Abnormal bradykinin signalling in fibroblasts deficient in the PIP$_2$ 5-phosphatase, ocrl1

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Summary The oculocerebrorenal syndrome of Lowe (Lowe syndrome) is an X-linked disorder of phosphatidylinositol metabolism characterized by congenital cataracts, renal proximal tubulopathy and neurological deficits. The disorder is due to the deficiency of the phosphatidylinositol 4,5-bisphosphate (PIP$_2$) 5-phosphatase, ocrl1. PIP$_2$ is critical for numerous cellular processes, including cell signalling, actin reorganization and protein trafficking, and is chronically elevated in patients with Lowe syndrome. The elevation of PIP$_2$ cells of patients with Lowe syndrome provides the unique opportunity to investigate the roles of this phospholipid in fundamental cellular processes. We previously demonstrated that ocrl1 deficiency causes alterations in the actin cytoskeleton. Since actin remodelling is strongly activated by [Ca$^{2+}$], which increases in response to IP$_3$ production, we hypothesized that altered calcium signalling might contribute to the observed abnormalities in actin organization. Here we report a specific increase in bradykinin-induced Ca$^{2+}$ mobilization in Lowe fibroblasts. We show that the abnormal bradykinin signalling occurs in spite of normal total cellular receptor content. These data point to a novel role for ocrl1 in agonist-induced calcium release.

Abbreviations
2-APB 2-aminoethoxydiphenylborate
EGF epidermal growth factor
GPCR G-protein-coupled receptor
PDGF platelet-derived growth factor
PIP$_2$ phosphatidylinositol 4,5-bisphosphate
TGN trans-Golgi network

Introduction

The oculocerebrorenal syndrome of Lowe (Lowe syndrome) is a rare X-linked disorder characterized by bilateral congenital cataracts, renal proximal tubulopathy including low-molecular-weight proteinuria, albuminuria, aminoaciduria, hypercalcemia, metabolic acidosis, phosphaturia and nephrocalcinosis (Bockenhauer et al. 2008) and neurological deficits. It is due to the deficiency of ocrl1, a type II phosphatidylinositol 4,5 bisphosphate (PIP$_2$) 5-phosphatase. This enzyme catalyzes the hydrolysis of PIP$_2$, which has a prominent role in a number of essential cellular processes including cell signalling, protein trafficking and actin polymerization.
Lowe syndrome is the first known human disorder caused by the deficiency of a PIP2 5-phosphatase. Thus the study of cells derived from patients with Lowe syndrome provides a unique opportunity to investigate the roles of PIP2 metabolism in cells and tissues.

The ocrl1 protein is ubiquitously expressed, except for haematopoietic tissues; its localization to the trans-Golgi network (TGN), endosomes (Choudhury et al. 2005; Dressman et al. 2000; Ungewickell et al. 2004) and the plasma membrane (Erdmann et al. 2007; Faucherre et al. 2005) suggest that ocrl1 plays a role in protein trafficking. The deficiency of ocrl1 leads to elevated cellular levels of PIP2 (Wenk et al. 2003; Zhang et al. 1998). However, it is unknown at present why a deficiency of this widely expressed protein primarily affects the lens, kidney and brain in Lowe syndrome. PIP2 plays an important role as a second messenger in regulating cell adhesion through the actin cytoskeleton (Raucher et al. 2000). Since actin reorganization is required for the formation and maintenance of cell–cell contacts (Lee et al. 2000; Shen and Turner 2005), the defects in lens epithelial cell differentiation and renal proximal tubule function in Lowe syndrome could result, in part, from abnormalities in the actin cytoskeleton. We have previously shown that actin remodelling is disrupted in Lowe fibroblasts (Suchy and Nussbaum 2002). Further evidence for a role for type II inositol polyphosphate 5-phosphatases in cell–cell contact formation, particularly in polarized cells, is suggested by work in the mouse. A mouse knockout of the closest paralogue to Ocr1l, Inpp5b, showed abnormal germ cell adhesion and abnormal Sertoli cell junctions (Hellsten et al. 2002). However, the loss of ocr1l by itself did not result in a detectable phenotype in the mouse as it does in humans (Janne et al. 1998).

Actin polymerization and the formation of actin-dependent structures such as tight junctions are calcium-dependent processes (Vasioukhin et al. 2000). Intracellular calcium concentrations are strongly influenced by the hydrolysis of PIP2, which results in the release of calcium from internal stores. We therefore hypothesized that a chronic PIP2 5-phosphatase deficiency and elevation of PIP2 in Lowe syndrome fibroblasts might also lead to increased calcium release from internal stores. This in turn might contribute to the observed abnormalities in actin remodelling, ultimately resulting in abnormal junction formation. Differences in calcium signalling between cell types might help explain the tissue specificity of the Lowe syndrome phenotype.

