Etiology of the membrane potential of rat white fat adipocytes

Donna C. Bentley, Pawitra Pulbutr, Sue Chan, and Paul A. Smith

School of Life Sciences, University of Nottingham, Nottingham, United Kingdom

Submitted 15 August 2013; accepted in final form 20 May 2014

Bentley DC, Pulbutr P, Chan S, Smith PA. Etiology of the membrane potential of rat white fat adipocytes. Am J Physiol Endocrinol Metab 307: E161–E175, 2014. First published May 20, 2014; doi:10.1152/ajpendo.00446.2013.—The plasma membrane potential (Vm) is key to many physiological processes; however, its ionic etiology in white fat adipocytes is poorly characterized. To address this question, we employed the perforated patch current clamp and cell-attached patch clamp methods in isolated primary white fat adipocytes and their cellular model 3T3-L1. The resting Vm of primary and 3T3-L1 adipocytes were −32.1 ± 1.2 mV (n = 95) and −28.8 ± 1.2 mV (n = 87), respectively. Vm was independent of cell size and fat content. Elevation of extracellular K+ to 50 mM by equimolar substitution of bath Na+ did not affect Vm, whereas substitution of bath Na+ with the membrane-impermeant cation N-methyl-D-glucamine−-hyperpolarized Vm by 16 mV, data indicative of a nonselective cation permeability. Substitution of 133 mM extracellular Cl− with glucose-depolarized Vm by 25 mV, whereas Cl− substitution with I− caused a −9 mV hyperpolarization. Isoprenaline (10 μM), but not insulin (100 nM), significantly depolarized Vm. Single-channel ion activity was voltage independent; currents were indicative for Cl− with an inward slope conductance of 16 ± 1.3 pS (n = 11) and a reversal potential close to the Cl− equilibrium potential, −29 ± 1.6 mV. Although the reduction of extracellular Cl− elevated the intracellular Ca2+ of adipocytes, this was not as large as that produced by elevation of extracellular K+. In conclusion, the Vm of white fat adipocytes is well described by the Goldman-Hodgkin-Katz equation with a predominant permeability to Cl−, where its biophysical and single-channel properties suggest a volume-sensitive anion channel identity. Consequently, changes in serum Cl− homeostasis or the adipocyte’s permeability to this anion via drugs will affect its Vm, intracellular Ca2+, and ultimately its function and its role in metabolic control.

adipocyte; white fat; 3T3-L1 cells; chloride; membrane potential

THE PLASMA MEMBRANE POTENTIAL (Vm) is a fundamental biological property for the survival and function of virtually every eukaryotic cell. In excitable tissues of animalia, such as the heart, muscles, and nerves, excursions in Vm in the form of action potentials represent the fastest known biological means of intraorganism communication. The action potential often acts to mediate intracellular signaling; for example, an associated influx of extracellular Ca2+ controls functions as diverse as contraction to secretion. In nonexcitable tissues, subtle changes in Vm are involved in the control of the transmembrane and transcellular fluxes of many solutes. Moreover, the membrane potential per se also has an important role to play in basic cell homeostasis: the regulation of intracellular ionic composition and the maintenance of osmotic equilibrium. A thorough understanding of the etiology of Vm permits us to accurately control it experimentally, a process that allows us to investigate associated physiological and pathological sequelae. Virtually all types of mammalian cell possess a negative membrane potential, ranging from −8 mV in red blood cells (26) to in excess of −90 mV in skeletal muscle (23). The resting membrane potential is a thermodynamic steady-state situation where there is no net flux of electrical charge across the plasma membrane. The Vm arises through a complex biological calculation performed at the level of the plasma membrane that involves the electrochemical ion gradients, transmembrane ion permeabilities, ion exchange, and electrogenic pump processes (22). For most cell types, the major ion species involved in control of the resting membrane potential are K+, Na+, Ca2+, and Cl−, with the selective ionic permeabilities and related molecular components, such as ion pumps and exchanges, of the plasma membrane reflecting this.

White fat cells are no exception to the possession of a Vm, although white fat adipocytes are reported to possess a resting membrane potential of between −17 and −69 mV (Table 1); although its exact ionic etiology is still undefined due to various shortfalls in the previous methodologies employed for its study (6, 7). Consequently, the assumption that the adipocyte Vm arises primarily due to a predominant plasma membrane permeability to K+ (6, 7) has led workers to use protocols where it is assumed that manipulation of the extracellular concentration of K+ will modulate adipocyte Vm and affect membrane transport processes, in particular the transmembrane flux of Ca2+ (15, 17, 29, 36, 50). Although such manipulations may indeed increase intracellular Ca2+ (29), this effect may not necessarily arise via depolarization of the plasma membrane and voltage-gated Ca2+ influx; an increase in intracellular Ca2+ can also arise through osmotic shrinkage, reverse-mode Na+/Ca2+ exchange, or necrosis. The possible inappropriate use of high extracellular K+ to elevate intracellular Ca2+ is especially pertinent when interpreting previous studies that have employed this methodology to investigate the role of intracellular Ca2+ signaling in the physiological functions of white fat adipocytes: insulin-stimulated glucose uptake and lipogenesis (29), β-adrenoceptor-mediated lipolysis (17, 50), and secretion of various adipokines such as leptin, adiponectin, and resistin (51), especially pertinent events given the clinical importance of understanding the function and physiology of white fat adipocytes in diabetes.

The aim of this study was to characterize the ionic mechanism of the Vm in unequivocally identified white fat adipocytes. In doing so, we sought to provide values of Vm for future reference. Furthermore, these data, when combined with published radioisotope ion flux data, allow quantification of the major ionic permeabilities of the adipocyte and provide a clearer picture of ion transport processes across the plasma membrane of this cell type. Moreover, changes that may occur on the Vm of these cells produced by the two main regulators of adipocyte function, insulin and epinephrine, can be identified and characterized. Finally, we aimed to determine robust
protocols that are appropriate for experimental modulation of adipocyte Vm and intracellular Ca\(^{2+}\).

**MATERIALS AND METHODS**

For this study, we used primary adipocytes isolated from epididymal fat pads and 3T3-L1 adipocytes, a well-established differentiated fibroblastic model of white fat (20, 21).

**Preparation of primary white fat adipocytes.** Primary adipocytes were isolated from the epididymal fat pads (41) of both Wistar and Sprague-Dawley strains of rat. Male rats, 250–340 g, fed ad libitum (Charles River Laboratory, Kent, UK) were killed by cervical dislocation after stunning in accordance with UK Home Office guidelines. The epididymal fat pads were excised, the epididymis and major blood vessels expurgated, and the fat pads minced with scissors. Adipocytes were liberated from the mince by 6- to 8-min collagenase digestion: 1 mg/ml Type II collagenase (Sigma cat. no. C6885) in Hank’s solution (0.5% BSA wt/vol) at 37°C with mild agitation. The digest was filtered through a 250 \(\mu\)M nylon mesh (Normesh, Oldham, UK), followed by 50 ml of Hank’s solution, and the filtrate was collected in an inverted 50-ml syringe. Adipocytes separated from the buffer by flotation to form a white layer on top of the filtrate, whereas cell debris and other cell types precipitated out. The infranatant and other cell types were removed by drainage, and the separation process was repeated twice more by further buffer additions and drainage. After the final drain, the adipocytes were resuspended in Hank’s solution and kept at room temperature (21–22°C) prior to use. For experiments, adipocytes were plated onto glass coverslips coated with poly-D-lysine (100 \(\mu\)g/ml) for 2 days in the differentiation medium but without dexamethasone and IBMX. Finally, the differentiated cells were maintained up to 5 days in the differentiation medium but with no supplements.

**3T3-L1 oil red O hematoxylin counterstain.** To confirm the presence and accumulation of lipid droplets following cell differentiation, we used Oil red O and hematoxylin counterstaining. Cells were washed with phosphate-buffered saline and then fixed in 10% (vol/vol) formalin. Oil drops were then stained by incubation in 2 mg/ml Oil red O in 60% (vol/vol) isopropanol. To visualize the nuclei, cells were counterstained with hematoxylin. Cells were observed and images taken using a phase contrast microscope at \(\times 10\) magnification.

