A mechanically activated TRPC1-like current in white adipocytes

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Running title: Mechanically activated Ca^{2+} current in white adipocytes

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Abbreviations: SOCE, store-operated Ca^{2+} entry; SOCC store-operate Ca^{2+} channel; STIM1, stromal interaction molecule 1; ORAI1, calcium release-activated calcium channel protein 1; TRPC, transient receptor potential (TRP) channel; ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; Ca_v, voltage-dependent Ca^{2+} channels; PLC, phospholipase C; 2-APB, 2-Aminoethoxydiphenyl borate; NPPB, 5-Nitro-2-(3-phenylpropylamino)benzoic acid.

Abstract

Ca^{2+} impacts a large array of cellular processes in every known cell type. In the white adipocyte, Ca^{2+} is involved in regulation of metabolic processes such as lipolysis, glucose uptake and hormone secretion. Although the importance of Ca^{2+} in control of white adipocyte function is clear, knowledge is still lacking regarding the control of dynamic Ca^{2+} alterations within adipocytes and mechanisms inducing intracellular Ca^{2+} changes remain elusive. Own work has recently demonstrated the existence of store-operated Ca^{2+} entry (SOCE) in lipid filled adipocytes. We defined stromal interaction molecule 1 (STIM1) and the calcium release-activated calcium channel protein 1 (ORAI1) as the key players involved in this process and we showed that the transient receptor potential (TRP) channel TRPC1 contributed to SOCE. Here we have aimed to further characterised SOCE in the white adipocyte by use of single cell whole-cell patch clamp recordings. The electrophysiological measurements show the existence of a seemingly constitutively active current that is inhibited by known store-operated Ca^{2+} channel (SOCC) blockers. We demonstrate that the mechanical force applied to the plasma membrane upon patching leads to an elevation of the cytoplasmic Ca^{2+} concentration and that this elevation can be reversed by SOCC antagonists. We conclude that a mechanically activated current with properties similar to TRPC1 is present in white adipocytes. Activation of TRPC1 by membrane tension/stretch may be specifically important for the function of this cell type, since adipocytes can rapidly increase or decrease in size.

Keywords

White adipocytes, ion channels, mechanical activation, intracellular Ca^{2+}, store-operated Ca^{2+} entry, TRPC1

Background

Several metabolic/endocrine processes in the white adipocyte have been proposed to be regulated by Ca^{2+}-dependent mechanisms. Ca^{2+} has been suggested to both augment [1-3] and inhibit [4] lipolysis (breakdown of stored triglycerides) and to have a role in insulin-stimulated glucose uptake [5, 6]. Secretion of both leptin [7-9] and adiponectin [10, 11] have been shown to comprise Ca^{2+}-dependent steps. Clearly, Ca^{2+} is important for white adipocyte function; however, the dynamic Ca^{2+} alterations within the adipocytes as well as mechanisms inducing intracellular Ca^{2+} changes remain inadequately investigated.

The Ca^{2+} concentration within a cells cytoplasm can be elevated via a number of pathways that may co-exist or be specific for a particular cell type. Endocrine cells typically control their hormone secretion via influx of Ca^{2+} through plasma membrane-bound voltage-dependent Ca^{2+} (Ca_v) channels [12]. Adipocytes have been suggested to express Ca_vs [13, 14] but functional evidence for their presence in this cell type is lacking. An alternative pathway that leads to an elevation of $[Ca^{2+}]_i$ is release of Ca^{2+} stored in the endoplasmic reticulum (ER), usually in response to activation of plasma membrane bound receptors [15]. The depletion of ER Ca^{2+} stores leads to activation of plasma membrane-bound store-operated Ca^{2+} channels (SOCCs) that allow influx of extracellular Ca^{2+} in a process termed store-operated Ca^{2+} entry (SOCE; also denoted capacitative calcium entry). The influx of Ca^{2+} via SOCCs may affect Ca^{2+}-dependent intracellular signalling processes [16]. In addition, mechanical stress may lead to activation of stretch-activated channels that are permeable to Ca^{2+} [17]. In particular channels of the transient receptor potential (TRP) channel family, non-selective cation channels with preferred selectivity for Ca^{2+}, have been suggested to be activated by stretch or tension [18].

By use of intracellular Ca^{2+} imaging and siRNA knockdown, we have recently shown that extracellular ATP or the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor

thapsigargin stimulates SOCE in white adipocytes. Our study pinpointed the stromal interaction molecule 1 (STIM1) and the calcium release-activated calcium channel protein 1 (ORAI1) as key players involved in mediating the Ca^{2+} influx. In addition, the transient receptor potential channel 1 (TRPC1) was shown to partake [19]. In the current work we aimed to further determine the function of SOCCs in white adipocytes by a single-cell electrophysiological approach. Our data show that the mechanical force, here introduced by patching itself, activates a Ca^{2+}-carried current with properties similar to TRPC1.

Material and methods

Reagents were obtained from Life Technologies; Tocris or Sigma Aldrich.

