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CaV1.2 and CaV1.3 voltage-gated L-type Ca²⁺ channels in rat white fat adipocytes

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Abstract

L-type channel antagonists are of therapeutic benefit in the treatment of hyperlipidaemia and insulin resistance. Our aim was to identify L-type voltage-gated Ca²⁺ channels in white fat adipocytes, and determine if they affect intracellular Ca²⁺, lipolysis and lipogenesis. We used a multidisciplinary approach of molecular biology, confocal microscopy, Ca²⁺ imaging and metabolic assays to explore this problem using adipocytes isolated from adult rat epididymal fat pads. CaV1.2, CaV1.3 and CaV1.1 α_1 , β and $\alpha_2\delta$ subunits were detected at the gene expression level. The CaV1.2 and CaV1.3 α_1 subunits were identified in the plasma membrane at the protein level. Confocal microscopy with fluorescent antibodies labelled CaV1.2 in the plasma membrane. Ca²⁺ imaging revealed that the intracellular Ca²⁺ concentration, [Ca²⁺]_i, was reversibly decreased by removal of extracellular Ca²⁺, an effect mimicked by verapamil, nifedipine and Co²⁺, all blockers of L-type channels, whereas the Ca²⁺ channel agonist BAY-K8644 increased [Ca²⁺]_i. The finding that the magnitude of these effects correlated with basal [Ca²⁺]_i suggests that adipocyte [Ca²⁺]_i is controlled by L-type Ca²⁺ channels that are constitutively active at the adipocyte depolarized membrane potential. Pharmacological manipulation of L-type channel activity modulated both basal and catecholamine-stimulated lipolysis but not insulin-induced glucose uptake or lipogenesis. We conclude that white adipocytes have constitutively active L-type Ca²⁺ channels which explains their sensitivity of lipolysis to Ca²⁺ channel modulators. Our data suggest CaV1.2 as a potential novel therapeutic target in the treatment of obesity.

Key Words

- ▶ adipocyte
- ▶ calcium channel
- ▶ lipolysis
- ▶ obesity

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Introduction

White fat adipocytes (WFA) are the major energy depot of the body, storing and releasing energy in response to the calorific demands of the body during periods of excess and need respectively (Arner *et al.* 2011). It is widely accepted that an impairment of WFA triglyceride metabolism in obesity is a major aetiological factor of the metabolic

syndrome and type 2 diabetes. Whereby the inability of WFA to appropriately store energy as fat leads to ectopic disposition of lipids in insulin-responsive tissues such as skeletal muscle, liver and pancreas to promote insulin resistance and associated systemic disorders (Sattar & Gill 2014). To address dysfunctional fat storage, it is first

necessary to understand the regulation of lipid storage in healthy WFA.

Extracellular Ca^{2+} influx is implicated in the processes of fat storage (Arruda & Hotamisligil 2015): lipolysis (Schimmel 1978, Izawa *et al.* 1983, Allen & Beck 1986) and lipogenesis (Avasthy *et al.* 1988). Indeed, studies in *Drosophila* mutants indicate that impaired adipocyte cytosolic Ca^{2+} is associated with increased lipid deposition (Baumbach *et al.* 2014). However, the identity of the Ca^{2+} pathway is unclear (Draznin *et al.* 1988).

Several routes by which Ca^{2+} enters primary WFA are identified; these include voltage-gated Ca^{2+} channels, VGCC (Clausen & Martin 1977, Pershadsingh *et al.* 1989) store-operated Ca^{2+} channels (El Hachmane & Olofsson 2018), and reverse mode $\text{Na}^{+}/\text{Ca}^{2+}$ exchange, NCX (Pershadsingh *et al.* 1989, Bentley *et al.* 2014). Although VGCCs play a prominent role in Ca^{2+} entry and function of electrically excitable cells (Lipscombe *et al.* 2004), this route of influx is ill-defined in non-excitable adipocytes.

Pharmacological investigations show that verapamil and nifedipine, blockers of L-type VGCCs, can inhibit WFA Ca^{2+} uptake (Martin *et al.* 1975, Pershadsingh *et al.* 1989) and impair glucose transport (Draznin *et al.* 1987), lipolysis (Izawa *et al.* 1983) and lipogenesis (Avasthy *et al.* 1988). However, this inference of an association between Ca^{2+} influx and lipid turnover has come from independent studies where animal species, adipose depot as well as experimental conditions differed. To date, no comprehensive synthetic study exists where changes in Ca^{2+} influx and WFA function have been examined in primary adipocytes for the same species under similar experimental conditions of similar age and weight. Furthermore, a detailed molecular and immunohistochemical investigation of L-type VGCC expression in WFA is absent.

The aim of this study was to use a combination of Ca^{2+} imaging, metabolic, immunocytochemical and molecular biology techniques to identify L-type Ca^{2+} channels in WAT and demonstrate their functional role in lipid turnover.

Materials and methods

Unless stated otherwise, adipocyte isolation and experiments were performed in Hank's buffer solution which composed of (in mM): NaCl 138, NaHCO_3 4.2, KCl 5.6, MgCl_2 1.2, CaCl_2 2.6, NaH_2PO_4 1.2, glucose 5 and HEPES 10 (pH 7.4 with NaOH). For nominally Ca^{2+} free solutions, CaCl_2 was equimolarly replaced with MgCl_2 .

All % values are weight per volume. Unless stated otherwise drugs and chemicals are from Sigma.

Ethical approval

Animal care and experimental procedures were carried out in accordance with either the UK Home Office Animals (Scientific Procedures) Act (1986) or Swedish Ethical Review Board. In both instances the local ethical committees approved the animal procedures. Animals were killed by cervical dislocation or CO_2 .

Isolation and preparation of adipocytes

Epididymal fat pads were taken from Wistar rats (fed *ad libitum*, 12 h dark/light cycle, weight 220–420 g; Charles River Laboratory). For some experiments tissue from Sprague–Dawley rats was used, but since no difference could be detected, data were pooled. Adipocytes were isolated as described previously (Bentley *et al.* 2014).

Polymerase chain reaction

For reverse transcriptase PCR (RT-PCR) total RNA was isolated by cell lysis in TRI Reagent. Genomic DNA was removed with RQ1 RNase-free DNase (Promega) with RNA quantity and purity determined by the A_{260}/A_{280} ratio (1.98 to 2.02). The RNA integrity numbers (6–8) ascertained samples were suitable for RT-PCR (Schroeder *et al.* 2006). First-strand cDNA synthesis used 1 μg of total RNA with 200 ng random hexamer primers and Avian Myeloblastosis Virus reverse transcriptase (10 units/ μL RNA, Promega).

