



HAL
open science

Increasing expression of calcium permeable TRPC3 and TRPC7 channels enhances constitutive secretion

Verna Lavender, Setareh Chong, Katherine Ralphs, Adrian J Wolstenholme,
Barbara J. Reaves

► **To cite this version:**

Verna Lavender, Setareh Chong, Katherine Ralphs, Adrian J Wolstenholme, Barbara J. Reaves. Increasing expression of calcium permeable TRPC3 and TRPC7 channels enhances constitutive secretion. *Biochemical Journal*, 2008, 413 (3), pp.437-446. 10.1042/BJ20071488 . hal-00478907

HAL Id: hal-00478907

<https://hal.science/hal-00478907>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Increasing expression of calcium permeable TRPC3 and TRPC7 channels enhances constitutive secretion

Verna Lavender, Setareh Chong*, Katherine Ralphs,
Adrian J. Wolstenholme and Barbara J. Reaves¹

Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK

Running Title: TRPCs and secretion

Current Address:

*Department of Biology, University of York, PO Box 373, York YO10 5YW, UK

¹To whom correspondence should be addressed (e-mail: b.j.reaves@bath.ac.uk)

ABSTRACT

The hTRPC (human transient receptor potential canonical) family of non-selective cation channels are proposed to mediate calcium influx across the plasma membrane via PLC-coupled receptors. Heterologously expressed hTRPC3 and hTRPC7 have been localised at the cell surface, however, a large intracellular component has also been noted but not characterised. In this study we have investigated the intracellular pool in COS-7 cells and have shown co-localisation with markers for both the trans-Golgi network (TGN) and the cis-Golgi cisternae by immunofluorescence microscopy. Addition of Brefeldin A to cells expressing hTRPC3 or hTRPC7 resulted in the redistribution of the Golgi component to the endoplasmic reticulum indicating this pool is present in both the Golgi stack and the TGN. Expression of either TRPC3 or TRPC7, but not TRPC1 or the cell surface marker CD8, resulted in a 2-4 fold increase of secreted alkaline phosphatase in the extracellular media. Based on these data, we propose that an additional function of these members of the hTRPC family may be to enhance secretion either by affecting transport through the Golgi stack or increasing fusion at the plasma membrane.

Keywords: Transient receptor potential, cation channel trafficking, secretory pathway, calcium

INTRODUCTION

The *transient receptor potential* (TRP) protein superfamily consists of calcium permeable non-selective cation channels first described in a *Drosophila* phototransduction mutant associated with a defect in light induced calcium entry [1]. The number of mammalian homologues of *Drosophila* TRP (dTRP) that have been identified over the past decade have expanded into a superfamily which has been further divided into seven subfamilies (reviewed in [2]). Except for the lack of a voltage sensor in S4, the structure all of the TRP channels resembles that of voltage-gated ion channels; it includes six predicted transmembrane segments with N-terminal and C-terminal cytoplasmic tails and a re-entrant pore loop between S5 and S6. In addition, analogously to Kv channels, TRPs most likely form tetrameric structures.

In contrast to other ion channel families, the TRP family members are predominantly grouped according to sequence homology and are diverse in terms of modes of activation, ion selectivity, tissue distribution and function both between and within each subfamily. TRPC or 'classical' (sometimes referred to as canonical) TRPs are most closely related to dTRP and are also subdivided into a further 4 groups based on sequence similarity: TRPC1, and TRPC2 (a pseudogene in humans) in their own categories, TRPC4 and TRPC5 forming the third grouping and TRPC3, TRPC6 and TRPC7 forming the fourth subfamily. All of the TRPC channels open in response to activation of PLC which leads to the production of IP₃ and diacylglycerol (DAG). The issue of whether (or which) TRPC family members are activated by IP₃ and subsequent depletion of intracellular stores, or an increase in DAG remains controversial [3]. Alternatively, or in combination with the mechanisms described above, activation of calcium influx through TRP channels also occurs by insertion of a vesicular pool into the plasma membrane [4-6].

Not only are there differences in tissue distribution, function and activation within TRP families, it is also becoming clear that there are differences in the subcellular distribution of these channels as well. Localization studies in conjunction with electrophysiological experiments have demonstrated the presence of both heterologously expressed and endogenous TRP proteins at the plasma as predicted by a role in calcium influx [7]. However, it is apparent that large intracellular pools of TRP proteins also exist. As would be predicted from the insertional activation model, a number of studies report localisation of TRP channels including the *C. elegans* TRP,

TRP-3 [8], TRPC3 [6], TRPV5 and TRPV6 [9], TRPC5[5] and TRPV1 [10] to vesicular pools which then fuse with the plasma membrane after stimulation.

More surprisingly, TRPs have recently been implicated in endosome/lysosome function after the gene responsible for the neurogenetic disorder mucopolipidosis type IV was cloned and found to encode the group 2 TRP, TRPML (mucopolipin-1). TRPML and its homologue in *C. elegans*, CUP-5, have both been localised to late endosomes/lysosomes and have been proposed to have a role either in lysosome biogenesis [11] and/or regulation of lysosomal [12]. Another subcellular compartment in which several types of calcium channels reside is the ER. Both folding and assembly of channels occurs in this compartment and consequently it has been difficult to establish whether TRP channels actually function in this organelle or are merely being assembled and transported; a recent report suggests that the cold/menthol-sensitive TRPM8 channel is not only resident in the ER but has a role as an ER calcium-release channel[13]. Three distinct locations, the ER, plasma membrane and Golgi complex, have been reported for another group 2 TRP, TRPP2 (polycystin-2), mutations in which cause polycystic kidney disease (reviewed in[14]). Thus, in addition to their function as calcium influx channels at the plasma membrane, an increasing number of TRP channels have been demonstrated to act intracellularly in a variety of different organelles.

In this study, we present a detailed analysis of the subcellular localisation of the intracellular pools of hTRPC1, 3 and 7 and propose a novel role for both hTRPC3 and hTRPC7 in enhancing constitutive secretion.

MATERIALS AND METHODS

Antibodies and Reagents

The following antibodies were used in immunofluorescence microscopy at a 1:300 dilution unless otherwise specified: anti-Myc monoclonal antibody (9E10) (a kind gift from Prof. J.Paul Luzio, University of Cambridge), anti-Flag monoclonal antibody M2 (Sigma, Poole, Dorset UK), rabbit polyclonal Gpp130 and mouse anti-mannosidase II (Covance, UK), and sheep anti-human TGN46 (Serotec). Goat anti-rabbit IgG-Alexa Fluor 594 and goat anti-mouse IgG-Alexa Fluor 488 were purchased from Molecular Probes. Anti-myc monoclonal antibody, clone 4A6, was purchased from Upstate UK and used at a dilution of 1:200 for immunoblotting. The polyclonal rabbit anti-hTRPC7 antibody has been described previously [15]. Specific antibodies were purified by affinity chromatography on Sepharose 4B coupled to the peptide, concentrated and stored at -20°C prior to use. The antibody was used at a dilution of 1:100 for immunofluorescence and 1:1000 for immunoblotting. All other reagents were obtained from Sigma unless otherwise specified.

Constructs

Myc-hTRPC3 in expression vector pcDNA3 and Flag-hTRPC1 β in pcDNA3.1 were kind gifts from Dr. Craig Montell, Johns Hopkins University, Baltimore, MD [16]. The full length cDNA coding sequence for hTRPC7 (accession number AJ272034) was subcloned from pCR-Blunt into the HindIII and KpnI sites of the pFLAG-CMV vector (Sigma) to produce an N-terminal epitope-tagged version of the polypeptide.

Δ pMep4-hTRPC3 was generated by the insertion of a 700 nt KpnI/XhoI fragment from the 5' end of pcDNA3-hTRPC3 into the Δ pMep4 mammalian expression vector [17]. The 3' XhoI fragment of hTRPC3 was then cloned into the Δ pMep4 XhoI site. Products containing this insert in the correct orientation were determined by restriction mapping and sequencing.

The pcDNA3-eGFP-hTRPC3 vector was constructed by inserting a Kpn I fragment containing eGFP into the KpnI site of pcDNA3-hTRPC3 to create a cDNA encoding an N-terminally eGFP-tagged hTRPC3.