In vitro 3T3-L1 adipocyte differentiation

3T3-L1 cells were cultured and maintained as previously described [19]. After 2-3 days (~70% confluency) cells were resuspended in 35mm diameter plastic dishes (Nunc) at a density of 1.5 x 10^5 cells/ml. 2 days after seeding (90-100% confluency) differentiation was initiated by a medium containing insulin (170 µM), dexamethasone (10 mM) and 3-isobutyl-1-methylxanthine (5 mM). After a further 2 days, the medium was changed to one containing only insulin. The cells were used day 8 and 9 after start of differentiation. Only cells that appeared well differentiated (lipid-filled) were used for experiments.

Electrophysiological recordings

Currents were recorded applying the perforated patch configuration of the whole-cell patch-clamp technique. The cell dish was superfused with a solution composed of: 118 mM NaCl, 20 mM TEACl (Tetraethylammonium Chloride), 5.6 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES and 5 Glucose (pH = 7.4). The pipette filling solution comprised:

76 mM Cs_2SO_4, 10 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES (pH=7.35; supplemented with the pore-forming antibiotic Amphotericine B at120 μg/ml). Glass pipettes had a resistance between 2.5 and 5 MΩ. Recorded currents were included for analysis only if the access resistance was <50 MΩ and the leak current was <200 pA at -130 mV. Voltage-independent calcium currents were studied by application of voltage steps from -130 to -80 mV (0.37 Hz) followed by a 150 s ramp depolarisation from -130 to +100mV . All current density analyses were performed by analysis of the current difference when stepping the voltage from -130 mV to -80 mV. Currents were recorded and analysed using Patchmaster software and OriginPro 8.5. For each cell, the current amplitude (pA) was normalized to the cell capacitance (pF). All data was collected at 32°C.

Ratiometric calcium imaging

Cells were loaded for 90 min at room temperature (19-22°C) with fura-2AM (2 μmol/l) and pluronic acid (0.007% wt/vol). The bath medium consisted of 138 mM NaCl, 5.6 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES and 5 mM Glucose (pH=7.4) Changes in $[Ca^{2+}]_i$ were recorded by dual-wavelength microfluorimetry. Using minimal light, fura-2AM was excited at 340 and 380 nm by use of a Lambda DG-4 filter system (Sutter Instruments). Emitted light was collected above 510 nm. The data were recorded using MetaFluor software and calculated into absolute concentrations using equation 5 of [20].

Results

Extracellular ATP stimulates SOCE and Ca^{2+} influx through ORAI1 with contribution of TRPC1

As we have recently demonstrated [19] and as shown in Fig. 1A, extracellular application of ATP (100 μM) generates a transient increase in $[Ca^{2+}]_i$ after which $[Ca^{2+}]_i$ declines to a

elevated level lasting throughout the duration of the nucleotide application. Our previous work has shown that the transient peak arises as a result of release of Ca^{2+} from intracellular stores whereas the steady increase is due to influx of Ca^{2+} from the extracellular space, via SOCE channels [19]. When ATP is applied in the absence of extracellular Ca^{2+}, the transient peak is still present but $[Ca^{2+}]_i$ thereafter declines to a level similar to that observed before addition of ATP. Subsequent substitution of the extracellular solution to a Ca^{2+}-containing one (2.6 mM) generates an increase in $[Ca^{2+}]_i$ to a stably elevated level. This elevation of $[Ca^{2+}]_i$ upon reintroduction of extracellular Ca^{2+} is indicative of Ca^{2+} entering the cell through SOCCs that have opened as a result of emptying of ER stores (depicted in Fig. 1B; [19]). SOCCs remain open until intracellular Ca^{2+} stores are again refilled [16]. As shown in Fig. 1B, the influx observed upon introduction of extracellular Ca^{2+} can be inhibited by the 2-Aminoethoxydiphenyl borate (2-APB) or Gd^{3+}, known inhibitors of SOCE. Results were similar using the SERCA-inhibitor thapsigargin (pharmacological depletion of ER Ca^{2+} stores). By use of siRNA knockdown, we confirmed the results using antagonists. We identified ORAI1 as the SOCC chiefly responsible for the Ca^{2+} influx and the contribution of TRPC1 [19].

Perforated-patch whole-cell recordings show the occurrence of a current that can be inhibited by 2-APB or Gd^{3+}

In order to functionally investigate white adipocyte SOCCs, we applied perforated patch whole-cell recordings to single 3T3-L1 adipocytes. The perforated patch configuration was chosen in order to minimally interfere with the intracellular milieu and thus largely maintain cell physiology. We detected a seemingly constitutively active SOCC-like current that could be inhibited by 2-APB (Fig. 2A-C) or Gd^{3+} (Fig. 2D-F).