**Electrophysiology.** For electrophysiology standard patch-clamp methods were used.

**PERFORATED PATCH STUDY OF PLASMA MEMBRANE POTENTIAL.** Membrane potential measurements were made using the perforated patch configuration of the whole cell patch clamp technique in current clamp mode. This technique has the advantage over conventional whole cell in that it maintains cellular metabolism and intracellular ionic composition, prevents ion channel rundown, and imparts little or no osmotic or shear stress on the studied cell (42). The pipette solution comprised (in mM): 76 K\(_2\)SO\(_4\), 10 KCl, 10 NaCl, 55 sucrose, and 10 HEPES (pH 7.4 with NaOH). For perforation, pipettes were backfilled with a solution that also contained 0.1 \(\mu\)g/ml of the polyene antibiotic amphotericin. Perfusion was considered adequate when the series resistance (Rs) was stable and less than 35 M\(\Omega\) as determined by electronic compensation. For the 3T3-L1 adipocytes, R\(_s\) and cell capacitance were 32 \(\pm\) 1 M\(\Omega\) and 15.6 \(\pm\) 1.3 \(\mu\)F (n = 47), respectively. Unfortunately, due to the large diameters of the primary adipocytes measured in these experiments as 75.2 \(\pm\) 1.3 \(\mu\)m, their cell capacitance could not be compensated by the amplifier (Axopatch 1D; Axion Instruments, Molecular Devices).

The input resistance (R\(_{in}\)) of the cells was monitored by the steady-state change in membrane potential (\(\Delta\text{Vm}\)) in response to steps of injected current (\(\Delta I\)) of up to \(\pm\) 40 pA applied for 0.1–20 s duration (12). R\(_{in}\) was calculated as the slope of the relationship between \(\Delta\text{Vm}\) and \(\Delta I\) at Vm. Cells with Vm’s depolarized to \(-\)10 mV were excluded from the analyses to prevent bias from leaky or damaged cells as also indicated by a low R\(_{in}\) values \(<\)100 M\(\Omega\).

**CELL-ATTACHED STUDY OF SINGLE ION CHANNEL ACTIVITY.** To investigate the properties of the ion channels responsible for Vm, cell-attached patch clamp experiments were performed. To reduce electrical capacitance and improve noise, pipettes were coated at their tips with dental wax (Sticky Wax, Kerr). To maximize discovery of either Cl\(^{-}\) or NS ion channels, the pipette solution comprised (in mM) 140 KCl, 1.2 MgCl\(_2\), 2.6 CaCl\(_2\), and 10 HEPES (pH 7.4 with NaOH; 147 mM Cl\(^{-}\)). Pipettes had resistances of 3–5 M\(\Omega\). Membrane currents were recorded with an Axopatch 1D patch clamp amplifier (Axon instruments, Molecular Devices), filtered by an 8-pole Bessel filter at 0.5–2 kHz, and digitized at 5 kHz. Since most patches had a high level of channel activity, often with channel substates, single-channel amplitudes were measured by cursors with the median amplitude used for further analyses to overcome misclassification of temporally coincident openings. Voltage dependency of channel-open probability was estimated from all-point histograms. Inward currents are displayed as downward deflections by convention and potentials as membrane potential: Vm = Vr – Vp, where Vr is the resting membrane potential (assumed to be \(-30\) mV) and Vp the pipette voltage.

**Intracellular Ca\(^{2+}\) imaging and analyses.** The concentration of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) was monitored with Fluo 4 using standard epifluorescent imaging techniques. Adipocytes were loaded with dye by incubation in Hank’s solution containing 1 \(\mu\)M Fluo 4-AM for 30 min in the dark at 21–23°C. Loaded cells were imaged using an inverted Axiovert 135TV microscope fitted with \(x\times 10\) FLUO objective (Carl Zeiss, Welwyn Garden City, UK). To monitor [Ca\(^{2+}\)]\(_i\), cells were continuously illuminated at an excitation wavelength of 450–490 nm. The emitted light was bandpass filtered at 515–565 nm and

**Table 1. Measurement of membrane potential in cells of rat epididymal fat pads**

<table>
<thead>
<tr>
<th>Vm (mV)</th>
<th>Technique</th>
<th>Preparation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>-29</td>
<td>(^{36})Cl distribution</td>
<td>Isolated cells</td>
<td>33</td>
</tr>
<tr>
<td>-17 to -69</td>
<td>Microelectrode</td>
<td>Explant</td>
<td>5</td>
</tr>
<tr>
<td>-27 to -29</td>
<td>Microelectrode</td>
<td>Explant</td>
<td>7</td>
</tr>
<tr>
<td>-46</td>
<td>Microelectrode</td>
<td>Explant</td>
<td>44</td>
</tr>
<tr>
<td>-34</td>
<td>Microelectrode</td>
<td>Explant</td>
<td>39</td>
</tr>
<tr>
<td>-30</td>
<td>Microelectrode</td>
<td>Isolated cells</td>
<td>28</td>
</tr>
</tbody>
</table>

For these studies, isolated cells, cells in intact segments, or explants of epididymal fat pads were used as indicated.
the signal detected using a Photonics Science ISIS camera with image
intensification. Images were captured at 1 Hz with an 8-bit frame
grabber (DT3155; Data Translation, Basingstoke, UK) and Imaging
Workbench software (Indec Biosystems, Santa Clara, CA). For image
analysis, a region of interest (ROI) was drawn around each cell, the
background fluorescence subtracted, and the time course of its mean
fluorescent intensity calculated. For some experiments, the fluores-
cence changes were calibrated by a two-point method: the maximum
fluorescence (Fmax), determined by permeabilization of the cells with
Triton X-100 (0.0125–0.1% vol/vol) was followed by perifusion of 10
mM EGTA to determine Fmin, the minimum fluorescence. To calcu-
late [Ca^{2+}], we used the following equation:
\[
[Ca^{2+}] = K_d \times \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)}
\]
where F is the background corrected fluorescence, and Kd is the
dissociation constant of Fluo 4 (345 nM, Molecular Probes). For other
experiments, fluorescence changes were left uncalibrated, with basal
values taken as 100%. Adipocytes were perfused for at least 10 min
before experimental intervention, with the time-averaged fluorescence
intensity over this period taken as the basal level. We assumed that the
dye was predominantly cytosolic in location.

**Solutions.** All experiments were performed under continuous per-
fusion in a HEPES-buffered Hank’s solution that contained (in mM):
10 glucose, 138 NaCl, 4.2 NaHCO3, 1.2 Na2HPO4, 5.6 KCl, 1.2
MgCl2, 2.6 CaCl2, 10 HEPES (pH 7.4 with NaOH), and 0.01% (wt/vol)
fatty acid-free bovine serum albumin (BSA) at 32°C for perforated patch (the highest temperature at which stability was
maintained), at room temperature (22–25°C) for single-channel re-
cording (to slow gating kinetics and aid comparison with previously
published biophysical data), and at 28°C for Ca^{2+} imaging (to limit
the dye extrusion that occurred at higher temperatures). For the Li+
experiments, 138 mM NaCl in the Hank’s solution was replaced with
138 mM LiCl, whereas for the Γ– experiments 138 mM NaCl was
replaced with 138 mM NaI. In the ion substitution experiments, we
corrected for the liquid junction potential (LJP) change that occurs at
the reference potential. LJPs were measured experimentally following
the methods as detailed in Smith et al. (43), and Vm was adjusted
accordingly. Osmolarities were measured in triplicate with a Roelbling
MicroOsmometer 12 (Camlab, Cambridge, UK).

**Drugs and reagents.** All drugs and reagents were obtained from
Sigma. The relevant vehicles used for the drugs used are given in the
results.

**Statistical analyses.** Analyses were performed using Origin soft-
ware with bespoke scripts written in Labtalk (OriginLab).