Cells and Transfections

African Green Monkey kidney cells (COS-7) were grown in Dulbecco's modified Eagles medium (DMEM, Sigma) containing 10% (v/v) foetal calf serum and

100 U/ml penicillin-streptomycin. Transfections were performed using Gene Juice transfection reagent (Novagen, UK) using 1 µg of construct DNA except for cotransfections wherein 0.5 µg of each plasmid was used. Further manipulations of the cells were carried out 24 hours post-transfection unless otherwise specified. For the generation of stable cell lines, COS-7 cells were transfected with the inducible expression vector ΔpMep4- hTRPC3 and stable cell lines isolated after selection in 50 µg/ml hygromycin B (Sigma) for approximately two weeks. Three clones were chosen for further study based on differential levels of expression in uninduced versus induced conditions. For inducible expression, 50 µM ZnCl₂ was added to the media and cells incubated for a further 24-48 hours prior to processing for immunocytochemistry or immunoblotting. The rat pituitary cell line, GH4C1, was a kind gift of Professor Priscilla Dannies, Department of Pharmacology, Yale University and was grown in a 1:1 mixture of DMEM and Ham's F-10 media supplemented with 15% horse serum.

Protein Extraction and Immunoblotting

Confluent cells were harvested by scraping on ice into lysis buffer consisting of 100 mM Tris HCl, pH 8.0, 1 mM MgCl₂, 0.1 mM PMSF and a protease inhibitor cocktail (Roche, Complete Mini, EDTA-free) (Buffer A) and frozen at -80°C until use. Frozen cells were thawed on ice, homogenized in a Dounce homogenizer and diluted in 0.25M sucrose, 10 mM Tris-HEPES, pH 7.4 containing protease inhibitors. The suspension was centrifuged at 3,000 x g for 15 minutes, 4°C and the supernatant then centrifuged at 50,000 x g for 30 minutes. The pellet was resuspended in Buffer A and protein determinations conducted using the BioRad protein assay. 20 µg of the crude membrane pellet was resolved on an 8% SDS Tris-glycine gel and the gel transferred to nitrocellulose. Monoclonal anti-myc 4A6 (Upstate, 1:1000) was used to detect hTRPC3 in COS-hTRPC3-1 cells and polyclonal anti-TRPC7 for detection in GH4C₁ cells (1:1000), followed by anti-mouse IgG-HRP or anti-rabbit IgG-HRP (Amersham Biosciences, LittleChalfont, Buckinghamshire, UK) respectively. Detection was carried out with ECL™ Western Blotting Detection reagent (Amersham, U.K.).

Densitometry was conducted using the Epi Chemi II Darkroom and captured luminescence imaged using LABWORKS software (UVP Lab Products, Cambridge, U.K.).

SEAP Assays

COS-7 cells and clonal COS-7 cells stably expressing N-terminal myc-tagged human TRPC3, were transiently transfected with pSEAP2-Control mammalian expression vector (BD Biosciences Clontech, Palo Alto, CA) using GeneJuice® Transfection Reagent (Novagen, La Jolla, CA) for 24 hours followed by induction with 50 μ M ZnCl₂ for a further 24 hours. Samples of media were harvested at 48 and 72 hours post-transfection, and the level of the heat-stable secreted placental alkaline phosphatase in 15 μ l aliquots was determined using BD Great EscAPe™ SEAP Chemiluminescent assay (BD Biosciences Clontech, Palo Alto, CA). Assays were conducted in transparent 96-well flat-bottomed microtitre plates, and luminescence was detected for 10 s integrals using a FARCyte™ plate luminometer (Amersham Pharmacia Biotech, Little Chalfont, Bucks). COS-7 cells were also transiently co-transfected with pSEAP2-Control mammalian expression vector and secondary recombinant mammalian expression plasmids. Extracellular media was harvested and assayed as described above. Immunofluorescence microscopy was performed on the pSEAP2-Control transfected cells to ensure the transfection efficiency was constant between the different sample groups.

Immunocytochemistry

Indirect immunofluorescence microscopy was performed as described previously [18]. Briefly, cells were washed with PBS and fixed for 20 min at room temperature using 2% (w/v) paraformaldehyde and permeabilised for 5 min with methanol at -20°C or 0.1% (v/v) Triton X-100 at room temperature, blocked with 10% (v/v) FCS in PBS for 20 min followed by addition of primary antibody at the dilutions indicated above. After washing, secondary antibody was added and the cells incubated 20 min followed by washing and mounting with Mowiol® (Calbiochem). When required, cultured cells were incubated with 5 μ g/ml Brefeldin A and incubated for 2 hrs at 37°C as previously described [18].

Imaging

Cells were visualised using either a Zeiss LSM 510 confocal microscope (Oberkochen, Germany), with a plan-apochromat 63x, 1.4 oil immersion objective lens or a Nikon TE2000 fluorescence microscope (Nikon, UK) equipped with a Hamamatsu ER-ORCA CCD camera and a Sutter Lambda 10-2 controller and filter wheel. In the latter case, images were captured using Perkin Elmer Ultraview

software (Perkin Elmer UK). The final images were processed using Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA).

Statistical analysis

Results are presented as means \pm S.E.M. with the number of experiments indicated in the legend.

Stage 2(a) POST-PRINT

RESULTS

Localisation of transiently expressed hTRPC1, hTRPC3 and hTRPC7 in COS-7 cells

A number of different approaches including immunocytochemistry have been used to analyse the expression and distribution of TRPC proteins in various tissues as well as heterologously expressing cell lines. Although expression at the plasma membrane, in vesicles and in the ER have been documented for many of the TRP family members, several reports allude to a large intracellular pool of hTRPC3 that also appears to colocalise with the Golgi complex [19-21]; this observation, however, has not been characterised further. In order to determine the exact localisation of the intracellular pool of hTRPC3, we first investigated the localisation pattern of N-terminally tagged constructs of hTRPC1, hTRPC3 and hTRPC7 in COS-7 cells. We used this cell line since the flattened morphology and large size enables clear identification of intracellular compartments and structures.

We transiently transfected COS-7 cells with a full-length construct of hTRPC3 containing an N-terminal myc epitope (Myc-hTRPC3) [16]. As shown in Figure 1A (middle image), over-expressed Myc-hTRPC3 is concentrated in a perinuclear location as well as in punctate structures throughout the periphery and at the plasma membrane. A similar distribution was seen for FLAG-hTRPC7 (Figure 1A, last image), however a more distinctive plasma membrane localisation was also observed. In contrast to the results obtained with hTRPC3 and hTRPC7, our results indicate that FLAG-hTRPC1 β [16, 22] localises predominantly to the endoplasmic reticulum (ER) in COS-7 cells (Figure 1A, first image).

In order to determine whether the large intracellular pool of hTRPC3 is merely due to overexpression and consequent saturation within the exocytic pathway resulting in the Golgi-like distribution, COS-7 cells were stably transfected with Myc-hTRPC3 under the regulation of the inducible metallothionein promoter contained in the expression vector Δ pMEP4 (COS-hTRPC3-1). Even under uninduced conditions some expression was detected by both immunofluorescence and immunoblotting (Figure 1B and C). However, at these low levels of expression the distribution of hTRPC3 in these cells is similar to that after transient transfection with little or no detectable signal apparent at the plasma membrane and the majority of signal appearing intracellularly (Figure 1B). With increasing expression levels, the

concentration in the perinuclear area and in punctate structures increases without a concomitant increase at the cell surface. These data suggest that the perinuclear localisation is not simply due to overexpression resulting in an increased concentration in the secretory pathway.

In order to confirm the distribution data described above we also conducted co-transfection experiments with hTRPC1 β and hTRPC3. Based on previous immunoprecipitation data [23], hTRPC1 and hTRPC3 would not be expected to interact and indeed the localisation of hTRPC1 did not appear to alter upon co-transfection with hTRPC3 [7]; the intracellular distribution of hTRPC3, however, was not investigated in these experiments. In contrast, recent reports based on immunoprecipitation data suggest TRPC1 and TRPC3 will heteromultimerize [24]. Results presented in Figure 2A demonstrate that COS-7 cells co-transfected with hTRPC1 β and hTRPC3 exhibited expression patterns for both proteins that resembled the pattern in singly-transfected cells. Minimal colocalization was apparent and the intracellular pool of hTRPC3 remained unaffected.