PCR reactions used Dream Taq PCR Master Mix (Thermo Fisher) and primers shown in Table 1. PCR was performed at 95°C for 10 min, followed by 40 cycles at 95°C for 45 s, 58°C for 45 s, 72°C for 45 s and terminated by a final extension step at 72°C for 10 min. PCR products were separated on 1% agarose gels, DNA stained with 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide and imaged with GeneSnap software (Syngene, UK). cDNA sequencing (DeepSeq, Nottingham, UK) checked PCR product identity.

Quantitative PCR (qPCR) reactions were performed in triplicate with SYBR® Green JumpStart™ Taq ReadyMix™. The thermal profile was 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s, annealing for 20 s at the primer-specific temperature, and elongation at 72°C for 35 s. The resultant mean threshold cycle (Ct) values were used for gene normalization and expression analyses. Three stable reference genes with the sequences

Table 1 Primer sequences.

Name	Accession number	Sequence	Product size (bp)
<i>β-Actin</i>	V01217	FWD: 5'-AGGCCAGAGCAAGAGAG-3' REV: 5'-CCTCATAGATGGGCACAGT-3'	333
<i>18s rRNA</i>	V01270	FWD: 5'-TCTGCCCTATCAACTTTCGATG-3' REV: 5'-AATTTGCGCGCCTGCTGCCTTCCTT-3'	137
<i>Cacna1s</i> (CaV1.1)	U31816.1	FWD: 5'-CAAGTCCTTCCAGGCCCTG-3' REV: 5'-CGTAGTCAGACTCCGGGTCG-3'	271
<i>Cacna1c</i> (CaV1.2)	M67515.1	FWD: 5'-CGCATTGTCAATGACACGATC-3' REV: 5'-CGGCAGAAAGAGCCCTTGT-3'	217
<i>Cacna1d</i> (CaV1.3)	M57682.1	FWD: 5'-TTGGTACGGACGGCTCTCA-3' REV: 5'-CCCCACGGTTACCTCATCAT-3'	156
<i>Cacna1f</i> (CaV1.4)	U31816.1	FWD: 5'-AGCACAAAGACCGTAGTGGTG-3' REV: 5'-ATACCCCAATGCCACACAG-3'	168
<i>Cacnb1</i>	NM_017346.1	FWD: 5'-AGTGCCAAACAGAAGCAGAAGT-3' REV: 5'-GTGTTTGTCTGGGGTTGTTGAG-3'	237
<i>Cacnb2</i>	NM_053851.1	FWD: 5'-CTCTTCTTCCCCTGCACCA-3' REV: 5'-GCCTCGGCTAAGAGCAGTTT-3'	237
<i>Cacnb3</i>	NM_012828	FWD: 5'-CCTACGCCGGGTTTGA-3' REV: 5'-CAAATGCCACAGGTTTGTGCT-3'	174
<i>Cacnb4</i>	NM_001105733.1	FWD: 5'-ATGCCAGGTCTGCATGTCTC-3' REV: 5'-ACATGGGGGTCTGGTGATCC-3'	231
<i>Cacna2d1</i>	NM_012919.3	FWD: 5'-CCAAATCTCAGGAGCCGGT-3' REV: 5'-GCAATACCAAGGCCAACTGT-3'	219
<i>Cacna2d2</i>	NM_175592.2	FWD: 5'-CTGCAGGTCAAGTTGCCAAT-3' REV: 5'-AGACGCGTTCCACTAACTGC-3'	262
<i>Cacna2d3</i>	NM_175595	FWD: 5'-TGGACGAGAGGCTGCTTTTG-3' REV: 5'-ATGTACGCTTCGGTCCACAC-3'	180
<i>Cacna2d4</i>	NM_001191751.1	FWD: 5'-ATCGCTTCGACTGCAGAAA-3' REV: 5'-CTCTCGGTTGTCTCGATCCG-3'	255

listed in Table 2 were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1) and hypoxanthine guanine phosphoribosyl transferase (HPRT1) (Gorzelniaik *et al.* 2001, Fink *et al.* 2008, Silver *et al.* 2008). Relative mRNA levels were quantified by the method of Pfaffl (Pfaffl 2001).

Western blot analysis

Cytosolic and solubilized plasma membrane proteins were prepared by differential centrifugation. Cell lysates were prepared at 4°C with a lysis solution that contained 10 µL of protease inhibitor cocktail per 1 mL of lysis buffer (10 mM Tris, 250 mM sucrose, 1 mM EDTA, pH 7.4). Protein content was determined by Lowry. 15–20 µg of

protein was resolved by SDS-PAGE on 4–20% gradient precast minigels TGX (BioRad) at 170 volts for 40 min.

Protein transfer onto nitrocellulose membranes was performed at 100 volts for 60 min in cold transfer buffer; Ponceau S staining confirmed transfer. Membranes were blocked in 5% milk in TBST (25mM Tris, pH 7.6; 125 mM NaCl; 0.1% Tween 20) for 3–4 h, at 20–22°C, followed by overnight incubation at 4°C with primary antibodies: rabbit anti-CaV1.3 at 1:200 (ACC-005, Alomone), anti CaV1.2 at 1:500 dilution (ACC-003, Alomone), anti-β-actin at 1:5000 and anti-Na,K-ATPase at 1:20,000 (Abcam). Membranes were washed with TBST and incubated for 1 h at room temperature with a 1:10,000 dilution of secondary antibodies: IRDye 800 CW goat anti-rabbit IgG (Li-COR™) and IRDye 680 CW goat anti-mouse IgG (Li-COR™). Antibody dilutions were made in 5% milk in TBST. Membranes were then washed with TBST, rinsed in H₂O and imaged with Li-COR Odyssey infrared Imaging System.

Band intensity was measured using the Li-COR Odyssey software, version 2.1 (Li-COR Bioscience, USA) and analyzed using Image Studio Lite, version 5.2. For each protein band the signal intensity was normalized to that of β-actin expression, which was then expressed relative to that in brain. In initial experiments GAPDH was used as a control, but since comparison of the ratios

Table 2 Primer sequences used for qPCR analysis.