We next investigated the role of cytoplasmic Ca^{2+} for the SOCC-like current. It should be emphasized that SOCE is activated by the emptying of Ca^{2+} stores and is independent of the cytoplasmic Ca^{2+} level [16]. Adipocytes were pre-loaded with the membrane permeable Ca^{2+} chelator BAPTA-AM (50 μM during 30 min) prior to patching. (The Ca^{2+}-buffering effect of BAPTA was verified by measurements of $[Ca^{2+}]_i$ upon application of ATP. In 4 separate experiments with BAPTA-loaded cells, ATP application did not affect $[Ca^{2+}]_i$; not shown). The current density at the start of the recording was similar in cells pre-treated with BAPTA compared with non-treated cells. 2-APB (Fig. 3A, C and E) or Gd^{3+} (Fig. 3B, D and F) inhibited the current by a similar magnitude in Ca^{2+}-depleted cells as in cells unexposed to BAPTA.

We investigated if extracellular application of ATP or thapsigargin during patching could, in agreement with our $[Ca^{2+}]_i$ recordings (*c.f.* Fig. 1A and [19]), activate an additional conductance (permeable to Ca^{2+}). However, neither ATP (100 μM) nor thapsigargin (1 μM; both added extracellularly to the cell dish during the electrophysiological recordings) affected the recorded current and the current density was similar before and 2 min after addition of the compounds (Fig. 3E-F).

The SOCC-like current is PLC-dependent

Our recent study show that ATP-induced SOCE occurs via stimulation of P2Y2 receptors and activation of phospholipase C (PLC) [19]. We therefore pre-treated 3T3-L1 adipocytes with the PLC inhibitor U73122 (10 μM during 30 min) prior to the electrophysiological recordings. Interestingly, as shown in Fig 4A, addition of Gd^{3+} (1 mM) during the recording reduced the currents by only ~30% in U73122-treated cells compared to ~70% in the non-treated cells in Figs. 2F and 3D. Our results thus signify that a PLC-dependent and Gd^{3+}-sensitive conductance is inhibited by U73122. As noticeable in Fig. 4A, the current density before

addition of Gd^{3+} was not markedly reduced in U73122-treated cells compared to non-treated cells (*c.f.* Figs. 2F and 3D). However, as also clear when looking at data in Fig. 2 and 3, the current density under control condition varies between experimental series (likely due to a variation of channel expression level). It is thus likely that cells used in the experiments in Fig. 4A would have had a large current density, if untreated with the PLC inhibitor. More importantly though, the observation that the percentage Gd^{3+} inhibition is markedly lower in U73122-treated cells strongly indicates the existence of a PLC-dependent step in activation of the measured current.

Patching activates a SOCC-like current

The finding that application of ATP or thapsigargin was unable to activate a current in the electrophysiological recordings together with the observation of a SOCC-like current in unstimulated cells, led us to hypothesize that patching of the cells mechanically activates a Ca^{2+}-permeable current. To study this, we measured alterations of $[Ca^{2+}]_i$ upon patch pipette sealing with the plasma membrane. Indeed, as shown in Fig. 4B and C, pipette sealing induced a stable increase of $[Ca^{2+}]_i$, averaging 78±29nM, thus a magnitude similar to the $\Delta[Ca^{2+}]_i$ elevation induced by application of ATP or thapsigargin (*c.f.* trace in Fig. 1A and [19]). The $[Ca^{2+}]_i$ elevation induced by pipette sealing was completely reversed by Gd^{3+}. Our results suggest that cell patching activates a SOCC-like, Ca^{2+}-permeable current, thus partly explaining the presence of a seemingly constitutively active 2-APB/Gd^{3+}-sensitive current in the patch recordings as well as the lack of ATP/thapsigargin effect in electrophysiological measurements.

The negative reversal potential is due to the presence of Cl^- currents

The collected results above are indicative of mechanical activation of a Ca^{2+} carried SOCC-like current. However, the reversal potential for non-selective cation channels, such as TRPC1, is close to 0 mV [21]; the measured current in Fig. 2 has a reversal potential of -33±4 mV in Fig. 2B and -36±4 mV in Fig. 2E, thus significantly more negative than expected. However, since we perform our experiments in cells endogenously expressing a diversity of ion channels, and not in an isolated over-expressing system, our recorded whole-cell current will naturally show the net effect of all currents active under the chosen experimental condition. In the majority of cell types, the resting membrane potential is chiefly determined by K^+ that has a reversal potential around -80 mV. However, a study by Bentley et al. has demonstrated that the white adipocyte resting membrane potential is largely set by passive diffusion of Cl- with a contribution of non-selective cation channels [22]. Noteworthy, our measured reversal potential in Fig. 2 is in fact very close to the reported 3T3-L1 adipocyte resting membrane potential of -32 mV [22]. To investigated the presence of a Cl^- currents, cells were exposed to 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; 50 μM). NPPB blocks both Ca^{2+}- and volume-regulated Cl^- channels. In agreement with the existence of Cl^- channels in white adipocytes, the measured current was decreased by NPPB, although the inhibition was not statistically significant (P=0.1; Fig. 4D. Successive inclusion of Gd^{3+} still decreased the remaining current by ~30%. Noteworthy, the total percentage decrease of current by the combination of NPPB and Gd^{3+} is similar to the magnitude of block seen by the lanthanide alone in Figs. 2 and 3. In agreement with the inhibition of Cl^- currents, the reversal potential was positively shifted in cells exposed to NPPB (-15.7±3 mV).

