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AWARD NUMBER: W81XWH-07-1-0040

TITLE: The Mechanosensitive Ca2+ Channel as a Central Regulator of Prostate Tumor Cell Migration and Invasiveness

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REPORT DATE: January 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 January 2010	Annual	18 Dec 2008 – 17 Dec 2009

4. TITLE AND SUBTITLE

The Mechanosensitive Ca2+ Channel as a Central Regulator of Prostate Tumor Cell Migration and Invasiveness

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0040

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas Medical Branch
Galveston, TX 77555

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our patch clamp studies indicate MscCa is expressed by the invasive prostate tumor cell PC-3. Anti-MscCa agents, Gd3+, GsmTx-4, and an anti-TRPC1 antibody block PC-3 cell migration. MscCa activity can be recorded over the surface of the PC-3 cell but is expressed at higher density on the rear compared with the front of the cell. This channel density gradient combined with a higher density of thapsigargin-sensitive Ca2+ stores in the rear of the cell enables the development of an intracellular Ca2+ gradient (low front –high rear) in migrating PC-3 cells that determines migration directionality. Gene silencing of TRPC1 and/or TRPC3, but not TRPC4 or TRPC6, blocks PC-3 cell migration. Permanently suppressing TRPC1 also reduces PC-3 cell proliferation and thereby blocks tumor invasion in vivo. The non-invasive human prostate tumor cell line LNCaP expresses MscCa but the channel undergoes rapid inactivation that prevents Ca2+ gradient development and directional cell migration. Our results indicate that specific forms of mechanical stimuli can switch the inactivating gating mode to the non-inactivating mode seen in PC-3 cells, and this switch is independent of the actin-cytoskeleton. These findings have specific implications regarding the possible role of the increases mechanical forces (e.g., solid stress and interstitial fluid compression) that develop within a growing prostate tumor in promoting its progression to malignancy.

15. SUBJECT TERMS
Prostate tumor cell migration, tumor invasion, mechanical forces on tumor progression, Ca2+ channels, transient receptor potential family

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	59	USAMRMC

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Introduction

A major challenge for treating prostate cancer (PC) is to discover new therapies that will prevent the spread of PC cells from the prostate to distal sites. Our research focuses on the mechanosensitive Ca^{2+} permeant channel (MscCa) as a central regulator of prostate tumor cell migration. Our experiments are designed to address the two most basic issues of the disease: the mechanism(s) that trigger progression of PC to malignancy and the urgent need for new therapeutic targets to block or reverse this progression. Our original experiments funded by DOD were aimed to test whether MscCa is expressed in human prostate tumor cells and whether MscCa activity is required for prostate tumor cell migration. We confirmed both results. In the course of these experiments we also discovered that the predominate gating mode of the MscCa differs between noninvasive and invasive PC cells, and this is the most powerful determinate of the $[Ca^{2+}]_i$ dynamics required to coordinate cell locomotion. The aims of the current award were three-fold. First, determine the mechanisms underlying MscCa gating. Second, determine the cancer-related processes that switch MscCa gating, and third determine whether anti-MscCa conditions that suppress PC migration in vitro also block PC cell invasion in vivo. Insights into these aspects would provide added motivation for developing more selective therapies that target MscCa and its regulatory mechanisms. The basic results supporting our hypothesis have been published (Maroto, R. & Hamill, O.P. MscCa regulation of tumor cell migration and metastasis. *Current Topics in Membranes*. 59, 485-509,2007). Also included in the Appendix are our manuscripts (Maroto & Hamill, 2010; currently under revision for submission and Maroto, Kurosky and Hamill, 2010, in preparation, results included in body of the text).

Body

As reported previously in our Year 2 progress report we completed Task 1-1, 1.2 and 1.3. In year 2 our work was interrupted by the mandatory evacuation of Galveston Island due to Hurricane Ike and its aftermath. We have subsequently regenerated the prostate cells lines that were lost and are now continuing to test them. However, because of the delays we have requested and been granted a 1 year no cost extension to complete the tasks of year 3.

During the course of Year 3 we have carried out additional experiments that were either recommended based on feedback from reviews or stemmed directly from our new observations. Although these experiments were not part of our original proposal they are a natural follow on and key for understanding/publishing the MscCa mechanisms that block PC-3 cell migration. In particular, we proposed (Maroto & Hamill, 2007) that another TRP family member in addition to TRPC1, TRPM7, because of its reported stretch sensitivity and expression at the leading edge of cells, may also participate in regulating tumor cell migration. In this case, in addition to the sustained Ca2+ gradient and transients that we observed in PC-3 cells due to MscCa/TRPC1 (Maroto & Hamill, 2010 see appendix) we also predicated that cells may express MscCa/TRPM7-dependent transients at the cell's leading edge. This idea has been confirmed by rapid confocal measurements of Ca^{2+} changes in migrating fibroblasts (Wei et al., 2009; see also Hamill & Maroto, 2009 included in appendix). We are now carrying our measurements to dissect the individual roles of TRPC1 and TRPM7 in PC-3 cell migration and in particular in "mechanically-ignited Ca2+ flickers" that may guide PC-3 cell migration to metastatic sites. First, we have tested for TRPM7 expression in PC-3 cells. However, we found the commercially available TRPM7 antibodies (including one monoclonal antibody) do not detect low level endogenous TRPM7 expression.

For this reason, we are using immunoprecipitation to concentrate TRPM7 that may then be detected by Westerns. Second, we are using rapid confocal microscopy to measure Ca2+ flickers in the lamellipodia of PC-3 cells in mock transfected PC-3 cells and in cell in which TRPM7 and/or TRPC1 expression have been suppressed. Third, in order to determine the location of TRPC1 and TRPM7 in PC-3 cells we have generated fusion proteins of yellow fluorescent protein (YFP). These experiments indicate that overexpressed YFP-TRPC1 is located at the leading edge but is also concentrated around the nucleus of the PC-3 cells. This result is consistent with our previous published results (Gottlieb et al., 2008) that indicate that indicate that a significant fraction of TRPC1 is not conducted the surface membrane but is retained intracellularly where may function as an internal release Ca2+ channel.

Our results in years 1 and 2 indicated that suppression of TRPC1 reduces PC-3 cell migration, this observation has now been confirmed in two other cell types, namely, canine kidney cells and rat myoblasts (Fabian et al., 2008; Louis et al., 2008). However, we also found that overexpression of TRPC1 blocked PC-3 cell migration as judged by three different methods: time-lapse video microscopy, wound closure assay and loss of the motile lamellipodia. According to this observation we proposed that there is an optimal level of expression required for cell migration and that either suppression or overexpression will block migration. However, in contradiction to this idea another study has reported that TRPC1 overexpression increases canine kidney cell migration (Fabian et al., 2008). To determine if this discrepancy reflects a difference in expression vectors or cell type (tumor versus nontumor cells) we have generated and tested over the last year two additional TRPC1 expression constructs. In addition to the original XOOM TRPC1 vector, a YFP-TRPC1 construct and a tetracycline-inducible TRPC1 vector. Our new experiments in indicate that all vectors increase TRPC1 expression in PC-3 cells as measured by Westerns and in all cases PC-3 cell migration is blocked. To investigate a possible alternative explanation for the discrepant results we are currently testing whether TRPC1 overexpression blocks or stimulates migration in cells other than PC-3 cells. Resolving this issue is important for the overall project because opposing effects of TRPC1 on normal versus prostate tumor cells would have important implications in terms of therapy.

We have also continued tasks 2.1 and 3.1. Our work for these tasks has been submitted as a manuscript and we are carrying out revisions suggested by the reviewers (Maroto & Hamill, 2010 see Appendix 1). In addition we have carried out additional experiments that relate to Tasks 1.2 described below. These results will be part of a second manuscript (Maroto, Kurosky & Hamill in preparation) and are described in the body of this report.

Task 1: Determine the mechanism(s) that regulate MscCa gating, expression and surface distribution in PC cells that display different invasiveness and metastatic potential.

1.1 Use patch-clamp/pressure clamp techniques, confocal immunofluorescence, Westerns and surface biotinylation techniques to measure gating, surface distribution and expression, respectively, of MscCa/TRPC1 in PC-3 and LNCaP cell lines.

Time line: Year 1, months 1-6.

Milestone: Establish a baseline for studying the effects of various agents and treatments that may alter these properties as described in tasks 1.2 to 1.4.

1.2 Use Westerns to establish the TRPCs (TRPC1-7) expressed in PC-3 and LNCaP cells. Use cDNA or short hair pin RNAs inserted in plasmid vectors in order to generate permanent PC-3 and LNCaP cell lines in which specific TRPCs have been either over expressed or silenced. Use methods of 1.1 to establish the functional properties of MscCa and how TRPC1 expression and surface distribution are alter. Use time-lapse $[Ca^{2+}]i$ imaging to study $[Ca^{2+}]_i$ dynamics and migration in the various PC cell sub-lines.

Time line: Year 1, months 1-12.

Milestone: Role of specific TRPCs in determining PC cell specific MscCa properties and their influence on $[Ca^{2+}]_i$ dynamics and cell migration.

Figure 1 show Western blot measurements of expression of TRPC1, 3, 4, 5 and 6 in the highly motile PC-3 cells and weakly motile LNCaP cells. We show for the first time TRPC1 is more highly expressed in LNCaP vs PC-3 cells consistent with the higher density of MscCa in LNCaP vs PC-3 cells; TRPC3 is also more highly expressed LNCaP vs PC-3 cells; TRPC4 although present in LNCaP cells was not detectable in PC-3 cells; TRPC5 was not detectable in either cell line even though the antibody detected the protein in Xenopus oocyte; TRPC6 was equally but only weakly expressed in PC-3 and LNCaP cells.

These results of Figure 1 are significant because in addition to TRPC1 (Maroto et al., 2005) recent reports indicate that TRPC5 and TRPC6 may also be involved in MscCa. However, at least in prostate tumor cells our results indicate that TRPC5 cannot serve this function because of its absence at the protein level. Furthermore, our patch clamp studies indicate that overexpression of TRPC6 in several mammalian cells lines does not result in increase in endogenous MscCa activity (Gotlieb et al., 2008).

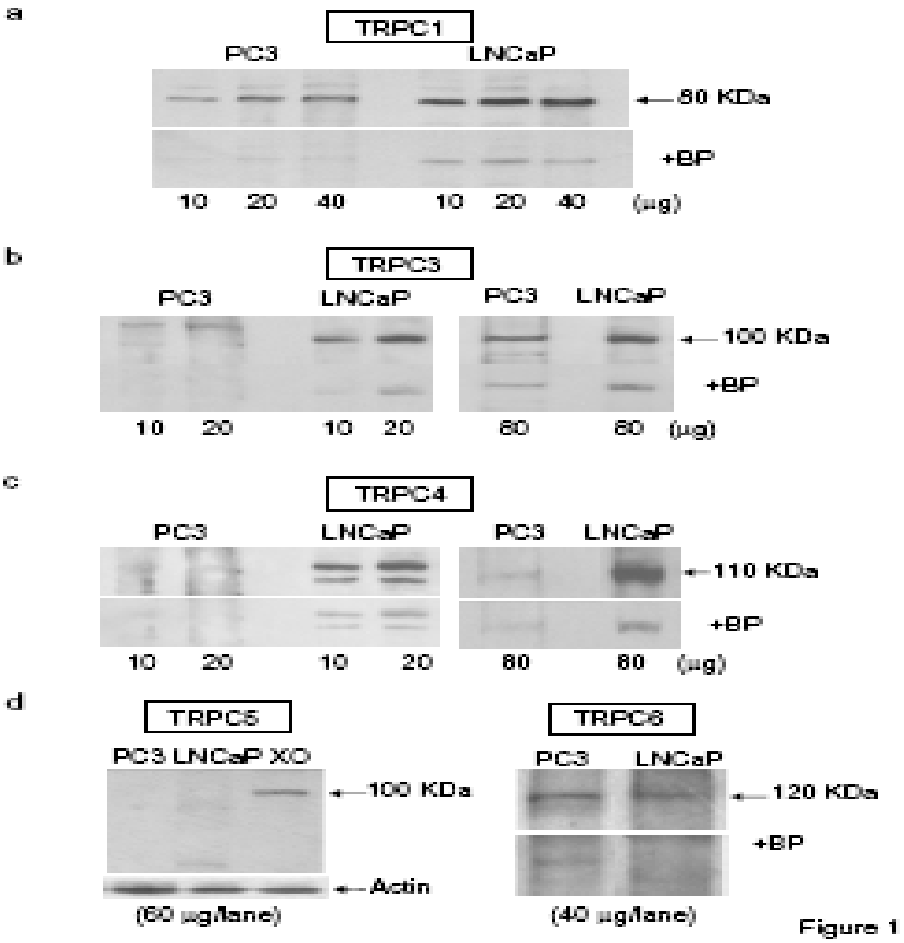

Figure 1. Westerns showing relative expression of TRPC1, 3, 4, 5 and 6 in PC-3 and LNCaP cells lines. The positive control for the absence of TRPC5 in prostate tumor cells is the demonstration that the anti-TRPC5 recognizes the protein expressed in *Xenopus* oocytes (XO).

To further test the role of specific TRPCs in prostate tumor cell function we generated permanent PC-3 and LNCaP cell lines in which TRPC1, TRPC3 or TRPC6 were selectively suppressed as confirmed in Westerns shown in Figure 2.

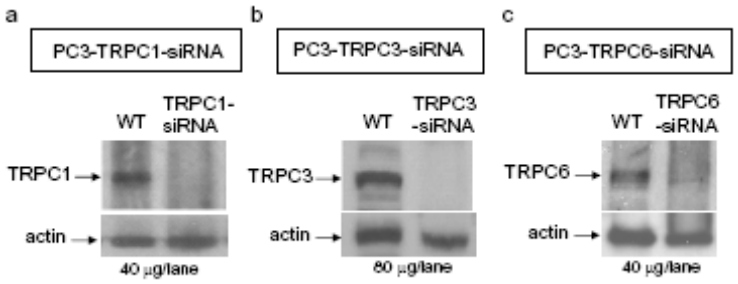

Figure 2. Western blots comparing TRPC1, TRPC3 and TRPC6 expression in wild type PC-3 cells and in PC-3 sublines that transfectecd with short hairpin RNAs to selectively and permanently suppress each TRPC1, TRPC3 or TRPC6.

We next measured PC-3 cell migration by video-microscopy and wound/scratch closure assay and found that siRNA suppression of TRPC1 and TRPC3 but not TRPC6 blocked PC-3 cell migration as shown in Figure 3 below.

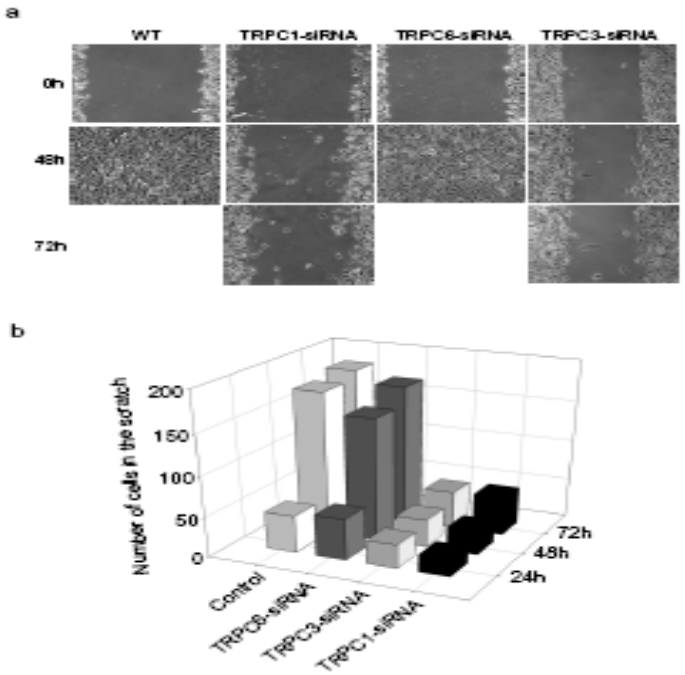

Figure 3. Wound/scratch closure assays used to measure the migratory function of wild type PC-3 cells and PC-3 sublines in which TRPC1, 3 or 6 were permanently suppressed.