Name	Sequence	Product size (bp)
Hprt1	FWD: 5'-CGAGGAGTCCTGTTGATGTTGC-3' REV: 5'-CTGGCCTATAGGCTCATAGTGC-3'	172
Pgk1	FWD: 5'-TAGTGGCTGAGATGTGGCACAG-3' REV: 5'-GCTCACTTCCTTTCTCAGGCAG-3'	166
GAPDH	FWD: 5'-GGCAAGTTCAATGGCACAGT-3' REV: 5'-TGGTGAAGACGCCAGTAGACTC-3'	183

obtained with that of β -actin expression showed no difference, the two set of data were pooled. For CaV1.2 in brain, bands spanning 130–250 kDa were combined before normalization to β -actin. Samples probed with the primary antibodies in the absence of primary antiserum indicated absence of non-specific binding.

Immunocytochemistry

Adipocytes were attached to poly-L-lysine (25–100 μ g/mL)-coated coverslips and fixed with 4% PFA for 10 min. Cells were permeabilised in blocking buffer (PBS with 3% BSA and 0.5% Triton-100x) for 10 min then stained with primary anti-Calcium Channel L-type α 1C subunit (Cacna1C) antibody conjugated with Atto 594 (ACC-003-AG, Alomone) at 1:200 dilution for 16 h (Raifman *et al.* 2017). To visualize nuclei cells were stained with Hoechst 33342 (8 μ M for 30 min). Positive controls were initially performed with pancreatic beta-cell that express CaV1.2 (Schulla *et al.* 2003). Images were captured on a Zeiss LSM880C confocal microscope with excitation wavelengths of 405nm for Hoechst33342 and 633 nm for the anti-CaV1.2-ATTO antibody.

Measurement of $[Ca^{2+}]_i$

Adipocytes attached to coverslips were incubated with the Ca^{2+} fluorophore Fluo-4 AM (1 μ M; Molecular Probes) in Hank's solution with 0.01% BSA for 30 min at 21–23°C in the dark. Coverslips were mounted in a perfusion chamber on an Axiovert 135 Inverted microscope equipped for epifluorescence (Carl Zeiss Ltd). Cells were focused to maximize equatorial circumference and fluorescence. Adipocytes were identified under Kohler illumination as 50–100 μ m diameter spheroids with a nuclear protuberance (Fig. 3). Experiments were performed 1–4 h post isolation.

Fluo-4 was excited at 450–490 nm, the emitted light band-pass filtered at 515–565 nm and the signal detected using a Photonics Science ISIS-3 camera with image intensification (luminous gain 8000:1). Fluorescence emission was integrated for 900 ms and captured at 1 Hz with an 8-bit frame grabber (DT3155, Data Translation, Basingstoke, UK) and Imaging Workbench, ver. 6.0 software (Indec Biosystems, Santa Clara, CA, USA). Cells were perfused at 1 mL/min. Since dye extrusion occurred at temperatures $>30^\circ\text{C}$, experiments were performed at 27–28°C.

For data analysis, a region of interest (ROI) was drawn around each cell, background corrected and the time course of its mean fluorescent intensity was calculated. Fluorescence was calibrated by a two-point method (Ni *et al.* 1994):

the maximum fluorescence value, $F_{\max}$; determined by permeabilization of the cells with Triton X-100 (0.0125–0.1%) followed by 10 mM EGTA to determine the minimum, $F_{\min}$. $[Ca^{2+}]_i$ was calculated with the equation:

$$[Ca^{2+}]_i = K_d \times \frac{(F - F_{\min})}{(F_{\max} - F)}$$

where F is the background corrected fluorescence, and K_d the dissociation constant of Fluo-4: 345 nM (Bentley *et al.* 2014). Over 75% of basal $[Ca^{2+}]_i$ values measured were below the K_d of the dye and thus within its linear region of sensitivity. Data were only used from cells in which $[Ca^{2+}]_i$ was stable and had a basal value within the 5–95% percentile range (60–380 nM).

To visualize spatial variation of $[Ca^{2+}]_i$ it is necessary to mitigate fluorescent signal heterogeneity due to uneven dye loading or/and differences in columnar volume. In the absence of extracellular Ca^{2+} , Ca^{2+} influx is abolished and heterogeneity in fluorescence was assumed to reflect variation in only cell volume and dye loading. Since these parameters proportionally affect Fluo-4 fluorescence, a ratio of the fluorescence signal in the presence of extracellular Ca^{2+} with that in its absence was undertaken using ImageJ2 with floating point arithmetic (Rueden *et al.* 2017) to normalize these confounders to reveal true differences in spatial $[Ca^{2+}]_i$.

Biochemical assays

To control for differences in adipocyte cell-density, biochemical data were normalized to paired values measured under basal or control conditions. Experiments were performed at 37°C.

Free fatty acid assay

Free fatty acid (FFA) release was used to measure lipolysis. Adipocytes were incubated for 60 min under different experimental conditions in 1 mL of Hank's. After which, 100 μ L of supernatant was removed and stored at -20°C prior to assay. FFA was assayed with a non-esterified FFA assay kit (WAKO chemicals). After subtraction of blank, data were normalized to the absorbance of the FFA standard (17 μ M oleic acid) and corrected for dilution.

Glucose uptake

Glucose utilization was measured via ^{14}C glucose uptake. After a 30-min pre-incubation under different

experimental conditions, ^{14}C glucose was added and the cells were incubated for a further 30 min, final volume 0.5 mL. For the assay, cells were separated from the medium by centrifugation and their ^{14}C content measured via scintillation counting. The GLUT-dependent glucose uptake was determined by subtraction of non-specific uptake measured in the presence of 10 μM Cytochalasin B. For analysis, ^{14}C uptake was normalized to that measured in the absence of insulin and drug additions under control conditions.

Lipogenesis

Lipogenesis was measured via $3\text{-}^3\text{H}$ -glucose incorporation. Adipocytes were incubated for 30 min under different experimental conditions with a fixed activity of $3\text{-}^3\text{H}$ -glucose. For the assay, cells were separated from the medium by centrifugation and the ^3H content determined via scintillation counting. Lipogenesis was determined as the difference of cellular counts to that measured in the absence of $3\text{-}^3\text{H}$ -glucose. For analysis, lipogenesis was normalized to that measured in the absence of insulin and drug additions under control conditions.