The intracellular pool of hTRPC3 and hTRPC7 localise to both the trans-Golgi network (TGN) and the Golgi stack

One model for TRP activation includes an insertion into the plasma membrane from an intracellular pool. There is a growing body of evidence to support this model (reviewed in [25]; however, although there is evidence for rapid insertion of TRPCs from a sub-plasmalemmal pool using TIRF microscopy [5], the possibility of contributions by other intracellular pools to this process has not been investigated. Recycling from the TGN through the plasma membrane has been well characterised for a number of proteins including TGN38 [26], and furin [27]. Precedence for transport of receptors from the TGN to the cell surface upon stimulation has also been demonstrated for two members of the tumour necrosis factor receptor family, TNFR1 and Fas [28]. It was therefore of interest to determine whether the perinuclear localisation of hTRPC3 and hTRPC7 corresponded to the TGN using double-immunofluorescence labelling of transiently transfected COS-7 cells with a marker for the TGN, TGN46 [29]. For comparison, cells were also transfected with hTRPC1 β and processed for IF using a monoclonal anti-FLAG antibody and sheep anti-TGN46. Limited if any co-localisation was detected for hTRPC1 and TGN46

(Figure 2B, top panel), however a high degree of colocalisation was seen between both hTRPC3 and hTRPC7 and TGN46 in the perinuclear region (Figure 2B, middle and lower panel). This data along with the co-transfection experiment supports the conclusion that these TRPC3/7 and TRPC1 isoforms reside in separate intracellular compartments.

Although the majority of the intracellular pool of hTRPC3 and hTRPC7 co-localised with TGN46, the co-localization was not complete. The possibility that some of the hTRPC3 concentrates in the Golgi stack was approached initially by conducting double labelling with hTRPC3 and a marker for the *cis* Golgi, Gpp130 [30]. As for TGN46, there was also extensive co-localization of hTRPC3 (Figure 3, top panel) with Gpp130. This approach, however, does not provide definitive means for the dissection of the TGN from the Golgi stack. Brefeldin A (BFA) is a fungal metabolite which inhibits ADF-ribosylation factors and consequently coat formation; the consequence of this inhibition is the disassembly and redistribution of the Golgi complex. In contrast to the TGN which collapses to a small dot around the microtubule organizing centre (MTOC), BFA causes the Golgi stack to be redistributed into the ER in COS-7 cells [18]. Surprisingly, after BFA treatment, hTRPC3 and hTRPC7 clearly redistribute with Gpp130 into the ER (Figure 3 panel 2 & 4) whereas TGN46 appeared in the classic 'BFA dot' (Figure 3, panel 3). This indicates that hTRPC3 localises not only to the TGN but also to the Golgi stack.

Endogenous TRPC7 localises to the Golgi complex in a rat pituitary cell line, GH₄C₁

A key question concerning possible effects of overexpression in transfected cells on subcellular distribution prompted us to examine various cell lines to see if we could detect endogenous protein using immunofluorescence microscopy. Unfortunately, none of the cell lines tested gave an unequivocal signal with either a commercially available or our own polyclonal anti-hTRPC3 antibody. However, as TRPC7 appears to have a similar distribution to TRPC3 and TRPC7 mRNA levels have been reported be highest in pituitary [31], we used a polyclonal anti-TRPC7 antibody raised to the extreme C-terminus [15] to assess distribution in a rat pituitary cell line, GH₄C₁. Consistent with the perinuclear concentration in COS-7 cells expressing FLAG-hTRPC7, the endogenous protein was detected in a Golgi-like area close to the nucleus (Figure 4A). Double-labelling experiments with the *cis*-Golgi

marker α -mannosidase II, indicate a high degree of colocalisation although not complete overlap. Immunoblotting of whole cell lysate revealed a positive signal at approximately 100kD corresponding to the same band detected in HEK293 cells overexpressing hTRPC7 which is not detected in untransfected cells (Figure 4B). This supports the finding that the signal detected by immunofluorescence was indeed TRPC7.

Expression of either hTRPC3 or hTRPC7, but not hTRPC1, enhances constitutive secretion

As a central organelle of the secretory pathway, one of the major functions of the Golgi complex is in sorting and modifying proteins destined for either the plasma membrane, endosomal compartments or specialized secretory granules. These processes are dependent on both the formation of vesicles and the fusion of these vesicles with a target membrane. Several studies implicate a role for luminal calcium in organellar fusion events, particularly endosome/lysosome fusion [32]. We therefore reasoned that one possible consequence of having a calcium channel in the Golgi complex might be to increase vesicle fusion and therefore constitutive secretion. In order to test this hypothesis, COS-7 control or COS-hTRPC3-1 cells were transfected with a plasmid encoding a secreted form of alkaline phosphatase (SEAP) [33]. The newly synthesised enzyme is translocated into the lumen of the ER, transported constitutively through the Golgi complex and then targeted for secretion at the plasma membrane. The amount of alkaline phosphatase present in the cell culture medium of untransfected control, untransfected control under inducing conditions (50 μ M ZnCl₂), uninduced or induced COS-hTRPC3 was measured 48 hours after transfection. As indicated in Figure 1C, hTRPC3 is expressed at low levels in this cell line even in the absence of induction. The 2-fold increase in secretion of the enzyme over control even in uninduced cells suggests that the presence of hTRPC3 has a significant effect on transport through the secretory pathway. A 3-fold increase was observed after induction of COS-hTRPC3 cells compared to control cells treated with 50 μ M ZnCl₂; there was also a significant increase when compared to uninduced cells (Figure 5A).

To examine whether this was specific for hTRPC3, we conducted similar experiments using hTRPC1, hTRPC7 and a resident cell surface protein that normally

traffics to the plasma membrane through the Golgi complex, CD8. In the absence of stable cell lines expressing these proteins it was necessary to carry out these studies on transiently transfected cells. In our hands, co-transfections often result in cells not only co-expressing but also expressing either one or the other protein. Hence, for all experiments, the number of cells expressing both the protein of interest and SEAP was determined by immunofluorescence to ensure comparable levels of both co-transfected and singly transfected cells (data not shown). Expression of each construct was compared to the expression of eGFP-N1 to control for effects of transfection alone. As can be seen in Figure 5B, expression of neither CD8 nor hTRPC1 had significant effects on the amount of SEAP in the medium; however, the expression of both hTRPC3 and its closely related family member, hTRPC7 increased constitutive secretion by at least 2.5 fold, and almost 3.5 fold in the case of hTRPC3. These findings suggest that not only are hTRPC3 and hTRPC7 expressed in the Golgi complex but they also have a significant effect on a major function of this organelle, constitutive secretion.

DISCUSSION

Despite an increasing number of studies focusing on hTRPC3 function and localisation, an in-depth analysis of its subcellular distribution has not previously been conducted. The initial hypothesis that TRPCs function as store-operated calcium channels predicts a plasma membrane localisation for the TRPC family. However a large intracellular pool of hTRPC3 is apparent in many immunofluorescence micrographs [19-21, 34]. Most dramatically, in immunofluorescence studies on the expression of TRPC isoforms in mouse skeletal muscle fibers, endogenous TRPC1,4 and 6 were clearly localised to the plasma membrane, however TRPC3 in particular was found almost exclusively intracellularly in perinuclear structures reminiscent of the Golgi [21]. We have characterised the distribution of hTRPC3 using double-label immunofluorescence microscopy in COS-7 cells and demonstrate that: 1) a major pool of hTRPC3 colocalises with markers for both the TGN and the Golgi stack, 2) it differs from hTRPC1 which is also found predominantly intracellularly but remains associated with the ER, and 3) a large proportion of the perinuclear pool redistributes after Brefeldin A treatment suggesting it localises throughout the Golgi stack. A similar distribution pattern is observed for hTRPC7; considering the close sequence homology of these subfamily members and their ability to interact as demonstrated by co-immunoprecipitation experiments this is not surprising [23, 35]. Although previous studies include evidence for TRPC1 on the plasma membrane, our results show a completely intracellular distribution which is consistent with results from subcellular fractionation of endogenous TRPC1 in platelets [36] and sperm [19] and overexpressed TRPC1 in HEK293 cells [7]. Potential ER exit motifs are present within the amino acid sequence of TRPC3/7 but are lacking in TRPC1. Putative ER retention signals are also present in TRPC1; these are now being investigated to determine whether the difference in trafficking between these subfamily members is due to specific targeting signals.

