A NAV2729-sensitive mechanism promotes adrenergic smooth muscle contraction and growth of stromal cells in the human prostate

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Voiding symptoms in benign prostatic hyperplasia (BPH) are driven by prostate smooth muscle contraction and prostate growth. Smooth muscle contraction in the prostate and other organs critically depends on activation of the small monomeric GTPase RhoA and probably Rac1. A role of another GTPase, ADP-ribosylation factor 6 (ARF6), for smooth muscle contraction has been recently suggested by indirect evidence but remains to be proven for any organ. Here, we report effects of NAV2729, an inhibitor with assumed specificity for ARF6, in human prostate tissues and cultured prostate stromal cells (WPMY-1). NAV2729 (5 μM) inhibited neurogenic and α1-adrenergic contractions of human prostate tissues. Constructions induced by endothelin-1, by the thromboxane A2 agonist U46619, or by high molar KCl were not inhibited. Correlation analyses suggested up-regulation of prostactic ARF6 expression with increasing degree of BPH, whereas ARF6 activity but not other GTPases (ARF1, RhoA, Rac1) in prostate tissues and in WPMY-1 cells. Proliferation of WPMY-1 cells was inhibited concentration-dependently by NAV2726, as reflected by decreased viability, 5-ethyl-2′-deoxyuridine (EdU) assay, colony formation assay, and expression of Ki-67. Silencing of ARF6 expression mimicked effects of NAV2729 on viability and in the EdU assay. Effects of NAV2729 on viability and proliferation were attenuated in cells with silenced ARF6 expression. Our findings suggest that a NAV2729-sensitive mechanism promotes adrenergic contraction and stromal cell growth in the human prostate, which is probably ARF6-mediated. Similar actions in other organs and urodynamic effects of NAV2729 appear possible.

Prostate smooth muscle tone and prostate growth may both be increased in benign prostatic hyperplasia (BPH) and commonly lead to urethral compression, to impaired bladder emptying, and finally to lower urinary tract symptoms (LUTS) (1). Estimates of at least 612 million patients being affected by LUTS suggestive of BPH worldwide in 2018 are contrasted by the low efficacy of available medications for treatment of LUTS suggestive of BPH (1–3). The option of first choice is α1-adrenoceptor antagonists (α1-blockers), which inhibit α1-adrenergic prostate smooth muscle relaxation, resulting in improvements of urethral obstruction, bladder emptying, and symptoms (1, 4, 5). Although they are the best available option, cumulative evidence demonstrates that their efficacy underlies certain ceilings; improvements of urinary flow (Qmax) and international prostate symptom scores are limited to 50%, whereas up to 30% can be expected from placebos (1, 3, 6–8). Restricted efficacy may contribute to high discontinuation rates, hospitalization, and high numbers of surgery for BPH (3, 9). In the face of the insufficient efficacy of available medications, together with the rapidly increasing case numbers due to the age dependence of prevalence and the demographic transition, development of novel options with higher efficacy represents a major challenge in urology.

The restricted efficacy of α1-blockers in LUTS treatment probably results from nonadrenergic mediators (thromboxane A2, endothelin-1), which induce prostate smooth muscle contraction in parallel with α1-adrenoceptors (1, 10, 11). These nonadrenergic contractions are supposed to maintain prostate smooth muscle tone, urethral obstruction, and symptoms under treatment with α1-blockers, in parallel with the contribution of prostate enlargement to urethral obstruction (1, 10, 11). Despite the high clinical relevance, little is known (a) about differences between α1-adrenergic and nonadrenergic smooth muscle contractions in the prostate and (b) about putative molecular connections linking prostate smooth muscle tone to

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1The abbreviations used are: BPH, benign prostatic hyperplasia; LUTS, lower urinary tract symptoms; ARF6, ADP-ribosylation factor 6; EdU, 5-ethyl-2′-deoxyuridine; GEF, guanosine exchange factor; PSA, prostate-specific antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ECL, enhanced chemiluminescence; EFS, electric field stimulation; RIPA, radioimmuno precipitation assay; FCS, fetal calf serum.

2The abbreviations used are: BPH, benign prostatic hyperplasia; LUTS, lower urinary tract symptoms; ARF6, ADP-ribosylation factor 6; EdU, 5-ethyl-2′-deoxyuridine; GEF, guanosine exchange factor; PSA, prostate-specific antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ECL, enhanced chemiluminescence; EFS, electric field stimulation; RIPA, radioimmuno precipitation assay; FCS, fetal calf serum.
**NAV2729 inhibits prostate contraction and growth**

hyperplastic growth. Overall, the understanding of prostate smooth muscle contraction is still incomplete, as reflected by the recent discovery of novel mechanisms involved in this contraction. However, current limits of medical therapy can only be understood and overcome by adequate knowledge of underlying molecular mechanisms.