To test the hypothesis that calcium signalling is disrupted in Lowe cells, we measured calcium release from internal stores in Lowe fibroblasts and controls. We found that Lowe fibroblasts had an augmented response to bradykinin stimulation but, surprisingly, we did not observe a generalized increase in cell signalling with other agonists known to induce IP3-mediated intracellular calcium release. These data point to a novel role for ocrl1 in altering only certain pathways in agonist-induced calcium release.

**Materials and methods**

**Patients and cell lines**

Normal human skin fibroblast cultures were obtained from American Type Culture Collection (Manassas, VA, USA), and from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ, USA). Fibroblast cultures from unrelated patients with Lowe syndrome, which had been obtained with the informed consent of a responsible parent or guardian, were used (NIH IRB protocol numbers 01-HG-0008, 01-HG-0095). Cells were grown in Dulbecco’s modified essential medium (Gibco; Grand Island, NY, USA) with 15% fetal bovine serum and 2 mmol/L glutamine at 37°C with 5% CO2.

**Calcium imaging**

Live cell imaging was performed with cell permeant, acetoxyethyl (AM) ester dyes, fluo 4 (0.5 μmol/L) and fura red (1.0 μmol/L), to measure calcium response to agonists. Upon binding calcium, fluo 4 fluorescence intensity increases and fura red intensity decreases (Molecular Probes; Carlsbad, CA, USA). Dyes were added to 20% pluronic solution (Molecular Probes and resuspended in nominally calcium free incubation buffer (1.06 mmol/L KH2PO4, 2.97 mmol/L NaHPO4, 155 mmol/L NaCl, 20 mmol/L Hepes, 25 mmol/L glucose, 5.3 mmol/L KCl, 1 mmol/L sodium pyruvate, 0.8 mmol/L magnesium sulfate, 100 μmol/L EGTA, 1.5 mg/ml BSA, pH 7.3) (Holtzclaw et al. 1995) with 0.08 mg/ml sulfinpyrazone to inhibit organic anion transport activity. To control for potential minor variations in buffer or dye prepared each day, a Lowe and control culture were run together as a pair. Seven different pairs of Lowe and control cultures were matched for passage number from four Lowe and four control cultures from different individuals. Each pair of cultures was plated two days prior to imaging, washed, and loaded into confocal chambers for a 20-min incubation with dyes at room temperature. Cultures were used at approximately 70% confluence.
Cells were rinsed three times, washed 20 min in incubation buffer, rinsed and loaded onto the confocal stage. A baseline calcium concentration was recorded for 5–10 frames, followed by stimulation with bradykinin (100 nmol/L), histamine (100 μmol/L) (Calbiochem, San Diego, CA, USA), or 50 ng/ml PDGF (platelet-derived growth factor; Sigma; St Louis, MO, USA). In other experiments, the cells were treated with the calcium ionophore A23187 (20 μmol/L) (Calbiochem) in order to measure stored calcium. Cells were imaged with a Zeiss Axiovert 100 M confocal microscope with LSM 510 software, using a Zeiss 20×/0.75 planapochromat objective. An excitation wavelength of 488 nm was used and images were collected with a 505–550 nm bandpass filter and a 650 nm long-pass filter, at a maximum pinhole setting, every 1–2 s for 3 min (for bradykinin, histamine and A23187) or 5 min (for PDGF and EGF). Data analysis was performed with the Kaleida-Graph software (Synergy Software, Reading, PA, USA).

**IP₃ receptor inhibition**

Cells were incubated with the [Ca²⁺]-sensitive dyes, as described above, washed and incubated for the final 5 min of the wash with 20–100 μmol/L 2-amino-ethoxydiphenylborate (2-APB) (Calbiochem/ EMD Biosciences, La Jolla, CA, USA), and maintained in the presence of 2-APB during imaging. Cells were stimulated with 100 nmol/L bradykinin and imaged as described above.

**Western blotting**

Fibroblasts from four different Lowe patients and four controls were harvested and 20 μg of total cell protein was loaded on 10% polyacrylamide gels. Proteins were separated by electrophoresis and transferred to a PDVF membrane (Immobilon P, Millipore Corporation, Bedford, MA, USA). The membrane was blocked for 30 min at 37°C in 5% non-fat dry milk, TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween 20), before overnight incubation with monoclonal anti-B2 bradykinin receptor antibodies (1/1000) (BD Biosciences, Pharmingen, San Jose, CA, USA). The membranes were washed, incubated for 1 h with peroxidase-conjugated anti-mouse IgG antibody, washed again, and detected by chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ, USA). The membranes were stripped and reprobed with a monoclonal anti-β-tubulin antibody (1/200), AB3194 (Abcam, Inc., Cambridge, MA, USA), and the receptor protein was quantified by densitometry using β-tubulin as a control.