Justification of drug concentrations employed

Verapamil and nifedipine were used at concentrations employed by others to block L-type VGCCs in this cell type (Martin *et al.* 1975, Begum *et al.* 1992, Ni *et al.* 1994). Moreover, concentrations were employed to mitigate their adsorption by serum (>90%) (Rumiantsev *et al.* 1989) and absorption by the adipocytes (Louis *et al.* 2014).

Statistical analysis

Data were checked with the D'Agostino and Pearson omnibus normality test with the appropriate inferential test given in the text. Graphical data are shown as box and whisker plots as median, interquartile range, 10, and 90% confidence intervals. Unless otherwise stated, experiments were performed as repeated measures. Numerical data are quoted as means $\pm$ S.E.M. or median with 5–95% confidence intervals (95% C.I.), where n is the number of determinations. Experimental data were collated from at least four animals. Fitting of equations to data used a least squares algorithm with the parameters given in text. Statistical analysis was performed using Graphpad PRISM, version 8.2. Data were considered statistically significant difference when $P < 0.05$ and in graphics is flagged as *, ** when $P < 0.01$, *** when $P < 0.001$ and **** when $P < 0.0001$.

Results

Molecular evidence and identification of VGCC in WAT

RT-PCR indicated the presence of mRNA for CaV1.1, CaV1.2 and CaV1.3 but not CaV1.4 L-type α_1 subunits in epididymal WFA (Fig. 1A); mRNA for L-type VGCCs β_2 subunits: *Cacnb2*, *Cacnb3* and *Cacnb4*, and $\alpha_2\delta$ subunits, *Cacna2d1*, *Cacna2d2* and *Cacna2d3* were also detected. cDNA sequence analysis of the PCR products for CaV1.1, CaV1.2 and CaV1.3 gave 98.7 ± 0.3 ($n=3$), $98.5 \pm 0.2\%$ ($n=6$) and $97.8 \pm 0.7\%$ ($n=6$) identity to the rat VGCC subunits $\alpha_1\text{S}$ (*Cacna1s*), $\alpha_1\text{C}$ (*Cacna1c*) and $\alpha_1\text{D}$ (*Cacna1d*) respectively. Quantification of mRNA levels by qPCR gave a rank expression order of CaV1.2 > CaV1.3 > CaV1.1 (Fig. 1B).

Western blots of the plasma membrane fraction revealed a 260 kDa CaV1.3 α -subunit (Fig. 2B) (N'Gouemo *et al.* 2015) and an extended form of CaV1.2 of >250 kDa (Fig. 2A); the latter bigger than the canonical neuronal isoform of 210 kDa (Raifman *et al.* 2017). CaV1.2 protein expression was 10-fold greater than that of CaV1.3 (Fig. 2C; $P < 0.001$, Mann–Whitney); a ratio comparable to the qPCR results (Fig. 1B).

Figure 1

CaV1.1, 1.2 and 1.3 mRNA are expressed in rat adipocytes. (A) RT-PCR products (*Rattus norvegicus*) of voltage-dependent L-type calcium channels CaV1.1, CaV1.2, CaV1.3 and CaV1.4 α_1 (*Cacna1s*, *Cacna1c*, *Cacna1d* and *Cacna1f*), β_2 (*Cacnb1*, *Cacnb2*, *Cacnb3* and *Cacnb4*) and $\alpha_2\delta$ (*Cacna2d1*, *Cacna2d2*, *Cacna2d3* and *Cacna2d4*) subunits for white fat adipocytes and for control: skeletal muscle for *Cacna1s* (CaV1.1) and whole brain for all other genes; L, DNA 50 bp ladder. PCR product sizes are in Table 1. (B) Relative expression of CaV1.1, CaV1.2 and CaV1.3 α_1 subunits as determined by qPCR. Data are normalized to mRNA expression of CaV1.2 in rat brain. Each point represents a different animal ($n = 10$), horizontal line is mean. Statistical significance is by one-way ANOVA, with Tukey's multiple comparison test.

Figure 2

White fat adipocytes express CaV1.2 and CaV1.3 protein in membrane fractions. Representative Western blots of CaV1.2 (A) and CaV1.3 (B) in adipocyte cell lysate fraction (ACF) and membrane fraction (AM) of white fat adipocytes. Note the larger molecular weight (MW) of the CaV1.2 protein in adipocytes: >250 kDa compared to the proteolytic cleavage product of 210 kDa in the whole brain cell lysate (BCL) positive control. (C) Relative protein expression of CaV1.2 ($n = 5$) to CaV1.3 ($n = 9$) in membrane fractions from white fat adipocytes. Data normalized to β -actin. Horizontal lines are the medians. Statistical significance is by Mann–Whitney.

Immunohistochemical evidence of VGCC in WAT

Confocal microscopy revealed that fixed adipocytes preserved their morphology (Fig. 3A) as demonstrated by retention of their nucleus and spherical form. Atto 594-labelled antibodies identified CaV1.2 in the plasma membrane; this was granular in appearance and densest near the nucleus (Fig. 3B). Given this finding, the spatial distribution of $[Ca^{2+}]_i$ was examined (Fig. 3C). Figure 3C shows six adipocytes with identifiable nuclei, all of which responded with a reversible decrease in $[Ca^{2+}]_i$ on removal of bath Ca^{2+} (Fig. 5A). Although the change in $[Ca^{2+}]_i$ was uniformly distributed in the extra-nuclear cell membrane for all six cells, three had a higher $[Ca^{2+}]_i$ in the perinuclear region (Fig. 3D); data which support a higher density of VGCCs in this region.

Evidence for constitutive Ca^{2+} influx

Epifluorescent imaging of adherent adipocytes revealed a skewed basal $[Ca^{2+}]_i$ and Gaussian cell diameter distributions with median values of 135 nM (129–145 nM, 95% C.I., $n = 555$) and 80 μ m (79–81 μ m, 95% C.I., $n = 580$) respectively (Fig. 4A and B). $[Ca^{2+}]_i$ values are similar to those previously reported (Schwartz *et al.* 1991, Hardy *et al.* 1992, Ni *et al.* 1994, Gaur *et al.* 1998).