Discussion

We here observe a SOCC-like current in white adipocytes studied by an electrophysiological approach. The recorded current partly shows functional characteristics resembling observations of SOCE (measured with ratiometric Ca^{2+} imaging, in [19] and in Fig. 1 of this paper.) However, whereas $[Ca^{2+}]_i$ recordings show that SOCE is triggered by application of extracellular ATP or thapsigargin, neither the nucleotide nor the SERCA inhibitor were able to induce a further conductance in patch-clamped cells. In the electrophysiological recordings, a current sensitive to blockage by 2-APB or Gd^{3+} was instead present in unstimulated cells (Fig. 2). By recordings of $[Ca^{2+}]_i$ in adipocytes simultaneously subjected to pipette sealing we show that the mechanical force introduced by patching itself elevates $[Ca^{2+}]_i$ and that subsequent inclusion of the SOCE inhibitor Gd^{3+} reverses the elevation (Fig. 4B and C). The current is PLC-dependent, thus analogous to ATP-induced SOCE via activation of P2Y2 receptors and the PLC/IP_3 pathway [19]. Our combined results suggest that mechanical force activates a SOCC-like current in white adipocytes and that this current, directly or indirectly, gives rise to Ca^{2+} influx via a pathway similar to that stimulated by ATP in un-patched cells.

Our findings in El Hachmane et al. [19] demonstrate that ORAI1 is the SOCC chiefly underlying white adipocyte SOCE (confirmed by a greatly diminished SOCE-dependent $[Ca^{2+}]_i$ elevation in cells where *Orai1* expression was reduced by siRNA knockdown). However, despite a large number of studies investigating the role of ORAI1 for SOCE and cell Ca^{2+} influx, mechanical activation of ORAI1 has not been reported. In the study by El Hachmane et al. we showed that TRPC1 contributes to STIM1-induced SOCE (the $[Ca^{2+}]_i$ elevation upon re-introduction of extracellular Ca^{2+} to thapsigargin-exposed cells was significantly reduced in cells knocked down for *Trpc1*) [19]. Non-selective cation channels, such as TRPC1, have been proposed to interact closely with SOCCs or even to themselves function as SOCCs [23-26]. TRPC1 channels have been shown to also be mechanically gated

[27, 28] and to be activated by STIM1 [29, 30]. The presence of *Trpc1* mRNA has been demonstrated in adipocytes [31] and we have confirmed its existence at the protein level [19]. Moreover, all TRPC channels are activated through Gq/11 receptors and downstream activation of PLC [32]. It is therefore of specific interest that the shown ATP-induced SOCE involves P2RY2 receptors, receptors that couple to Gq11 and trigger $[Ca^{2+}]_i$ increases via the PLC/IP_3 pathway [19]. In the work by Sukumar et al. TRPC1 channels were suggested to be constitutively active [31]. It is however possible that TRPC1 was also here mechanically activated in the patch-clamp experiments. To conclude, there is considerable evidence for that TRPC1 is involved in white adipocyte PLC-dependent SOCE and that the channel may also be mechanically gated. We thus suggest that mechanically activated TRPC1 channels, via interaction with ORAI1 channels, leads to Ca^{2+} influx from the extracellular space.

The group of Dr Paul Smith has shown the presence of Cl^- currents in white adipocytes and that Cl^- channels, in combination with non-selective cation channels (permeable to Ca^{2+}), account for a resting membrane potential around -30 mV [22]; our own recordings confirmed a reversal potential around -30 mV (Fig. 2B and E). It may appear puzzling that addition of 2-APB or Gd^{3+} does not shift the reversal potential towards more negative values (equilibrium potential for Cl^- is ~-60 mV and for non-selective cation channels around 0 mV). However, both 2-APB [33] and Gd^{3+} [34] have been shown to also block different types of Cl^- channels. Thus, our recordings likely show the net effect of the combined inhibition of Cl^- and SOC channels. The presence of Cl^- currents is confirmed in Fig. 4D showing that the inhibitor NPPB decreases the current density. Subsequent addition of Gd^{3+} still inhibits the remaining current. The SOCC-like current is evidently small. Out of a cell physiological perspective, given the constrained cytoplasmic space of an adipocyte (majority of cell filled with lipids), activation of a minute number of Ca^{2+} permeable channels is likely required in order to elevate $[Ca^{2+}]_i$ of the magnitude shown here and in [19].