**Results**

Lowe fibroblasts show increased response to bradykinin

Agonist-induced intracellular calcium release was measured in primary cultures of Lowe and control fibroblasts. The studies were performed with the cells in calcium-free buffer in order to focus on the release of calcium from internal stores without having the results confounded by agonist-induced influx of extracellular calcium. Two cell-permeant calcium-sensitive fluorescent indicator dyes, fluo-4 and fura red, were used for ratiometric assessment of calcium release. Fluo-4 fluorescence increases upon binding of free calcium, whereas fura-red fluorescence decreases on free calcium binding. Following a baseline assessment in untreated cells, fibroblasts were stimulated with bradykinin, resulting in increased ratio of fluo 4/fura red that peaked approximately 15 s after stimulation (Table 1), then declined to baseline levels. Bradykinin was used to stimulate seven pairs of cultures of Lowe and control fibroblasts. The response was assessed by measurement of the peak calcium release in an average of 34 cells per genotype. In all seven pairs of cultures, Lowe cells had a higher mean calcium release than control (Table 1). This difference was statistically significant in six of seven pairs. Overall, Lowe cells showed a 26% increase in calcium release over controls. Lowe cells also tended to reach peak calcium concentrations faster than controls; the mean time to peak calcium in Lowe cells 12.4 seconds versus 18.3 seconds in control fibroblasts, although this was not statistically significant (paired t-test, t = 2.10, p = 0.08). The ratiometric method employed was not sensitive enough to detect baseline differences in intracellular free calcium between Lowe and control fibroblasts in the absence of agonist stimulation.

It is well established that bradykinin signalling occurs via the IP₃ receptor. We confirmed the calcium release we observed in response to bradykinin was IP₃ receptor-mediated by using 2-APB, a non-competitive inhibitor of the IP₃ receptor (Maruyama et al 1997; Missiaen et al 2001). Cells were preincubated with increasing concentrations of 2-APB (20–100 μmol/L) and then stimulated with 100 nmol/L bradykinin. We found that 2-APB inhibited agonist-stimulated calcium release in a dose-dependent manner (Fig. 1). At lower doses of 2-APB, there was a reduced peak response to bradykinin stimulation, as well as a reduction in the number of cells that responded to the agonist. Increasing concentrations of 2-APB diminished both the level of response and the number of cells that responded. At
the lowest dose of 2-APB tested, the peak calcium release in response to bradykinin was reduced to 59.3% and 88.3% of that observed in untreated fibroblasts in Lowe and control cells, respectively. At higher doses of the compound very few cells responded to bradykinin stimulation after treatment with the inhibitor. In the present study, concentrations of 2-APB \leq 100 \, \text{μmol/L} were used to avoid additional effects beyond inhibition of the IP_3 receptor, which have previously been reported to occur at higher 2-APB concentrations (Missiaen et al. 2001).

Many cells have two major types of intracellular calcium release channels: those gated by the IP_3 receptor and those gated by the ryanodine receptor. Ryanodine receptor activation requires a higher baseline calcium level than that present in the calcium free media in experiments reported here. To confirm that we were measuring IP_3 receptor-mediated Ca^{2+} release, we tested whether we could observe ryanodine receptor activity in fibroblasts under the same conditions (nominally calcium-free medium) used here for measuring the IP_3-receptor-mediated responses. We stimulated cells with caffeine, an activator of the ryanodine receptor. We found no detectable calcium release in these fibroblasts even after stimulation with 100 mmol/L caffeine, confirming that the calcium release we were measuring in our experiments was IP_3-mediated.

Calcium stores are not abnormal in Lowe fibroblasts

Table 1 Release of calcium from internal stores in Lowe and control fibroblasts in response to 100 nmol/L bradykinin

| Experiment Number of cells analysed | Peak Ca^{2+} released (Fluo 4 / Fura red) | \( \Delta \) | \( p \) |
|------------------------------------|------------------------------------------|--------|--------|
| | Lowe | Control | Lowe Mean | SE | Control Mean | SE | \( \Delta \) Mean | SE | \( p \) |
| 1 | 29 | 24 | 2.37 | 0.09 | 1.69 | 0.08 | 5.76 | \(<0.0001\) |
| 2 | 28 | 22 | 2.15 | 0.07 | 1.87 | 0.05 | 3.35 | \(0.002\) |
| 3 | 48 | 48 | 1.44 | 0.04 | 0.92 | 0.04 | 8.57 | \(<0.0001\) |
| 4 | 39 | 49 | 1.49 | 0.03 | 1.40 | 0.05 | 1.73 | \(0.087\) |
| 5 | 26 | 15 | 2.84 | 0.16 | 2.26 | 0.15 | 2.67 | \(<0.020\) |
| 6 | 41 | 30 | 2.00 | 0.04 | 1.50 | 0.07 | 5.86 | \(<0.0001\) |
| 7 | 34 | 41 | 2.23 | 0.13 | 1.94 | 0.05 | 4.58 | \(<0.0001\) |

*aPairs of Lowe and control fibroblast cultures derived from different individuals.

\( t \)-test.

\( p \), significance level.

SE, standard error.

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Fig. 1 Bradykinin stimulation caused an IP_3 receptor-dependent release of stored calcium. 2-Aminoethoxydiphenyl borate (2-APB), 20–100 μmol/L, a non-competitive inhibitor of the IP_3 receptor, reduced the response to 100 nmol/L bradykinin in both Lowe (solid line, squares) and control fibroblasts (broken line, circles) in a dose-dependent manner. The standard error of each data point is displayed as a vertical line, thick solid line for the standard error of measurement of the Lowe cells, thin line for the standard error of measurement of the control cells.