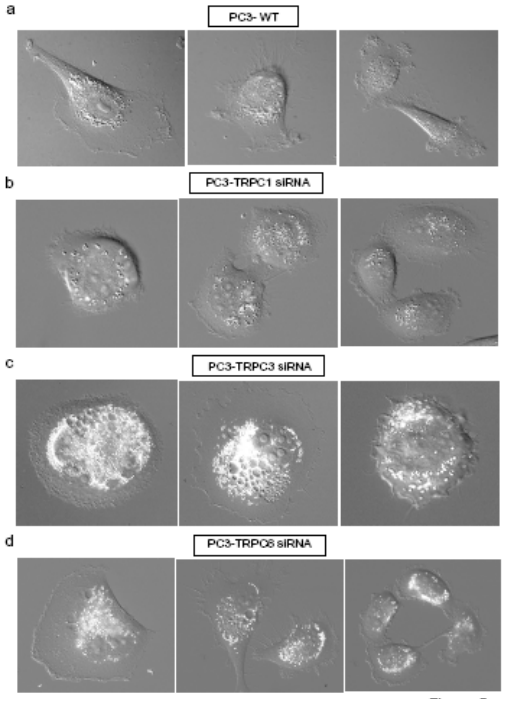

Figure 4. Morphology of wild-type and TRPC-suppressed PC-3 cell sublines.

In addition to the effects on cell migration TRPC1 and TRPC3 suppression, but not with TRPC6, also induced changes PC-3 cell morphology as shown in Figure 4 above. In

particular, following TRPC1 and TRPC3 suppression PC-3 cells no longer display the pronounced lamellipodia that are clearly evident in the wild type and TRPC6-suppressed PC-3 cells. Instead, the sTRPC1 and siTRPC3 cells display a nonpolarized morphology or "fried-egg" morphology that is most likely underlies their inability to migrate unlike the siTRPC6-PC-3 cells that show normal migration (see Task 3).

We have also generated permanent PC-3 cell lines that over express TRPC1 (H-TRPC1) and TRPC3 (H-TRPC3) as indicated in Western Blots in Figure 5 below.

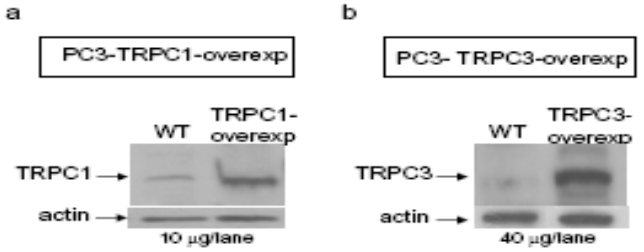

Figure 5. Western blots comparing TRPC1 and TRPC3 protein levels in wild type and in PC-3 sublines permanently transfected with TRPC1 or TRPC3.

HTRPC1 PC-3 cells are similar to the siTRPC1 PC-3 cells in that they fail to develop lamellepodia. In contrast, H-TRPC3 cells show a normal polarized morphology and develop lamellipodia as indicated in Figure 6.

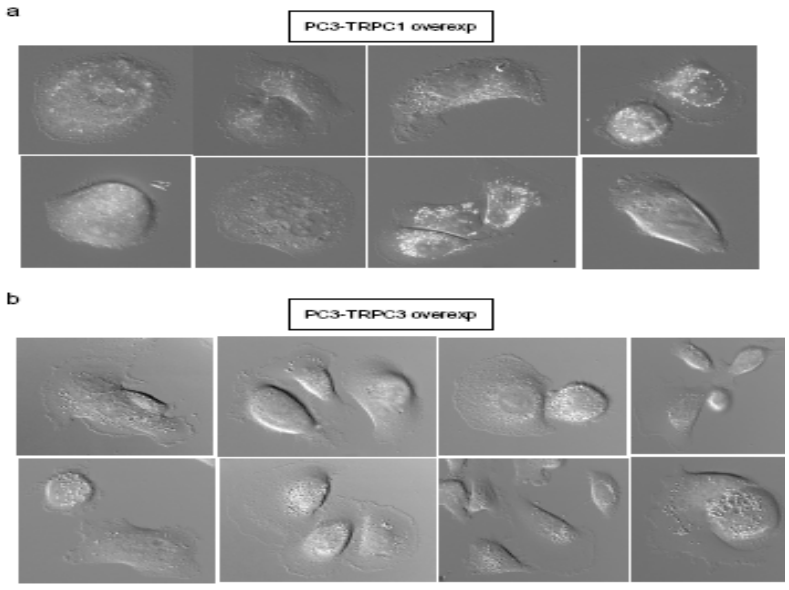

Figure 6. Morphological changes induced in PC-3 cells by over expression of TRPC1 and TRPC3. HTRPC1-PC3 cells do not develop lamellipodia and fail to migrate. HTRPC3-PC-3 show a normal morphology and migrate.

We have carried out patch clamp studies of the PC-3 sub-lines with altered TRPC1 and TRPC3 expression. As shown in Figure 7 below suppression of either TRPC1 or TRPC3 results in a significant decrease in MscCa patch density whereas overexpression of neither

TRPC1 or TRPC3 does not significantly increase MscCa expression. The lack of effect of TRPC1 overexpression on MscCa density may be due to inefficiency in trafficking of TRPC1 to the surface membrane when expressed alone (see Gottlieb et al. 2008 in appendix 1). When TRPC3 is over expressed another channel is evident that shows a larger single channel conductance than MscCa, and is constitutively open but not mechanosensitive.

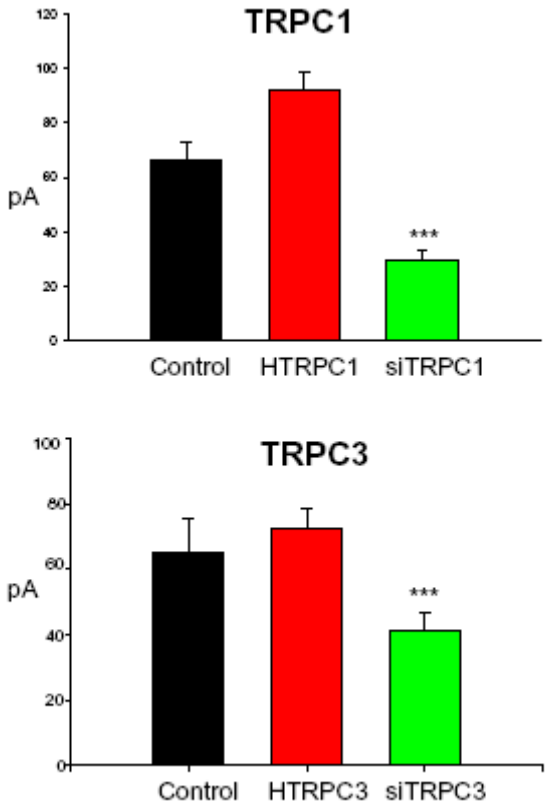

Figure 7. Histograms showing MscCa peak currents in PC-3 sublines that have suppressed or over expressed TRPC1 and TRPC3.

These results are consistent with the idea that TRPC1 combines with TRPC3 to form a heteromeric channels that is mechanosensitive (i.e, MscCa). Suppression of either TRPC1 or TRPC3 results in reduction in MscCa. However, when TRPC1 is overexpressed it forms a homomeric TRPC1 channel in the ER that may be mechanosensitive but cannot be measured with patch clamp recording. When TRPC3 is overexpressed it forms a homomeric TRPC3 channels that is inserted in the membrane and shows some spontaneous opening in patches but is not mechanosensitive (Hamill & Maroto, 2007).

Tasks 1.1 and 1.2 has been completed in years 1 and 2 and the results form the manuscripts (Maroto & Hamill, 2010, Appendix 1; Maroto, Kurosky & Hamill, in preparation).

Deliverables: We have generated permanent PC-3 cells lines in which TRPC1, 3 were suppressed or over expressed and another PC-3 cell line in which TRPC6 was suppressed. As previously mentioned after IKE we have spent the last two months regenerating some of the most critical lost sublines.

1.3 Use specific agents that either promote actin depolymerization (Latrunculin A) or polymerization (jasplakinolide) to study the effects on MscCa properties and $[Ca^{2+}]_i$ dynamics and cell motility on PC cell lines.

Time line: Year 1, months 9-12; Year 2, months 1-6.

Milestone: The role of the actin-CSK in regulating MscCa properties.

1.4 Use treatments (methyl-β-cyclodextrin with/without cholesterol) in order to deplete or enrich the bilayer with cholesterol and measure the effects on MscCa properties.

Time line: Year 2 months 6-12.

Milestone: The role of lipid bilayer structure in determining MscCa properties and PC cell motility.

Deliverables: Agents that regulate MscCa properties and thereby the motility of PC cells.

Our results indicate that the transient gating of MscCa of LNCaP cells is highly sensitive to specific forms of mechanical disruption. In particular, whereas repetitive 100 ms suction pulses has no effect on the transient gating, the application of 1 second pulses causes a progressive and irreversible shift of the transient gating mode to the sustained gating mode that is more PC-3 cell-like (Maroto & Hamill, 2009 see appendix). Based on this mechanical fragility we proposed that sustained mechanical stimuli may act by disrupting the actin cytoskeleton directly underlying the membrane. However, our experiments do not support our original hypothesis, using cytochalasin D (Figure 8) and latrunculin at concentrations (5-10 micromolar) and incubation times(> 1 hour) known to cause extensive actin deplolymerization left the transient gating mode intact. Similarly, jasplakinolide (200 nM for 2 hours), an agent reported to promote actin polymerization also did not alter MscCa transient gating in LNCaP cells. However, curcumin (60 micromolar for 30 minutes) which also promotes actin polymerization reduced the transient gating mode. This result did not fit with our predictions and does not fit with lack of effect of jasplakinolide. Our preliminary experiments indicate that methyl-β-cyclodextrin which alter membrane cholesterol does not significantly alter MscCa gating. We are continuing these measurements with a range of concentrations.

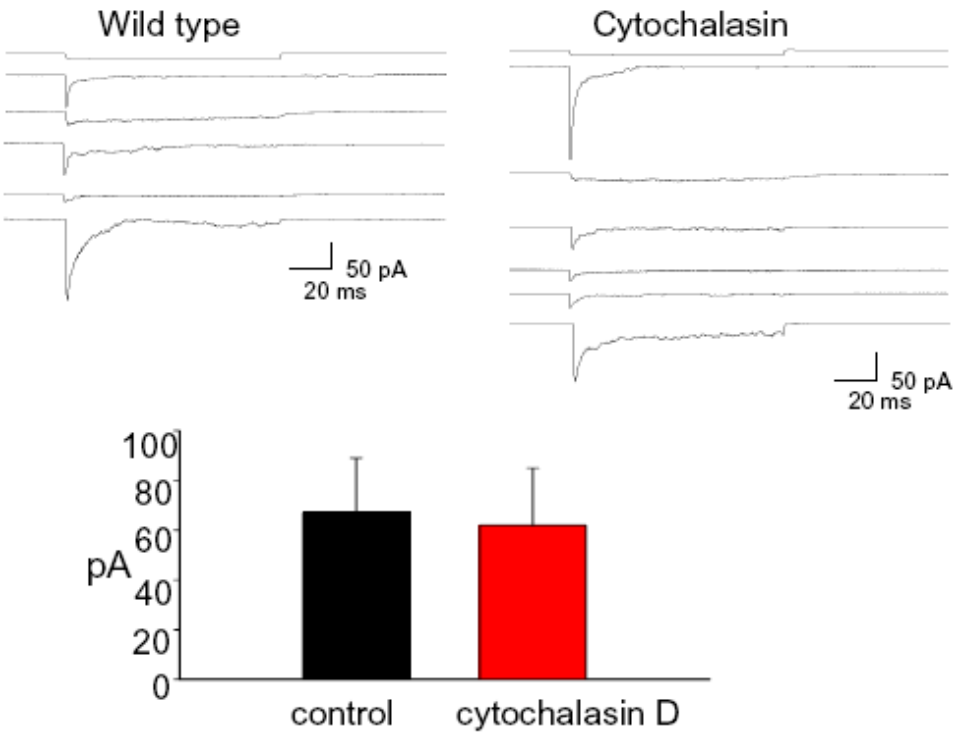

Figure 8. Cytochalasin treatment (10 μM for 4 hours) which is know to causes significant actin depolymerization but does not affect the peak amplitude or fast inactivation of MscCa recorded in LNCaP cells. The current traces are from 5 control cells and 6 cytochalasin treated cells.

We are also planning new experiments to test the role of the lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) on MscCa gating based on recent finings that PIP_2 inactivates TRP channels and also affects mechanosensitive K+ channels. In particular, it has been reported that moderate to severe depletion of PIP_2 in the membrane can lead to different levels of TRP current inactivation and that changes in the apparent affinity of the ion channel for PIP_2 can dramatically alter the sensitivity of the channel to PIP_2 depletion. Based on the absence of effects of actin and cholesterol targeting agents on MscCa our plan is to test a range [PIP_2] on MscCa activity in inside-out patches from LNCaP cells in order to determine whether the fast inactivation of the MscCa seen in the patch is due to a reversible depletion of the lipid PIP_2 and whether the fragile irreversible loss of fast inactivation is due to mechanical inactivation of the enzymes that replenish the depleted PIP_2.

Task 1.4 is partially completed. We have excluded a dominant role for actin and membrane cholesterol in regulating MscCa gating and we are in the process of testing a PIP2 as a promising candidate for regulation of MscCa activation and gating dynamics.

Task 2: Determine the effects of cancer-related conditions that promote tumor progression to increased invasiveness on MscCa properties.

2.1 Use Westerns, immunofluorescence and patch-clamp recording to examine the influence of TNF-α, a transcriptional regulator of TRPC1 expression, and TGF-β both known to promote the EMT on MscCa properties in NPE and PC cells.

Time line: Year 2, months 1-9.

Milestone: TNF-α and TGF-β regulation of MscCa/TRPC1 expression in PT cells.

2.2 Use time-lapse Ca^{2+} imaging to determine the effects of transforming factors on the functional properties, motility and $[Ca^{2+}]_i$ dynamics of NPE and PC cells.

Time line: Year 2, months 6-12, Year 3, months 1-12.

Milestone: TNF-α and TGF-β effects on $[Ca^{2+}]_i$ dynamics and cell migration.

The commencement of these experiments in year 2 (Tasks 2.1 and Tasks 2.2) was delayed because we began the *in vivo* studies (Task 3.1 and 3.2) early in Year 1, and continued these experiments in year 2. We felt it was of highest priority to determine if TRPC1 suppression and overexpression blocks PC-3 cell invasion and metastasis in nude mice. We will complete Tasks 2.1 and 2.2 in Year 3.

2.3 Use various conditions know to trigger increased invasiveness in the normally noninvasive LNCaP cell line to study the role of MscCa in triggering progression in these sub-lines.

Time line: Year 1, months 1-12; year 2 months 1-9.

Milestone: Identify cancer-linked progression factors that act on MscCa.