The best characterised trafficking routes for proteins recycling through the plasma membrane back to an intracellular store is to and from the TGN or from the TGN through the plasma membrane to recycling endosomes or specialised pools such as for the glucose transporter, GLUT4, or neurotransmitters. Hence, our first hypothesis as to a possible function for the TGN/Golgi-localised pool of hTRPC3 was as a 'readily releasable' storage pool. A number of studies have recently demonstrated translocation of a variety of TRP family members from an intracellular pool to the

plasma membrane including a *C. elegans* TRPC homolog, TRP-3 [8], TRPV1 [10], TRPC3 [37] and TRPC5 [5]. Most of these studies postulate that the translocation occurs from a vesicular pool close to the plasma membrane. Our finding that the majority of the intracellular pool was localised in a perinuclear localisation overlapping the Golgi stack was therefore surprising. An intriguing possibility, however, is that the translocatable pool is at least partially Golgi-derived. Singh et al (2004) clearly demonstrate a decrease in the agonist stimulated plasma membrane localisation of TRPC3 after treatment of cells for 30-60 minutes with Brefeldin A, a compound which disrupts the Golgi stack and intra-Golgi transport but does not inhibit post-Golgi transport to the cell surface [38]. Since the pool of TRPC3 that was being translocated was reported to be a subplasmalemmal vesicular pool, BFA should not have inhibited this translocation step but it would inhibit translocation from the Golgi stack. Precedence for transport occurring along this trafficking route in response to external stimuli comes from studies on the stimulation of Fas from the Golgi to the cell surface which occurs upon stimulation by p53 [39]. Although this could be one possibility for our finding that a large pool of TRPC3 resides in the Golgi stack, it raised the question as to whether TRPC3 may have functions in the Golgi in addition to, or instead of, acting as a calcium entry channel at the plasma membrane.

A variety of Ca^{2+} -dependent functions occur within the Golgi which include post-translational modifications of secretory proteins [40], formation of COPI coats on Golgi-ER transport vesicles [41] and both anterograde and retrograde vesicular transport through and to the Golgi complex [42, 43]. In addition to having a role in coat formation, release of luminal calcium from vesicles has also been suggested to play a role in vesicle fusion as shown for insulin containing secretory granules with the plasma membrane [44]. Thus, we hypothesised that an alternative function for TRPC3 could be mediating vesicle fusion, either between the ER and the cis-Golgi, transport from the trans-Golgi/TGN and/or increasing fusion at the plasma membrane. Indeed, TRPC3 has already been shown to interact by yeast two hybrid with VAMP2, NSF and α -SNAP [6] which normally reside in vesicles and play a role in vesicle fusion. TRPM7 has also recently been localised to synaptic vesicle membranes where it forms complexes with SNAREs and their associated proteins, e.g. snapin, as well as the scaffolding protein synapsin. Synapsin has been postulated to anchor vesicles in a

DISCUSSION

Despite an increasing number of studies focusing on hTRPC3 function and localisation, an in-depth analysis of its subcellular distribution has not previously been conducted. The initial hypothesis that TRPCs function as store-operated calcium channels predicts a plasma membrane localisation for the TRPC family. However a large intracellular pool of hTRPC3 is apparent in many immunofluorescence micrographs [19-21, 34]. Most dramatically, in immunofluorescence studies on the expression of TRPC isoforms in mouse skeletal muscle fibers, endogenous TRPC1,4 and 6 were clearly localised to the plasma membrane, however TRPC3 in particular was found almost exclusively intracellularly in perinuclear structures reminiscent of the Golgi [21]. We have characterised the distribution of hTRPC3 using double-label immunofluorescence microscopy in COS-7 cells and demonstrate that: 1) a major pool of hTRPC3 colocalises with markers for both the TGN and the Golgi stack, 2) it differs from hTRPC1 which is also found predominantly intracellularly but remains associated with the ER, and 3) a large proportion of the perinuclear pool redistributes after Brefeldin A treatment suggesting it localises throughout the Golgi stack. A similar distribution pattern is observed for hTRPC7; considering the close sequence homology of these subfamily members and their ability to interact as demonstrated by co-immunoprecipitation experiments this is not surprising [23, 35]. Although previous studies include evidence for TRPC1 on the plasma membrane, our results show a completely intracellular distribution which is consistent with results from subcellular fractionation of endogenous TRPC1 in platelets [36] and sperm [19] and overexpressed TRPC1 in HEK293 cells [7]. Potential ER exit motifs are present within the amino acid sequence of TRPC3/7 but are lacking in TRPC1. Putative ER retention signals are also present in TRPC1; these are now being investigated to determine whether the difference in trafficking between these subfamily members is due to specific targeting signals.

The best characterised trafficking routes for proteins recycling through the plasma membrane back to an intracellular store is to and from the TGN or from the TGN through the plasma membrane to recycling endosomes or specialised pools such as for the glucose transporter, GLUT4, or neurotransmitters. Hence, our first hypothesis as to a possible function for the TGN/Golgi-localised pool of hTRPC3 was as a 'readily releasable' storage pool. A number of studies have recently demonstrated translocation of a variety of TRP family members from an intracellular pool to the

effective than EGTA at inhibiting many of the organellar fusion and transport events within the cell most likely due to a much faster on rate for calcium binding, it has been proposed that calcium transients or gradients are more important than steady state calcium levels. This would also suggest the necessity for either a vesicular or organellar calcium release channel which could mediate rapid but localised increases in calcium; TRPC3 could fulfil this function. Since a number of agents which affect the ability of TRPC3 to mediate calcium entry at the plasma membrane are also integral to the Golgi complex, e.g. DAG, PLC γ , and PLD, an intriguing possibility is that these agents may affect channel function at both locations. It has recently been shown that diacylglycerol levels in the Golgi are critical for Golgi secretory function [47]. DAG also activates mitochondrial cationic channels and elicits release of sequestered calcium in this organelle [48] so it is possible that this may also occur in the Golgi mediated via TRP channels. Indeed, release of Golgi calcium in response to menthol, an activator of TRPM8, has recently been reported but surprisingly this response could also be elicited in cells without endogenous TRPM8 suggesting that other TRP channels may be responsible for this release from Golgi stores [49]. TRPC3/7 could be ideal candidates.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Craig Montell for the generous gift of the hTRPC1 and hTRPC3 constructs and Professor J. Paul Luzio for kindly supplying the 9E10 ascites, helpful discussions and much appreciated support. This work was supported by The Wellcome Trust, Grant #069934.

REFERENCES

- 1 Hardie, R.C. and Minke, B. (1992) The *trp* gene is essential for a light-activated Ca²⁺ channel in Drosophila photoreceptors. *Neuron* **8**, 643-651
- 2 Montell, C. (2005) Drosophila TRP channels. *Eur.J. Physiol.* **451**, 19-28
- 3 Putney, J.W. (2007) Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here). *Cell Calcium* **42**, 103-110
- 4 Cayouette, S., Lussier, M. P., Mathieu, E. L., Bousquet, S. M. and Boulay, G. (2004) Exocytotic insertion of TRPC6 channels into the plasma membrane upon Gq Protein-coupled receptor activation. *J. Biol. Chem.* **279**, 7241-7246
- 5 Bezzerides, V. J., Ramsey, I. S., Kotecha, S., Greka, A. and Clapham, D. E. (2004) Rapid vesicular translocation and insertion of TRP channels. *Nat. Cell Biol.* **6**, 709-720
- 6 Singh, B. B., Lockwich, T. P., Bandyopadhyay, B. C., Liu, X., Bollimuntha, S., Brazer, S. C., Combs, C., Das, S., Leenders, A. G., Sheng, Z. H., Knepper, M. A., Ambudkar, S. V. and Ambudkar, I. S. (2004) VAMP2-dependent exocytosis regulates plasma membrane insertion of TRPC3 channels and contributes to agonist-stimulated Ca²⁺ influx. *Mol. Cell* **15**, 635-646
- 7 Hofmann, T., Schaefer, M., Schultz, G. and Gudermann, T. (2002) Subunit composition of mammalian transient receptor potential channels in living cells. *Proc. Natl. Acad. Sci. USA* **99**, 7461-7466
- 8 Xu, X.-Z.S. and Sternberg, P.W. (2003) A *C. elegans* sperm TRP protein Xu, X. Z. and Sternberg, P. (2003) A *C. elegans* sperm TRP protein required for sperm-egg interactions during fertilization. *Cell* **114**, 285-297
- 9 Hoenderop, J. G., Voets, T., Hoefs, S., Weidema, F., Prenen, J., Nilius, B. and Bindels, R. J. (2003) Homo- and heterotetrameric architecture of the epithelial Ca²⁺ channels TRPV5 and TRPV6. *EMBO J.* **22**, 776-785
- 10 Morenilla-Palao, C., Planells-Cases, R., Garcia-Sanz, N. and Ferrer-Montiel, A. (2004) Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. *J. Biol. Chem.* **279**, 25665-25672
- 11 Piper, R. C. and Luzio, J. P. (2004) CUPpling calcium to lysosomal biogenesis. *Trends Cell Biol.* **14**, 471-473
- 12 Soyombo, A. A., Tjon-Kon-Sang, S., Rbaibi, Y., Bashllari, E., Bisceglia, J., Muallem, S. and Kiselyov, K. (2005) TRP-ML1 regulates lysosomal pH and lysosomal lipid hydrolytic activity. *J. Biol. Chem.* **281**, 7294-7301