To determine whether there was an increase in calcium stores in Lowe fibroblasts that might account for the increased response in Lowe cells, a calcium ionophore, A23187, was used to empty calcium stores. The relative total calcium released from the stores was measured as described above for agonist stimulation, and total stored calcium was assessed from the integrated area under the calcium release curve. Using...
four pairs of cell cultures, we found no elevation in the total stored calcium in Lowe cells (Table 2). In fact, there was a trend for lower stored calcium in Lowe fibroblasts, with three of the four experiments showing a statistically significant reduction in stored calcium in Lowe fibroblasts. Therefore, higher calcium stores in Lowe cells is not the explanation for the increase in calcium release in response to bradykinin.

**Table 2** Total Ca$^{2+}$ released from internal stores in response to the calcium ionophore, A23187 (20 μmol/L)

| Experiment | Pairs of Lowe and control fibroblast cultures derived from different individuals. |
|------------|----------------------------------------------------------------------------------|
| Experiment | t-Test.                                                                          |
| Experiment | p, significance level.                                                           |
| Experiment | SE, standard error.                                                               |

| Experiment | Number of cells analysed | Total Ca$^{2+}$ released | $t^b$ | $p^b$ |
|------------|--------------------------|--------------------------|-------|-------|
|            | Lowe                     | Control                  |       |       |
| 1          | 38                       | 34                       | 52.82 | 1.66  | 59.13 | 2.62  | 2.03  | <0.05 |
| 2          | 36                       | 41                       | 92.27 | 3.92  | 93.76 | 3.51  | 0.28  | 0.78  |
| 3          | 30                       | 30                       | 47.63 | 2.00  | 63.11 | 3.52  | 3.82  | <0.001|
| 4          | 32                       | 37                       | 79.52 | 4.30  | 96.21 | 3.56  | 3.02  | <0.005|

Fig. 2 The total cellular bradykinin receptor concentration was not increased in Lowe fibroblasts. Shown are the results of quantitative western analysis of two experiments, the first showing four different Lowe patient fibroblast cultures and four controls (experiment 1; panels a, c). Proteins were separated by electrophoresis and western blotting was performed using a monoclonal antibody to the bradykinin receptor and to β-tubulin protein, used as a loading control (panel a). The concentrations of receptor protein and β-tubulin protein were quantified by densitometry and the concentration of receptor protein was expressed relative to the β-tubulin protein. The mean and standard error of the relative bradykinin concentrations are shown in the histogram in panel b; the standard error is represented by the vertical bar. There was no significant difference in bradykinin receptor concentration observed between Lowe and control cells ($t = 0.249$, $p = 0.81$). These results were replicated in experiment 2 (panels b, d) using three different Lowe and control cell cultures, with similar results ($t = 1.04$, $p = 0.36$)

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The concentration of bradykinin receptor is not increased in Lowe fibroblasts

We next investigated the possible cause for the abnormal bradykinin-induced calcium release in Lowe cells. The total bradykinin receptor expression was measured in four different Lowe patient fibroblast cultures and four control fibroblast cultures (Fig. 2a). Western analysis of total cell lysates, and quantification by densitometry relative to a β-tubulin control, revealed no difference in the total number of bradykinin receptors present in Lowe and control cells (t = 0.249, p = 0.81) (Fig. 2a and c). The results of a second set of experiments using three pairs of Lowe and control fibroblast cultures provided similar results (Fig. 2b and d).

Increased signalling in Lowe cells was not generalized

Our hypothesis was that the elevated calcium response to IP₃ was the result of elevated levels of PIP₂, the metabolic precursor of IP₃. This would imply that stimulation with other agonists that activate the phospholipase C-mediated hydrolysis of PIP₂ would also result in an increased response in Lowe cells. We therefore tested the response of Lowe and control cells to stimulation with a number of additional agonists. Histamine, like bradykinin, signals through a G-protein-coupled receptor (GPCR). PDGF signals through a tyrosine–kinase coupled receptor. We observed that, unlike the response to bradykinin, the response of Lowe fibroblasts to histamine was not increased over that of controls. Instead, Lowe fibroblasts tended to show a decreased response to histamine. In two experiments this decreased response to histamine was statistically significant (Table 3). The time to peak calcium concentration after histamine stimulation was 19 s in Lowe cells and 15 s in control cells, which was not significantly different (t = 0.384, p = 0.72). Thus, we found no evidence for an increase in response to histamine stimulation in Lowe cells. Furthermore, the response of Lowe fibroblasts to PDGF stimulation was not significantly different from controls in four experiments (Table 4).