The data presented here and in [19] demonstrate an $[Ca^{2+}]_i$ elevation in white adipocytes that is stimulated mechanically or by agents depleting ER Ca^{2+} stores. We propose that the common denominator is TRPC1. What is then the role of Ca^{2+} influx and the different gating mechanisms in white adipocytes? As already mentioned, several metabolic or endocrine processes within the white adipocyte are affected by changes of $[Ca^{2+}]_i$ levels [1-9]. Studies from our group have defined that white adipocyte adiponectin vesicle release is triggered by cAMP/catecholamines and that secretion of the hormone is potently augmented by Ca^{2+}-dependent processes [10, 11, 35]. Own unpublished findings show that externally applied ATP induces adiponectin exocytosis, an effect largely dependent on $[Ca^{2+}]_i$ dynamics (Musovic, Komai, Olofsson, unpublished). It is tempting to speculate that Ca^{2+} influx via ATP-activated SOCCs potentiates the release of adiponectin vesicles residing close to the plasma membrane and the Ca^{2+} influx sites. The fact that SOCCs have been shown to function in cAMP-generating microdomains are of particular interest in this context [36].

White adipocytes are able to rapidly both increase and decrease in size upon overfeeding and starvation respectively and it has been suggested that cell size variations may alter intracellular signalling [37, 38]. The findings in the current study showing that SOCC-like currents are mechanically activated suggest that Ca^{2+}-dependent processes in the adipocyte are impinged by cell expansion/membrane tension. In fact, lipolysis, glucose uptake and hormone secretion, processes involving Ca^{2+}-dependent signalling steps, are perturbed in diabesity, a condition associated with enlarged white adipocytes [39, 40]. Interestingly, there is evidence for that TRPC1 is linked to the regulation of liver cell volume [41]. Further investigations are needed in order to clarify a possible similar role for TRPC1 in regulation of white adipocyte size as well as the role of mechanical regulation of SOCC-like channel in obesity-induced metabolic/endocrine disturbances.

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Conflict of interest

None of the authors have any conflicts of interests.

Author contributions

M.F.E and C.S.O. planned and designed the study. M.F.E carried out experiments, collected and analysed the data. M.F.E and C.S.O. interpreted the results and wrote the manuscript.

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Figure legends

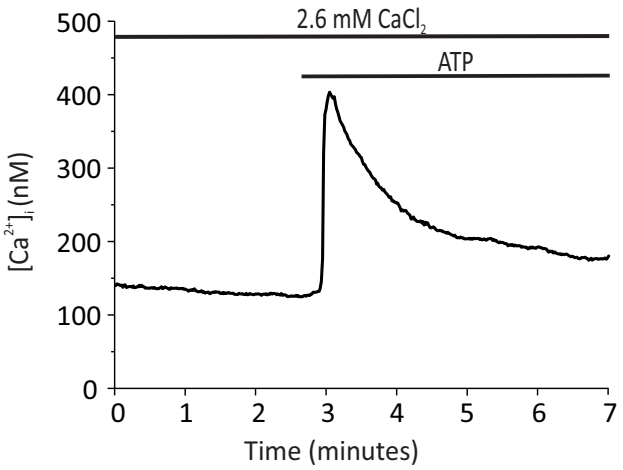
Figure 1: **Effects of extracellular ATP on adipocyte $[Ca^{2+}]_i$.** ***A)*** Representative trace of effect of ATP (100 μM) applied extracellularly to a 3T3-L1 adipocyte. Note the first transient elevation, du to Ca^{2+} release from intracellular stores, followed by a sustained increase owing to influx via SOCCs [19]. ***B)*** Left: Illustration of experimental design for investigation of SOCE upon $[Ca^{2+}]_i$ imaging. SOCE is analysed as the elevation observed after store depletion induced by, in this case, extracellular application of 100 μM ATP (marked by red double arrow). Right: Magnitude of inhibition of SOCE-dependent $[Ca^{2+}]_i$ elevation by pre-treatment with the antagonists 2-APB and Gd^{3+}, measured upon changing from a Ca^{2+} free to a Ca^{2+} containing solution, in agreement with illustration to the left. $P<0.5$ for all concentrations (Students t-test). The data in *B* constitutes part of results included in [19].

Figure 2: Patch clamp recordings of SOCC-like currents. ***A)*** Representative trace of current density before and after application of 50 μM 2-APB. ***B)*** Average currents elicited by ramp depolarisation before and after application of 2-APB. ***C)*** Average density of current before and after application of 50 μM 2-APB. Data in *B* and *C* are representative of 10 recordings in each experimental series. ***D-F)*** As in *A-C* but applying indicated concentrations of Gd^{3+}. Data in Data in *E* and *F* are representative of 9 recordings. * $P<0.05$; *** $P<0.001$ (Students t-test in *C*; one way-ANOVA with Bonferroni post hoc in *D*.)

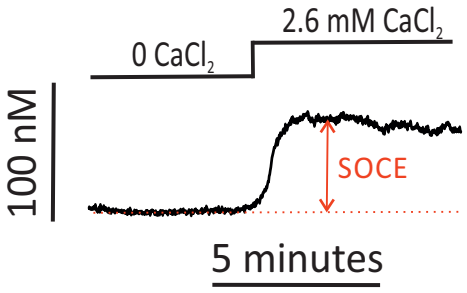
Figure 3: Current density under intracellular Ca^{2+} free conditions and after application of ATP or thapsigargin. Typical recordings of **c**urrent density in 3T3-L1 adipocytes pre-exposed to BAPTA-AM, before and after addition of 2-APB ***(A)*** or Gd^{3+} ***(B).***Average current density shown in ***(C)*** and ***(D)*** are from 11 experiments respectively. Extracellular addition of 100 μM ATP ***(E)*** or 1 μM thapsigaring ***(F)*** was without effect on the measured current. Average current density before and after addition of ATP or thapsigargin is shown in in insets (ATP and thapsigargin was applied in 7 and 11 experiments respectively. ****P<0.001* (Students t-test).