- 13 Thebault, S., Lemonnier, L., Bidaux, G., Flourakis, M., Bavencoffe, A., Gordienko, D., Roudbaraki, M., Delcourt, P., Panchin, Y., Shuba, Y., Skryma, R. and Prevarskaya, N. (2005) Novel role of cold/menthol-sensitive TRPM8 in the activation of store-operated channels in LNCaP human prostate cancer epithelial cells. *J. Biol. Chem.* **204**, 320-328
- 14 Kottgen, M. and Walz, G. (2005) Subcellular localization and trafficking of polycystins. *Pflugers Arch.* **451**, 286-293
- 15 Sekaran, S., Lall, G. S., Ralphs, K. L., Wolstenholme, A. J., Lucas, R. J., Foster, R. G. and Hankins, M. W. (2007) 2-Aminoethoxydiphenylborane is an acute inhibitor of directly photosensitive retinal ganglion cell activity *In Vitro* and *In Vivo*. *J. Neurosci.* **27**, 3981-3986
- 16 Xu, X. Z., Li, H. S., Guggino, W. B. and Montell, C. (1997) Coassembly of TRP and TRPL produces a distinct store-operated conductance. *Cell* **89**, 1155-116417
- 17 Roquemore, E.P. and Banting, G. (1998) Efficient trafficking of TGN38 from the endosome to the *trans*-Golgi network requires a free hydroxyl group at position 331 in the cytosolic domain. *Mol. Biol. Cell* **9**, 2125-2144
- 18 Reaves, B. and Banting, G. (1992) Perturbation of the morphology of the *trans*-Golgi network following Brefeldin A treatment: redistribution of a TGN-specific integral membrane protein, TGN38. *J. Cell Biol.* **116**, 85-94
- 19 Trevino, C. L., Serrano, C. J., Beltran, C., Felix, R. and Darszon, A. (2001) Identification of mouse *trp* homologs and lipid rafts from spermatogenic cells and sperm. *FEBS Letts.* **509**, 119-125
- 20 McKay, R. R., Szymeczek-Seay, C. L., Lievremont, J. P., Bird, G. S., Zitt, C., Jungling, E., Luckhoff, A. and Putney, J. W., Jr. (2000) Cloning and expression of the human transient receptor potential 4 (TRP4) gene: localization and functional expression of human TRP4 and TRP3. *Biochem. J.* **351**, 735-746
- 21 Vandebrouck, C., Martin, D., Colson-Van Schoor, M., Debaix, H. and Gailly, P. (2002) Involvement of TRPC in the abnormal calcium influx observed in dystrophic (mdx) mouse skeletal muscle fibers. *J. Cell Biol.* **158**, 1089-1096
- 22 Wes, P. D., Chevesich, J., Jeromin, A., Rosenberg, C., Stetten, G. and Montell, C. (1995) TRPC1, a human homolog of a *Drosophila* store-operated channel. *Proc. Natl. Acad. Sci. U S A* **92**, 9652-9656
- 23 Goel, M., Sinkins, W. G. and Schilling, W. P. (2002) Selective association of TRPC channel subunits in rat brain synaptosomes. *J. Biol. Chem.* **277**, 48303-48310

- 24 Yuan, J. P., Zeng, W., Huang, G. N., Worley, P. F. and Muallem, S. (2007) STIM1 heteromultimerizes TRPC channels to determine their function as store-operated channels. *Nat. Cell Biol.* **9**, 636-645
- 25 Cayouette, S. and Boulay, G. (2007) Intracellular trafficking of TRP channels. *Cell Calcium* **42**, 225-232
- 26 Reaves, B. and Banting, G. (1992) Perturbation of the morphology of the trans-Golgi network following Brefeldin A treatment: redistribution of a TGN-specific integral membrane protein, TGN38. *J. Cell. Biol.* **116**, 85-94
- 27 Thomas, G. (2002) Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat. Revs. Mol. Cell Biol.* **10**, 753-766
- 28 Storey, H., Stewart, A., Vandenaabeele, P. and Luzio, J. (2002) The p55 tumour necrosis factor receptor TNFR1 contains a trans-Golgi network localization signal in the C-terminal region of its cytoplasmic tail. *Biochem. J.* **366**, 15-22
- 29 Ponnambalam, S., Girotti, M., Yaspo, M., Owen, C., Perry AC, Suganuma, T., Nillson, T., Fired, M., Banting, G. and Warren, G. (1996) Primate homologues of rat TGN38: primary structure, expression and function. *J. Cell Sci.* **109**, 675-685
- 30 Linstedt, A. D., Mehta, A., Suhan, J., Reggio, H. and Hauri, H. (1997) Sequence and overexpression of GPP130/GIMPc: evidence for saturable pH-sensitive targeting of a type II early Golgi membrane protein. *Mol. Biol. Cell* **8**, 1073-1087
- 31 Riccio, A., Medhurst, A. D., Mattei, C., Kellsell, R. E., Calver, A. R., Randall, A. D., Benham, C. D. and Pangalos, M. N. (2002) mRNA distribution analysis of human TRPC family in CNS and peripheral tissues. *Mol. Brain Res.* **109**, 95-104
- 32 Pryor, P. R., Mullock, B. M., Bright, N. A., Gray, S. R. and Luzio, J. P. (2000) The role of intraorganellar Ca(2+) in late endosome-lysosome heterotypic fusion and in the reformation of lysosomes from hybrid organelles. *J. Cell Biol.* **149**, 1053-1062
- 33 Towler, M. C., Prescott, A. R., James, J., Lucocq, J. M. and Ponnambalam, S. (2000) The manganese cation disrupts membrane dynamics along the secretory pathway. *Exp. Cell Res.* **259**, 167-179
- 34 Wang, W., O'Connell, B., Dykeman, R., Sakai, T., Delporte, C., Swaim, W., Zhu, X., Birnbaumer, L. and Ambudkar, I. S. (1999) Cloning of Trp1beta isoform from rat brain: immunodetection and localization of the endogenous Trp1 protein. *Am. J. Physiol.* **276**, C969-979
- 35 Vannier, B., Zhu, X., Brown, D. and Birnbaumer, L. (1998) The membrane topology of human transient receptor potential 3 as inferred from glycosylation-scanning mutagenesis and epitope immunocytochemistry. *J. Biol. Chem.* **273**, 8675-8679