### Table 3 Release of Ca²⁺ from internal stores upon stimulation with histamine (100 μmol/L)

| Experiment | Number of cells analysed | Peak Ca²⁺ released (Fluo 4 / Fura red) | tᵇ | pᶜ |
|------------|--------------------------|-----------------------------------|----|----|
|            | Lowe        | Control     | Lowe | Control |
|            | Mean        | SEᵈ        | Mean | SE |
| 1          | 22          | 50         | 0.89 | 0.06 | 0.85 | 0.06 | 1.14 | 0.25 |
| 2          | 32          | 42         | 1.46 | 0.10 | 1.86 | 0.08 | 3.13 | 0.003 |
| 3          | 40          | 42         | 1.40 | 0.04 | 1.48 | 0.06 | 1.16 | 0.25 |
| 4          | 48          | 50         | 1.41 | 0.06 | 1.98 | 0.06 | 8.18 | <0.0001 |

ᵃPairs of Lowe and control fibroblast cultures derived from different individuals.
bᵗ, t-test.  
cᵖ, significance level.  
dSE, standard error.

### Table 4 Released of Ca²⁺ from internal stores upon stimulation with PDGF (50 ng/ml)

| Experiment | Number of cells analysed | Peak Ca²⁺ released (Fluo 4 / Fura red) | tᵇ | pᶜ |
|------------|--------------------------|-----------------------------------|----|----|
|            | Lowe        | Control     | Lowe | Control |
|            | Mean        | SEᵈ        | Mean | SE |
| 1          | 29          | 29         | 1.08 | 0.07 | 1.12 | 0.04 | 0.58 | 0.56 |
| 2          | 17          | 32         | 0.93 | 0.08 | 0.80 | 0.05 | 1.39 | 0.17 |
| 3          | 32          | 32         | 0.83 | 0.07 | 0.81 | 0.05 | 0.20 | 0.85 |
| 4          | 35          | 41         | 0.79 | 0.09 | 0.80 | 0.05 | 0.06 | 0.95 |

ᵃPairs of Lowe and control fibroblast cultures derived from different individuals.  
bᵗ, t-test.  
cᵖ, significance level.  
dSE, standard error.

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observe a difference in time to peak calcium in PDGF stimulated cells; the mean time to peak calcium concentration was 111 s in Lowe cells and 112 s in controls ($t = 0.055, p = 0.96$). We also tested the response of Lowe and control fibroblasts to bombesin (signalling through a GPCR) and epidermal growth factor (signalling via a tyrosine-kinase coupled receptor) in several pairs of Lowe and control fibroblast cultures. Although only 50–70% of the fibroblasts responded to EGF or bombesin stimulation, compared with 92–100% with bradykinin or histamine and greater than 90% after PDGF stimulation, we observed no evidence for a consistent difference in the peak response of Lowe versus control cells (data not shown). These results indicate that the observed increased response to bradykinin stimulation in Lowe fibroblasts was not indicative of a generalized increase in the cell signalling response.

Discussion

We show here that Lowe patient fibroblasts have increased intracellular $[Ca^{2+}]$ mobilization in response to bradykinin stimulation. This indicates that PIP$_2$ 5-phosphatases can play a role in $[Ca^{2+}]$ signalling and that abnormal $[Ca^{2+}]$ signalling may contribute to the phenotype in Lowe syndrome. Bradykinin and the other agonists used in this study trigger calcium release from internal stores by the rapid phospholipase C-stimulated hydrolysis of PIP$_2$, producing IP$_3$ (Cruzblanca et al. 1998). IP$_3$ binds to IP$_3$ receptors on several intracellular organelles, most notably the endoplasmic reticulum. A subsequent wave of calcium release that occurs by the parallel production of diacylglycerol, which activates protein kinase C and stimulates the influx of extracellular calcium, was not considered here, as the cells were incubated in nominally calcium-free medium.

We predicted a generalized increase in calcium signalling in Lowe cells due to increased PIP$_2$ substrate availability for IP$_3$ production. However, the defect in signalling was observed only with bradykinin and not with any of the other G-protein-coupled receptors or the tyrosine kinase-coupled receptor agonists that we tested. Furthermore, we found no increase in stored calcium in Lowe cells. We conclude that the increased bradykinin-stimulated $[Ca^{2+}]$ mobilization in Lowe syndrome fibroblasts must occur by a mechanism in addition to simply increasing the availability of PIP$_2$ substrate for IP$_3$ production and is specific to bradykinin signalling per se. We show here that the augmented response to bradykinin stimulation in Lowe cells was not due to a change in the total receptor content or to an increase in calcium stores. We hypothesize, therefore, that the observed response may be due to disrupted endocytosis/trafficking of the bradykinin receptors. Bradykinin receptors are trafficked by caveolae- or non-clathrin-mediated mechanisms (De Weerd and Leeb-Lundberg 1997; Haasemann et al. 1998; Lamb et al. 2002), whereas the other receptors tested here, PDGF and histamine, are not (Sato et al. 2003; Self et al. 2005; Newton et al. 2005). The caveolin-1 binding motifs, $\Phi X \Phi (X)_4 \Phi$ and $\Phi (X)_4 \Phi X \Phi$, (where $\Phi$ is an aromatic amino acid (W,Y,F) and $X$ is any amino acid) (Couet et al. 1997) are present in ocrl1 at amino acids 223–230, 345–352, 555–562 of the ocrl1 protein sequence, U57627 (numbering from the putative OCRL1 start site, corresponding to the second methionine in the reference sequence) (Suchy et al. 1995). Overexpression of ocrl1 has been reported to block Shigatoxin b trafficking (Choudhury et al. 2005), a process mediated by caveolae (Nichols and Lippincott-Schwartz 2001). Furthermore, the cellular localization of ocrl1 has implicated it in protein trafficking. Thus, an investigation of abnormalities in caveolar trafficking would be a logical next step in attempting to understand the cellular abnormalities caused by a deficiency in ocrl1.