Both basal $[Ca^{2+}]_i$ (Spearman $r = 0.17$, 0.084–0.25 95% C.I., $P < 0.0001$, Fig. 4C) and body weight (Spearman

Figure 3

CaV1.2 in the plasma membrane. (A and B) Confocal images of fixed rat epididymal white fat adipocytes. (A) Blue, nucleus stained with Hoechst 33342; Magenta, Atto 594-labelled antibody to CaV1.2. Image captured over 62 s. (B) Magenta only channel to highlight CaV1.2 labelling which appears densest in the nuclear region. Arrows indicate associated nuclei. (C and D) Greyscale epifluorescent Ca^{2+} images of a field of six rat epididymal white fat adipocytes. (C) Adipocytes under basal conditions, arrows indicate nuclei. Note nuclear protuberances and brighter circumferential fluorescence where the cytoplasm has the deepest volume parallel to the plane of illumination. (D) Image shown in C is ratioed to that observed in the absence of extracellular Ca^{2+} to normalize dye loading and columnar volume. The brighter fluorescence in the perinuclear region of cells i, ii, and iii indicates a higher Ca^{2+} level. (C) and (D) are averages of 100 frames.

$r = 0.13$, 0.01–0.26 95% C.I., $P < 0.05$, Fig. 4D) were positively correlated with adipocyte diameter, although $[Ca^{2+}]_i$ did not correlate with individual animal weight (Spearman, Fig. 4E).

To explore Ca^{2+} influx, the effect of equimolar substitution of extracellular Ca^{2+} with Mg^{2+} was investigated. Removal of bath Ca^{2+} reversibly decreased $[Ca^{2+}]_i$, $\Delta[Ca^{2+}]_i$, by 30% (25–34%, 95% C.I.; $P < 0.0001$, Friedman; Fig. 5A and B). $\Delta[Ca^{2+}]_i$ was negatively correlated with basal $[Ca^{2+}]_i$; that is cells with the highest basal values underwent the largest percentage decrease on Ca^{2+} removal (Spearman $r = -0.32$, $P < 0.001$; Fig. 5C). $\Delta[Ca^{2+}]_i$ was unrelated to cell diameter or body weight (Spearman). Prolonged exposure to Ca^{2+} removal for over 3 h did not affect cell integrity which suggest that this intervention is not cytotoxic.

Doubling the extracellular Ca^{2+} concentration produced a 9% increase in $[Ca^{2+}]_i$ (4–16%, 95% C.I.;

Figure 4

Measurement of intracellular $[Ca^{2+}]_i$ in isolated adipocytes. (A) Distribution of basal $[Ca^{2+}]_i$ ($n = 588$). (B) Distribution of adipocyte diameters ($n = 547$). (C) Scatter plot of $[Ca^{2+}]_i$ versus cell diameter ($n = 495$). (D) Scatter plot of cell diameter for 233 adipocytes with each vertical data set taken from a given weighed animal (45 in total). Solid lines in (C) and (D) are drawn by linear regression with slopes of 0.65 ± 0.29 nM/ μ m ($P < 0.03$) and 0.058 ± 0.02 μ m/g ($P < 0.01$) respectively. Dotted lines are 95% C.I. for the fits shown. (E) Individual distributions of basal $[Ca^{2+}]_i$ for epididymal adipocytes from 13 different rats ($n = 7-40$). Note the variation in $[Ca^{2+}]_i$ within any given animal was greater than between animals (ANOVA).

$P < 0.0001$ Wilcoxon signed-rank test; Fig. 5D and E). Conversely, addition of 2.5 mM Co^{2+} , an inorganic inhibitor of VGCCs, irreversibly decreased $[Ca^{2+}]_i$ by 13% (8–17%, 95% C.I.; $P < 0.0001$ Wilcoxon signed-rank; Fig. 5D); a magnitude comparable to that seen with removal of extracellular Ca^{2+} (Fig. 5E).

Pharmacological manipulation of Ca^{2+} influx

We next tested a variety of pharmacological agents that affect VGCC activity. Although both verapamil and nifedipine significantly decreased $[Ca^{2+}]_i$ relative to DMSO control (Fig. 6) ($P < 0.001$ and $P < 0.0001$ respectively, Wilcoxon signed-rank test), the effect of nifedipine was small (Fig. 6B and F). Verapamil decreased $\Delta[Ca^{2+}]_i$ to a similar extent to Ca^{2+} removal (Kruskal–Wallis, Fig. 6F). In the presence of nifedipine Ca^{2+} removal further decreased $[Ca^{2+}]_i$ (Fig. 6B and G). In any given cell, the amount by which verapamil decreased $[Ca^{2+}]_i$ positively correlated with that seen with extracellular Ca^{2+} removal (Pearson $r = 0.77$, slope 0.66 ± 0.13 , $P < 0.001$; Fig. 6H).

We next explored the pharmacology of $[Ca^{2+}]_i$ recovery following re-addition of Ca^{2+} to the bath after its removal. Both verapamil (Fig. 7B, F and J) and nifedipine (Fig. 7C, G and J) significantly affected $[Ca^{2+}]_i$ recovery (Fig. 7A, E and J). The NCX inhibitor SN-6 neither affected basal $[Ca^{2+}]_i$ nor its recovery (Fig. 7H and J). BAY-K8644, an agonist of L-type VGCCs, significantly enhanced recovery (Fig. 7D, I and J).

L-type Ca^{2+} influx stimulates basal lipolysis

Since Ca^{2+} modulates the lipolytic cascade in WFA (Schimmel 1978, Izawa *et al.* 1983, Allen & Beck 1986), we investigated if L-type VGCCs affected lipolysis (Fig. 9). Isoprenaline stimulated lipolysis with a pEC_{50} of 7 (6.7–7.3, 95% C.I.) and Hill coefficient of 0.8 (0.43–1.2, 95% C.I.; Fig. 9A). At 10 μ M, isoprenaline stimulated lipolysis 9.8 ± 0.63 fold ($P < 0.001$, one sample t test; $n = 39$). Consistent with previous reports (Izawa *et al.* 1983, Allen & Beck 1986), removal of extracellular Ca^{2+} neither affected the EC_{50} nor Hill coefficient for isoprenaline, but decreased lipolysis by ~30% ($P < 0.0004$; Fig. 8A and C). Insulin inhibited the beta-adrenoceptor-stimulated lipolysis with a pEC_{50} of 9.4 (9.6–9.3, 95% C.I.; Fig. 9B).