All datasets were tested for normality with the D’Agostino and
Pearson omnibus normality test. Statistical analysis was performed
using Graphpad PRISM v. 6 (San Diego, CA), and the statistical test
used is given in the text. Data are given in the text either as means ±
SE or median with 5–95% confidence intervals (CI), with n the
number of determinations. To show the range and distribution of
values obtained, data are expressed in the figures as box and whisker
representations, which show the median, interquartile range, and 5 and
95% CIs. The fitting of equations to the data used a least squares
algorithm as supplied with PRISM. Statistical significance is defined
as P < 0.05 and is flagged as * in graphics, ** when P < 0.01, or ***
when P < 0.001.

**RESULTS AND DISCUSSION**

Identification of differentiated 3T3-L1 and primary adipocytes.
Figure 1 shows representative images of 3T3 fibroblasts both prior
to and at various stages of the differentiation process.
Differeniation of 3T3-L1 fibroblasts to adipocytes is charac-
terized by the accumulation of lipid droplets, clearly visible in
some cells by the presence of stain (Fig. 1B). As the cells
accumulate lipid, they become less flat and adopt a more
rounded shape (Fig. 1C). Our differentiated 3T3-L1 adipocytes
clearly adopted an appearance that is similar, but not identical,
to that of the mature adipocytes (20, 21). 3T3-L1 fibroblasts at
an intermediate stage of differentiation show multiple lipid
droplets and a centralized nucleus. Fully differentiated 3T3-L1
adipocytes are spherical and contain a singular coalesced fat
droplet (Fig. 1C, black arrows). Some cells within the popu-
lation failed to differentiate (Fig. 1C), a previously noted
phenomenon (21). Only fully differentiated single adipocytes
were selected for electrophysiological study.

Figure 2 illustrates a typical pattern of fluorescence observed
for primary adipocytes loaded with Rh-123. In this particular
field, four of five of the adipocytes viewed were evenly loaded
with dye and were considered suitable for patch clamp. The
fifth cell was excessively fluorescent; such cells were occa-
sionally observed, the reason for which was not explored and
they were not considered for patch clamp.

Resting membrane potential of adipocytes. Both the primary
and the differentiated 3T3-L1 adipocytes had median Vm
values that were not significantly different from each other at
−31 mV (−32 to −27 95% CI, n = 95) and −28 mV (−30 to
−24 95% CI, n = 87), respectively (P > 0.05, Mann-Whitney;
Fig. 3), data that suggest that under resting conditions the
plasma membranes of these two cell types possess common ion
transport properties. The Vm for the primary and differentiated 3T3-L1 adipocytes share a similar, negatively skewed frequency distribution (Fig. 3A), with median values almost identical to their mean values at $-32.1 \pm 1.2$ and $-28.8 \pm 1.2$ mV, respectively (Fig. 3B). The values of Vm reported here, using the perforated patch clamp technique, compare very favorably with those previously reported for adipocytes from rat epididymal fat pads measured with other techniques (Table 1), being almost identical to that directly measured in white fat adipocytes with sharp electrodes ($-34$ mV, 39) or identified isolated adipocytes with whole cell patch clamp ($-30$ mV, 28), as well as that estimated from the passive distribution of Cl$^-$ ($-29$ mV) based on an intracellular Cl$^-$ concentration, [Cl$^-$], of 43 mM estimated from 36Cl$^-$ efflux studies (34). Although, cell damage and current leakage across the pipette-cell seal may explain the spread of Vm toward depolarized values, in order to account for the more hyperpolarized Vm values, additional mechanisms are sought such as variation in ion permeability or intracellular ion concentration. Since the primary adipocytes had a resting Vm distribution similar to that of the 3T3-L1 adipocytes, the underlying biophysical mechanisms are likely to be common to both cell types. Furthermore, the similar distribution of Vm values between the two cell types excludes phenotypic heterogeneity as a source of variation in the primary cell data.

**Input resistance of adipocytes and its relationship to cell size.** Figure 4A shows representative measurements of Vm and Rin in a primary adipocyte under normal, control conditions and after equimolar substitution of 138 mM bath Cl$^-$ with I$^-$. The primary adipocytes had plasma membrane time constants in excess of 100 ms, values consistent with their large surface area (Fig. 4B). Rin varied with the ionic composition of the bath (Fig. 4B), a phenomenon explored later. Figure 4C illustrates that, under control conditions, both primary fat cells and differentiated 3T3-L1 adipocytes had similar median Rin at 0.6 G$\Omega$ (0.42 to 0.72 95% CI, n = 40) and 0.64 G$\Omega$ (0.37 to 0.78 95% CI, n = 38), respectively ($P > 0.95$, Mann-Whitney). These values of Rin are far larger than that previously reported with sharp electrodes (0.3 G$\Omega$, 39) but similar to those measured with standard whole cell patch clamp ($\sim 1$ G$\Omega$, 28).

Given that primary adipocytes, assumed to be perfect spheres, had a significantly larger cell surface area at 17,700 ± 613 $\mu$m$^2$ ($n = 47$) than 3T3-L1 adipocytes, at 4,190 ± 500 $\mu$m$^2$ ($n = 24$, $P < 0.001$, Mann-Whitney), suggests that primary adipocytes have one-quarter of the channel density as the cell line. In the primary adipocytes, Rin was negatively (Spearman $r = -0.4$, $P < 0.01$) correlated with cell diameter (Fig. 4D), data that suggest that larger adipocytes possess greater ion channel activity. Vm was negatively (Spearman $r = -0.48$, $P < 0.001$) correlated to Rin (Fig. 4E). Since Rin is clearly related to cell size (Fig. 4D) and Vm (Fig. 4C), it could be inferred that larger cells may possess a more depolarized Vm, although this did not seem to be the case for 46 cells for which cell diameter was measured, where Fig. 4F clearly shows that the magnitude of Vm appears to be independent of cell diameter, even though their Rin ($n = 31$) was still negatively correlated with cell diameter (Spearman $r = -0.42$, $P < 0.01$) and Vm (Spearman $r = -0.5$, $P < 0.01$). Due to the uncertainty of the exact cell geometry (Fig. 1C) of the differentiated 3T3-L1 adipocytes, they were not subjected to the above analyses.

**Ionic dependence of membrane potential.** Figure 5 shows the effect of extracellular ion composition on Vm for both types of adipocytes. Since K$^+$ ions have a major role to play in the
control of membrane potential for many classes of cell and also have been shown to affect both $V_m$ (6, 7) and $[Ca^{2+}]_i$ (36, 38) of white fat tissue, the effect of this ion on the membrane potential of adipocytes was first to be tested.

Elevation of the extracellular concentration of potassium ions ($[K^+]_o$) from 5.6 to 50 mM by equimolar substitution of bath $Na^+$ had no significant effect (paired $t$-test) on the membrane potential of either type of adipocyte, with a mean difference of $1 \text{ mV}$ in both cases (Fig. 5, A and B) (Friedman’s test), although others have reported the $V_m$ of white fat tissue to be affected by changes in extracellular $K^+$ (6, 7), an observation suggested to be limited to rats weighing less than 215 g (6), which generally have smaller adipocytes (14). Although this may be proposed as the reason why we failed to see an effect of $K^+$ on $V_m$, our data refute this idea, since we observed no correlation between $V_m$ and adipocyte diameter (Fig. 4F); furthermore, no such relationship was observed in the differentiated 3T3-L1 adipocytes, which were variable in size. One possible reason for this discrepancy in the $K^+$ ionic dependency of adipocyte $V_m$, is that in our study isolated adipocytes were clearly identified as such, whereas in previous studies of the ionic dependency of $V_m$ sharp electrodes were impaled blindly into white fat explants, where it was assumed that $V_m$ was recorded from adipocytes. This is a pertinent point, since only a narrow band of cytoplasm circumnavigates the lipid droplet (11, 49), whereas other cells of the adipose tissue, such as smooth muscle and endothelia of its vasculature, being larger, have a higher likelihood of actual impalement and have $V_m$ values quite similar to those reported for identified adipocytes; for example, the resting membrane potential of smooth muscle myocytes from a variety of arterial tissues range from $-32 \text{ mV}$ in pulmonary (3) to $-39 \text{ mV}$ in renal (8), whereas the resting $V_m$ for endothelial cells range from $-26 \text{ mV}$ from pulmonary artery (52) to $-43 \text{ mV}$ from aorta (9). Another possible reason for this discrepancy is that in previously published work (6), although not explicitly given, the
authors state the composition of their high \([K^+]\) solutions were the same as those used by Hodgkin and Horowicz (23). Inspection of these original methods (23) reveals that these contain varying or nominal \([Cl^-]\). In a sequential study (7), this problem was resolved, since \([Cl^-]\) was given; moreover, the same study went on to demonstrate that \([Cl^-]\) did indeed contribute to adipocyte \(V_m\), an anion we later show to be predominant in the control of adipocyte \(V_m\).