How might defective bradykinin signalling contribute to the Lowe syndrome phenotype? The peptide hormone bradykinin is a common cellular agonist and the bradykinin receptor is expressed in most tissues, including the lens, kidney and brain (Vio et al. 1996), the tissues primarily affected in Lowe syndrome. Disruptions in calcium signalling can affect cells and tissues in a way that can resemble the Lowe syndrome pathology. Abnormal calcium signalling has been shown to alter cell adhesion in polarized renal epithelial cells (De Blasio et al. 2004; Stuart et al. 1996) and appears to play a role in the development of lens cataracts including posterior subcapsular cataracts that occur in Lowe patients (Churchill and Louis 2002; Gupta et al. 2004). Calcium is also critical in regulating the release of neurotransmitters and has been shown to play a role in initiating seizures, which occur in 50% of Lowe syndrome patients (Fletcher et al. 1996; Pal et al. 2001).

While abnormal bradykinin signalling may contribute to the phenotype in susceptible tissues, we suspect that the signalling defect may be one facet of a broader problem in actin reorganization and/or endocytosis in Lowe cells. PIP$_2$ is a second messenger affecting the reorganization of the actin cytoskeleton (Raucher et al. 2000) and has a direct effect on endocytosis (Martin 2001; Cremona and De Camilli 2001). The phenotype of Lowe syndrome points strongly to a defect in epithelial tight junction formation that requires actin...
polymerization. Interestingly, it has recently been reported that caveolae-mediated endocytosis is required for the formation of tight junctions and that the mechanism by which actin depolymerization disrupts tight junctions is by inhibiting caveolae-mediated endocytosis (Shen and Turner 2005). Caveolae are present in most tissues, including lens epithelial and fiber cells, and participate in signalling and endocytosis (Hnasko and Lisanti 2003; Lo et al 2004). Not all epithelial cells are affected in Lowe syndrome, indicating that some types of epithelial cells are more susceptible than others to the effects of ocrl1 deficiency. Ultimately, the explanation for how a deficiency of ocrl1 leads to the Lowe syndrome phenotype must elucidate both why the target tissues are affected and why other tissues are not. The explanation may lie in the differential concentration of PIP<sub>2</sub> and PIP<sub>3</sub> in the apical or basolateral membranes of epithelial cells (Gassama-Diagne et al 2006; Pilot et al 2006), in the differential organization of the actin cytoskeleton in different cell types (Lee et al 2000; Yonemura et al 2004), or in the selective expression and trafficking of endocytic proteins in different tissues (Lutcke et al 1994). Further studies on the role of ocrl1 in various epithelial cell types may lead to a better understanding of the complex phenotype of Lowe syndrome.

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References

Bockenhauer D, Bokenkamp A, van’t Hoff W, et al (2008) Renal phenotype in Lowe syndrome: a selective proximal tubular dysfunction. *Clin J Am Soc Nephrol* 3: 1430–1436. doi:10.2215/CJN.00520108.

Choudhry R, Diao AP, Zhang F, et al (2005) Lowe syndrome protein OCRL1 interacts with clathrin and regulates protein trafficking between endosomes and the trans-Golgi network. *Mol Biol Cell* 16: 3467–3479. doi:10.1091/mbc.E05-02-0120.

Churchill GC, Louis CF (2002) Ca<sup>2+</sup> regulation in differentiating lens cells in culture. *Exp Eye Res* 75: 77–85. doi:10.1006/expr.2002.1184.

Couet J, Sargiacomo M, Lisanti, MP (1997) Interaction of a receptor tyrosine kinase, EGFR-R, with caveolins. *J Biol Chem* 272: 30429–30438. doi:10.1074/jbc.272.48.30429.

Cremona O, De Camilli P (2001) Phosphoinositides in membrane traffic at the synapse. *J Cell Sci* 114: 1041–1052.

Cruzblanca H, Koh DS, Hille B (1998) Bradykinin inhibits M current via phospholipase C and Ca<sup>2+</sup> release from IP<sub>3</sub> sensitive Ca<sup>2+</sup> stores in rat sympathetic neurons. *Proc Natl Acad Sci USA* 95: 7151–7156.