Interventions that promoted Ca^{2+} influx: BAY-K8644 or elevation of $[K^+]_o$ (Bentley *et al.* 2014), stimulated basal, but not isoprenaline-stimulated, lipolysis (Fig. 8C and D). Interventions that decreased Ca^{2+} influx: removal of extracellular Ca^{2+} or verapamil inhibited both isoprenaline-stimulated and basal lipolysis (Fig. 8C and D). Insulin stimulated basal lipolysis (Fig. 8D), an effect consistent with its ability to elevate $[Ca^{2+}]_i$ (Clausen & Martin 1977). The capacity of 20 nM insulin to block beta-adrenoceptor-stimulated lipolysis was unaffected by interventions that affected $[Ca^{2+}]_i$ (Fig. 8E).

We checked if intracellular Ca^{2+} handling affected lipolysis. 1 μ M oxytocin, which mobilizes intracellular Ca^{2+} (Kelly *et al.* 1989), did not affect basal lipolysis

Figure 5

Extracellular Ca^{2+} removal decreases intracellular $[Ca^{2+}]_i$. (A) $[Ca^{2+}]_i$ time courses measured for six adipocytes within a single field in response to removal of bath Ca^{2+} (Ca-free), followed by 0.1% DMSO. (B) $[Ca^{2+}]_i$ in control (Ctrl), after removal of extracellular Ca^{2+} (Ca-free) and recovery (Wash) ($n = 138$). Statistical inference by Friedman with Dunn's multiple comparison tests. (C) Relationship between the decrease in $[Ca^{2+}]_i$, $\Delta[Ca^{2+}]_i$, on removal of bath Ca^{2+} and basal $[Ca^{2+}]_i$. Solid line drawn by linear regression with a slope of $-0.17 \pm 0.01\%/nM$ ($P < 0.0001$). Dotted lines are the 95% C.I. for the fit shown. (D) Mean time course of $[Ca^{2+}]_i$ for five adipocytes in response to 5 mM $CaCl_2$ (HiCa) followed by 2.5 mM $CoCl_2$ (Cobalt) added to the bath. (E) $\Delta[Ca^{2+}]_i$ responses to HiCa ($n = 17$), Cobalt ($n = 19$) and removal of extracellular Ca^{2+} (Ca-free; $n = 140$). Dashed line indicates no effect. Data are from seven animals. Statistical inference was by Kruskal–Wallis with Dunn's multiple comparison tests. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0493>.

(Fig. 8G), but consistent with others (Fain *et al.* 1997) inhibited isoprenaline-stimulated lipolysis (Fig. 8F). Neither store depletion with 1 μM thapsigargin nor increased cytosolic Ca^{2+} buffering with 10 μM BAPTA-AM affected isoprenaline-stimulated lipolysis (Fig. 8F); however, both treatments impaired basal lipolysis (Fig. 8G); actions consistent with the greater sensitivity of basal lipolysis to $[Ca^{2+}]_i$ compared to that stimulated by isoprenaline (Fig. 8C and D).

Although alpha-adrenoceptor activation mobilizes intracellular Ca^{2+} stores in WFA (Hardy *et al.* 1992), like others (Blackmore & Augert 1989, Seydoux *et al.* 1996), we did not see an increase in $[Ca^{2+}]_i$ with beta-adrenoceptor activation ($n = 7$).

Figure 6

Verapamil and nifedipine decrease the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. (A) Mean time course of $[Ca^{2+}]_i$ measured for 11 adipocytes within a single field in response to removal of bath Ca^{2+} (Caf), followed by 20 μM verapamil (V). (B) Mean time course of $[Ca^{2+}]_i$ measured for a field of five adipocytes in response to removal of bath Ca^{2+} (Caf) in the absence and then presence of 20 μM nifedipine (Nif). Note block by nifedipine is countered by a positive DMSO effect on fluorescence. (C) $[Ca^{2+}]_i$ in control (Ctrl), after removal of extracellular Ca^{2+} (Ca-free) and recovery (Wash) followed by 20 μM verapamil (Ver) ($n = 19$ from six animals). (D) $[Ca^{2+}]_i$ in control (Ctrl), after removal of extracellular Ca^{2+} (Ca-free) and recovery (Wash) followed by 20 μM nifedipine (Nif) ($n = 15$ from four animals). (E) $[Ca^{2+}]_i$ in control (Ctrl), after removal of extracellular Ca^{2+} (Ca-free) and recovery (Wash) followed by 0.1% DMSO ($n = 36$ from six animals). (F) Comparison of $\Delta[Ca^{2+}]_i$ produced by removal of bath Ca^{2+} (Ca-free), 20 μM nifedipine (Nif; $n = 15$) and 20 μM verapamil (Ver; $n = 19$). Data are from C and D and are corrected for DMSO effects shown in E ($n = 137$). (G) $[Ca^{2+}]_i$ in the continuous presence of 20 μM nifedipine (Nif1) where Ca^{2+} is removed from the bath (Nif Ca-free) and added back again (Nif2) ($n = 9$ from two animals). For C, D, E, F and G, statistical comparison between groups was by Kruskal–Wallis with Dunn's multiple comparison tests. (H) Relationship between $\Delta[Ca^{2+}]_i$ produced by 20 μM verapamil and removal of bath Ca^{2+} ($n = 21$). Solid line is linear regression of data with a slope of 0.66 ± 0.13 . The dotted lines are the 95% C.I. of the fit.

L-type Ca^{2+} influx does not affect glucose uptake or lipogenesis

Insulin at 2 nM maximally stimulated glucose uptake fivefold ($P < 0.001$, one sample *t* test) (Fig. 9A). Insulin-stimulated glucose uptake was unaffected by either 5 μM