- 36 Hassock, S. R., Zhu, M. X., Trost, C., Flockerzi, V. and Authi, K. S. (2002) Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. *Blood* **100**, 2801-2811
- 37 Singh, B.B. et al. (2001) Trp1-dependent enhancement of salivary gland fluid secretion: role of store-operated calcium entry. *FASEB J.* **15** (9), 1652-1654
- 38 Klausner, R., Donaldson, J. and Lippincott-Schwartz, J. (1992) Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **116**, 1071-1080
- 39 Bennett, M., Macdonald, K., Chan, S. W., Luzio, J. P., Simari, R. and Weissberg, P. (1998) Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science* **282**, 290-293
- 40 Austin, C. D. and Shields, D. (1996) Prosomatostatin processing in permeabilized cells. Calcium is required for prohormone cleavage but not formation of nascent secretory vesicles. *J. Biol. Chem.* **271**, 1194-1199
- 41 Ahluwalia, J. P., Topp, J. D., Weirather, K., Zimmerman, M. and Stamnes, M. (2001) A role for calcium in stabilizing transport vesicle coats. *J. Biol. Chem.* **276**, 34148-34155
- 42 Porat, A. and Elazar, Z. (2000) Regulation of intra-Golgi membrane transport by calcium. *J. Biol. Chem.* **275**, 29233-29237
- 43 Chen, J. L., Ahluwalia, J. P. and Stamnes, M. (2002) Selective effects of calcium chelators on anterograde and retrograde protein transport in the cell. *J. Biol. Chem.* **277**, 35682-35687
- 44 Mitchell, K. J., Pinton, P., Varadi, A., Tacchetti, C., Ainscow, E. K., Pozzan, T., Rizzuto, R. and Rutter, G. A. (2001) Dense core secretory vesicles revealed as a dynamic Ca²⁺ store in neuroendocrine cells with a vesicle-associated membrane protein aequorin chimera. *J. Cell Biol.* **155**, 41-51
- 45 Venkatachalam, K. and Montell, C. (2007) TRP Channels. *Ann. Rev. Biochem.* **76**, 387-417
- 46 Mathie, A., Sutton, G.L., Clarke, C.E. and Veale, E. L. (2006) Zinc and copper: Pharmacological probes and endogenous modulators of neuronal excitability. *Pharmacology & Therapeutics* **111** (3), 567-583
- 47 Litvak, V., Dahan, N., Ramachandran, S., Sabanay, H. and Lev, S. (2005) Maintenance of the diacylglycerol level in the Golgi apparatus by the Nir2 protein is critical for Golgi secretory function. *Nat. Cell Biol.* **7**, 225-234
- 48 Chinopoulos, C., Starkov, A. A., Grigoriev, S., Dejean, L. M., Kinnally, K. W., Liu, X., Ambudkar, I. S. and Fiskum, G. (2005) Diacylglycerols activate mitochondrial cationic channel(s) and release sequestered Ca²⁺. *J. Bioenerg. Biomembr.* **37**, 237-247

- 49 Mahieu, F., Owsianik, G., Verbert, L., Janssens, A., De Smedt, H., Nilius, B. and Voets, T. (2007) TRPM8-independent menthol-induced Ca²⁺ release from endoplasmic reticulum and Golgi. *J. Biol. Chem.* **282**, 3325-3336

Stage 2(a) POST-PRINT

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071488

FIGURE LEGENDS

Figure 1. Expression of FLAG-hTRPC1, Myc-hTRPC3 and FLAG-hTRPC7 in COS-7 cells. A) COS-7 cells transiently expressing FLAG-hTRPC1, Myc-hTRPC3 or FLAG-hTRPC7 were processed for immunofluorescence microscopy using antibodies to the epitope tags. B) A clonal cell line expressing myc-hTRPC3 downstream of the metallothionein promoter (COS-hTRPC3-1) was grown for 48 hours either in the absence (uninduced) or presence of 10 μ M or 50 μ M ZnCl₂ to induce increasing levels of expression. Cells were fixed and processed for immunofluorescence microscopy using a monoclonal antibody to the myc epitope tag. C) Untransfected COS-7 and COS-hTRPC3-1 cells were treated as in (B) and processed for immunoblotting. The immunoblot obtained using a monoclonal anti-myc antibody is shown on the left. The filled arrow indicates the position of hTRPC3 which is absent in the untransfected cells. The dashed arrow indicates the position of a cross-reacting band which appears in the untransfected cells. Increases in expression levels were quantitated and are expressed as arbitrary units in the right hand panel. Bars, 10 μ m.

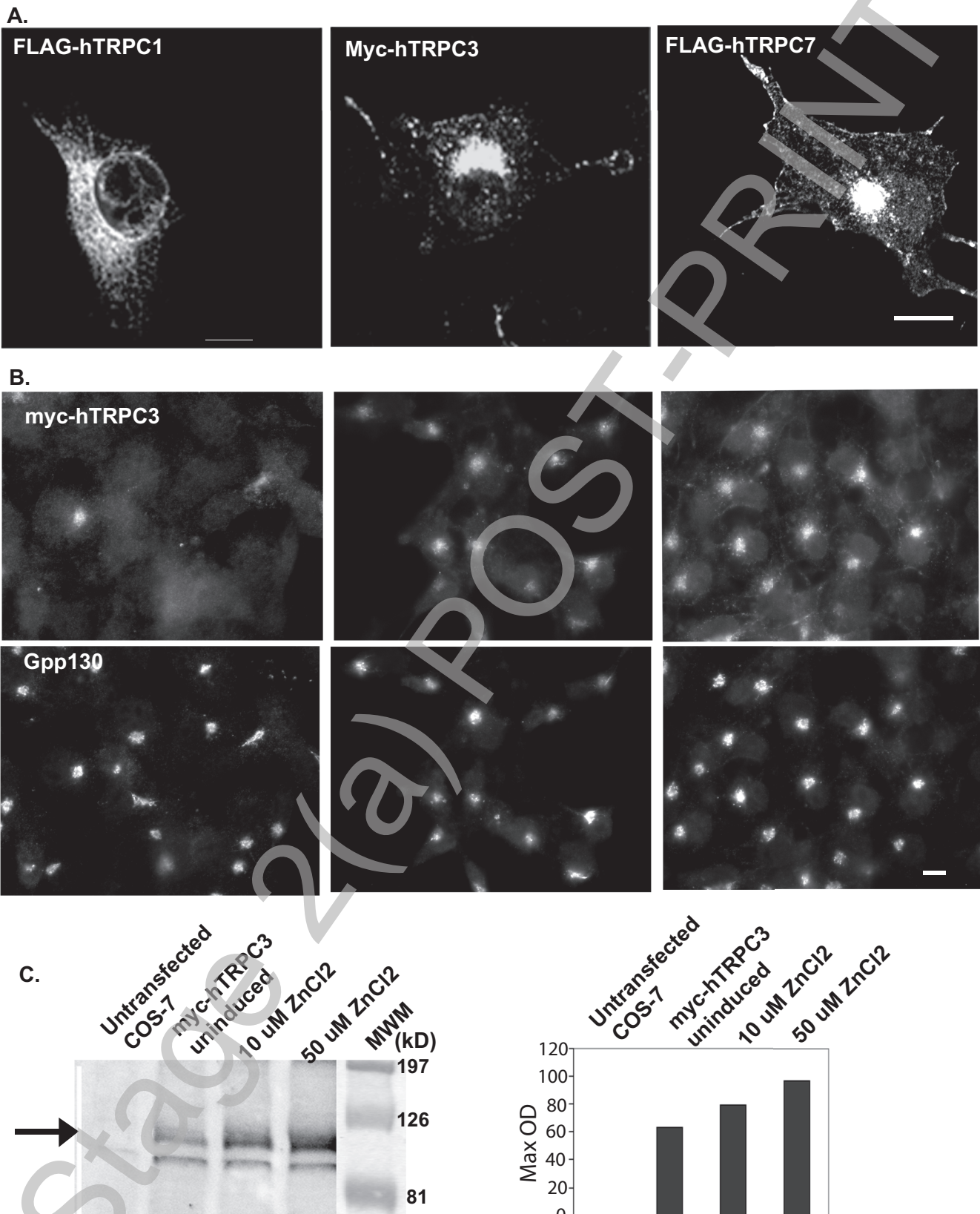
Figure 2. (A) Co-expression of TRPC1 and TRPC3 do not alter their subcellular distribution. COS-7 cells were co-transfected with both Flag-hTRPC1 and Myc-hTRPC3 constructs. Distribution of the tagged proteins was determined using a monoclonal anti-Flag antibody and a rabbit polyclonal antiserum to TRPC3. Co-expression of the two constructs did not result in redistribution of either isoform. (B) hTRPC3 and hTRPC7, but not hTRPC1 colocalise with TGN46. Double-label immunofluorescence studies were performed on COS-7 cells transiently transfected with hTRPC1 (top panel) using a monoclonal antibody to the FLAG epitope tag and a sheep polyclonal antibody to a marker of the *trans*-Golgi network (TGN), TGN46. Minimal overlap is seen as represented by a lack of yellow in the merged image. Cells transiently expressing hTRPC3 (middle panel) and hTRPC7 (bottom panel) were processed for immunofluorescence microscopy using a mouse anti-myc epitope antibody 9E10 (hTRPC3) or monoclonal anti-FLAG (hTRPC7) and sheep anti-TGN46 (middle panels). Overlap between hTRPC3 and both TGN46 and Gpp130 is seen in yellow in the merged images. Bar, 10 μ m