In fact, many other factors could pervade in cell suspensions and intact tissue that may affect ion channel behavior and are not present in our sanitized, perfused, single isolated cell paradigm, such as paracrine modulation and physical interaction.

As we (38) have previously demonstrated that white fat adipocytes have a background \(Ca^{2+}\) influx pathway, the effect of removal of this ion by substitution with equimolar \(Mg^{2+}\)

Fig. 5. Effect of different ions on the membrane potential of primary and 3T3-L1 adipocytes. Effect of changes in extracellular ion concentrations as indicated on the \(V_m\) of primary adipocytes (left) and 3T3-L1 adipocytes (right). Values represent individual measurement of \(V_m\) once a steady-state value was reached after bath exchange. Statistical significance was determined relative to initial ionic conditions via Wilcoxon. The number of experiments is explicitly represented by the number of before-after lines: 7–12 for each experimental paradigm.
was tested; no significant effect on \( V_m \) was detected (Fig. 5, C and D). These results indicate that the plasma membrane of both types of adipocyte do not appear to have a selective permeability to either K\(^+\) or Ca\(^{2+}\) ions or, if they do, the contribution of these ions to \( V_m \) is so small that our experimental paradigm does not have the statistical power to detect it. Since white fat adipocytes have a negative membrane potential, that appears to be independent of the concentration of [K\(^+\)]\(_o\), our data suggest that \( V_m \) must be predominantly controlled by the passive distribution of Cl\(^-\) ions. This hypothesis was tested by equimolar substitution of 134 mM bath Cl\(^-\) with the gluconate anion (Fig. 4). After correction for the change in LJP at the reference electrode (8.3 mV), a reduction in extracellular Cl\(^-\) from 152 mM (300 \( \pm \) 2.7 mOsm) to 18 mM (282 \( \pm \) 2.3 mOsm) resulted in a significant and reversible depolarization in \( V_m \) of +24 mV (18 to 36 mV, 95% CI, \( P < 0.002 \), Wilcoxon) and +26 mV (20 to 28 mV, 95% CI, \( P < 0.0002 \), Wilcoxon) for primary and 3T3-L1 differentiated adipocytes, respectively or, for pooled data, +25 mV (20 to 28 mV, 95% CI, \( P < 0.0001 \), Wilcoxon, \( n = 21 \)); from –28 mV (–33 to –25 mV, 95% CI) to –4.7 mV (–2.7 to –8.3 mV, 95% CI). These data are consistent with the work of Beigelman and Shu (7), who demonstrated that the \( V_m \) of white fat adipocyte has a Cl\(^-\) dependent component, as well as that of Hales and Perry (36), who suggest that \( V_m \) is solely Nernstian in behavior for Cl\(^-\) and is given by the diffusion potential for Cl\(^-\): \( E_{CI} \). If the latter is true, then we estimate from our values of \( V_m \) that [Cl\(^-\)]\(_i\) is 50 \( \pm \) 3 mM (\( n = 21 \)). However, the fact that on reduction of [Cl\(^-\)]\(_o\), \( V_m \) depolarized to a value that was significantly different from the \( V_m \) predicted (+26.0 \( \pm \) 1.6 mV) using our [Cl\(^-\)]\(_i\) estimate and a Nernstian behavior for Cl\(^-\) (\( P < 0.001 \), paired \( t \)-test) implies that other ionic permeabilities must also be involved in the generation of adipocyte \( V_m \).

To test for the contribution of monovalent cation permeability to the generation of \( V_m \), the majority of extracellular monovalent cations were removed via equimolar substitution of 138 mM bath Na\(^+\) with NMDG\(^+\) (N-methyl-d-glucamine; 291 \( \pm \) 0.3 mOsm), a large organic cation considered impermeant through monovalent cation channels (1). This ionic substitution resulted in a significant and reversible hyperpolarization in \( V_m \) for the primary and 3T3-L1 differentiated adipocytes of –7.2 mV (–28.2 to –4.7 mV, 95% CI, \( P < 0.008 \), Wilcoxon) and –16.7 mV (–34.7 to –9.7 mV, 95% CI, \( P < 0.008 \), Wilcoxon), respectively (Fig. 4, G and H). Since the magnitude of hyperpolarization was insignificantly different between the two cell types (\( P = 0.45 \), Mann-Whitney), data were pooled to give –16.2 mV (–21.7 to –7.2 mV, 95% CI, \( P < 0.0001 \), Wilcoxon). The fact that removal of Na\(^+\) but not its substitution with K\(^+\) affected \( V_m \) supports the existence of nonselective, monovalent cation permeability, \( P_{NS} \), in the control of adipocyte \( V_m \) with a \( P_{K}/P_{Na} \) ratio of –1.

**Exploration of Cl\(^-\) dependence of \( V_m \).** Since Cl\(^-\) appeared to be the predominant ion that controls the membrane potential for both types of adipocytes, the dependency of \( V_m \) on the external chloride concentration, [Cl\(^-\)]\(_o\), was explored further. Figure 6A shows typical changes in \( V_m \) recorded from a 3T3-L1 differentiated adipocyte in response to eight consecutive decrements in [Cl\(^-\)]\(_o\). A new stable \( V_m \) was reached within 1 min of each solution change. Complete recovery of the original resting value of \( V_m \) was achieved on return to 152 mM [Cl\(^-\)]\(_o\). The Goldman constant field model (22) was used to quantitate the relationship between ion concentration and \( V_m \) via the Goldman-Hodgkin-Katz membrane potential equation (GHK), with the assumption that electronic processes did not contribute to the generation of \( V_m \):

\[
V_m = -\frac{RT}{F} \ln \left[ \frac{P_{NS}[K^+]_o + P_{NS}[Na^+]_o + P_{CI}[Cl^-]_o}{P_{NS}[K^+]_o + P_{NS}[Na^+]_o + P_{CI}[Cl^-]_i} \right]
\]

where \( R \) is the universal gas constant (8.314 J·mol\(^{-1}\)·K\(^{-1}\)), \( T \) the absolute temperature (305K) and \( F \), Faraday constant (96.4 kC/mol). \([K^+]_o, [Na^+]_o, \) and \([Cl^-]_o\), are the intracellular and \([K^+]_i, [Na^+]_i, \) and \([Cl^-]_i\), the extracellular concentrations for potassium, sodium, and chloride, respectively. \( P_{NS} \) is the monovalent cation permeability (nonselective) and \( P_{Cl} \) is the permeability to Cl\(^-\). If we assume no contribution from divalent cations and the major passive permeability for monovalent cations is \( P_{NS} \), then the intracellular and extracellular cations can be lumped together and the relative permeabilities of Cl\(^-\) and monovalent cations, \( P_{Cl}/P_{NS} \), defined as Alpha. For [K\(^+\)]\(_i\), \([Na^+]_i, \) and [Cl\(^-\)]\(_i\), in white fat cells with radioisotopes, as well as that of 152 mM calculated from the sum of [K\(^+\)], and [Na\(^+\)], measured with ion-sensitive microelectrodes in white fat adipocytes by Stark et al. (44). The value of [Cl\(^-\)], given by Perry and Hales (34) is the mean from a cell population study, whereas here individual cells are being investigated that reveal cell-to-cell variation. To account for the latter, we varied both Alpha and [Cl\(^-\)]; during fitting GHK to our data. To account for any passive permeability of the gluconate anion though the chloride permeability pathway, [Cl\(^-\)]\(_o\) was substituted by:

\[
[Cl^-]_o + \Delta [Gluc^-]_o
\]

where [Gluc\(^-\)]\(_o\) is the extracellular concentration of gluconate and \( \Delta [\text{Cl}^-]_o \) its relative permeability to that of chloride: \( P_{Gluc}/P_{Cl} \). For [K\(^+\)]\(_o\), [Na\(^+\)]\(_o\), and [Cl\(^-\)]\(_o\), we used 152.6 mM, 134 mM for [K\(^+\)]\(_i\), and 18.6 mM for [Na\(^+\)]\(_i\), as determined from the radioisotope efflux studies of Perry and Hales (34). A similar value of 150 mM is argued by Thoresteinsson et al. (47), who independently measured [K\(^+\)]\(_i\) and [Na\(^+\)]\(_i\), in white fat cells with radioisotopes, as well as that of 152 mM calculated from the sum of [K\(^+\)] and [Na\(^+\)], measured with ion-sensitive microelectrodes in white fat adipocytes by Stark et al. (44). The value of [Cl\(^-\)], given by Perry and Hales (34) is the mean from a cell population study, whereas here individual cells are being investigated that reveal cell-to-cell variation. To account for the latter, we varied both Alpha and [Cl\(^-\)]; during fitting GHK to our data. To account for any passive permeability of the gluconate anion though the chloride permeability pathway, [Cl\(^-\)]\(_o\) was substituted by:

\[
[Cl^-]_o + \Delta [Gluc^-]_o
\]
conate, is given by \( \Delta G = G_{Cl} - G_{Cl}' \), where \( G_{Cl} \) and \( G_{Cl}' \) are the chloride conductances in 152 mM and 18 mM extracellular \( Cl^- \), respectively. \( \Delta G \) can be estimated from

\[
\Delta G = \frac{R_{in} - R_{in}'}{R_{in}R_{in}'}
\]

where \( R_{in} \) and \( R_{in}' \) are the input resistances measured in normal and low \( [Cl^-]_o \), respectively; this gives an estimated \( \Delta G \) of \(-1.18 \pm 0.26 \) nS, or \( 0.058 \pm 0.013 \) S m\(^{-2} \) for primary adipocytes considered as a sphere of diameter \(-80 \) μm.

With the Goldman constant field model, the membrane conductance for any ion, \( G_i \), is obtained by differentiation of the Goldman flux equation with respect to \( V_m \) (13):

\[
G_j = P_j\left[\frac{-a_j F}{1 - \exp[-aEf]}\right] (C_o - C_i) \{\exp[a Ef] + (C_o - C_i \exp[a Ef])(1 - \exp[a Ef])\}
\]

where \( P_j \) is the passive permeability for ion \( j \), \( C_o \) and \( C_i \) are the respective extracellular and intracellular concentrations of ion \( j \), \( \alpha = z_j F/RT \) (\( z_j \) the valency of ion \( j \)), and \( E \) is the membrane potential. Since we know \( E \), \( C_o \), and \( C_i \) under two different extracellular \( Cl^- \) conditions, by using \( \Delta G \) we estimate \( P_{Cl} \) as \( 0.364 \) nm/s with \( 27 \) mM \( [Cl^-]_i \) or \( 0.345 \) nm/s with \( 43 \) mM \( [Cl^-] \).

It is also possible to calculate the permeability to an ion from its efflux rate constant, if it is assumed to be totally passive in nature, by using the Goldman constant field model (22, 13):

\[
P_j = k_j \frac{V}{A} \left[\frac{1}{aE}(1 - \exp[-aEf])\right]
\]

where \( k_j \) is the efflux rate constant for ion \( j \) and \( V/A \) the volume of cell water to surface area ratio for a single cell. Since the cell volume of adipocytes is predominantly composed of the lipid droplet (11), it is necessary to know the percentage of the cell

---

Fig. 6. Determination of the permeability ratio \( \text{Alpha} \) for primary and 3T3-L1 adipocytes. A: \( V_m \) (thick black line) in response to changes in bath extracellular \( Cl^- \) concentration \( [Cl^-]_o \) (thin gray line), recorded from a 3T3-L1 adipocyte. B: representative plots of the membrane potential determined at different \( [Cl^-]_o \) for a primary (○) and differentiated 3T3-L1 adipocytes (□). Solid lines are best fits to the Goldman-Hodgkin-Katz membrane potential equation (GHK) with \( \text{Alpha} \) values of 4.9 and 27, and \( [Cl^-]_o \) of 48 and 59 mM for primary and differentiated 3T3-L1 cells, respectively. Dotted lines describe the 95% confidence intervals (CI) for respective fits. C: effect of substitution of 133 mM bath \( Cl^- \) with \( I^- \) on the \( V_m \) of primary adipocytes. D: effect of substitution of 138 mM bath \( Na^+ \) with \( Li^+ \) on the \( V_m \) of primary adipocytes. E: estimates of \( \text{Alpha} \), the permeability ratio of \( P_{Cl}/P_{Na} \) determined from fits of the GHK when either \( [Cl^-]_o \) or \( [Na^+]_o \) was allowed to vary. F: estimates of \( [Cl^-]_o \), from fits of the GHK equation to the dependency of \( V_m \) on either changes in \( [Cl^-]_o \) \( (\text{CI suffix}) \) or \( [Na^+]_o \) \( (\text{Na suffix}) \) for the 2 types of adipocytes as indicated. Median values are given by solid horizontal bars.
volume that is occupied by water to gain an accurate estimate of V/A. The percentage of water content of adipocytes has been reported to be between 2.3% (19) and 2.75% (47). However, since this value is negatively correlated with rat weight and cell size (14), a revised value of ~2% was estimated for the mean V of 268 pl of our primary adipocytes. This gives a V/A of 0.26 μm, a volume manifested as the thin ring of cytoplasm of ~0.3 μm average thickness around the central lipid droplet (11, 49). Using kCl of 0.158 min⁻¹ from Perry and Hales (34), PCl is calculated to be 0.4 nm/s, a value within 20% of those we estimate from the electrophysiological changes in Rin andVm.

In white fat adipocytes, 36Cl⁻ efflux is observed to decrease with a reduction in extracellular anion concentration; these data were originally used to support the notion that ~30–40% of the 36Cl⁻ efflux from adipocytes occurred by exchange with external anions (35). Such a result, however, can also be simply explained as due to a change in the electrochemical gradient for Cl⁻, in particular the change inVm that occurs on removal of [Cl⁻]o. This idea can be checked by comparison of the predicted ratio of passive efflux rate constants with those actually measured. The expected passive efflux ratio (4) is given by

\[
\frac{k_{j,1}}{k_{j,2}} = \frac{E_1(1 - \exp[-aE_2])}{E_2(1 - \exp[-aE_1])}
\]

where \(k_{j,1}/k_{j,2}\) is the expected ratio with membrane potentials E₁ and E₂ in low and normal [Cl⁻]o, respectively. From ourVm data, we predict the \(k_{j,1}/k_{j,2}\) ratio to be 0.65, which is similar to the ratio of 0.7 calculated from the actual 36Cl⁻ efflux rate constants (0.157 and 0.110 min⁻¹) measured under similar experimental conditions by Perry and Hales (35). Consequently, our data support the notion that the PCl in adipocytes represents a predominantly passive diffusion pathway via ion channels rather than one mediated by anion exchange.

Our demonstration that white fat adipocytes have aVm that is predominantly dependent on \(P_{Cl}\) is contrary to previous suggestions that \(P_{K}\) is predominant in the control of adipocyte Vm (6). Although we have already discussed this at length, the calculations above yield results that are entirely and internally consistent with our finding.