Figure 7

Pharmacological exploration of Ca^{2+} recovery. (A, B, C and D) Representative mean time courses of $[\text{Ca}^{2+}]_i$ in response to removal of bath Ca^{2+} (Ca-free) followed by the addition of drugs as indicated. (A) 0.1% v/v DMSO; (B) 20 μM verapamil (V); (C) 20 μM nifedipine (Nif); (D) 10 μM BAY-K8644 (BAYK) all in Ca^{2+} free. (E) $[\text{Ca}^{2+}]_i$ in control (Ctrl), after removal of extracellular Ca^{2+} (Ca-free), after addition of 0.1% DMSO (Ca-fDMSO), and then re-addition of bath Ca^{2+} in the presence of DMSO ($n = 19$ from 12 animals). (F) $[\text{Ca}^{2+}]_i$ in control (Ctrl), after removal of extracellular Ca^{2+} (Ca-free), followed by 20 μM verapamil in Ca^{2+} free (Ca-fVer), then re-addition of bath Ca^{2+} in verapamil (Ver) ($n = 67$ from 12 animals). (G) $[\text{Ca}^{2+}]_i$ in control (Ctrl), after removal of extracellular Ca^{2+} (Ca-free), followed by 20 μM nifedipine in Ca^{2+} free (Ca-fNif), then re-addition of bath Ca^{2+} in nifedipine (Nif) ($n = 33$ from six animals). (H) $[\text{Ca}^{2+}]_i$ in control (Ctrl), after removal of extracellular Ca^{2+} (Ca-free), followed by 10 μM SN6 in Ca^{2+} free (Ca-fSN6), then re-addition of bath Ca^{2+} in SN6 (SN6) ($n = 18$ from five animals). (I) $[\text{Ca}^{2+}]_i$ in control (Ctrl), after removal of extracellular Ca^{2+} (Ca-free), followed by 10 μM BAY-K8644 in Ca^{2+} free (Ca-fBAYK), and then re-addition of bath Ca^{2+} in BAY-K8644 (BAYK) ($n = 18$ from eight animals). All data uncorrected for DMSO effect. (J) Percentage change in basal $[\text{Ca}^{2+}]_i$ after recovery on re-addition of bath Ca^{2+} with the various treatments as shown. Statistical comparison was by Friedman with Dunn's multiple comparison multiple comparison.

nifedipine or 1 μM BAY-K8644 (Fig. 9A and B); outcomes that were independent of insulin concentration. Lipogenesis was also maximally stimulated by 2 nM insulin (~ninefold, $P < 0.001$, one sample t test) (Fig. 9C and D). However, neither 5 μM nifedipine nor 1 μM BAY-K8644 significantly affected lipogenesis (Fig. 9C and D).

Figure 8

Extracellular Ca^{2+} -influx potentiates lipolysis. (A) Lipolysis as a function of isoprenaline in the presence (black circles) and absence (open circles) of bath Ca^{2+} . Solid lines are fits of the data with sigmoidal dose-response curves with parameters given in the text. Data means $\pm$ s.e.m. ($n = 4-5$). (B) Inhibition of lipolysis by insulin. Solid line is a fit of the data to dose-response curve with parameters given in the text. Data are means $\pm$ s.e.m. ($n = 5-9$). (C) Effects of interventions on lipolysis stimulated by 10 μM isoprenaline: Ca free, removal of bath Ca^{2+} ; Ver, 5 μM verapamil; Nif, 20 μM nifedipine; Ins, 20 nM insulin; BAYK, 1 μM BAY-K8644; HiK, 50 mM bath $[\text{K}^+]_o$ ($n = 6-16$). (D) Effects of interventions on basal lipolysis. Key as for C ($n = 6-16$). (E) Effects of interventions on beta-adrenoceptor-mediated lipolysis inhibited by 20 nM insulin: Key as for C ($n = 6-10$). (F) Effects of interventions on 10 μM isoprenaline-stimulated lipolysis: OXY, 1 μM oxytocin; THAP, 10 μM thapsigargin; BAPTA, 10 μM BAPTA-AM ($n = 9-21$). (G) Effects of interventions as shown in F on basal lipolysis ($n = 6-10$). DMSO the solvent for verapamil, nifedipine, and BAY-K8644 was without effect on lipolysis. Statistics are Wilcoxon sign test relative to 100%.

Discussion

Molecular evidence for L-type Ca^{2+} channels

Our Western blot, PCR and *in situ* immunolabelling data demonstrate expression of $\text{CaV}1.2$ and $\text{CaV}1.3$ L-type VGCCs in primary white fat adipocytes. The presence of $\text{CaV}1.2/\text{CaV}1.3$ in WFA is consistent with transcriptomic data published for human (Fagerberg *et al.* 2014) and mouse fat tissue (Yue *et al.* 2014); however, in contrast to these, we failed to detect $\text{CaV}1.4$. Although the 250 kDa $\text{CaV}1.2$ protein band went undetected in our brain tissue control we did detect its 210 kD proteolytic cleavage product (Buonarati *et al.* 2017, Shi *et al.* 2017). Using the same antibody, others have also detected just a 210 kDa $\text{CaV}1.2$ band (N'Gouemou *et al.* 2015). The 140 kDa band may relate to the 130 kDa or 150 kDa

Figure 9

Ca²⁺-influx via L-Type VGCC does not affect glucose uptake and lipogenesis. (A) Effect of 5 μ M nifedipine on glucose uptake. (B) Effect of 1 μ M BAY-K 8644 on glucose uptake. (C) Effect of 5 μ M nifedipine on lipogenesis. (D) Effect of 1 μ M BAY-K 8644 on lipogenesis. Drug additions are indicated by filled bars, controls by open bars, conditions are as indicated. Ins, insulin, glu, glucose. Data are all paired with $n = 5$ for each condition. Statistical inference by Friedman's test.

non-CaV1.2 epitope that this antibody recognized in CaV1.2-knockout mice (Bavley *et al.* 2017, Buonarati *et al.* 2017). Though adipocytes possessed a CaV1.2 immunopositive band, they did not show any further proteolytic cleavage products, data that suggest that this protein has post-translational modification with a polymorphic proteolytic cleavage site. For CaV1.3, we only obtained positive immunoblots with early batches of antibody, a recognized problem with commercial antibodies to CaV1.3 (Buonarati *et al.* 2017). In WFA, CaV1.3 was comparable in molecular weight to that observed in our brain controls and heart (268 kDa) (Le Scouarnec *et al.* 2008). The presence of mRNA for L-type VGCC α_1 , β_2 and $\alpha_2\delta$ subunits suggests that adipocytes have the capacity to traffic and assemble functional VGCCs (Dolphin 2016).

Constitutive Ca²⁺ influx through L-type Ca²⁺ channels

Our finding that both Co²⁺ and verapamil decreased [Ca²⁺]_i by similar amounts to Ca²⁺ removal is indicative of a Ca²⁺-influx pathway mediated by L-type VGCCs. This notion is reinforced by enhancement of Ca²⁺ influx by the L-type VGCCs dihydropyridine agonist BAY-K8644. Our data contrast to studies where neither nitrendipine nor verapamil affected [Ca²⁺]_i under basal conditions (Gaur *et al.* 1996a,b). One possible reason for this discrepancy is that we corrected for the effect of DMSO on fluorescence to reveal a block, whereas previous studies had not.