Figure 4: Patching activates a PLC-dependent Ca^{2+}-permeable current. A) Current density in cells pre-treated with the PLC inhibitor U73122 (10 μM during 30 min) before and after application of Gd^{3+} (1 mM). ***B)*** Imaging of the $[Ca^{2+}]_i$ change in a cell upon sealing with a patch pipette at the time pint indicated by the arrow. Inclusion of Gd^{3+} (1 mM added as marked by the arrow) reversed the mechanically induced $[Ca^{2+}]_i$ elevation. ***C)*** Average $[Ca^{2+}]_i$ increase induced by pipette sealing and inhibition by Gd^{3+} from 5 separate experiments. D) Effect of the Cl- blocker NPPB on the measured current and subsequent addition of Gd^{3+} (1 mM) in 10 experiments. * *P<0.05; **P<0.01; ***P<0.001* (Students t-test in *A*; one way-ANOVA with Bonferroni post hoc in *C* and *D*.)

A



B



	Concentration	% inhibiton
2-APB	10 µM	~30
	50 µM	~65
	100 µM	~70
Gd^{3+}	10 µM	~20
	100 µM	~15
	1 mM	~85

Figure 1

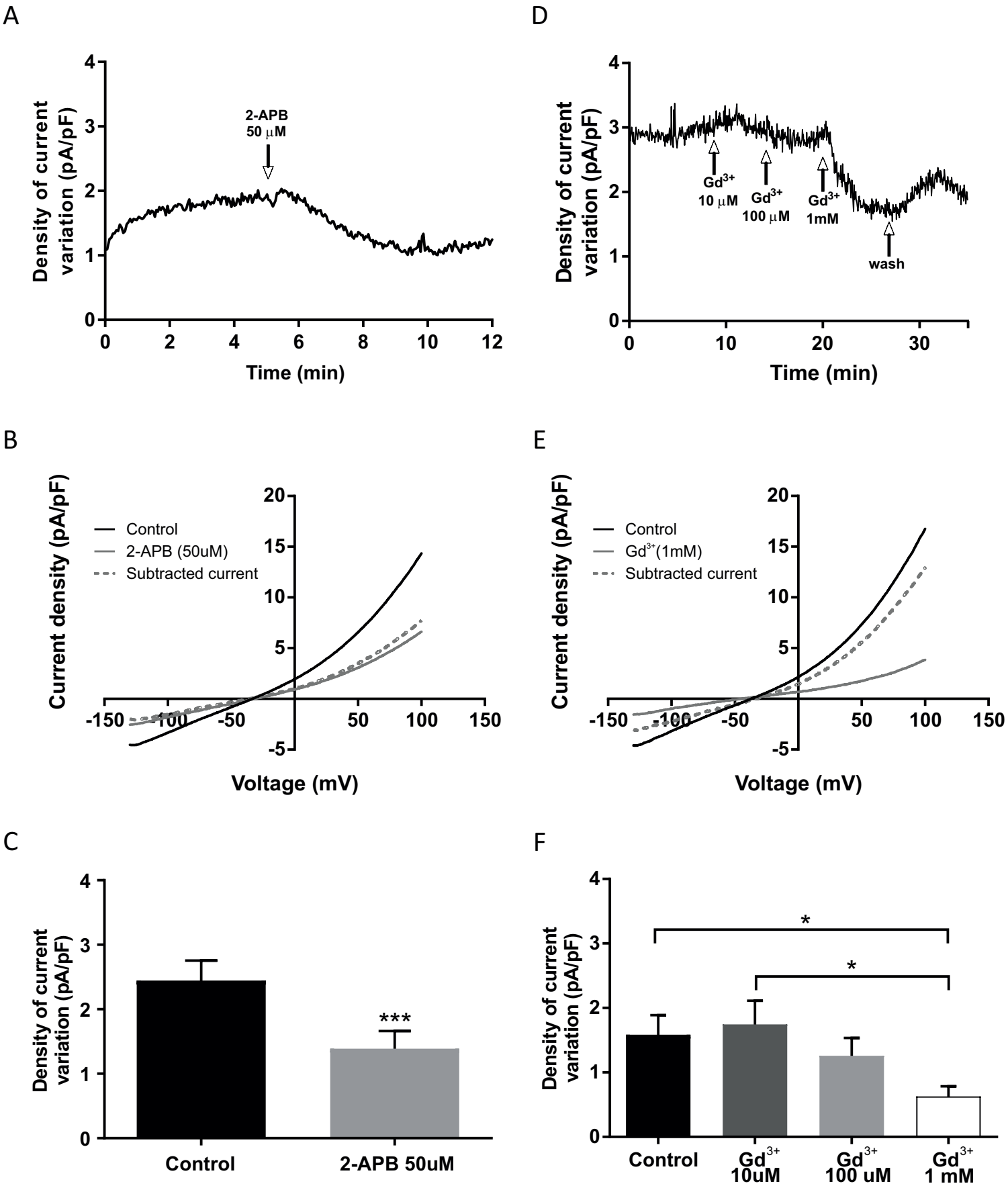

A