De Blasio BF, Rittinger J-A, Sand KL, Giaever I, Iversen J-G (2004) Global, synchronous oscillations in cytosolic calcium and adherence in bradykinin-stimulated Madin-Darby canine kidney cells. *Acta Physiol Scand* 180: 335–346. doi:10.1111/j.1365-201X.2004.01261.x.

De Weerd WFC, Leeb-Lundberg LMF (1997) Bradykinin sequesters B<sub>2</sub> bradykinin receptors and the receptor-linked G<sub>α</sub> subunits G<sub>αq</sub> and G<sub>αi</sub> in caveolae in DDT<sub>1</sub> MF-2 smooth muscle cells. *J Biol Chem* 272: 17858–17866. doi:10.1074/jbc.272.28.17858.

Di Paolo G, De Camilli, P (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443: 651–657. doi:10.1038/nature05185.

Dressman MA, Olivos-Gliander IM, Nussbaum RL, Suchy SF (2000) Ocrl1, a PtdIns(4,5)P<sub>2</sub> 5-phosphatase, is localized to the trans-Golgi network of fibroblasts and epithelial cells. *J Histochem Cytochem* 48: 179–189.

Erdmann KS, Yuxin M, McCrea HJ, et al (2007) A role of the Lowe syndrome protein OCRL1 in early steps of the endocytic pathway. *Dev Cell* 13: 377–390. doi:10.1016/j.devcel.2007.08.004.

Faucherre A, Desbois P, Nagano F, et al (2005) Lowe syndrome protein Ocr11 is translocated to membrane ruffles upon Rac GTPase activation: a new perspective on Lowe syndrome pathophysiology. *Hum Mol Genet* 14: 1441–1448. doi:10.1093/hmg/ddi153.

Fletcher CF, Lutz CM, O’Sullivan TN, et al (1996) Absence epilepsy in tottering mutant mice is associated with calcium channel defects. *Cell* 87: 607–617. doi:10.1016/S0092-8674(00)81381-1.

Gassama-Diagne A, Yu W, Beest M, et al (2006). Phosphatidylinositol-3,4,5-trisphosphate regulates the formation of the basolateral plasma membrane in epithelial cells. *Nat Cell Biol* 8: 963–970. doi:10.1038/ncb1461.

Gupta PD, Johar K, Vasavada A. (2004) Causative and preventive action of calcium in cataractogenesis. *Acta Pharmacol Sin* 25: 1250–1256.

Haasemann M, Cartaud J, Muller-Esterl W, Dunin I (1998) Agonist-induced redistribution of bradykinin B2 receptors in caveolae. *J Cell Sci* 111: 917–928.

Hellsten E, Bernard DJ, Owens JW, Echkaus M, Suchy SF, Nussbaum RL (2002) Perturbations in cell adhesion and endocytosis in mice deficient in the inositol polyphosphate 5-phosphatase (*Inpp5b*). *Biol Reprod* 66: 1522–1530. doi:10.1095/biolreprod.65.1522.

Hnasko R, Lisanti MP (2003) The biology of caveolae: lessons from caveolin knockout mice and implications for human disease. *Mol Interv* 3: 445–464. doi:10.1124/mi.3.8.445.

Holtzclaw FL, Gallo V, Russell, JT (1995) AMPA receptors shape Ca<sup>2+</sup> responses in cortical oligodendrocyte progenitors and CG-4 cells. *J Neurosci Res* 42: 124–130. doi:10.1002/jnr.490420114.

Janne PA, Suchy SF, Bernard D, et al (1998) Functional overlap between murine *Inpp5b* and Ocr11 may explain why deficiency of the murine ortholog for OCRL1 does not cause Lowe syndrome in mice. *J Clin Invest* 101: 2042–2053. doi:10.1172/JCI2414.

Lamb ME, Zhang CW, Shea T, Kyle DJ, Leeb-Lundberg LMF (2002) Human B1 and B2 bradykinin receptors and their agonists target caveola-related lipid rafts to different degrees in HEK293 cells. *Biochemistry* 41: 14340–14347. doi:10.1021/bi020235d.
Lee A, Fischer RS, Fowler VM (2000) Stabilization and remodeling of the membrane skeleton during lens fiber cell differentiation and maturation. Dev Dynam 217: 257–270. doi:10.1002/(SICI)1097-0177(200003)217:3<257::AID-DVDY-3.0.CO;2-5.

Lo W-K, Zhou C-J, Reddan J (2004) Identification of caveolae and their signature proteins caveolin 1 and 2 in the lens. Exp Eye Res 79: 487–498. doi:10.1016/j.exer.2004.06.019.

Lutcke A, Parton RG, Murphy C, et al (1994) Cloning and subcellular localization of novel rab proteins reveals polarized and cell type-specific expression. J Cell Sci 107: 3437–3448.

Martin TFJ (2001) PI(4,5)P2 regulation of surface membrane traffic. Curr Opin Cell Biol 13: 493–499. doi:10.1016/S0955-0674(00)00241-6.

Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K (1997) 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P3-induced Ca2+ release. J Biochem 122: 498–505.