We have tested 3 different LNCaP sub-lines that have been reported in the literature to show increased invasiveness over the parent LNCaP cell. One sub-line was generated in our laboratory by long term growth in the absence of androgen using charcoal-depleted serum over several months (Tso et al., 2000). Under these conditions many of the LNCaP did not survive and those that did developed long processes. However, they did not show significant locomotion and patch clamp studies indicated MscCa currents with similar peak amplitudes and kinetics as untreated LNCaP cells. We have also tested a LNCaP cell sub-line generated by in vivo selection (Wu et al., 1998) and by transfection with β3 integrin subunit (Zheng et al., 1999). However, again neither sub-line showed significant migration as judged by time-lapse video microscopy and the MscCa activity was indistinguishable for the parent LNCaP sub-line. Our negative results so far are hard to explain in terms of the previous reports. However, the literature indicates that still no condition that have been able to generate a robust and reproducible LNCaP sub-line that is highly motile/invasive.

Deliverables: Regulator of MscCa expression in PT cells.

This part of the project has so far provided negative results and will be continued in this year.

Task 3: Quantify the ability of treatments that selectively target MscCa/TRPC1 and that block PC-3 cell migration in vitro to block PC-3 cell invasion when orthotopically implanted in nude mice.

3.1 Use time-lapse videomicroscopy and patch-clamp techniques to characterize the motility of eGFP-transfected PC-3 cells in which MScCa/TRPC1 has been selectively over-expressed or silenced.

Time line: Year 1, months 1-12.

Milestone: Genetic block of PC-3 cells migration.

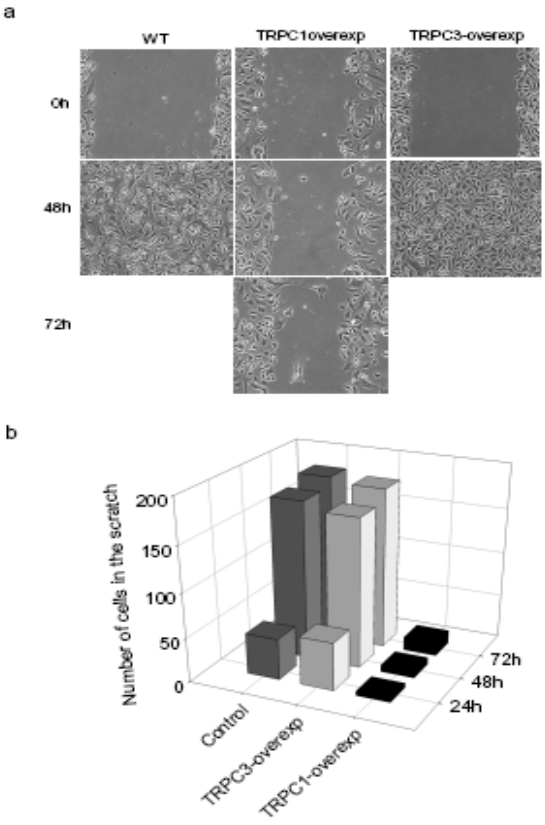

Figure 9. Wound/scratch closure assays of control PC-3 cells and PC-3 sublines that permanently over expresss TRPC1 or TRPC3. Overexpression of TRPC1 but not TRPC3 blocks PC-3 cells migration.

The results shown in Figures 3 and 8 indicate the successful generation of PC-3 cell sub-lines in which specific TRPC expression is altered. We have completed the characterization of these sub-lines in terms of MscCa expression and motility.

Carry out orthotopic implantation of eGFP-labeled human PC-3 cells in which MscCa expression and test the effects on tumor invasion and metastasis as measured using fluorescence.

Time line: Year 2, months 6-12, year 3, months 1-12.

Milestone: Block of the PC and invasion and metastasis disease *in vivo*.

Deliverables: New gene constructs that can block PC invasion and metastasis.

We implanted siTRPC1-PC-3 cells subcutaneously in nude mice as a first step in carrying out orthotopic implants in mice. However, although the PC-cell tumors grew their fluorescence was less than the autofluorescence.

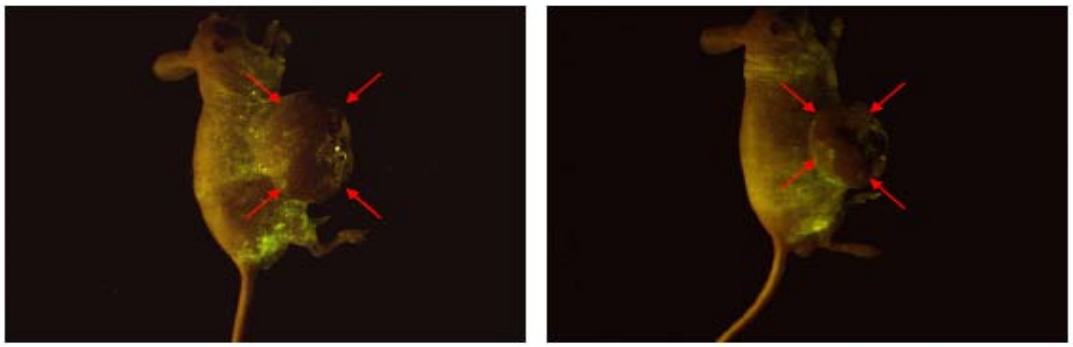

Figure 10. Two mice that were subcutaneously injected with the eGFP-shTRPC1-PC3 cell subline. The red arrows delineate the tumor that does not show significant fluorescence above background fluorescence.

Figure 11 show the brightness of PC-3 cell tumors that were generated by AntiCancer using PC-3 cells virally transfected with the cDNA for GFP.

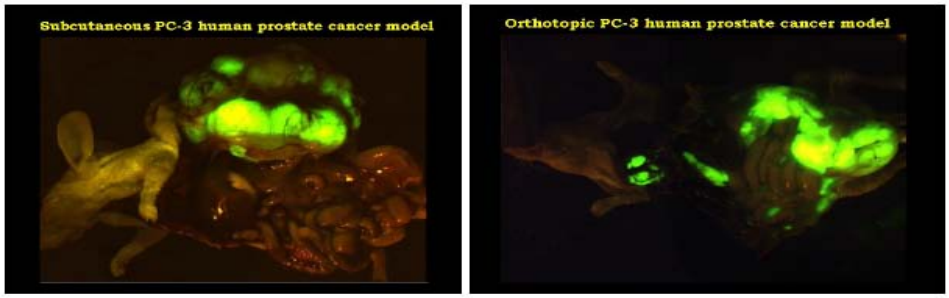

Figure 11. Shows the bright fluorescence of PC-3 cells virally-transfected with cDNA of GFP and injected subcutaneously (left) or implanted orthotopically (right) by Anticancer inc.

To overcome this limitation we have transfected the short hairpin TRPC1 construct into a virally transfected GFP PC-3 cell sub-line, and have selected a sub-clone using antibiotic resistance. Figure 12 compares the fluorescence of our new eGFP-siTRPC1 PC3 sub-line with the previous sub-line. We are currently testing this cell line for migration and MscCa activity before again attempting orthotopic implantation.

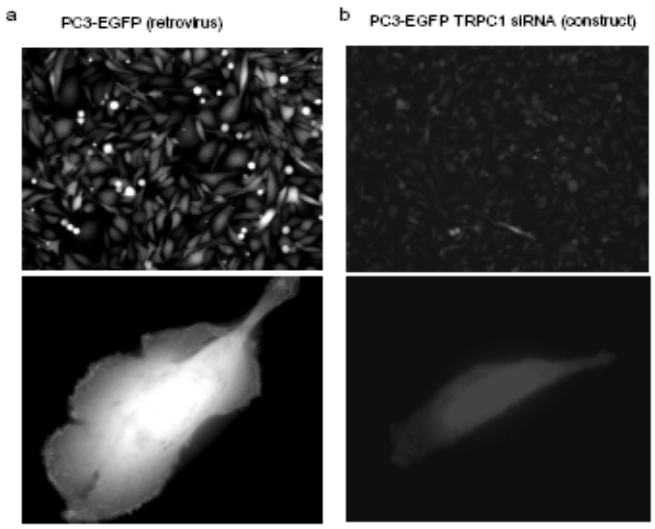

Figure 12. Comparison of the fluorescence of a siTRPC1 PC-3 cell (left panels) that was permanently transfected by retrovirus to express GFP and our earlier PC-3 cell line transfected with GFP-siTRPC1 using lipofection.

Task 3.2 was originally planned for year 2 and to be completed in year 3. We have already collaborated with AntiCancer Inc. in the in vivo studies using TRPC1-suppressed GFP-PC-3 cell line. However, in the initial step it was found that the proliferation rate of the TRPC1 may have suppressed PC-3 cells so that tumor growth was suppressed compared with control PC-3 cells. However, through further feedback and our own examination of the subcutaneous tumors under fluorescence (see figure 10) and comparison of the individual cells (Figure 12) indicates that the main problem was the relatively weak fluorescence of our PC-3 cell subline. We have now increased the fluorescence of the siTRPC1-PC-3 cell by using retrovirus transfection of the cDNA for GFP.

KEY RESEARCH ACCOMPLISHMENTS.

Our key research accomplishments in years 1 to 3 are as follows:

1. MscCa is expressed in human prostate tumor cells.

2. MscCa differs in its gating, density and surface distribution between invasive and noninvasive prostate tumor cells.

3. MscCa as measured by patch recording is expressed on both the front lamellipodia and the rear tether of migrating prostate tumor cells but can occur at higher density on the cell rear.

4. MscCa is expressed at a higher and uniform distribution on the non-migratory LNCaP cell line and its sublines.

5. Internal Ca2+ stores located in the endoplasmic reticular are more concentrated in the cell body than in the front lamellipodia of the migratory PC-3 cell but uniformly with the non invasive LNCaP cell.

6. The polarized distribution of MscCa, the sustained opening of MscCa in response to maintained stretch and the distribution of internal Ca2+ stores can account for the intracellular Ca2+ gradient (high rear-low front) that develops in migrating prostate tumor cells and determines migration directionality.

7. Suppression or over expression of TRPC1 blocks prostate tumor cell migration as measured by time-lapse video microscopy of wound closure assay.

8. Suppression of TRPC1 and TRPC3 (but not TRPC4, 6 or 7) also blocks prostate tumor cell migration.

9. Both the magnitude and temporal characteristics of mechanical stimuli applied to the tumor cells can affect the transition between the non motile and motile MscCa gating mode.

10. Our original hypothesis that changes in the actin cytoskeleton is critical in mediating the transition in MscCa gating from the noninvasive to the invasive mode was not supported by our experiments that tested various agents known to promote either actin deploymerizaion or actin polymerization.

11. Different LNCaP cells sublines that have been reported to show increased invasiveness failed to show migratory behavior when measured in vitro by either wound closure assay or time lapse video microscopy. Consistent with this, all LNCaP cell sublines displayed the same inactivating MscCa gating mode characteristic of the parent cell line.

12. In addition to blocking prostate tumor cell migration, TRPC1 suppression also inhibits prostate tumor proliferation thereby blocking tumor growth when implanted subcutaneously in nude mice.

13. Overexpressed YFP-TRPC1 is localized at the leading edge of PC-3 cells but is also concentrated around the cell nucleus.

14. Overexpression of TRPC1as verified by three different transfection vectors and by three independent motility assays reduces PC-3 cell motility.

REPORTABLE OUTCOMES

The above research findings represent our reportable outcomes and major manuscript outlining these findings has been submitted for publication (Maroto & Hamill., see Appendix 1). This manuscript is currently under revision and being prepared for resubmission with another manuscript (Maroto, Kurosky & Hamill, in preparation). The first manuscript focuses on MscCa and the second manuscript focuses on the role of TRPCs. The planned submission of joint papers is in response to a reviewer's requested that we carry out in vivo measurements testing the effect of TRPC1 suppression on PC-3 cell invasiveness in mice. In addition to these two unpublished manuscript we have also published four other manuscripts that describe 1) the role of different TRPCs in forming channels that are directly or indirectly activated by mechanical stimuli (Hamill & Maroto, 2007 a,b). The role of MscCa in cancer progression (Maroto & Hamill, 2007). Finally, in a collaborative effort we have shown that additional protein subunits are likely needed to enable efficient trafficking of TRPC1 in mammalian cell lines (Gottlieb et al., 2008). We also presented our results at invited talks at a Howard Hughes Medical Institute sponsored meeting "Force-gated ion channels" at Janella Farm, DC May 18-21, 2008 and at the Keystone Symposium "Mechanotransduction in Physiology and Disease" at Taos, New Mexico, January, 18-23, 2009.

CONCLUSIONS

Our ongoing experiments confirm that both MscCa and specific TRPCs (TRPC1 and TRPC3) are required for prostate tumor cell migration. In particular, their channel activity is required for the development of the intracellular Ca2+ gradient (high rear-low front) that determines migration directionality. Our results provide new mechanistic insight into the roles MscCa gating and surface distribution plays in maintaining the [Ca2+]i gradient in the migrating tumor cell and indicate that the MscCa gating mode and surface distribution differs between invasive and noninvasive prostate tumor cells, and that mechanical forces applied to the noninvasive cell may be able to switch gating seen in the noninvasive cell to that expressed by the invasive cell. In contradiction of our original hypothesis this mechanical switch does not depend upon changes in the underlying actin-cytoskeleton and indicates the possibility that mechanical forces act directly on the lipid bilayer and/or the channel protein itself. Experiments outlined in the original proposal will test these possibilities. Our experiments testing TRPC1 –suppression on prostate tumor cell invasion in living animals indicates that TRPC suppression may have multiple effects and reduce cell proliferation in addition to blocking cell migration. We have also determined that our original EGF-siTRPC1-transfected PC-3 cells are not sufficiently fluorescent to be discriminated from the auto-fluorescence. As a consequence it was necessary to use a PC-3 subline that has enhanced fluorescence (i.e., by viral transfectionof the GFP cDNA) to carry out permanent TRPC1 suppression..

The completion of the experiments of years 1 and 2 confirms that MscCa is a promising new target to block prostate cancer progression and provides added motivation to further

understand its detailed mechanisms of action in vitro and in vivo. In particular, because Ca2+ influx via MscCa appears important in regulating all major modes of cell migration (i.e., mesenchymal, amoeboid and collective) it may be more effective than other strategies targeting integrins and metalloproteinases that appear to fail in vivo as a consequence of migration mode plasticity (Wolf & Friedl, 2006, Maroto & Hamill, 2007).

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LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

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A stretch-activated Ca^{2+} channel regulates human prostate tumor cell migration[1]

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[1]This work was supported by grant PC030021 from the USAMRM (Prostate Cancer Research Program) and grant CA106629 from the National Cancer Institute.

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[3]The abbreviations used are: CSK, cytoskeleton; ECM, extracellular matrix; GsmTX-4 *Grammastola* spider venom peptide 4 kDa; MscCa, mechanosensitive Ca^{2+}-permeant channel; PC, prostate cancer; SAC, stretch activated channel; TRPC, canonical transient receptor potential channel.

ABSTRACT

In its early stages prostate cancer stays in the prostate and is not life-threatening, but without treatment it spreads to other parts of the body and eventually causes death. Because the acquisition of cell motility is a critical step in the metastatic cascade it is important to identify the mechanisms that regulate tumor cell migration. Based on studies of fast moving fish keratocytes (25) it has been proposed that the stretch-activated mechanosensitive Ca^{2+}-permeable channel (MscCa) regulates cell migration by coordinating forward extension with mechanisms that lead to cell retraction. Here we report that MscCa is expressed in the highly migratory/invasive human prostate tumor cell line PC-3, and that sustained Ca^{2+} influx via MscCa activity is required to generate an intracellular [Ca^{2+}] gradient that determines directed migration. The nonmigratory human prostate tumor cell line LNCaP also expresses MscCa. However, in this cell the channel undergoes rapid (< 100 ms) inactivation with stretch precluding sustained Ca^{2+} influx, $[Ca^{2+}]_i$ gradient and directed cell migration. Our results indicate that MscCa and the physical/biochemical mechanisms that regulate channel gating are promising targets to block prostate tumor cell migration.

