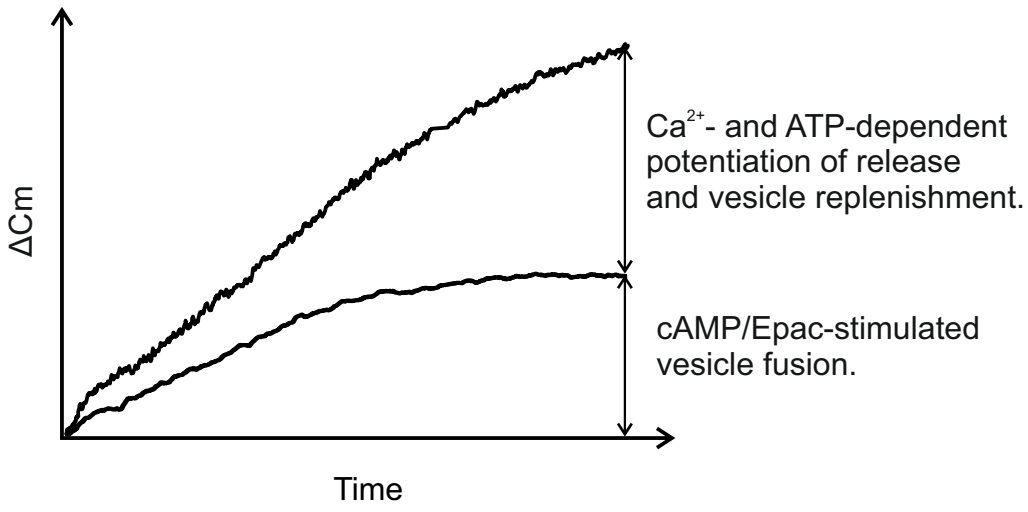

ΔCm
Time
Ca2+- and ATP-dependent
potentiation of release
and vesicle replenishment.
cAMP/Epac-stimulated
vesicle fusion.

Fig. 8