We observed a very negative spread of Vm with 25% of all values (n = 182) hyperpolarized beyond ~39 mV, and 18 values beyond ~47 mV (Fig. 3A). AVm solely Nernstian for Cl⁻, i.e., with no \(P_{NS}\) component, based upon previously published (43 mM, 34) or even our own (27 mM) value of [Cl⁻]i can muster maximum Vm’s of only ~33 and ~45 mV, respectively. These data suggest that in order to obtain the most negative of Vm values (~72 mV) either those cells have a substantially lower [Cl⁻]i than the population mean ~10 mM with little or no \(P_{NS}\), or/and these cells have an extra \(P_{K}\) component that contributes to Vm. Hyperpolarized adipocytes with an extra \(P_{K}\) component would be expected to exhibit increased and decreased Vm responses to changes in [K⁺]o and [Cl⁻]i, respectively; however, since neither was observed, the presence of a \(P_{K}\) to explain the more negative values of Vm is unsupported.

During Rin measurement we assumed that the adipocytes had a large electrical space constant and reached isopotential within a few seconds of current injection. Since the cytoplasm exists as a thin 0.3-μm-thick submembrane sheet, due to internal resistance considerations, Rin and \(\Delta G\) may be considered to be under- and overestimated, respectively (45). The fact that our estimate of \(P_{Cl}\), determined from Rin and \(\Delta G\), was close to that obtained from radioisotope flux studies is strong evidence in support of the assumption of isopotential.

Effects of insulin and isoprenaline on membrane potential. Insulin and (nor)epinephrine are the two main modulators of adipocyte function. With this in mind, we sought to test whether they affected adipocyte Vm. Figure 7 shows that perfusion of 100 nM insulin at 32°C did not affect the membrane potential of primary or of 3T3-L1 adipocytes. The

---

Fig. 7. Effects of insulin and isoprenaline on Vm. Effect of 100 nM insulin on Vm of primary (A) and 3T3-differentiated adipocytes (B). C: effect of 10 μM isoprenaline (iso), on the Vm of 3T3-differentiated adipocytes. AA, acetic acid control. Statistical significance is relative to control (Student’s paired t-test).
failure of insulin to affect \( V_m \) or membrane permeability (Alpha) is supported by previous biophysical studies that also showed an absence of acute peptide effect on \( V_m \) measured epididymal fat pad explants with sharp electrodes (5, 44) as well as \(^{42}\text{K}^+\) efflux from isolated adipocytes (35). However, Beigelman and Hollander (5) report a depolarization of \( V_m \) with insulin in adipocytes from young animals, whereas, conversely, Cheng et al. (10) reports that the peptide hyperpolarizes \( V_m \) of white fat cells from similar aged animals. Overall, this body of evidence refutes an effect of the peptide on \( V_m \).

Perfusion of 10 \( \mu \text{M} \) isoprenaline, a \( \beta \)-adrenoceptor agonist, significantly and reversibly depolarized the plasma membrane by 5 ± 0.7 mV (\( n = 8 \), \( P < 0.001 \), Student’s paired \( t \)-test; Fig. 7C). Furthermore, this was associated with a significant decrease in \( R_m \) from 0.35 \( \text{M} \Omega \) (0.30–0.85, 95% CI) to 0.33 \( \text{M} \Omega \) (0.23–0.79, 95% CI, \( P < 0.05 \), Wilcoxon test). The depolarization in \( V_m \) produced by isoprenaline can be explained by a decrease in Alpha from 4.8 to 3.0 ([\( \text{Cl}^- \])] \text{in} \), = 27 mM), as a result of either a decrease in \( P_{\text{Cl}} \) or an increase in \( P_{\text{NS}} \), or both. However, since the change in \( V_m \) was associated with a decrease in input resistance, an increase in \( P_{\text{NS}} \) is supported. Using the analytical approaches above, we estimate that the change in \( \Delta G \) observed with isoprenaline is +0.0135 \( \text{S} \text{m}^2\) (0.0042 to 0.051, 95% CI): an increase in \( P_{\text{NS}} \) from 0.076 nm/s, calculated from Alpha (4.7) and \( P_{\text{Cl}} \) (0.49 nm/s) under control conditions, to 0.10 nm/s in the presence of the drug, which gives a new Alpha of 3.6, close to that estimated above to explain the change in \( V_m \) observed with this drug. Perry and Hales (35) demonstrated that epinephrine increased cation (\(^{42}\text{K}^+\)) efflux, an event consistent with biophysical changes that we observe: an increase in cation permeability. However, the actual change in \( P_K \) associated with epinephrine, estimated from radioisotope efflux data, was 0.13 nm/s (35), 0.1 nm/s larger than the change in \( P_{\text{NS}} \) that we determined electrophysiologically: 0.03 nm/s. This is a finding that suggests that ~0.1 nm/s cation permeation is not mediated by ion channels but is probably due to ion exchange. Importantly, the same authors (35) also reported an increase in \(^{36}\text{Cl}^-\) efflux with epinephrine, which we estimate from their data to be associated with an increase in \( P_{\text{Cl}} \) of 0.1 nm/s, a phenomenon apparently contradictory to that expected with our observed depolarization in \( V_m \). The nearly identical values for the increases in \( P_K \) and \( P_{\text{Cl}} \) that are unaccounted for electrophysiologically suggests that some form of electroneutral K–Cl cotransporter (\( \text{K},\text{Cl}\)) is activated in adipocytes with \( \beta \)-adrenoceptor activation, a phenomenon that is well established in many other cell types (2).

The ability of isoprenaline to depolarize \( V_m \) is similar to that previously reported, an action also blocked by the nonselective \( \beta \)-adrenoceptor antagonist propranolol (10). Furthermore, using the cell-attached patch clamp technique, Ringer et al. (40) demonstrated a \( \beta_1 \)-adrenoceptor-mediated activation of a nonselective cation channel on white fat adipocytes, an observation wholly consistent with the increase in nonselective cation permeability that we infer. The role of this phenomenon is unclear; it may be simply coincidental with \( \beta \)-adrenoceptor-mediated lipolysis, or alternatively, it may serve a role to regulate cell volume during the act of lipolysis.

**Cell-attached single-channel studies.** To characterize the identities of the ion channels responsible for the resting membrane potential of the adipocytes, the cell-attached configuration of the patch clamp technique was used. Figure 8A shows one type of single-channel current activity typical for that measured in 11 of 19 patches that possessed single-channel activity. At a \( V_m \) of ~90 mV, distinct inward-current single-channel activity was observed characterized by relatively long open states with occasional substates. The opening probability, \( NP_0 \), was voltage independent over the \( V_m \) range tested (Fig. 8C). The relationship between single-channel current amplitude and membrane potential, \( i-V \), demonstrates outward rectification (Fig. 8E) with a single-channel slope conductance, \( \gamma \), of 16 ± 1.3 pS and an extrapolated reversal potential, \( R_p \), of ~29 ± 1.6 mV (\( n = 11 \)). The similarity of \( R_p \) to the estimated \( E_{\text{Cl}} \) of ~32 ± 1.8 mV supports \( \text{Cl}^- \) as the predominant permeant ion species for this channel. To further characterize the single-channel \( i-V \) relationship, the data were fitted with the following form of the Goldman flux equation, assuming the intracellular and extracellular surface potentials were identical (43, 37):

\[
i_j = P_j^i \frac{-E(F_i)^2}{(1 - \exp[aE])} \left( C_o - C_{\text{exp}}[aE] \right)
\]

where \( i_j \) is the single-channel current amplitude and \( P_j^i \) is the single-channel permeability for ion \( j \). Since \( E (V_m) \) and \( C_o (147 \text{ mM}) \) are known, the \( i-V \) were fitted with a \( C_i \) and \( P_{\text{Cl}} \) of 41 ± 3 mM and 7.1 ± 0.9 \( \times \) \( 10^{-14} \) \text{cm}^3\text{s}^{-1}, respectively (\( n = 5 \)). The close agreement between \( C_i \) and the measured values for [\( \text{Cl}^- \)], further supports \( \text{Cl}^- \) as the permeant ionic species for these single-channel currents. Although \( \gamma \) and \( P_{\text{Cl}} \) are about twice as large as those described for the CLC family and cystic fibrosis transmembrane regulator (CFTR) \( \text{Cl}^- \) channels recorded under similar conditions in recombinant cell models (16, 37, 46, 48), the observation of substates suggests that these adipocyte \( \text{Cl}^- \) channels may have a multimeric molecular identity or multiple configurations. Alternatively, they may be an adipocyte homolog of the volume-regulated anion channel (VRAC), which has similar biophysical properties to the channel we describe: an inward \( \gamma \) of 10–20 pS under similar ionic gradients, voltage-independent gating, and slow gating kinetics (32). Indeed, these channels may be the same as those responsible for the volume-sensing outward-rectifying (VSOR) chloride currents described in mature white fat adipocytes (24, 25).