INTRODUCTION

Prostate cancer (PC) is a progressive disease involving transformation to unlimited cell growth, immortalization to escape the limits of senescence/apoptosis, and the ability to spread to distal sites (invasion and metastasis). In order for PC^3 to spread, tumor cells must migrate from the prostate, pass through blood vessels, penetrate into the secondary tumor site (typically bone), and migrate through its tissue to establish a metastasis (33). Cell migration is therefore necessary although not sufficient for invasion and metastasis, which also require the additional steps of barrier matrix breakdown, and tumor cell adherence, growth and angiogenesis at the secondary sites (12). Nevertheless, because metastasis will only be achieved if the tumor cell completes every step in this cascade, identifying the most sensitive and susceptible step in tumor cell migration should provide a promising target to block PC metastasis (21).

Although the rates and patterns of cell migration vary among normal and cancer cells (9) they also share a basic cycle of steps involving (24,35,39) protrusion and adhesion of the front of the cell, a contraction of the cell body that leads to cell extension and finally rear retraction. A key question about this cycle concerns the mechanosensitive mechanisms that coordinate forward protrusion with rear retraction. Lee and colleagues (25) proposed from their studies of fast moving fish keratocytes that the stretch-activated MscCa (also referred to as SAC), could serve this function by its ability to "sense" and transduce membrane stretch into Ca^{2+} influx and thereby provide feedback between mechanisms that cause cell forward protrusion and those Ca^{2+}-dependent mechanisms (e.g., cell

contractility and adhesion disassembly) that promote rear retraction. Since the process of cell migration is conserved, we thought that MscCa activity might also be important for coordinating PC cell migration. In order to test this hypothesis we use patch-/pressure clamp techniques to determine if MscCa is expressed in the highly motile/invasive human prostate tumor cell line PC3, and whether the channel activity is required for PC3 cell migration. We also use high resolution intracellular Ca^{2+}-imaging techniques to measure changes in intracellular Ca^{2+} during PC-3 cell migration, and determine the role of MscCa in modulating these changes. Finally, we compare MscCa expression and intracellular Ca^{2+} changes between motile PC-3 cells and the nonmotile human prostate cell line LNCaP.

MATERIALS AND METHODS

Cultures. The human PC cell lines (ATCC, Manassa, VA) studied included the PC-3 (20), LNCaP (19) and DU-145. Cell cultures were grown in RPMI 1640 medium with 25 mM Hepes and glutamine, 8 % FCS, 1 mM Na pyruvate, 4.5 g/L glucose and antibiotics at 37ºC in a humidified 95% O_2-5% CO_2 atmosphere.

Patch-clamp recording. Standard cell-attached, patch-clamp recording was used to record single-channel currents. A custom-built pressure clamp was used to apply a gentle and reproducible suction protocols (< 10 mmHg for 10 s) in order to achieve the initial tight seal and then to mechanically stimulate the patch (15). A standardized sealing protocol was important in order to minimize any mechanically-induced changes in

channel properties before recording. The standard pipette solution contained, in mM: 100 KCl, 2 EGTA (KOH), 5 Hepes (KOH) at pH 7.4. To measure Ca^{2+} block and permeation 1 mM Ca^{2+} replaced the 2 mM EGTA. Gd^{3+} was added to the pipette solution without EGTA. In order to monitor MscCa activity before exposure to the agents, the pipette tip was filled (~300 μm) by capillary action with agent-free pipette solution, then backfilled with the agent-containing solution. The standard bath solution contained, in mM: 150 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes (NaOH) at pH 7.4. Patch currents were filtered at 500 Hz with an 8-pole Bessel filter and digitized at 1 kHz on an IBM clone using pCLAMP (Axon Instruments, Union City, CA). Chemicals in general were purchased from Sigma (St Louis, MO) except for GsmTx-4 (Peptides International, Louisville, KY) and fluorescent agents (Invitrogen/Molecular Probes, Carlsbad, CA).

Videomicroscopy and Ca^{2+}-imaging. Cell migration was monitored at 37°C by time-lapse videomicroscopy using Nomarski optics with an Epifluorescent microscope (Nikon). Fura-2 AM (5μM, 20 min) was loaded for calcium imaging assays. Images were captured at 340 and 380 nm, at 30 s and/or 1 min intervals. Images acquired with Metafluor (Universal Imaging Corp. Sunnyvale, CA) and a Photometrics Coolsnap HQ camera (Roper Scientific). Metamorph (Version 6.2, Universal Imaging Corp. Sunnyvale, CA) and Excel 2000 (Microsoft Excel, WA) were used for analysis. Conversion of pixels to μm was based on a calibrated slide. Wound assays were carried on sub confluent PC3 cells (90%) seeded on 35 mm dishes. Three scratches (~500 μm across) per dish were made using a sterile 200 μl pipette tip. As indicated, 3 μM GsMTx-4 was added to the

culture and replaced by fresh solution after 24 h. The same procedure was used in controls.

Confocal Immunofluorescence. A Zeiss LSM 510 META confocal system configured on an Axiovert 200M inverted microscope (63X 1.4 objective) was used to acquire the images (543nm excitation, green He/Ne laser) later processed with Metamorph. The distribution of ER in PC cells was studied by 10 min incubation with 200 nM BODIPY FL-thapsigargin (excitation 488, emission 510-600).

RESULTS

MscCa activity in migrating PC-3 cells. Figure 1a is a photomicrograph of a migrating PC-3 cell showing three distinct morphologically regions — a leading zone that includes the lamellipodium and lamellum, a thicker somatic region that encloses the nucleus, and an extended/stretched rear tether. Cell-attached recordings from these regions (arrowed) on over 120 different PC-3 cells indicate stretch-activated currents can be recorded in all regions (Fig. 1a). Figure 1b shows single stretch-activated channel currents recorded at -50 and 50 mV, and indicates the open channel undergoes more frequent brief openings and closings at negative compared with positive potentials. Figure 1c shows current-voltage relations measured under different ionic conditions, and indicates an inwardly rectifying channel that is permeable to both Na^+ and K^+, and shows reduced conductance in the presence of 1 mM Ca^{2+} (Fig. 1c; chord conductance measured at -50 mV was 55 pS (100 K^+:0 Ca^{2+}); 42 pS (100 Na^+:0 Ca^{2+}); 25 pS (100 K^+:1 Ca^{2+}) and

20 pS (100 Na^+:1 Ca^{2+}). Interestingly, the channel in human tumor cells displays similar gating and conductance properties as MscCa expressed in frog oocytes (44, 46, 49). In order to test if functionally significant Ca^{2+} flows through the channel under physiological conditions (~1 mM Ca^2) we used Ca^{2+}-activated K^+ (K_{Ca2+}) channel activity to assay for stretch-induced $[Ca^{2+}]_I$ changes. Figure 1d shows single outward channel currents that followed the stretch-activated inward currents. The conductance of the outward currents was ~20 pS, which is similar to the intermediate conductance K_{Ca2+} channel reported in PC-3 cells (31). Most importantly, the outward currents were never activated by stretch when Ca^{2+} was removed from the pipette solution. These results indicate that Ca^{2+} flow through MscCa under physiological conditions is sufficient to raise local $[Ca^{2+}]_i$ to levels that activate Ca^{2+}-sensitive mechanisms.

Anti-MscCa agents block PC cell migration. Testing different anti-MscCa agents on PC-3 cells, we found that 5 μM Gd^{3+}, a relatively nonspecific MS channel blocker (49), abolished MscCa activity (supplementary Fig.1a). A similar effect was seen with 3 μM GsMTx-4, which is a more specific MS channel blocker (43; supplementary Figs. 1b). Gd^{3+} and GsMTx-4 also blocked PC-3 cell migration as measured by time-lapse video microscopy and wound/scratch closure assays. Figure 2a shows video frames of PC-3 cells selected at 0, 30, and 60 minutes during migrating out from a cluster of 8 cells. Figure 2b shows representative PC-3 cell trajectories measured for periods of ≥ 1 h before, during, and after exposure to Gd^{3+} (5 μM) and GsMTx-4 (3 μM) (see also supplementary video 1). Histograms summarizing several experiments are shown in Figure 2c. Apart from blocking directional migration, the agents also altered PC-3 cell

morphology. For example, instead of a prominent ruffled lamellipodia, PC-3 cells in Gd^{3+} and GsMTx-4 lost their polarized morphology and took on a smooth, flattened "fried egg" appearance (see supplementary video 1). These effects on migration and cell morphology were rapidly reversed by washing out Gd^{3+} and GsMTx-4. Cell migration assayed by wound/scratch closure assays also confirmed the ability of GsmTx-4 to block PC-3 cell migration (supplementary Fig. 2a).

$[Ca^{2+}]_i$ dynamics in migrating PC cells. To address how MscCa activity might regulate cell migration, we measured the spatial and temporal $[Ca^{2+}]_i$ dynamics in the absence and presence of anti-MscCa agents. Figure 3a shows time-lapse fluorescent images of migrating PC-3 cell loaded with fura-2, and indicate that as the migrating cell becomes progressively extended it develops a $[Ca^{2+}]_i$ gradient increasing from the front to the rear of the cell (see also Supplementary Videos 2 and 3). This form of $[Ca^{2+}]_i$ gradient was a common trait seen in over 200 migrating PC-3 cells. The gradient collapsed with rear retraction, presumably as membrane stretch was relieved, and reversed when cells spontaneously reversed direction (Fig. 3b, supplementary video 4). Furthermore, the $[Ca^{2+}]_i$ gradient only developed in migrating PC-3 cells. For example, Fig. 3b shows two neighboring PC-3 cells in which only the migrating cell develops a gradient (see also supplementary video 4). A small proportion of migrating PC-3 cells (~10%) also exhibited fast $[Ca^{2+}]_i$ transients that spread rapidly ($\leq$ 2 min) throughout the cell and occurred with an average frequency of 2 $\pm$ 0.8 transients/h (range 1-4 transients/h in 15 cells). In some, but not all cases, the transients were immediately followed by retraction of the rear tether (see Fig. 3b & c, and supplementary video 4).

GsMTx-4 and Gd^{3+}, at the same concentrations that block PC-3 cell migration, also prevented the development of $[Ca^{2+}]_i$ gradients and transients (Fig. 3d and supplementary video 5, 10-20 cells tested in each condition). The dependence of these $[Ca^{2+}]_i$ changes on Ca^{2+} influx, was also indicated by block with 10 mM external BAPTA (supplementary video 6). Similar blocking effects were observed when internal $[Ca^{2+}]_i$ stores were depleted with 5 μM thapsigargin (data not shown) indicating the $[Ca^{2+}]_i$ dynamics depend upon Ca^{2+} influx and Ca^{2+} release from internal stores (1). We have also carried out preliminary studies measuring $[Ca^{2+}]_i$ changes in PC-3 cells plated on elastic substrate that could be stretched using glass micropipettes (25,30). The response of PC-3 cells to stretch was variable and dependent on the stage in the migratory cycle — PC-3 cells that were not extended (i.e., had no trailing tether) showed a stretch-induced global $[Ca^{2+}]_i$ elevation, whereas 3 out of 5 cells that were already extended lost their $[Ca^{2+}]_i$ gradient as their trailing tether retracted during the applied stretch (Maroto & Hamill, unpublished observations). The latter finding indicates that the relaxation of intrinsically generated forces can overcome extrinsically applied stretch.

Mechanisms that generate the $[Ca^{2+}]_i$ gradient in migrating PC cells. Several mechanisms may support the spatial $[Ca^{2+}]_i$ gradient that develops in migrating PC-3 cells. First, there is a higher probability of recording MscCa active patches towards the rear of the cell. Overall ~15% of patches showed no stretch channel activity (i.e., with suctions up to 100 mmHg). However, the percentage of null patches was highest on the lamella (24%: 10 out 42 patches) compared with the cell body (15%: 8 out 54) or the

trailing tether (11%: 3 out 28). This polarized distribution could generate a gradient of Ca^{2+} influx increasing from front to back as reported previously (38). Second, the distribution of fluorescently labeled-thapsigargin (BODIPY FL-thapsigargin) indicates ER Ca^{2+} stores are more concentrated in the cell body and rear than in the front of the cell (Fig. 4a); this would tend to further amplify any effects of polarized Ca^{2+} influx by increasing Ca^{2+}-induced Ca^{2+} release (CICR) and/or Ca^{2+} leak from internal Ca^{2+} stores (38). Finally, it has been proposed that apparent $[Ca^{2+}]_i$ gradients may develop in cells due to mitochondrial sequestration of fura-2 (34). However, our results using Mitotracker Red to measure mitochondria distributions indicate the mitochondria were concentrated around the cell nucleus and in the front half on the PC-3 cell, but were excluded from the trailing tether (Figs. 4b & 4c). In this case, the distribution would appear opposite to that required to explain the $[Ca^{2+}]_i$ gradient.

MscCa properties in migratory vs nonmigratory PC cell lines. We next asked whether MscCa is expressed in the nonmigratory PC cell LNCaP. These cells do not display the polarized morphology of PC-3 cells, but instead either are either stellar- or spindle-shaped (Fig. 5a. Supplementary Fig. 4). LNCaP cells also do not migrate as measured by wound/scratch closure assay (Supplementary Fig. 2b) or by time-lapse video-microscopy (data not shown). However, they do undergo other forms of motility that include pulsating motions, as well as multiple mini-lamellipodia and blebs that transiently protrude around the perimeter of the cell (Supplementary video 7). Surprisingly, LNCaP cells express an even higher and more uniform MscCa channel density than PC-3 cells (Figs. 5b, c) with a similar single channel conductance as seen in

PC-3 cells (Fig. 5d). However, whereas LNCaP cell channels are gated predominately in a transient mode (TM) in which channels close rapidly (i.e., in ≤ 100 ms) at the onset of a pressure pulse (117 out of 135 (87%) patches), the PC-3 cell channels show sustained gating (SM) and can remain open even after the pressure pulse (100 out of 118 (85%) patches) (compare Figs. 6a & 6b). These gating differences should have profound effects on the Ca^{2+} influx, which can be most clearly demonstrated by comparing responses to pressure steps and ramps (c.f., Figs. 6a & 6c with Figs. 6b & 6d). In LNCaP cells, a pressure step activates a large peak current of ~120 pA, whereas a ramp to the same pressure level activates a much smaller peak current of only ~ 2 pA (Fig. 6a & c). In contrast in the PC-3 cell patch shown in Figs 6b & 6d, steps and ramps activate similar amplitude sustained currents of ~40 pA. The MscCa activity induced by ramps and steps displayed the same reversal potential of ~ 0 mV and could be abolished with Gd^{3+} and GsMTx-4 irrespective of the stimuli waveform.