Missiaen L, Callewaert G, De Smedt H, Parys, JB (2001) 2-Aminoethoxydiphenyl borate affects the inositol 1,4,5 trisphosphate receptor, the intracellular Ca2+ pump and the non-specific Ca2+ leak from non-mitochondrial Ca2+ stores in permeabilized A7r5 cells. Cell Calcium 29: 111–116. doi:10.1054/ceca.2000.0163.

Newton CS, Loukinova E, Mikhaileiko I, et al (2005) Platelet-derived growth factor receptor-β (PDGFR-β) activation promotes its association with the low density lipoprotein receptor-related protein (LRP) evidence for co-receptor function. J Biol Chem 280: 27872–27878. doi:10.1074/jbc.M505410200.

Nichols BJ, Lippincott-Schwartz J (2001) Endocytosis without clathrin coats. Trends Cell Biol 11: 406–412. doi:10.1016/S0962-8924(01)02107-9.

Pal S, Sun D, Limbrick D, Rafiq A, DeLorenzo RJ (2001) Epileptogenesis induces long-term alterations in intracellular calcium release and sequestration mechanisms in the hippocampal neuronal culture model of epilepsy. Cell Calcium 30: 285–296. doi:10.1016/S0962-8924(01.02136-3).

Pilot F, Philippe J-M, Lemmers C, Lecuit T (2006) Spatial control of actin organization at adherens junctions by the synaptotagmin-like protein Btsz. Nature 442: 580–584. doi:10.1038/nature04935.

Raucher D, Stauffer T, Chen W, et al (2000) Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulates cytoskeleton-plasma membrane adhesion. Cell 100: 221–228. doi:10.1016/S0092-8674(00)81560-3.

Sato M, Ueda Y, Takagi T, Umezawa Y (2003) Production of PtdInsP2 at endomembranes is triggered by receptor endocytosis. Nat Cell Biol 5: 1016–1022. doi:10.1038/ncb1054.

Self TJ, Oakley SM, Hill SJ (2005) Clathrin-independent internalization of the human histamine H1-receptor in CHO-K1 cells. Brit J Pharmacol 146: 612–624. doi:10.1038/sj.bjp.0706337.

Sheetz MP, Sable JE, Dobereiner HG (2006) Continuous membrane-cytoskeleton adhesion requires continuous accommodation to lipid and cytoskeleton dynamics. Annu Rev Biophys Biomol Struct 35: 417–434. doi:10.1146/annurev.biophys.35.040405.102017.

Shen L, Turner JR (2005) Actin depolymerization disrupts tight junctions via caveolae-mediated endocytosis. Mol Biol Cell 16: 3919–3936. doi:10.1091/mbc.E04-12-1089.

Stuart RO, Sun A, Bush KT, Nigam SK (1996) Dependence of epithelial intercellular junction biogenesis on thapsigargin-sensitive intracellular calcium stores. J Biol Chem 271: 13636–13641. doi:10.1074/jbc.271.23.13636.

Suchy SF, Nussbaum RL (2002) The deficiency of PIP2 5-phosphatase in Lowe syndrome affects actin polymerization. Am J Hum Genet 71: 1420–1427. doi:10.1086/344517.

Suchy SF, Olivos-Glander IM, Nussbaum RL (1995) Lowe syndrome a deficiency of a phosphatidyl-inositol 4,5-bisphosphate 5-phosphatase in the Golgi apparatus. Hum Mol Genet 4: 2245–2250. doi:10.1093/hmg/4.12.2245.

Ungewickell A, Ward ME, Ungewickell E, Majerus PW (2004) The inositol polyphosphate 5-phosphatase Ocr1 associates with endosomes that are partially coated with clathrin. Proc Natl Acad Sci U S A 101: 13501–13506. doi:10.1073/pnas.0405664101.

Vasiukhin V, Bauer C, Yin M, Fuchs E (2000) Directed actin polymerization is the driving force for epithelial cell-cell adhesion. Cell 100: 209–219. doi:10.1016/S0092-8674(00)81559-7.

Vio CP, Velarde V, Muller-Esterl W (1996) Cellular distribution and fate of the bradykinin antagonist HOE140 in the kidney: colocalization with the bradykinin B2 receptor. Immunopharmacology 33: 146–150. doi:10.1016/0162-3109(96)00031-8.

Wenk MR, Lucaet D, Di Paolo G, et al (2003) Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. Nat Biotechnol 21: 813–817. doi:10.1038/nbt837.

Yonemura S, Hirao-Minakuchi K, Nishimura Y (2004) Rhodopsin localization in cells and tissues. Exp Cell Res 295: 300–314. doi:10.1016/j.yexcr.2004.01.005.

Zhang X, Hartz PA, Philip E, Racusen LC, Majerus PW (1998) Cell lines from kidney proximal tubules of a patient with Lowe syndrome lack OCRL1 inositol polyphosphate 5-phosphatase and accumulate phosphatidylinositol 4,5 bisphosphate. J Biol Chem 273: 1574–1582. doi:10.1074/jbc.273.3.1574.