Figure 3. hTRPC3 and hTRPC7 localise to the Golgi stack. COS cells transiently transfected with Myc-hTRPC3 or FLAG-hTRPC7 were treated with (bottom 3 panels, 'Brefeldin A') or without (top panel, 'control') 5 $\mu\text{g/ml}$ BFA for 2 hours, fixed and processed for immunofluorescence microscopy using monoclonal antibodies to either the myc- or FLAG-epitopes respectively and either rabbit anti-Gpp130 or sheep anti-TGN46 as indicated. Overlap between the redistributed Myc-hTRPC3 and Gpp130 is shown in yellow in the merged image. Bar, 10 μm

Figure 4. Endogenous TRPC7 colocalises with a marker for the cis-Golgi, mannosidase II, in a rat pituitary cell line GH₄C₁. A) GH₄C₁ cells were fixed and processed for immunocytochemistry using a polyclonal rabbit anti-hTRPC7 antibody detected by a goat anti-rabbit Alexa 488 (green) and a monoclonal anti-mannosidase II antibody detected by a goat anti-mouse Alexa 594 (red). Overlap is depicted by yellow (merge). B) Immunoblot demonstrating the specificity of the polyclonal rabbit antisera raised to TRPC7. Whole cell lysate from either untransfected (negative control) or hTRPC7-transfected HEK (positive control) and GH₄C₁ cells were subjected to SDS-PAGE and then immunoblotted using the affinity-purified anti-TRPC7 antiserum. A band of approximately 100kD is detected in both the hTRPC7 transfected HEK cells and the GH₄C₁ cells but is absent in the untransfected controls. Bar, 10 μm

Figure 5. hTRPC3 and hTRPC7, but not hTRPC1, increase constitutive secretion. A) Control untransfected and COS-hTRPC3-1 cells were transfected with a construct which expresses a soluble secreted form of alkaline phosphatase. At 24 hours post-transfection they were cultured in the presence or absence of 50 μM ZnCl₂ for a further 48 hours and assayed in triplicate for SEAP activity. The data are presented as a percentage of enzyme activity (luminescence) in cells expressing SEAP alone. The results are means \pm S.E.M. for three independent experiments. B) COS-7 cells were co-transfected with the SEAP vector and EGFP-N1, CD8, FLAG-hTRPC1, eGFP-TRPC3 or FLAG-hTRPC7 and grown 48 hours prior to assaying for SEAP activity. Values are presented as a percentage of the total enzyme activity (luminescence) in the cells expressing eGFP. The results are means \pm S.E.M. for three independent experiments.