The TM gating that predominates in LNCaP cells arises though inactivation rather than adaptation since closed channels cannot be reopened by simply increasing the stimulus (Fig. 6a, see also 13, 15, 18). Instead, the stimulus must be turned off and reapplied, indicating the existence of an inactivated state that requires a finite time for recovery (Fig. 6b). Because the channel can enter the inactivated state from a closed state (i.e., without opening), this would account for why most channels are not available to be activated during the slower ramp stimulation. Unlike Na^{+} channel inactivation the pressure-dependent inactivation of MscCa is not strongly voltage dependent (Fig. 6c) but is subject to mechanical modulation (15, 42). For example, repetitive pressure pulses of 80 mmHg

of 1 s (but not 0.1 s) duration can result in irreversible loss of the transient current without increasing the sustained current. This response to over mechanical stimulation is similar to that reported for MscCa in other cells types and has been proposed to arise from decoupling of the membrane from the underlying CSK (15, 42). In PC-3 cells MscCa gating is more resistant to run down. As discussed below this cell-type difference in response to mechanical stimulation is consistent with the previously reported intrinsic difference in membrane-CSK decoupling and bleb formation seen between PC-3 and LNCaP cells (17).

$[Ca^{2+}]_i$ dynamics in LNCaP cells. We next asked whether the non-migrating LNCaP cells display $[Ca^{2+}]_i$ fluctuations, and if so, whether MscCa plays a role in shaping them. Of the 20 LNCaP cells studied in detail, none of the cells developed the sustained $[Ca^{2+}]_i$ gradient characteristic of migrating PC-3 cells. The lack of sustained $[Ca^{2+}]_i$ gradients is consistent with the uniform distribution of MscCa and ER/Ca^{2+} stores (Fig. 5) as well as the inability of MscCa to transduce stretch into sustained Ca^{2+} influx (Fig. 6). On the other hand, 16 out of 20 LNCaP cells did show repetitive $[Ca^{2+}]_i$ transients that spread throughout the cell (supplementary Fig. 4a). These transients were faster ($\leq$ 0.5 min) and occurred with a higher frequency (i.e., 8 $\pm$ 4.3 transients/h) than the transients observed in some PC-3 cells. Application of GsMTx-4 (3 μM) inhibited the Ca^{2+} transients/waves (supplementary Fig. 4b). The other cells that did not show Ca^{2+} transients displayed localized regions of elevated $[Ca^{2+}]_i$ that were associated with contractile or "tugging" activity at the end of the cell or with membrane protrusive "blebbing" activity (supplementary Fig. 4c). These local $[Ca^{2+}]_i$ elevations did not

develop in 10 out of 10 LNCaP cells that were incubated in 3 μM GsMTx-4 (data not shown).

DISCUSSION

Our results show that MscCa (also known as SAC) is expressed in the two most commonly studied human PC cell lines — the motile PC-3 line, originally isolated from a PC patient's bone metastasis, and highly invasive when implanted in nude mice (20, 48), and the nonmigratory LNCaP line, originally isolated from a patient's lymph node metastasis and noninvasive in nude mice (8, 19). We demonstrate that MscCa activity is essential for PC-3 cell migration based on reversible block produced by Gd^{3+} and GsMTx-4. Recently, it has been reported that GsMTx-4 also blocks directional migration in transformed MDCK-F cells (6). However, because MscCa is widely expressed in both motile and nonmotile cells (14, 36), it has not been clear what additional mechanisms, channel or otherwise, might be required to confer directional migration on non-motile cells. Our results indicate a specific mode of MscCa gating and consequent form of $[Ca^{2+}]_i$ dynamics, may be required for directed migration. In particular, because MscCa in PC-3 cells can remain fully open during stretch, the channel can support a sustained Ca^{2+} influx that is required to maintain a $[Ca^{2+}]_i$ gradient. This contrasts with the LNCaP cells in which the channel rapidly inactivates (< 100 ms) and therefore incompatible of the sustained Ca^{2+} influx required for any long term $[Ca^{2+}]$i gradient (1). We also find that the third major human PC cell line, the migratory DU-145 (isolated from a brain metastasis) expresses MscCa with a sustained gating mode (supplementary Fig. 5).

A key issue for MscCa is whether the extrinsic forces applied to activate the channel in the patch or the whole cell (25, 26) can also be generated intrinsically by the CSK. Clearly cells undergoing the mesenchymal form of migration become hyper-extended (i.e., up to 5 times their length), and this extension/spreading results in a visible smoothing out of membrane folds and microvilli (5). Furthermore, some migrating cells show elastic recoil as stretching forces exceed the strength of adhesions (27). Even more dramatic consequences occur when the retraction mechanisms are blocked and the stretching forces exceed the elastic limits of the bilayer, resulting in membrane rupture and cell fragmentation (45). Presumably, activation of MscCa and down-stream retraction mechanisms normally prevents this catastrophic event. Regarding the mechanism of MscCa gating it has been demonstrated that the force-generating CSK in migrating cells is equally linked to contacts within the dorsal and ventral matrix (10). In this case one would expect an isotropic increase in bilayer tension and activation of channels. However, while several lines of evidence support direct gating of MscCa by bilayer tension, similar to the various stretch-activated channels in bacteria (23, 29, 50), a recent elegant study carried out by Sokabe and colleagues has shown that direct tugging on actin stress fibers can cause local MscCa activation at the ventral cell surface (16). It still remains to be determined whether this localized channel activation occurs through direct actin-channel interactions or indirectly via increase in local bilayer tension, and whether similar local events are associated with cell migration.

Functionally, the $[Ca^{2+}]_i$ gradient seen in PC-3 cells would be expected to polarize the activity of Ca^{2+}-dependent molecules (e.g., transporters, enzymes, motors and adhesions) which in turn would coordinate polarization of the cell, and in particular promote the development of a single prominent and persistent lamellipodium essential for directional locomotion. On the other hand, the absence of this $[Ca^{2+}]$i gradient and Ca^{2+}-induced polarization in LNCaP cells may be permissive for the multiple mini-lamellipodia that arise transiently and randomly around the perimeter of the LNCaP cell. Although our results indicate that the MscCa-dependent $[Ca^{2+}]_i$ transients expressed by LNCaP cells are not sufficient to coordinate locomotion in this cell, they do play different roles in coordinating migration in other cell types (2, 3, 22, 25, 29, 28, 30). One view is that they activate proteins (e.g., calpain and/or mysosin II) that promote rear retraction (25), while another view is that they are more important in the development of traction forces at the cell front (30). In some of our PC-3 cell recordings we did see $[Ca^{2+}]_i$ transients that spread though out the cell, and on occasion they immediately preceded retraction (see supplementary video 4). However, their low frequency (or complete absence) in other PC-3 cells did not prevent cell migration, nor did their higher prevalence and frequency in LNCaP cells promote migration. It therefore seems that a sustained $[Ca^{2+}]_i$ gradient is key in determining directed migration in PC cells. This also seems to be the case for migrating cerebellum granule cells in which experimentally induced reversal of the $[Ca^{2+}]_i$ gradient is always accompanied by reversal in migration direction whereas the occasional occurrence of $[Ca^{2+}]_i$ transients cannot be causally related to directed migration (47). Most recently it has been reported (6) that in migrating Mardin-Darby kidney cells the highest $[Ca^{2+}]_i$ is seen at the very leading edge (see also 22), and this region of elevated

$[Ca^{2+}]_i$ appears to be superimposed on a less prominent $[Ca^{2+}]_i$ gradient that increases from the front to the rear of the cell. In this case, several forms of spatial $[Ca^{2+}]_i$ polarization may be involved in directing cell migration, which may vary cell type and/or mode of migration (4, 22, 28).

Our results also raise the possibility that a shift in Ca^{2+} dynamics mediated by changes in MscCa gating could switch PC cells from a nonmigratory to a migratory mode and vice versa. Indeed, previous studies indicate that MscCa can undergo a shift gating mode in response to mechanically-induced changes in interactions between the membrane and CSK. In particular, it has been shown that either mechanical over stimulation of the patch or membrane "blebbing" of the cell (15, 42, 50) can shift MscCa gating from the TM to the SM. In this case, it seems highly relevant that PC-3, but not LNCaP cells, exhibit spontaneous membrane blebbing, as well as "cytochalasin-induced" membrane blebbing on their apical surface (17). This apparent difference in the strength of membrane-CSK interactions may account for why SM gating predominates in PC-3 cells, and why mechanical over-stimulation of LNCaP cell patches, which itself promotes membrane blebbing, can switch gating from the transient to the sustained mode (15). However, a simple gating switch may not alone be sufficient to trigger directed migration since MscCa surface expression also differs between PC-3 and LNCaP cells, as presumably does the expression and/or coupling of the channel with downstream signaling pathways. Nevertheless, the idea of a mechanically-induced switch in cell motility is interesting given that during tumor progression, the cortical CSK does become more depolymerized (41) and Rho-induced membrane blebbing can lead to a switch in tumor cell motility

mode (37). Furthermore, it has been proposed that the elevated solid stresses and interstitial fluid pressures associated with a proliferating tumor, may actually promote increased tumor cell motility and cellular escape mechanisms from the tumor (32). At least consistent with this idea, is the demonstration that external mechanical forces can directly stimulate CSK polarization and persistent locomotion in cells and cell fragments (7, 45).

Clearly the identification of the protein subunits that form MscCa will be critical in more detailed understanding of its role in migration. However, at this time the exact protein composition of MscCa remains unresolved (11, 28, 40). So far two different members of the canonical transient receptor potential (TRPC) family, TRPC1 (28 & 29) and TRPC6 (40) have been proposed to form MscCa. However, there is also conflicting evidence on whether either subunit w expressed alone can form a directly gated MS channel (11). On the other hand, a recent report published online indicates that TRPC1 suppression blocks and TRPC1 overexpression stimulates migration (6). This compares with our observations indicating that either TRPC1 suppression or overexpression blocks PC-3 cell migration, whereas TRPC6 suppression is without effect (R. Maroto, A. Kurosky & Hamill, unpublished observations). Studies to resolve these discrepancies and determine the effects on functional expression of MscCa and other channels are currently underway.

Acknowledgements

We thank Miriam Falzon and Ana Pajor for providing the PC cell lines and Leoncio Vergara of the UTMB optical imaging laboratory.

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Figure legends

Figure 1. MscCa properties in PC-3 cells. **a:** Left panel is a photomicrograph indicating three distinct morphological regions: the front region that includes the lamellipodium and lamella (L), the cell body (B), and the rear tether (R). The right panels show cell-attached patch recordings made from each region on different PC-3 cells. Over all regions ~15% of patches were null for stretch-activated currents with a decreasing % of null patches towards the cell rear (L = 24%:10 out 42 patches; B= 15%: 8 out 54; T= 11%: 3 out 28). The mean current amplitudes (at -50 mV) excluding null patches were L= 23.5 ± 3.58 pA (n = 22); B = 24.5 ± 2.56 pA (n = 38); and R = 26.0 ± 3.8173 (n = 22). **b:** Suction step applied to a cell-attached patch activated single channel currents measured at -50 mV and 50 mV. Note the more frequent fast closures and reopenings at the negative potential. **c:** Single-channel current-voltage relations measured on cell-attached PC-3 patches (solid symbols) and *Xenopus* oocyte patches (hollow symbols) with zero Ca^{2+} (circles, pipette solution in mM: 100 KCl (or 100 NaCl), 5 Hepes, 2 EGTA) and 1 mM Ca^{2+} (triangles, pipette solution in mM 100 NaCl (or 100 KCl), 5 Hepes 1, $CaCl_2$). In the various pipette solutions the chord conductance at -50 mV was 55 pS (100 K^+:0 Ca^{2+}), 42 pS (100 Na^+:0 Ca^{2+}) 25 pS (100 K^+:1 Ca^{2+}) and 20 pS (100 Na^+: 1 Ca^{2+}) based on 4-10 patches for each

cell type and each ionic condition. **d:** Consecutive current traces showing that pressure activation of inward current results in delayed activation of unitary outward channel currents of ~1 pA (pipette solution; 100 NaCl, 5 Hepes and 1 mM $CaCl_2$, patch potential: ~ -10 mV). The same delayed activation of outward currents was seen in 5 other patches but was absent in 3 patches recorded with Ca^{2+} removed from the pipette solution. Ca^{2+} influx via the stretch-activated MscCa is able to selectively activate the intermediate conductance (~ 20 pS) Ca^{2+}-activated K^+ (IK) channel without activating a larger (~200 pS) conductance $Ca2^+$-activated K^+ (BK) channel. The BK channels could be activated in all PC-3 cell patches so far studied by either strong depolarization (i.e., more positive than 20 mV) or by inclusion of 10 μM A238187 in the bath solution, which also activated the IK channels (Maroto & Hamill, unpublished observations). We take this selective activation to indicate that IK and MscCa channels are in close proximity and form a functional unit. BK channels may be located further apart from MscCa and/or require higher $[Ca^{2+}]_i$ elevations possibly involving CICR from Ca^{2+} stores that are displaced during tight seal formation. The selective coupling between MscCa and IK channels was also seen in patches formed on LNCaP cells (Maroto & Hamill, unpublished observations).

Figure 2. PC-3 cell migration and the effects of Gd^{3+} and GsmTx-4. **a:** Selected video frames 30 min apart showing PC3 cells migrating out of a cluster. **b:** Representative trajectories (monitored every 5 minutes) before, during, and after application of 5 μM Gd^{3+} and 3 μM GsMTx-4. **c:** Histograms based on 25 or more cells (mean ± SEM) showing reversible block of migration by Gd^{3+} and GsMTx-4.

Figure 3. Intracellular Ca^{2+} gradients and transients in migrating PC-3 cells. **a: [**$Ca^{2+}]_i$ fluorescent images from left to right of a PC-3 showing regions of high $[Ca^{2+}]_i$ that develop initially in the rear half of the cell and spread later to the front of the cell. This particular cell was monitored for 3.5 h and shown in supplementary video 2. **b:** $[Ca^{2+}]_i$ images of two PC-3 cell initially migrating in opposite directions and with opposite $[Ca^{2+}]_i$ gradients (30 min frame) (arrows). In the next frame (125 min) cell 1 had reversed its $[Ca^{2+}]_i$ gradient and migration direction. The cell then showed a Ca^{2+} transient (136 min frame) that was followed by retraction of its tether (145 min) and continued cell movement (186 min). The original recording was made over 4.8 h and is shown in supplementary video 4. **c:** Fast Ca^{2+} transients in a migrating PC-3 cell. Images from left to right show two PC-3 cells, in which the migrating cell (#1) undergoes $[Ca^{2+}]_i$ transients while the stationary cell (#2) does not (see also supplementary video 5). For all images a 100X 1.3 NA objective was used. **d:** GsMTx-4 reversibly reduced $[Ca^{2+}]_i$ elevations in PC-3 cells. Three $[Ca^{2+}]_i$ images showing PC-3 cells before, after 5 min exposure to 3 μM GsmTx-4 solution and 30 minutes following GsmTx-4 washout in which the $[Ca^{2+}]_i$ had overshot levels before GsMTx-4 exposure. These images were taken with a 20X 0.75 objective. The ratiometric fura-2 measurements shown here permits the monitoring of free $[Ca^{2+}]_i$ independent of uneven dye distribution due to changes in cell thickness and/or dye accumulation in organelles. In particular, the apparent low $[Ca^{2+}]$ seen in the PC-3 cell nucleus ($[Ca^{2+}]_n$) compared with the cytosol ($[Ca^{2+}]_c$) is not due to low nuclear dye because the raw fluorescence images excited by 340 and 380 nm actually showed higher nuclear fluorescence indicating higher dye presence. Moreover the nucleus has a

relatively large free volume for fura-2 to occupy. However, the ratiometric measurements, by correcting for any "false" fluorescence due to the thickness and dye accumulation, indicates a relatively low free $[Ca^{2+}]_n$. Although there has been debate on the significance of $[Ca^{2+}]_{n/c}$ gradients, and it is possible that the nuclear environment changes dye properties, previous studies have reported the same or even opposite N/C gradients in other cell types.