Density of current variation (pA/pF)
2-APB
50 μM
Time (min)
D
Density of current variation (pA/pF)
Gd3+ 10 μM
Gd3+ 100 μM
Gd3+ 1mM
wash
Time (min)
B
Current density (pA/pF)
Control
2-APB (50uM)
Subtracted current
Voltage (mV)
E
Current density (pA/pF)
Control
Gd3+(1mM)
Subtracted current
Voltage (mV)
C
Density of current variation (pA/pF)
***
Control
2-APB 50uM
F
Density of current variation (pA/pF)
*
*
Control
Gd3+ 10uM
Gd3+ 100 uM
Gd3+ 1 mM

Figure 2

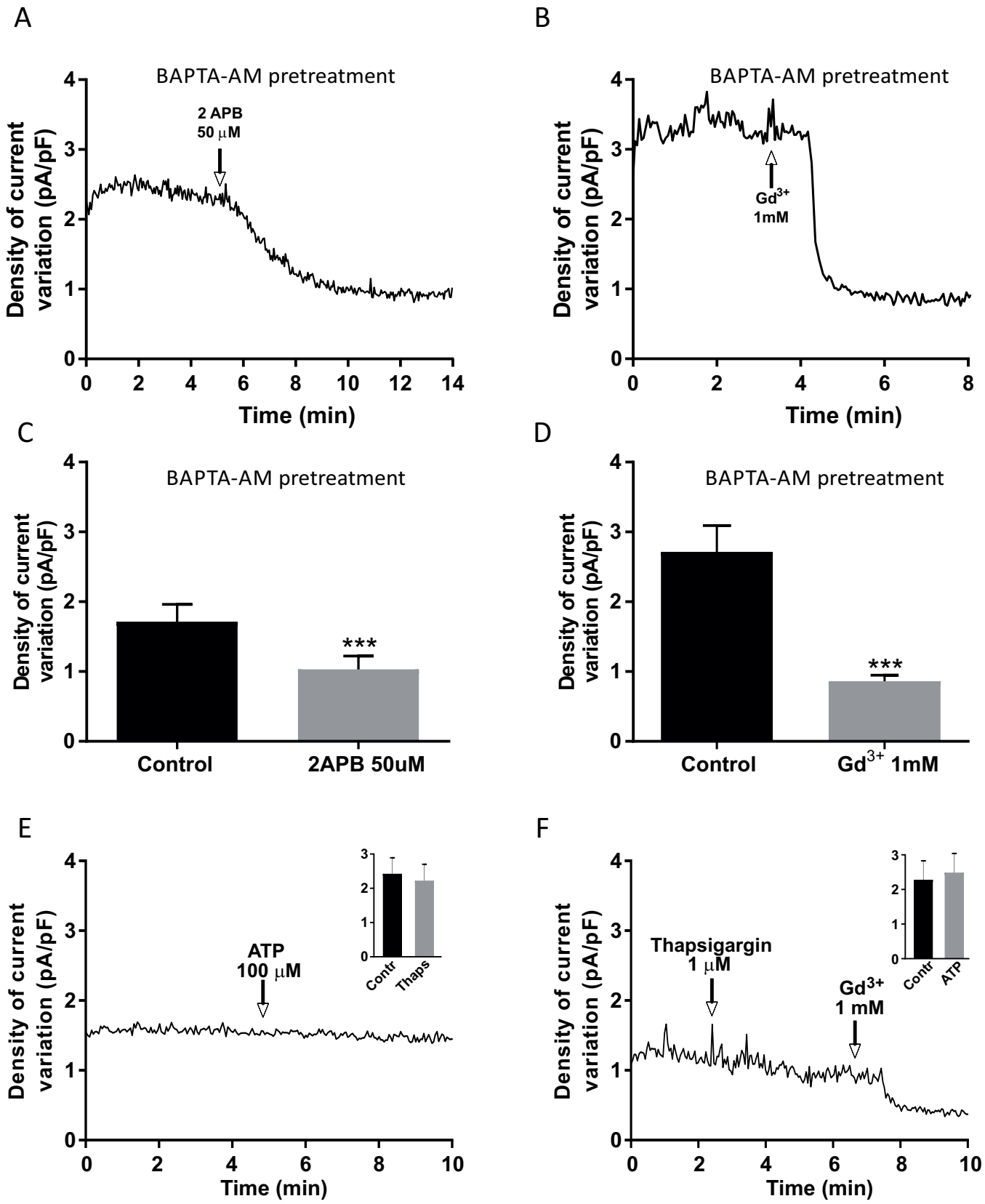

A
BAPTA-AM pretreatment
2 APB
50 μM
Density of current variation (pA/pF)
Time (min)
B
BAPTA-AM pretreatment
Gd3+
1mM
Density of current variation (pA/pF)
Time (min)
C
BAPTA-AM pretreatment
***
Density of current variation (pA/pF)
Control
2APB 50uM
D
BAPTA-AM pretreatment
***
Density of current variation (pA/pF)
Control
Gd3+ 1mM
E
ATP
100 μM
Contr
Thaps
Density of current variation (pA/pF)
Time (min)
F
Thapsigargin
1 μM
Gd3+
1 mM
Contr
ATP
Density of current variation (pA/pF)
Time (min)