The fact that removal of bath Ca²⁺ had a larger effect in adipocytes with higher basal [Ca²⁺]_i suggests that basal [Ca²⁺]_i is set by the prevalent VGCC activity. These findings, combined with the ability of verapamil and nifedipine to prevent, and BAY-K8644 to enhance Ca²⁺ recovery, suggest that basal [Ca²⁺]_i in WFA is maintained by a constitutive Ca²⁺ influx via L-type VGCCs – an idea supported by impairment of ⁴⁵Ca²⁺ uptake in WFA by L-type VGCCs antagonists (Martin *et al.* 1975).

The resting membrane potential of primary white adipocytes, measured by ourselves (Bentley *et al.* 2014) and others (Ramírez-Ponce *et al.* 1990, Lee & Pappone 1997), is around –30 mV. As this voltage is within the activation range of L-type VGCCs (Xu & Lipscombe 2001) a 'window Ca²⁺ current' (Fleischmann *et al.* 1994) is expected. Constitutive L-type VGCC activity in electrically non-excitable cells is not unique; for example, it has been recorded at a similar V_m (–30 mV) in osteoclasts (Miyachi *et al.* 1990). To be constitutively active, inactivation of these VGCCs must be incomplete.

Although whole-cell voltage-clamp would have been ideal for electrophysiological characterization of the VGCCs we did not attempt this for technical reasons. First, the adipocyte cytoplasm is a relatively thin ~0.3 μ m layer wrapped around a lipid droplet of ~80 μ m diameter (Bentley *et al.* 2014); this creates a membrane time constant of 100's of ms (Bentley *et al.* 2014), compared to ~2 ms for a neuron (Coombs *et al.* 1956) of similar diameter (Henneman & Mendell 2010). This difference precludes voltage-clamping of VGCCs under physiological conditions due to space clamp considerations and 'voltage escape' (Armstrong & Gilly 1992). Secondly, although Ca²⁺ influx can affect [Ca²⁺]_i, we have previously shown that it is too small to affect V_m (Bentley *et al.* 2014), a result indicative of low channel density. Indeed, ⁴⁵Ca²⁺ tracer studies (Martin *et al.* 1975) have measured the DHP-sensitive Ca²⁺ influx in WFA at ~0.024 pmol/s/cm²: ~1.5 amol/s/cell or ~0.3 pA of whole-cell inward Ca²⁺ current, a value too small to measure with whole-cell voltage clamp.

Constitutive Ca²⁺ influx modulates lipid metabolism

Our data confirm that both basal and stimulated lipolysis requires extracellular Ca²⁺ (Bleicher *et al.* 1966, Ziegler *et al.* 1980, Allen & Beck 1986). However, we now show that lipolysis is sensitive to agents that modulate L-type VGCC activity. Although, isoprenaline-stimulated lipolysis could not be enhanced by interventions that increase [Ca²⁺]_i, presumably because it was already maximal, like basal lipolysis, it was impaired by verapamil and Ca²⁺ removal.

The idea that Ca^{2+} -influx promotes lipolysis is also supported by the action of high K^+ , where substitution of bath Na^+ with K^+ elevates $[\text{Ca}^{2+}]_i$ by reverse Na^+ - Ca^{2+} exchange (Bentley *et al.* 2014).

Multiple targets exist for extracellular Ca^{2+} during beta-adrenoceptor-stimulated lipolysis. Extracellular Ca^{2+} enhances beta-adrenoceptor-stimulated cAMP production (Ziegler *et al.* 1980) and is also required for undefined lipolytic processes downstream of cytosolic cAMP (Allen & Beck 1986). Indeed, lipolysis can be potentiated by increasing $[\text{Ca}^{2+}]_i$ with Ca^{2+} ionophores in the absence of cAMP elevation (Gaion & Krishna 1982) or conversely, as shown here, depressed with Ca^{2+} chelators (Efendić *et al.* 1970). However, the exact mechanisms by which Ca^{2+} , and indeed Ca^{2+} influx modulates basal lipolysis remains unknown; it is unlikely to involve the Ca^{2+} -sensitive, isoform III, of adenylyl cyclase found in WFA (Wang *et al.* 2009) since changes in Ca^{2+} influx do not affect adipocyte cAMP levels under basal conditions (Ziegler *et al.* 1980). However, adipocyte lipoprotein lipase is positively modulated by Ca^{2+} (Efendić *et al.* 1970, Soma *et al.* 1989, Carmen & Víctor 2006).

Translational context

The idea that Ca^{2+} influx via $\text{CaV}1.2/\text{CaV}1.3$ regulates basal lipolysis agrees with translational data. Rats with chronic renal failure are hyperlipidaemic and possess WFA with elevated intracellular Ca^{2+} levels (Ni *et al.* 1995). The observation that verapamil reversed these phenomena suggests that exacerbated Ca^{2+} -influx via L-type VGCCs may be responsible for the elevated basal lipolysis. This notion is also supported by the ability of nifedipine to decrease plasma levels of FFA in spontaneously hypertensive rats (Cignarella 1994). Moreover, in humans, verapamil can also decrease basal plasma FFA levels, again data suggestive of VGCC-mediated Ca^{2+} -influx-dependent lipolysis (Hvarfner *et al.* 1988). Such effects do not arise through impairment of beta-adrenoceptor activation since antihypertensives such as nifedipine and verapamil actually promote catecholamine levels through activation of baroreflex-mediated sympathetic output. The widespread failure of the DHP L-type channel antagonists to affect plasma FFA in man can be reconciled by the binding of these drugs to $\text{CaV}1.2/1.3$ being compromised by FFA (Pepe *et al.* 1994).

Conclusion

Our study provides direct, corroborative evidence for the existence of $\text{CaV}1.2/\text{CaV}1.3$ L-type voltage-gated Ca^{2+}

channels in white adipocytes. The basal concentration of intracellular Ca^{2+} appears to reflect the ambient level of VGCC activity on an individual cell basis, apparently unrelated to adipocyte size or animal weight. Importantly, $\text{CaV}1.x$ channels contribute to a persistent state of Ca^{2+} influx in WFA, without the need for cell excitability, and appear to have a key role in lipolysis. Consequently, dysregulation of $\text{CaV}1.x$ in WFA may contribute to lipid storage disorders that may contribute to, or indeed precipitate, the metabolic syndrome. As such, these cation channels may be a potential therapeutic target for the treatment of hyperlipidaemia, peripheral insulin resistance and obesity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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