Figure 4. The distribution of ER and mitochondria in PC-3 cells. **a & b:** Transmission and immunofluorescent confocal images of a PC-3 cell labeled with 200nM BODIPY FL-thapsigargin indicates a higher distribution of the ER/internal Ca^{2+} stores within the cell body compared with the lamella and lamellipodium. The images represent the maximum intensity projection reconstructed from a stack of 25 confocal sections obtained at 0.2 µm intervals. **c**. Confocal image of a PC3 cell loaded with Mitotracker-Red (100 nM) to visualize mitochondria distribution. The fluorescent image represents the maximum intensity projection reconstructed from a stack of 20 confocal sections obtained at 0.2 µm interval using a 63X1.4 objective (excitation 581, emission 644). DAPI (emission 457) was simultaneously applied to visualize the nuclei. 14 out of 16 cells showed a similar pattern.

Figure 5. The non-migratory LNCaP cell also expresses MscCa. **a**. Photomicrogaphs showing transmission and confocal fluorescent images of an LNCaP cell labeled with BODIPY FL-thapsigargin to visualize ER distribution. The fluorescent image represents

the maximum intensity projection reconstructed from a stack of 30 confocal sections obtained at 0.2 μm intervals. Both transmission and fluorescent images are overlapped indicating an almost uniform ER distribution within the cytoplasm. **b.** Current response of a cell-attached patch from a LNCaP cell to a 60 mmHg suction step which showed a peak current of approximately 140 pA indicating ~70 channels in the patch that inactivated within 100 ms of the step. **c**: Histogram showing that LNCaP cells express relatively larger peak currents in response to pressure steps (based on 118 patches) compared with responses of patches on PC-3 cells (based on 135 patches). **d**: Single channel current-voltage relations measured for LNCaP and PC3 cells (with 100 mM KCl 2 EGTA (KOH) and 5 mM Hepes (KOH) in the pipette solution) superimpose indicating the same or closely-related pore structure. Data points based on 10-20 patches for each PC cell type.

Figure 6. Comparison of LNCaP and PC-3 cell responses to pressure steps versus ramps. **a:** Responses of the same membrane patch on an LNCaP cell to increasing pressure steps and ramps. The largest step of 100 mmHg activated a peak current of ~130 pA, compared with only ~2 pA for the ramp up to 100 mmHg. Similar discrepant responses were seen whether ramps were applied before or after steps. **b:** Similar protocols as in a applied to a PC-3 cell membrane patch in which both increasing steps and ramps produced similar maximal sustained currents of ~ 40 pA. **c:** Expanded records of ramp responses on a different LNCaP cell patch. At the very beginning of the ramp currents representing 2 channels were briefly activated but then inactivated with increasing ramp pressure. Towards the peak of the ramp pressure additional noisy currents were seen and both types

of currents were equally blocked by Gd^{3+} and GsMTx-4 (data not shown). The noisy currents may represent the inactivated channels reopening as lower sub-conductance states as has been reported for the inactivated MscCa in astrocytes (42). **d:** Expanded ramp responses on another PC-3 cell patch showing the sustained opening during the ramp and delayed closing after the ramp. Although the experimentally applied ramps here are clearly not as long as that expected to develop during the cell during migratory cycle which can last several hours, the absence of MscCa inactivation would allow for sustained Ca^{2+} influx over this time scale.

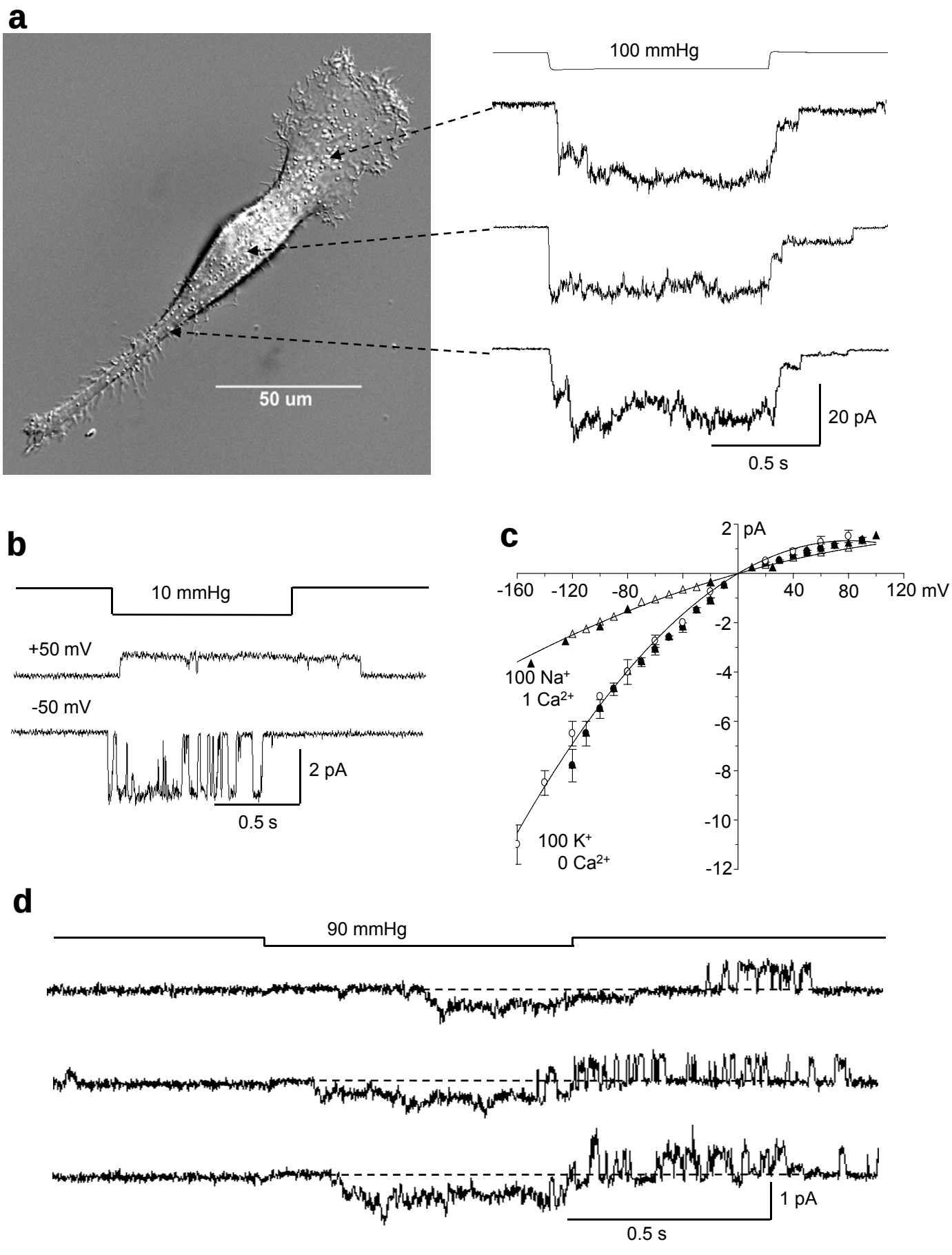
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Fig 1

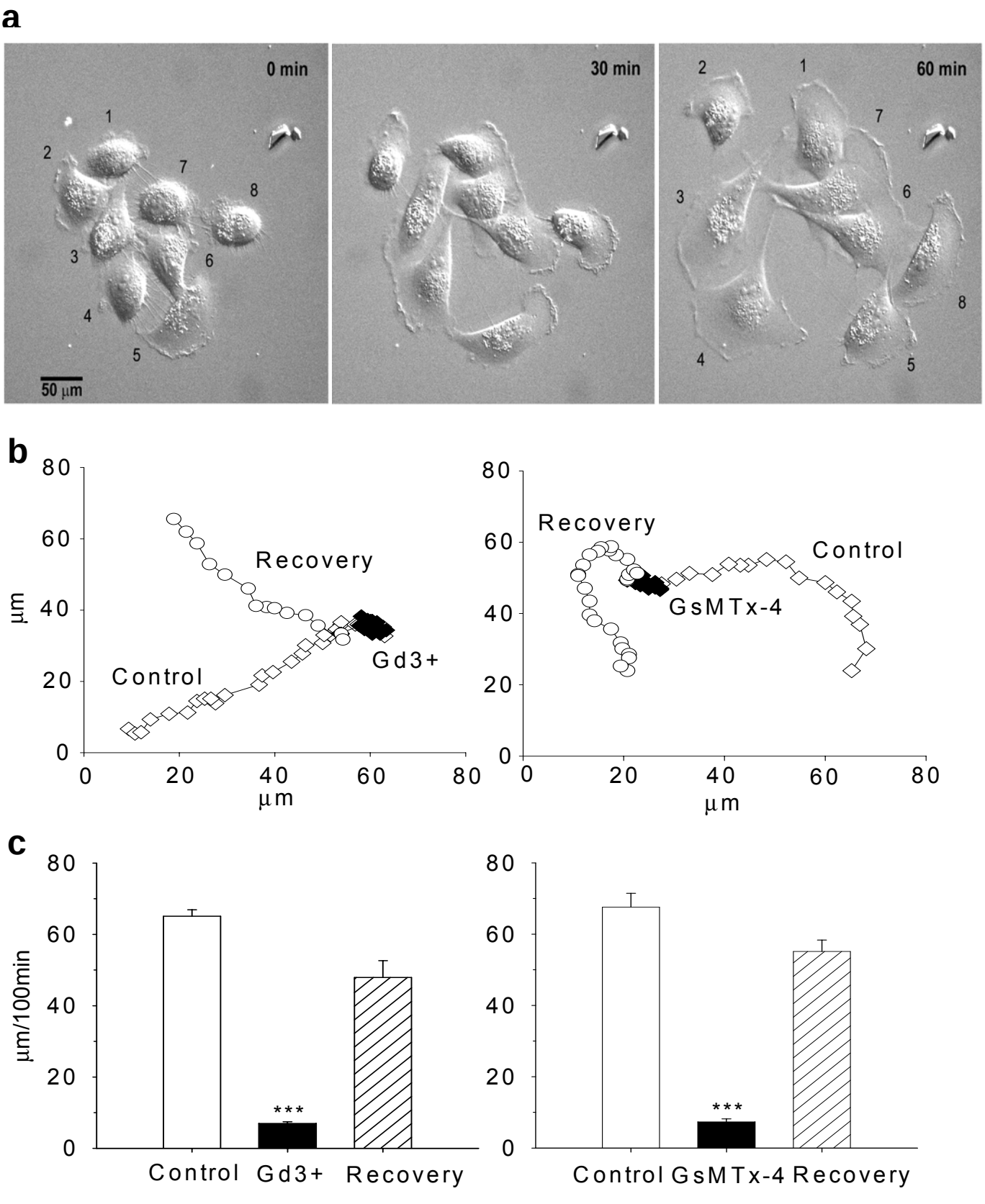
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Fig 2

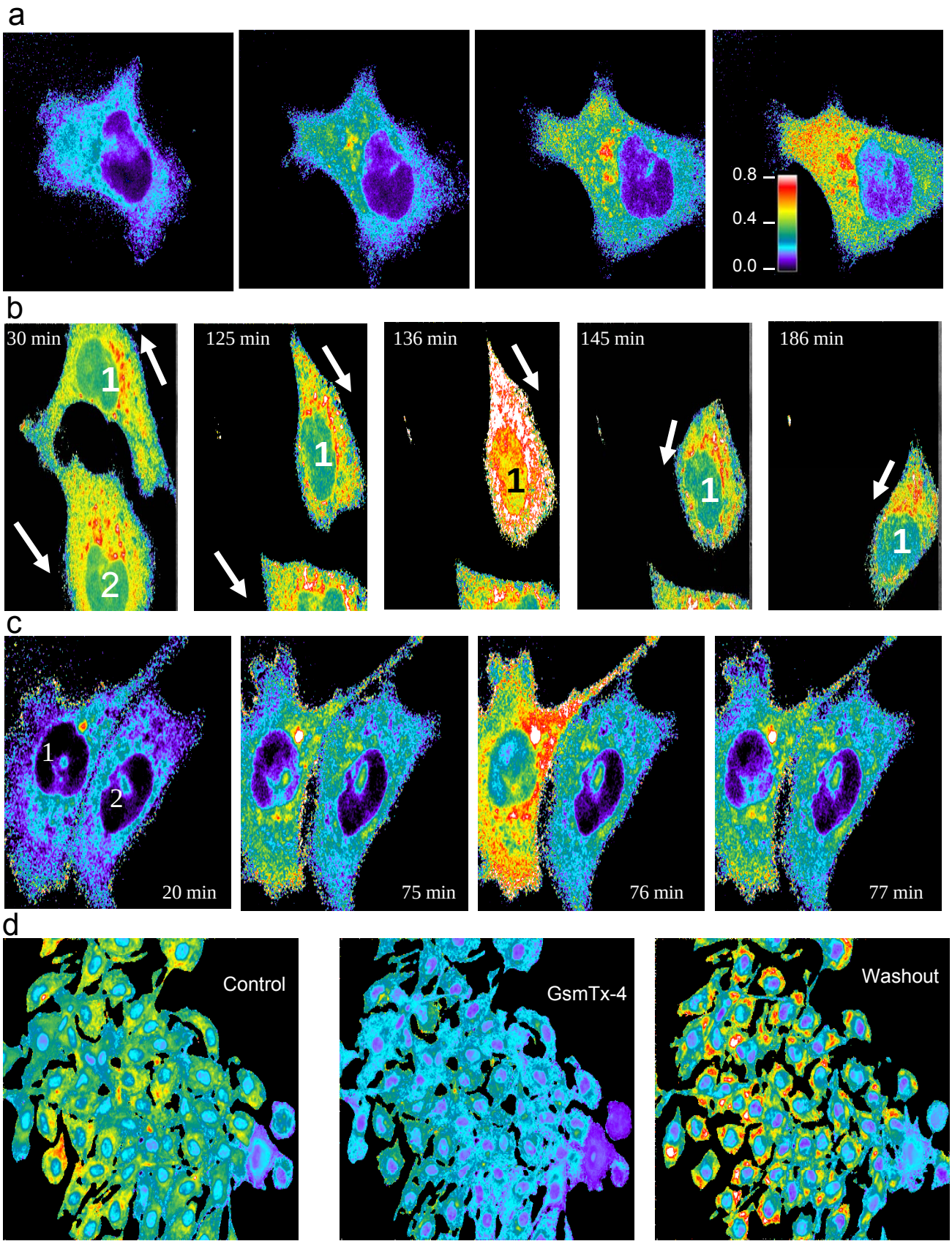
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Fig 3