Figure 3

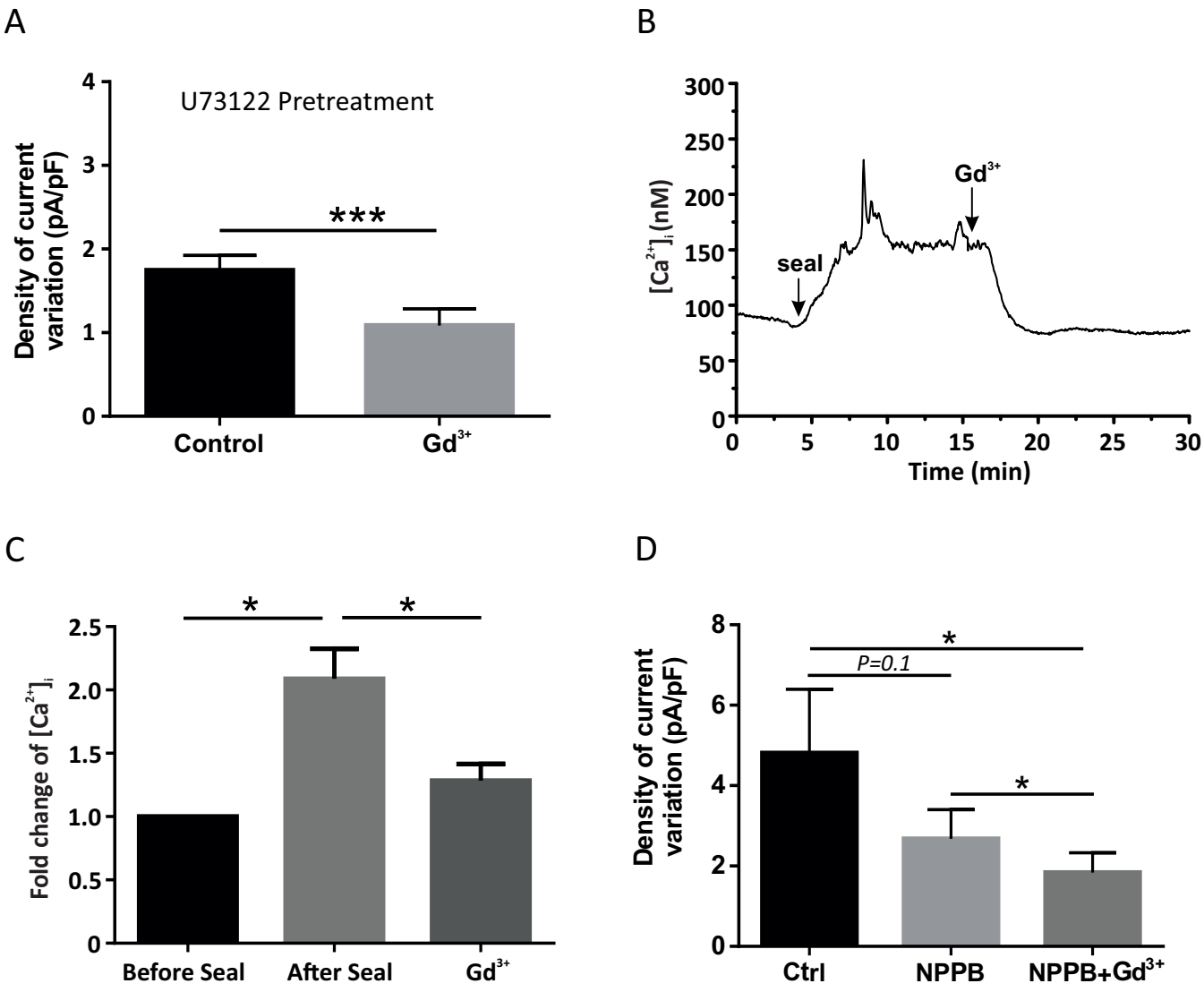

A
U73122 Pretreatment
Density of current variation (pA/pF)
***
Control
Gd3+
B
[Ca2+]i (nM)
seal
Gd3+
Time (min)
C
Fold change of [Ca2+]i
*
*
Before Seal
After Seal
Gd3+
D
Density of current variation (pA/pF)
P=0.1
*
*
Ctrl
NPPB
NPPB+Gd3+

Figure 4