In the other eight patches, single-channel currents similar in kinetics to those above were also observed (Fig. 8B); however, their inward slope conductance was significantly smaller at 9.8 ± 0.4 pS (\( P < 0.002 \), Student’s \( t \)-test) with a significantly depolarized \( R_p \) of 8.9 ± 4.5 mV (\( P < 0.0001 \), Student’s \( t \)-test; Fig. 8F). Moreover, they often possessed transitions between substates as well as noisy fluctuations in current amplitude that were greater than the unitary (sub)event (Fig. 8B). These, too, had a voltage-independent \( NP_0 \) (Fig. 8D). Although these properties are typical of those often observed for single-channel \( \text{Cl}^- \) currents (37, 47), the possession of an \( R_p \) positive to 0 mV contradicts a \( \text{Cl}^- \) identity. It is possible that these channels represent a different mode of opening from the former ones described above, a change
possibility brought about by cell damage to give a depolarized and erroneous estimate of \( V_m \) and \( R_p \) coupled with impairment of cellular signals that normally modulate channel gating. These channels were not characterized any further. We failed to see ion channel activity indicative of the nonselective ion channels with a \( \gamma \) of 25 pS, as previously described in white fat adipocytes by Ringer et al. (40).

**Iodide permeability of the chloride channel.** Since both the CFTR, a member of the ABC family of transporters (31), and the CLC family of chloride channels (46) have a far lower permeability to \( I^- \) than to the \( Cl^- \) anion, whereas the opposite is true for other \( Cl^- \) channels such as VSOR and VRAC (32), experiments were almost isosmotic mitigates against the idea that the different extracellular solutions used in our experimental conditions. Figure 4A shows that the substitution of 133 mM bath \( Cl^- \) with \( I^- \) (307 \pm 4.2 mOsm) led to a stable and significant 9 \pm 1.3 mV (\( n = 11 \)) hyperpolarization (\( P < 0.0001 \), paired \( t \)-test) of \( V_m \) (Fig. 6C). These data clearly show that primary adipocytes have a \( V_m \) that is predominantly controlled by \( P_{Cl} \) with a greater ionic permeability to \( I^- \) compared with \( Cl^- \) and also dismisses a major role for either CFTR or CLC in the control of \( V_m \). Fitting the following form of the GHK equation to our data

\[
V_m = \frac{-RT}{F} \ln \left[ \frac{P_{NS}[K^+]_o + P_{NS}[Na^+]_o + P_{i}[I^-]_o + P_{Cl}[Cl^-]_o}{P_{NS}[K^+]_o + P_{NS}[Na^+]_o + P_{i}[Li^+]_o + P_{Cl}[Cl^-]_o} \right]
\]

where \( P_i \) is the membrane permeability to \( I^- \), yielded a \( P_i/P_{Cl} \) ratio for the adipocyte membrane of 1.6 \pm 0.12 (\( n = 10 \)), a value almost identical to that of 1.5 previously described for VRAC (32). Moreover, our value of \(-0.13-0.14\) for the \( P_{Glost}/P_{Cl} \) ratio Delta is also similar to that of 0.17 also previously described for VRAC (32). Together, these data provide strong evidence in support of a VRAC/VSOR identity for the predominantly active ion channel in white fat adipocytes. That the different extracellular solutions used in our experiments were almost isosmotic mitigates against the idea that the \( Cl^- \) permeability we observed was activated by our experimental conditions.

**Lithium permeability of the nonselective cation channel.** To control for any nonspecific effects of NMDG+ on \( V_m \), and to further characterize \( P_{NS} \), we tested the effect of replacement of bath Na+ with Li+ on the \( V_m \) of primary adipocytes. Substitution of 138 mM bath Na+ with Li+ (292 \pm 0.6 mOsm) produced a stable and significant 8.5 \pm 1.3 mV (\( n = 10 \)) hyperpolarization (\( P < 0.0001 \), paired \( t \)-test) of \( V_m \) (Fig. 6F). These data suggest that the \( P_{NS} \) of primary adipocytes has a lower ionic permeability to Li+ than to Na+. Fitting the following form of the GHK equation to our data:

\[
V_m = \frac{-RT}{F} \ln \left[ \frac{P_{NS}[K^+]_o + P_{NS}[Na^+]_o + P_{Cl}[Cl^-]_o}{P_{NS}[K^+]_o + P_{NS}[Na^+]_o + P_{i}[Li^+]_o + P_{Cl}[Cl^-]_o} \right]
\]

where \( P_{Li} \) is the membrane permeability to Li+, gave a \( P_{Li}/P_{NS} \) ratio for \( P_{NS} \) of 0.49 \pm 0.08 (\( n = 7 \)), a value almost identical to that of 0.5 for a nonselective monovalent cation channel described in placental apical membrane (30), for which the \( P_{K}/P_{Na} \) ratio was also 1, a property we observed earlier for the plasma membrane of adipocytes.

**Effect of \([Cl^-]_o \) on \([Ca^{2+}]_o \).** Depolarization of the plasma membrane by elevation of \([K^+]_o \), is a well-established paradigm used with excitatory tissues to increase cytosolic calcium via activation of voltage-gated calcium channels (VGCCs). This same methodology has also been extensively applied to white fat adipocytes under the premise that they, too, have a \( V_m \) predominantly controlled by potassium, possessing VGCCs, with a resultant increase in \([Ca^{2+}]_o \), with membrane depolarization (15, 18, 27, 36). However, we have previously showed that only 21% of cells give an increase in \([Ca^{2+}]_o \), under similar conditions (38), evidence that suggests the majority of adipocytes either do not possess VGCCs or/and have a \( V_m \) that is not controlled by \( K^+ \); in this study, we have clearly demonstrated the latter. In primary adipocytes, median basal \([Ca^{2+}]_i \) was 131 nM (103 to 164 nm, 96% CI, \( n = 54 \); Fig. 9A). Figure 9 shows that elevation of \([K^+]_o \), from 5.6 to 50 mM by equimolar substitution of bath Na+, produced a 29 nM (10 to 45 nm, 96% CI, \( n = 40 \)) increase in \([Ca^{2+}]_o \) (\( P < 0.0001 \), Wilcoxon test), whereas a decrease in \([Cl^-]_o \), from 152 to 18 mM produced only a 16 nM (10 to 24 mM, 96% CI, \( n = 40 \)) increase in \([Ca^{2+}]_o \) (\( P < 0.005 \), Wilcoxon test), an effect significantly smaller than that observed with the elevated \([K^+]_o \), (\( P < 0.02 \), Mann-Whitney; Fig. 9, B and C). Moreover, when the two experimental paradigms were conducted back to back on the same cell, a larger increase in \([Ca^{2+}]_o \), was still brought about by the elevation of \([K^+]_o \), compared with the decrease in \([Cl^-]_o \); 30 nM (7 to 62 nM, 98% CI, \( n = 27 \), \( P < 0.0001 \), Wilcoxon signed rank; Fig. 9D). That an elevation of \([K^+]_o \) does not depolarize \( V_m \) suggests that the increase in \([Ca^{2+}]_o \), observed most likely results from reverse Na-Ca exchange (NCX), driven by the low \([Na^+]_o \), concomitant with the elevation of \([K^+]_o \), and not by activation of VGCCs. To test this idea, \([Na^+]_o \) was decreased from 138 to 93.6 mM, independently of bath \( K^+ \), by equimolar substitution with 44.4 mM NMDG+.