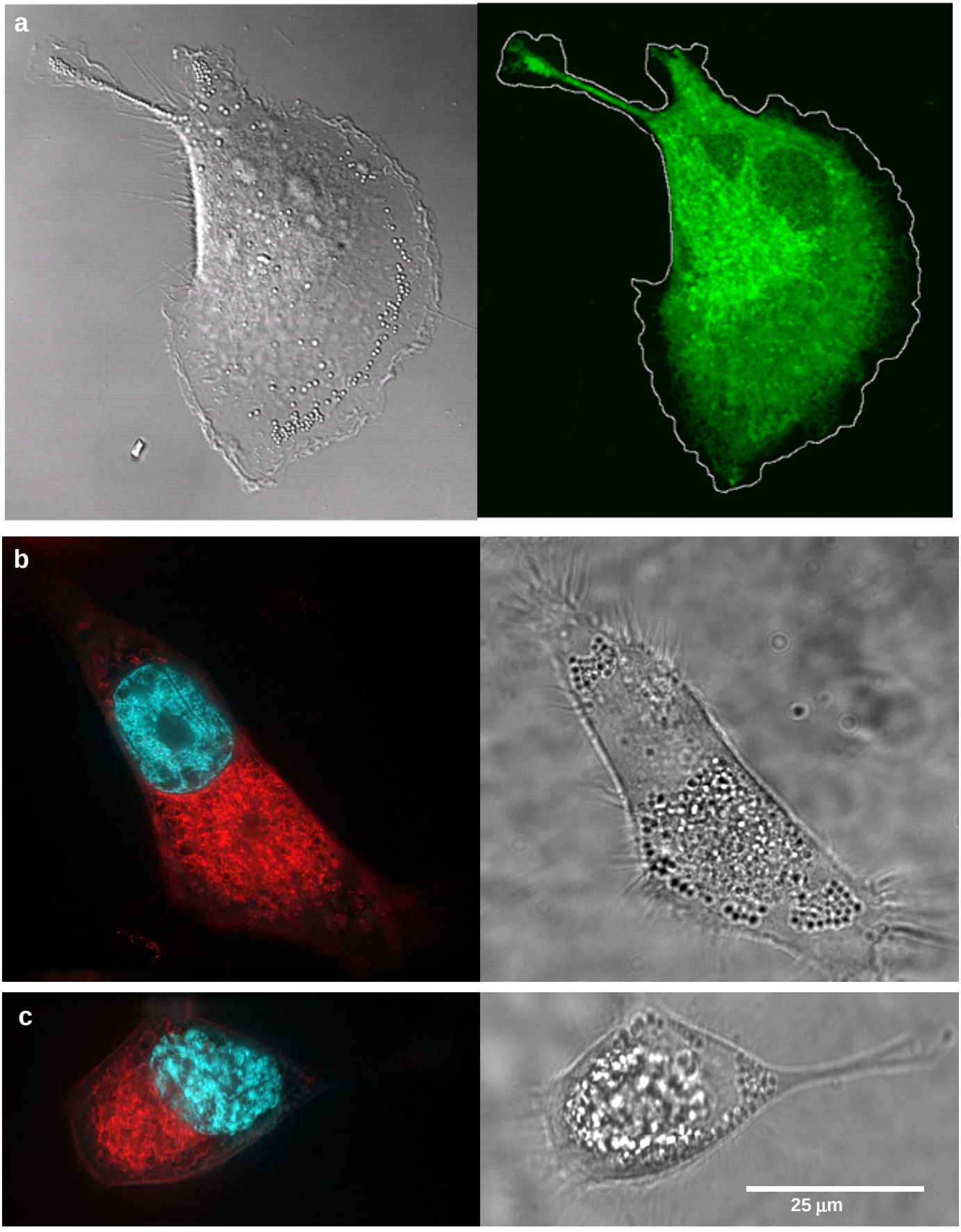
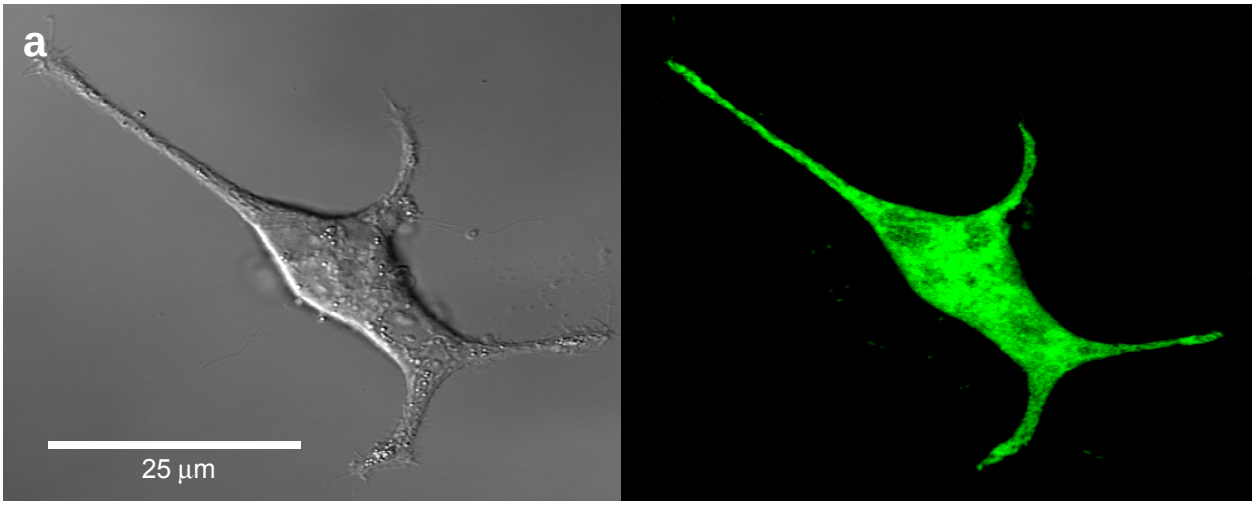


Fig 4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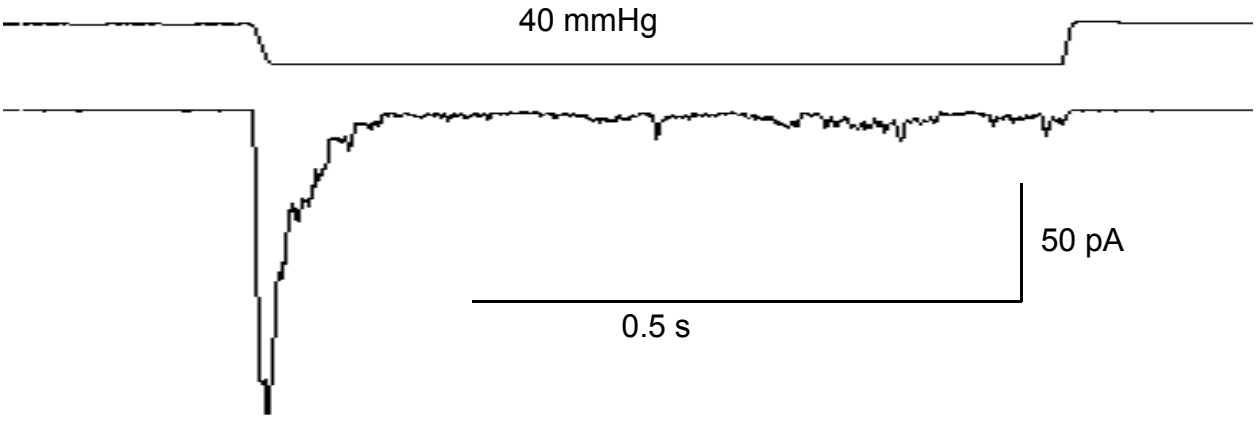
40 mmHg
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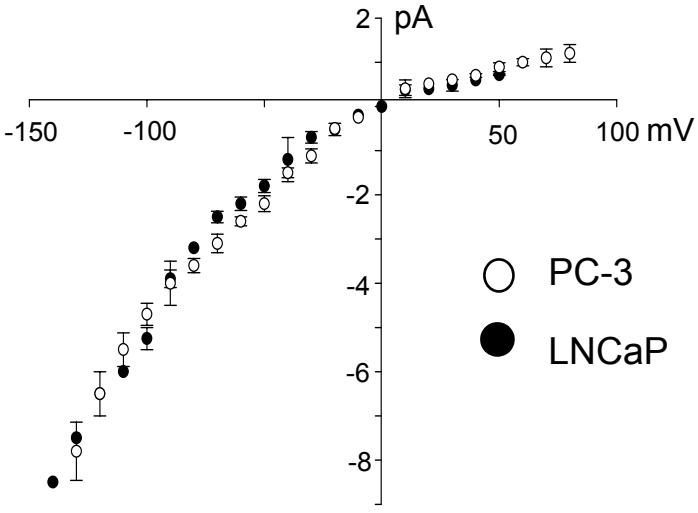
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-4
-6
-8
PC-3
LNCaP

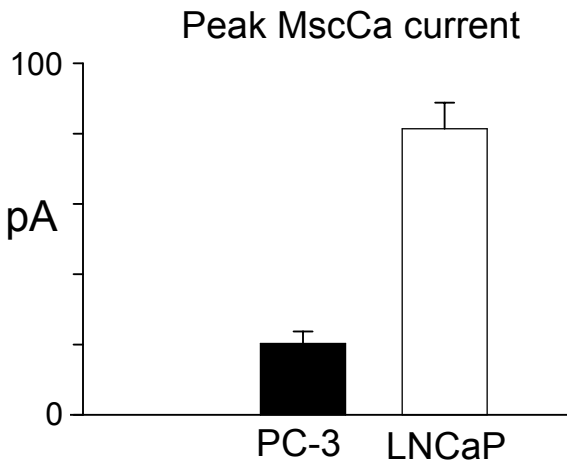
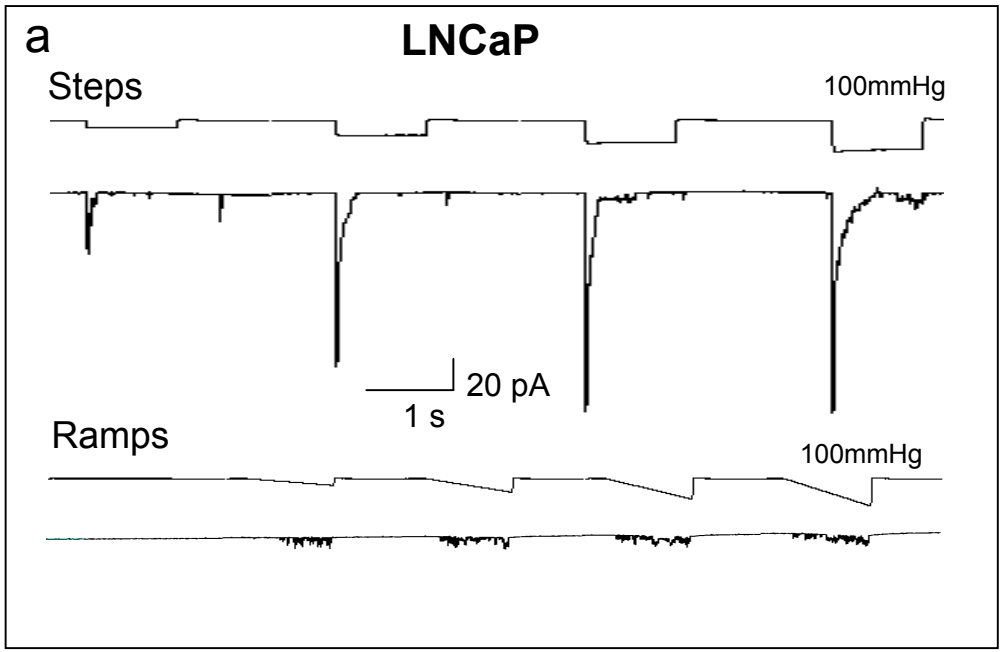
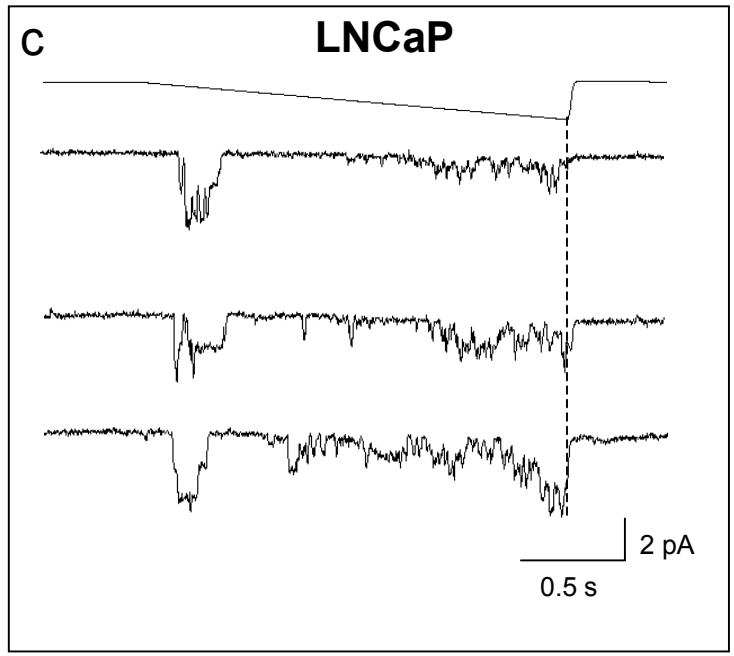
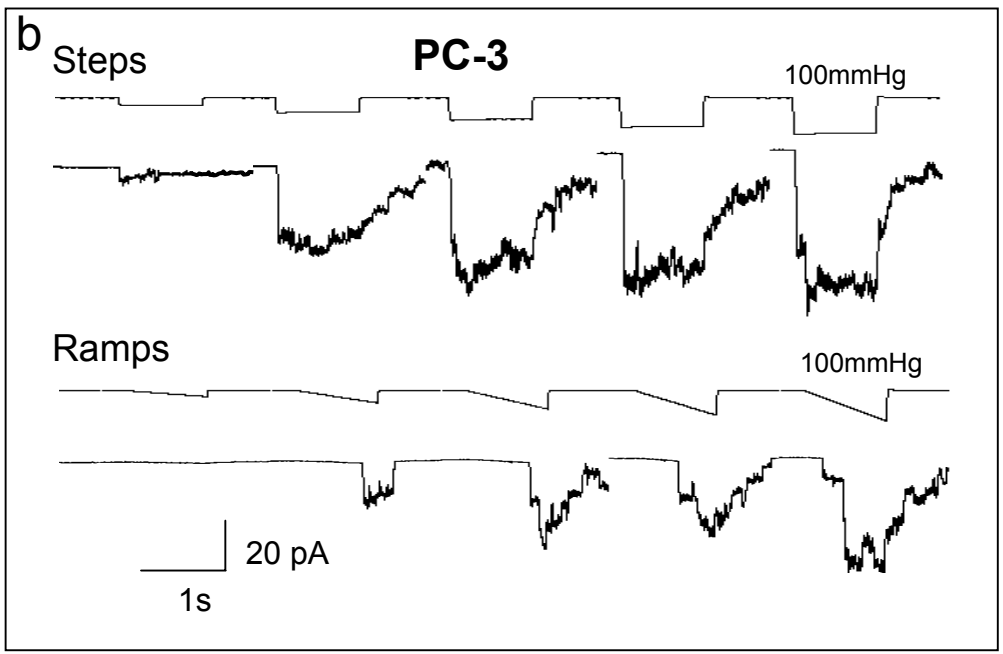

Peak MscCa current
100
pA
0
PC-3
LNCaP

Fig 5


a
LNCaP
Steps
100mmHg
20 pA
1 s
Ramps
100mmHg


c
LNCaP
2 pA
0.5 s


b
Steps
PC-3
100mmHg
Ramps
100mmHg
20 pA
1s

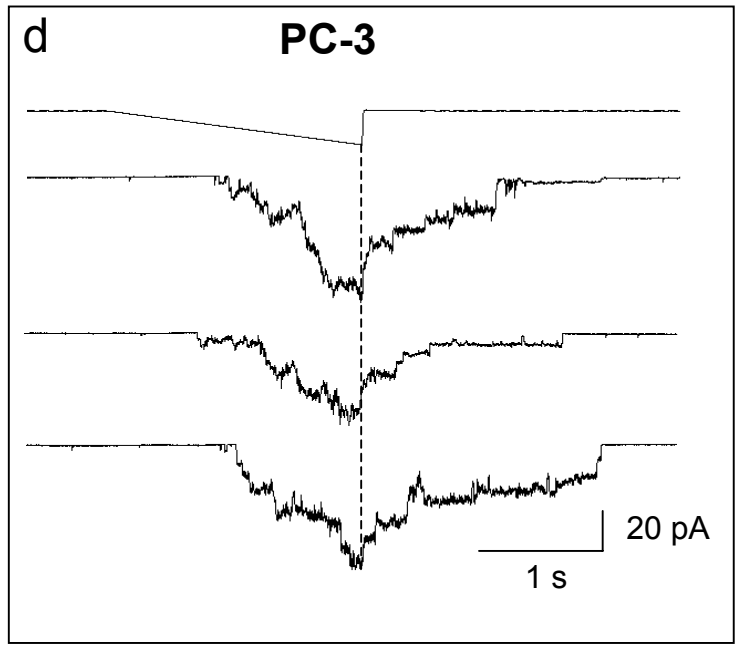

d
PC-3
20 pA
1 s

Fig 6

CELLSCIENCE
Commentary
Cell Science Reviews Vol 6 No 2
ISSN 1742-8130

Mechanically-ignited calcium flickers: Reemergence of an old role in mechanosensitive regulation of cell migration and the discovery of a new role in chemotaxis

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Received 16th October © Cell Science 2009

Ca^{2+} flickers represent a newly recognized phenomenon in migrating cells. They arise from the preferential activation of individual mechanosensitive Ca^{2+} channels at the front of the cell by the protrusive/traction forces that move the cell forward. Interestingly, chemo-attractants also increase Ca^{2+} flicker frequency presumably via biochemical signals that regulate force generation. This discovery highlights the important feedback role that mechanosensitive Ca^{2+} influx plays in regulating the biochemical signaling pathways that determine cell migration and chemotaxis.