Figure 1



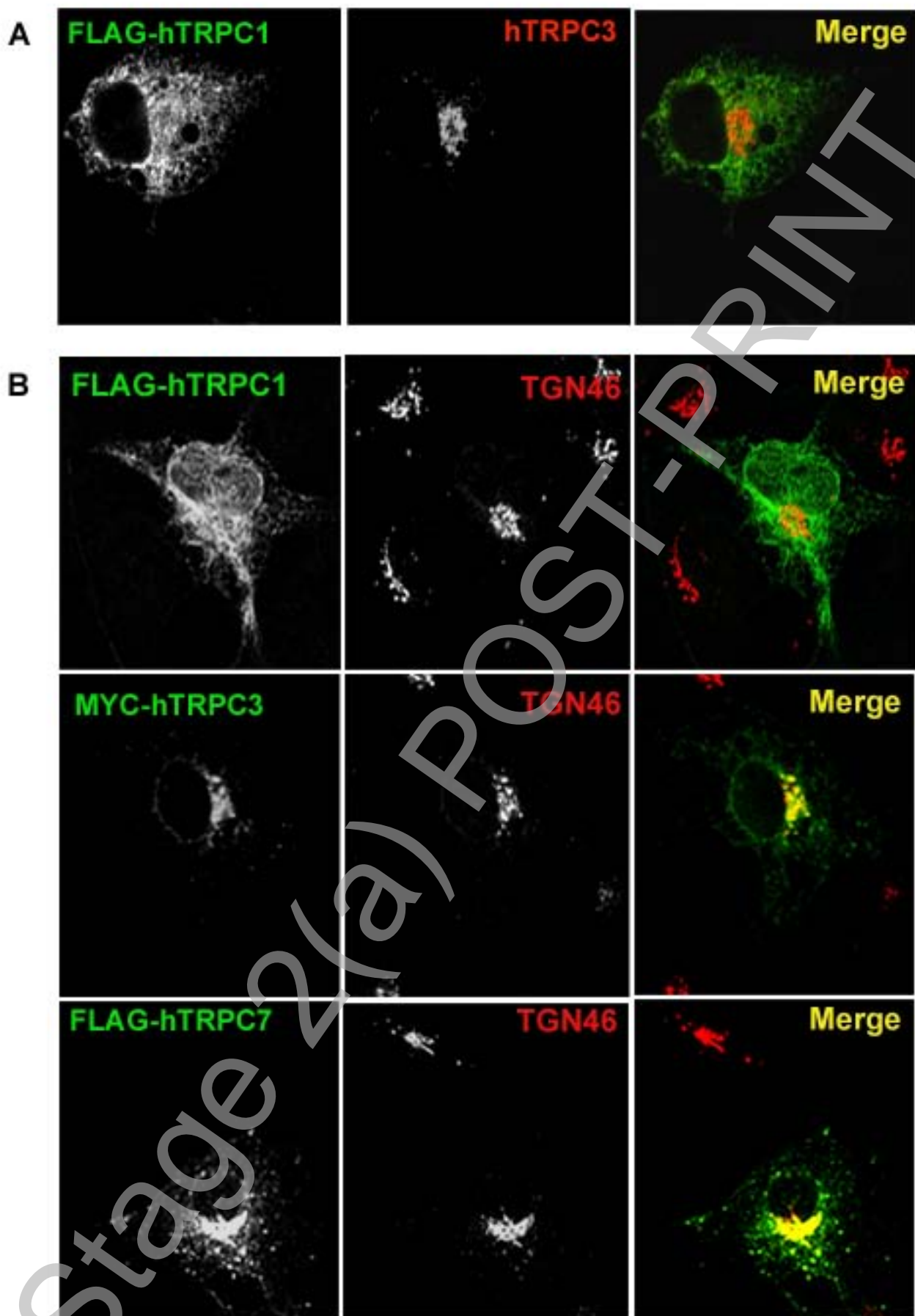


Figure 2

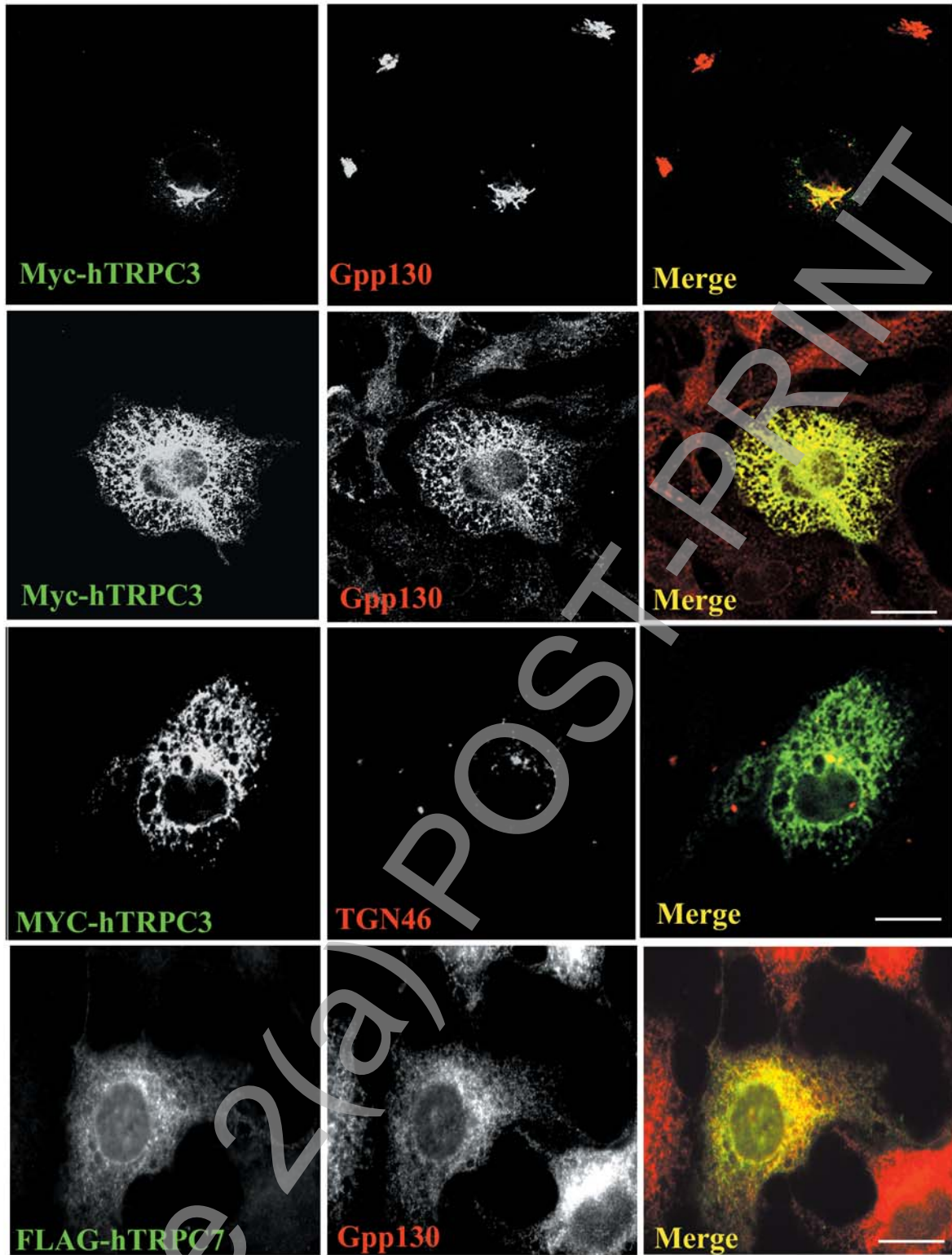
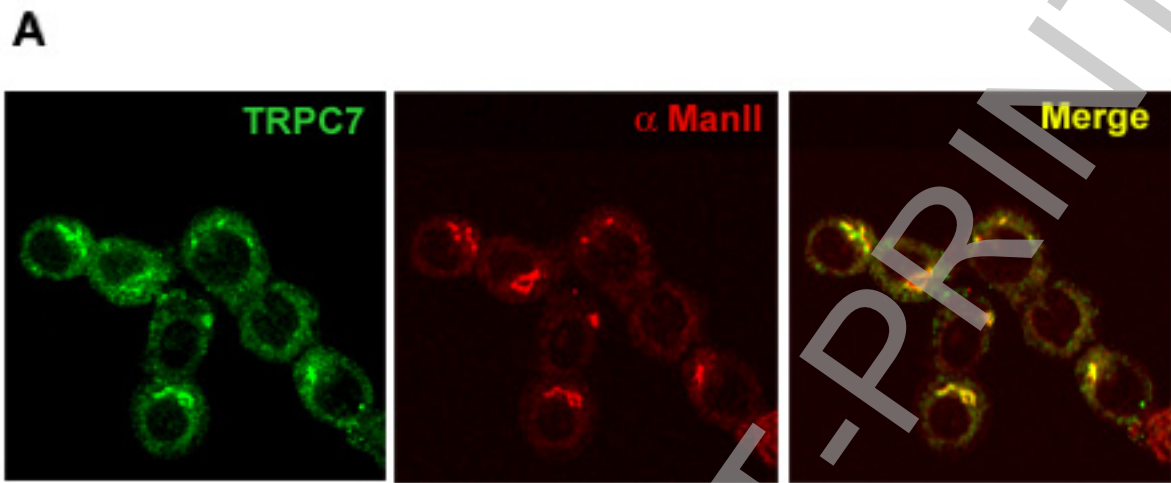


Figure 3

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071488

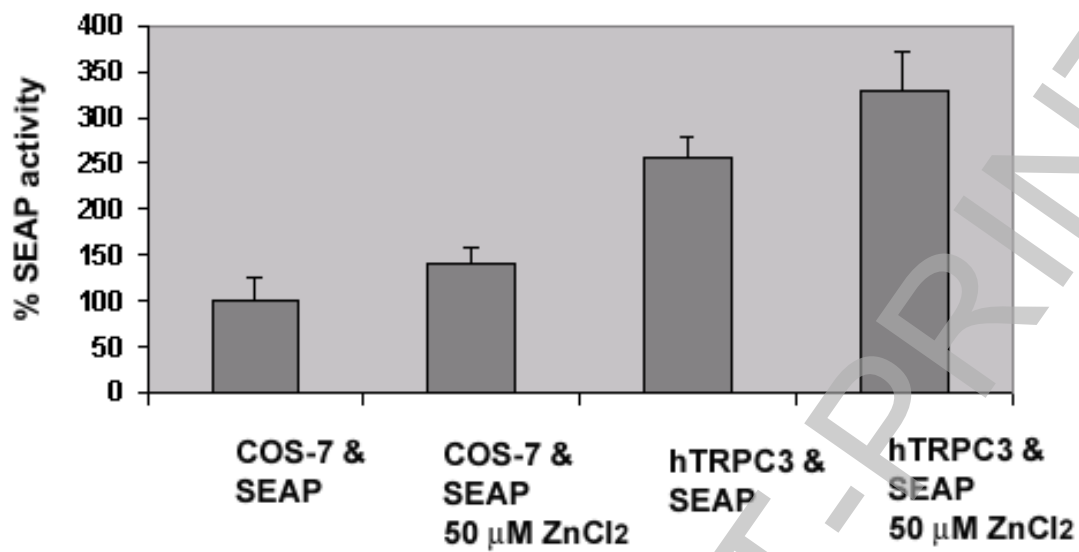


B



Figure 4

A



B

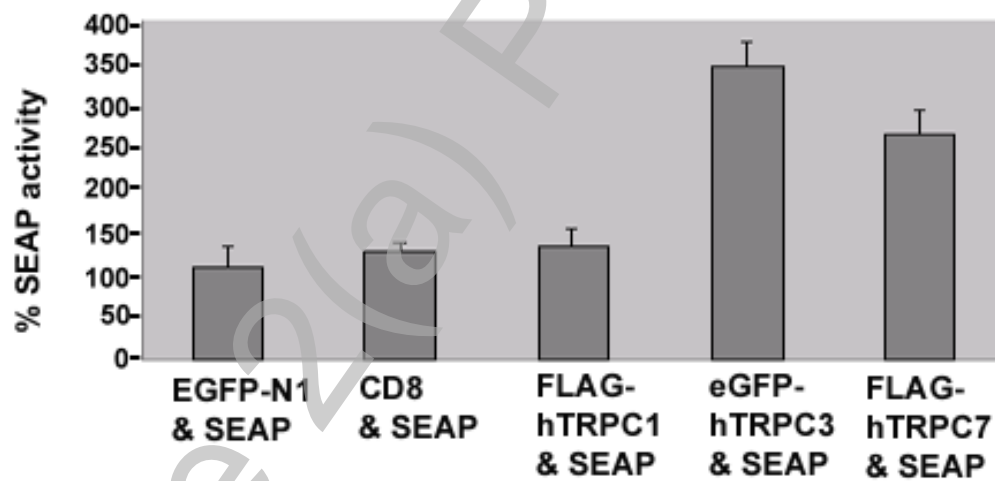


Figure 5