Figure 9E illustrates that a decrease in bath \( Na^+ \) independent of \([K^+]_o \), increased adipocyte \([Ca^{2+}]_o \), comparable in magnitude to that produced during elevation of bath \( K^+ \) to 50 mM (Fig. 9F). Moreover, in neither case did 20 \gum\ nifedipine, a specific dihydropyridine inhibitor of VGCC, or 10 \gum\ KB-R7943, an inhibitor of reverse NCX, prevent the increase in \([Ca^{2+}]_o \), brought about by a reduction of bath \( Na^+ \) either alone or coupled with an increase in \([K^+]_o \), concentration (Fig. 9,G, H, I and J). The identity of the mechanism responsible for the small increase in \([Ca^{2+}]_o \), brought about the reduction of \([Cl^-]_o \), and the depolarization of \( V_m \) remains unknown. The depolarization of the \( V_m \) associated with \( Cl^- \) removal was expected to cause reverse NCX and increase \([Ca^{2+}]_o \), similar in magnitude to that seen with the partial removal of extracellular \( Na^+ \). However, since the increase in \([Ca^{2+}]_o \), observed was only a fraction of that expected suggests that either counter regulatory mechanisms for \([Ca^{2+}]_o \), homeostasis were affected by the change in extracellular anion. Alternatively, the NCX in white fat adipocytes (38) is a unique isofonn with a nonelectrogenic 2Na+:1Ca2+ stoichiometry, that is insensitive to voltage, and is also insensitive to KB-R7943.

**A simple model of adipocyte \( V_m \).** At steady state, the net flux of ionic charge across the plasma membrane of the adipocyte is zero. For simplicity, both \( Na^+ \) and \( K^+ \) are
assumed to permeate via nonselective cation channels. However, since the adipocyte membrane potential is dictated by the passive permeability of both Cl\(^-\) and monovalent cations, at the resting Vm no ion is at equilibrium; consequently, there will be a net influx of NaCl and an efflux of K\(^+\). For ionic homoeostasis it is envisaged that Na\(^+\) is extruded via the Na\(^+\)-K\(^+\)-ATPase, whereas excess Cl\(^-\) is removed via co-transport with K\(^+\) via KCC, where [K\(^+\)] is replenished by the Na\(^+\)-K\(^+\)-ATPase. Since the Na\(^+\)-K\(^+\)-ATPase is electrogenic, it is necessary to incorporate its 

Fig. 8. Single-channel ion activity and related parameters. A and B: representative recordings of the 2 types of inward single-channel currents’ activity measured at a Vm of –90 mV; dotted line, closed channel state; dashed line, predominant open-channel state; dot-dashed line, substate. C and D: open-channel activity, NPo, plotted against holding membrane potential, for the 2 types of channels. Note the lack of voltage dependence in both cases. E and F: representative single-channel current amplitude-voltage relationship for channel types illustrated above. Solid lines are best fits to the GHK single-channel current equation in E and linear regression in F, with parameters given in text. Data are shown as means ± SE (n = 8–11).

Fig. 9. Effect of ion substitution on [Ca\(^{2+}\)] of primary adipocytes. A: distribution of intracellular calcium concentration, [Ca\(^{2+}\)], under basal conditions (n = 54). B: effect of reduction of [Cl\(^-\)], from 152 to 18 mM followed by a wash and then elevation of [K\(^+\)], from 5 to 50 mM on the mean [Ca\(^{2+}\)], from a field of 6 cells. C: box and whisker plot comparison for 3 different conditions as indicated on [Ca\(^{2+}\)], (n = 27). D: effect of reduction of [Cl\(^-\)], from 152 to 18 mM directly followed by elevation of [K\(^+\)], from 5 to 50 mM on the mean [Ca\(^{2+}\)], from a field of 4 cells. E: effect of increased [Na\(^+\)]\(^-\) independent of bath K\(^+\) and DMSO on the mean [Ca\(^{2+}\)], from 4 experiments. F: effect of increased [K\(^+\)], and DMSO on the mean [Ca\(^{2+}\)], from 6 experiments. G: effect of decreased [Na\(^+\)]\(^-\) independent of bath K\(^+\) in the presence of KB-R7943 on the mean [Ca\(^{2+}\)], from 8 experiments. H: effect of increased [K\(^+\)], in the presence of KB-R7943 on the mean [Ca\(^{2+}\)], from 13 experiments. I: effect of decreased [Na\(^+\)]\(^-\) independent of bath K\(^+\) in the presence of nifedipine on the mean [Ca\(^{2+}\)], from 8 experiments. J: effect of increased [K\(^+\)], in the presence of nifedipine on the mean [Ca\(^{2+}\)], from 7 experiments.
contribution to the membrane potential via $r$, the pump ratio in the GHK voltage equation:

$$V_m = -\frac{RT}{F} \ln \left( \frac{[K^+]_{in}}{[K^+]_{out}} + \frac{[Na^+]_{in}}{[Na^+]_{out}} + \frac{[Cl^-]_{out}}{[Cl^-]_{in}} \right)$$

where $\alpha$ is the actual permeability ratio of $P_{Cl}/P_{NS}$ at 4.2, and $r$ is the pump ratio of 1.5 for the $Na^+-K^+\text{-ATPase}$ (which is taken to have a 3$Na^+\cdot2K^+$ stoichiometry). Since KCC is electroneutral, it is omitted from the GHK equation.

**Conclusion**

In conclusion, we have unequivocally demonstrated that the resting membrane potential of isolated identified white fat adipocytes is predominantly controlled by the passive diffusion of $Cl^-$, most likely via spontaneously active VSOR/VRAc with a minor contribution from $K^+$ and $Na^+$ via nonselective cation channels. Somewhat controversially, our novel data are in contrast to the previous idea that the $V_m$ of this cell type is predominantly controlled by $K^-$ (6, 7). Indeed, we now provide a new model from which previous, as well as new data that relate to the membrane physiology of white adipose tissue can be explained. Although insulin did not affect $V_m$, isoprenaline depolarized adipocyte $V_m$, an observation consistent with an activation of nonselective cation channels. Whether the latter is causal or consequential of $\beta$-adrenoceptor-mediated lipolysis is unknown.

The demonstration that manipulation of the transmembrane $Cl^-$ flux causes membrane depolarization has important clinical implications with regard to both the control of the Ca-dependent functions of white fat adipocytes: lipogenesis (29), lipolysis (17, 50), and adipokine secretion such as that of leptin (51). Evidence in support of this idea arises from studies on the use of tamoxifen to treat breast cancer. Tamoxifen is an established inhibitor of VRAc (32) and has already been shown to block this channel in human white fat adipocytes (24). Given our demonstration of the key role that anion channels, which we identify to be most probably VRAc, play in the membrane physiology of white adipose tissue, we expect that tamoxifen should also lead to a depolarization of white fat adipocytes, elevate their $[Ca^{2+}]_i$, and affect adipokine secretion. Indeed the serum leptin level of patients on tamoxifen is nearly twice that of controls (33), evidence that strongly supports our hypothesis. Since VRAc is in fact a multiple target for a plethora of therapeutic drugs (32), metabolic complications that result from aberrant adipocyte function may be explained in part as due to the effects that these drugs have on adipocyte $V_m$ and $[Ca^{2+}]_i$.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: D.C.B., P.P., and P.A.S. performed experiments; D.C.B., P.P., and P.A.S. analyzed data; D.C.B., P.P., and P.A.S. interpreted results of experiments; D.C.B., P.P., and P.A.S. approved final version of manuscript; S.C. and P.A.S. conception and design of research; S.C. and P.A.S. edited and revised manuscript; P.A.S. prepared figures; P.A.S. drafted manuscript.

**REFERENCES**