The role of calcium in regulating cell motility has been long recognized (1, 2). Intracellular Ca^{2+} transients and gradients have been shown to be important in determining that rate of cell locomotion, as well as regulating neurite extension and steering nerve growth cones (3-5). In one study carried out 30 years ago, Taylor and colleagues (6) used the Ca^{2+}-sensitive photoprotein aequorin to visualize $[Ca^{2+}]_i$ fluctuations in locomoting amoebae. They observed brief, spatially-discrete pulses of $[Ca^{2+}]_i$ at the front of the cell, together with a more sustained elevated $[Ca^{2+}]_i$ at the cell rear. By removing external Ca^{2+} or by adding the Ca^{2+} channel blocker, La^{3+}, they could abolish the $[Ca^{2+}]_i$ pulses and block cell locomotion, without immediately reducing the elevated rear $[Ca^{2+}]_i$, which they proposed was more dependent upon Ca^{2+} release from internal stores. Furthermore, they demonstrated that gentle mechanical stimuli applied to the front of the cell increased the frequency of Ca^{2+} pulses and induced pseudopod extension towards the stimulating probe.

The above study now seem quite prescient in relation to recent observations made on migrating human fibroblasts using more sophisticated Ca^{2+} imaging techniques. In particular, Wei and colleagues (7) employed real time confocal microscopy to study $[Ca^{2+}]_i$ fluctuations in migrating cells loaded with Ca^{2+}-sensitive fluorescent dyes, and also saw brief, spatially-confined $[Ca^{2+}]_i$ pulses ('Ca^{2+} flickers') concentrated at the front of the cell, which occurred on

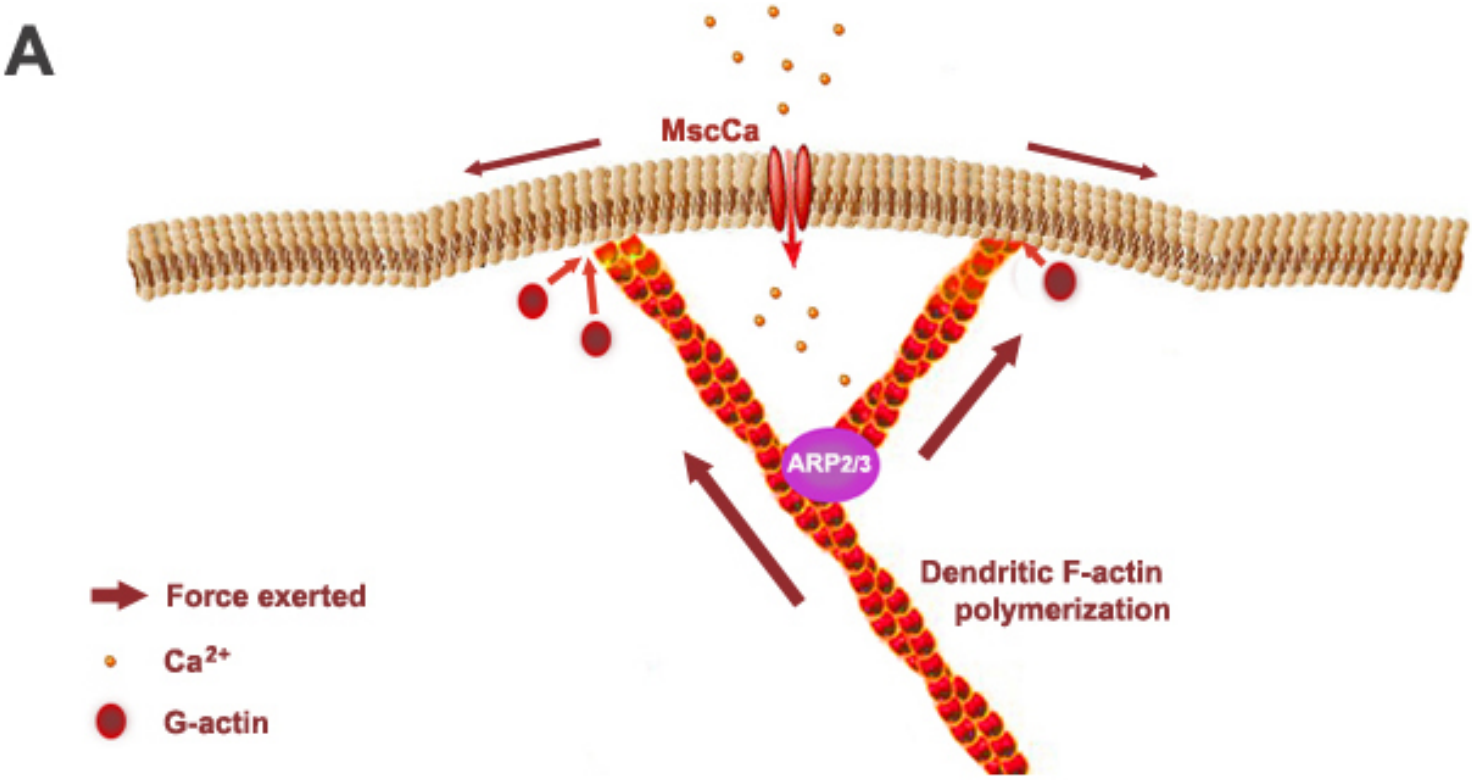
top of a sustained gradient of $[Ca^{2+}]_i$ that increased towards the back of the cell. As in the amoeba, the Ca^{2+} flickers were abolished by removal of external Ca^{2+} or by the addition of Ca^{2+} channel blockers (Gd^{3+} and streptomycin), and their frequency was modulated by direct mechanical stimulation applied to the front of the cell.

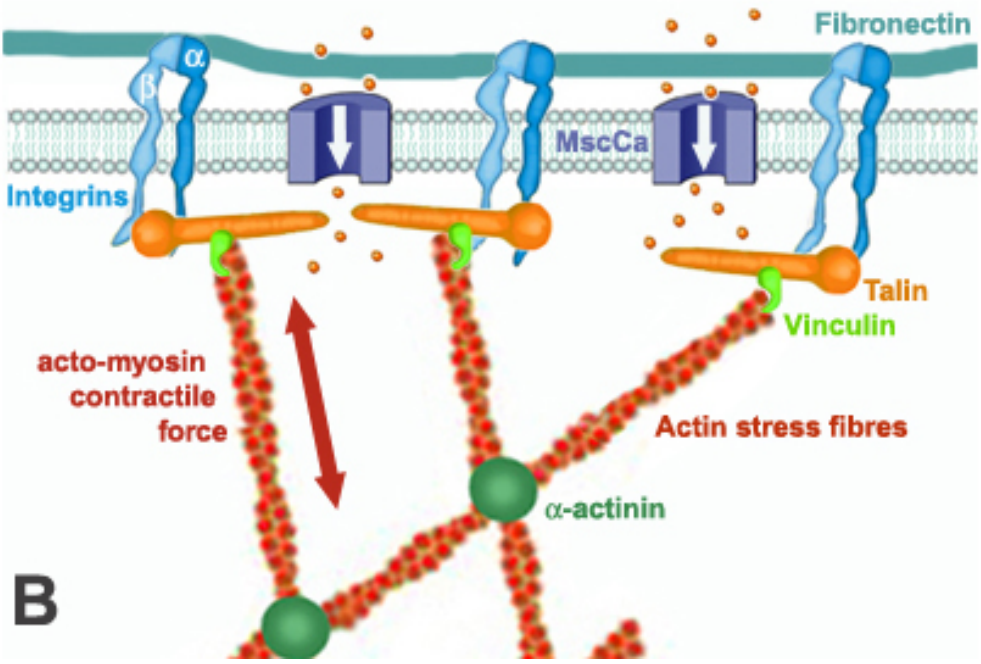
Therefore, two markedly different cell types - spanning two billion years of evolution, and using distinctly different modes of migration (‘amoeboid’ vs ‘mesenchymal’) - appear to utilize mechanosensitive Ca^{2+} channels (MscCa) preferentially activated at the cell front to steer cell migration. Several new insights have now been added to this story (7). In particular, it is now known the Ca^{2+} flickers are mediated by a transient receptor potential melastatin subfamily channel (TRPM-7) that can activated by integrin engagement as well as by directly applied membrane stretch (7, 8). The flickers were also shown to be enhanced by Ca^{2+}-induced Ca^{2+} release from IP_3-sensitive stores. These features are significant because they provide mechanisms that can link the mechanically-ignited Ca^{2+} flickers to biochemical signaling pathways that regulate protrusion/traction forces and chemotaxis. Indeed, Wei et al. (7) demonstrated that disruption of the frontal F-actin or inhibition of myosin contractility inhibited flicker production in the lamella. On the other hand, when the chemoattractant platelet derived growth factor (PDGF) was preferentially applied to one side of the lamella, it increased Ca^{2+} flicker frequency on that side and turned the cell towards the PDGF source. Furthermore, agents that blocked MscCa/TRPM7 activity also blocked the Ca^{2+} flickers and chemotaxis.

The biochemical pathway by which a PDGF ignites the Ca^{2+} flickers may involve Ras/PI3K/PIP3/Rac signaling that acts to regulate the dendritic F-actin polymerization force applied to the leading edge of the cell (9, Figure 1A). Significantly, two different studies indicate that Ca^{2+} may act in a feedback loop to reinforce Rac-signaling and thereby sustain the leading edge and steer cell migration (10, 11). This feedback depends upon Ca^{2+}-dependent recruitment of protein kinase C to the cell’s leading edge where it promotes translocation of cytosolic Rac to the plasma membrane. Another signaling pathway that may ignite Ca^{2+} flickers beyond the protruding lamellar region may involve RhoA/ROCK/myosin II-actin stress fiber activation. In this case, the increased traction force transmitted to focal adhesions would provide the force to activate Ca^{2+} flickers (12-14, Figure 1B). In direct support of this idea it has been shown that TRPM7 is localized to focal adhesion complexes (15), and Hayakawa and colleagues have used optical tweezers to selectively pull on actin stress fibers and ignite Ca^{2+} flickers at or near focal adhesions (16). In the case of Ca^{2+} flickers seen in cells undergoing amoeboid type migration (6), the ignition force at the front of the cell may be generated by hydrostatic pressure via acto-myosin gel contraction, which pushes out and blebs the front of the cell (17, 18, Figure 1C).

Additional specific features of TRPM7, which still need to be reconciled with Ca^{2+} flickers, point to the versatility of the channel and its different downstream effectors in regulating other aspects of cell motility. In particular, TRPM7 has been shown to activate m-calpain in vinculin-containing peripheral cell adhesion complexes and promote their disassembly (15). TRPM7 also produces a kinase-dependent inhibition of myosin II by phosphorylating the

myosin heavy chains, thereby producing a loss of cortical tension and promoting *de novo* podosome formation (19). To add even more complication to the story, other mechanosensitive TRP Ca^{2+} channels also regulate cell migration and/or neurite extension - including TRPC1, a putative MscCa (20, 21) and intracellular Ca^{2+} release channel (22) that has been shown to be necessary for the migration of several cell types (23-26) and also appears to elevate $[Ca^{2+}]_i$ at the cell's leading edge (26) - and TRPC5, a MscCa that mediates Ca^{2+} transients (27) and negatively regulate neurite extension (28) and possibly the filopodial calcium transients that promote growth cone turning (5).





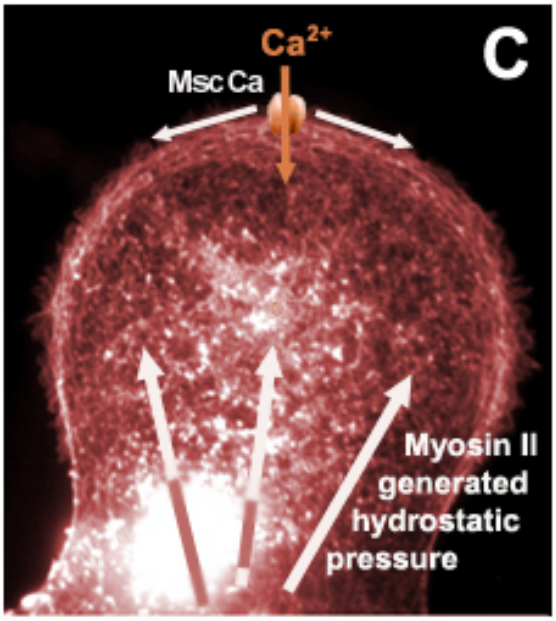


Figure 1. A schematic summary of three different mechanisms by which force may be generated at the front of the cell to increase membrane tension and mechanically activate Ca^{2+} flickers. A. Dendritic F-actin polymerization force applied at the leading edge of the cell deforms and increases local membrane tension. B. Actin-stress fibers apply contractile forces to adhesive complexes that generate traction force as well as increasing local membrane tension. C. Local hydrostatic pressure transients generated by local recruitment and activation of myosin II triggers increased membrane tension and causes membrane blebbing.

In conclusion, MscCa - which is typically measured in patch clamp experiments at millisecond time scales and traditionally thought to function in rapid sensory/transduction events - can act as a versatile mediator of Ca^{2+} signals that regulate migratory events ranging over the time scale of seconds to the several hours it can take to complete the motility cycle. It achieves this feat by performing in at least three distinct Ca^{2+} signaling roles:

1. As a short range, stochastic signal (Ca^{2+} flickers) that can promote activation of high threshold Ca^{2+}-sensitive mechanisms important for the regulation of protrusive forces and/or transmission of traction forces at discrete sites at the cell's leading edge (6,7).

2. As a long term global signal ($[Ca^{2+}]_i$ gradients) that can act to promote cell polarization and maintain differential localization of Ca^{2+}-sensitive activities at the front and rear of the cell (3, 24).

3. As a conveyor signal (Ca^{2+} waves) that can transmit information across the cell to coordinate Ca^{2+}-sensitive protrusive mechanisms with Ca^{2+}-sensitive retraction mechanisms (29).

The future challenge will be to fully understand how these different Ca^{2+} signals interact in terms of their initiation, amplification and propagation, and how each signal interacts with the various biochemical signaling pathways that regulate cell migration and chemotaxis.

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