Small monomeric guanosine triphosphate–hydrolyzing proteins (GTPases) with supposed functions in smooth muscle contraction include RhoA and Rac GTPases. Promotion of smooth muscle contraction in the cardiovascular system, airways, lower urinary tract, and gastrointestinal system by RhoA is widely accepted, whereas Rac-mediated smooth muscle contraction was proposed quite recently for the prostate, vessels, and airways (12–17). Smooth muscle contraction depends basically on actin dynamics, so regulators of actin organization may be involved in smooth muscle contraction (1, 18). ADP-ribosylation factor 6 (ARF6) is a monomeric GTPase, with proven roles for proliferation in malignant and nonmalignant cells, which is known to promote actin organization in the context of different actin-dependent cellular functions (19–21). Consequently, a role of ARF6 in smooth muscle contraction appears principally possible but has not, to the best of our knowledge, been explicitly considered to date.

Recently, it has been speculated that ARF6 may promote smooth muscle contraction in the human prostate (22). This was based on the observation that the cytohesin inhibitor secinH3 inhibited ARF6, but not Rac1 or RhoA, activity in prostate tissues, which was paralleled by inhibition of prostate smooth muscle contraction and went along with actin breakdown in prostate stromal cells (22). Consequently, a role of ARF6 in smooth muscle contraction was supposed for the first time, but this remains to be proven (22). NAV2729, a small molecule inhibitor with assumed specificity for ARF6, has recently become available (21, 23, 24). Consequently, we here examined effects of NAV2729 in human prostate tissues and in cultured stromal cells.

**Results**

**Detection of ARF6 in human prostate tissues**

Western blot analysis of human prostate tissues using an antibody raised against ARF6 revealed bands with a size matching the expected molecular mass for ARF6 (20 kDa) (Fig. 1A). These bands occurred in seven of eight prostate samples included to this analysis, with obvious variation in band intensity (Fig. 1A). Similarly, Western blot analysis demonstrated varying content of PSA in samples of different patients, reflecting a divergent degree of BPH in different prostates (Fig. 1A). Detection of calponin demonstrated the presence of smooth muscle in all prostate samples included in Western blot analysis, whereas cytokeratins reflected the presence of glandular epithelial cells in most samples (Fig. 1A). RT-PCR confirmed ARF6 expression in prostate tissues at the mRNA level (Fig. 1B).

Correlation analysis for ARF6 with PSA was performed for mRNA data and for bands from Western blotting. Correlation values consistently suggested an up-regulation of ARF6 expression with increasing degree of BPH (Fig. 1C), which was significant for bands from Western blotting, and showed a trend for values from RT-PCR. Correlation analysis for ARF6 with calponin and pan-cytokeratin detected by Western blot analysis suggested expression of ARF6 in smooth muscle cells and in glandular epithelial cells (Fig. 1C).

**Detection of ARF6 in WPMY-1 cells**

ARF6 mRNA was detectable by RT-PCR in WPMY-1 cells, an immortalized line of stromal cells from a human prostate (Fig. 1B). Possible protein expression of ARF6 and other GTPases was suggested by Western blot analysis of WPMY-1 cells using antibodies for ARF6, ARF1, Rac1, and RhoA, which revealed bands with sizes matching the corresponding expected molecular weights (Fig. 1E). WPMY-1 cells show expression of the smooth muscle marker calponin, whereas PSA and cytokeratins (both typical features of glandular epithelial cells in the prostate) are undetectable, together confirming the smooth muscle cell–like phenotype of WPMY-1 cells (Fig. 1E).

**Effects of NAV2729 on prostate smooth muscle contraction**

Recently, the cytohesin inhibitor secinH3 inhibited prostate smooth muscle contraction, which was paralleled by inhibition of ARF6 but not of RhoA or Rac (22). Therefore, we speculated that NAV2729 may inhibit contractions of human prostate tissues. Electric field stimulation (EFS) induced frequency-dependent contractions of prostate tissues, whereas noradrenaline, the α1-adrenoreceptor agonists phenylephrine and methoxamine, the thromboxane A2 analog U46619, and endothelin-1 induced concentration-dependent contractions (Fig. 2).

NAV2729 (5 μM) inhibited EFS-, noradrenaline-, phenylephrine-, and methoxamine-induced contractions. Two-way ANOVA was conducted to compare inhibitor and control groups and confirmed that these inhibitions were significant (p < 0.04 between controls versus NAV2729 for EFS, p < 0.001 for noradrenaline, p < 0.002 for phenylephrine, p < 0.001 for methoxamine). Multivariate analysis confirmed that inhibitions were significant for EFS with 32 Hz in EFS and for noradrenaline, phenylephrine, and methoxamine at 10, 30, and 100 μM (Fig. 2). In contrast to EFS-induced and α1-adrenergic contractions, NAV2729 did not inhibit contractions induced by U46619 or endothelin-1 (Fig. 2).

To examine whether a reduced viability of smooth muscle cells resulting from NAV2729 may account for the inhibition of EFS- and agonist-induced contractions, effects of NAV2729 on high molar KCl-induced contractions were examined. Contractions by high molar KCl were induced before application of NAV2729 (5 μM, 1 h) or solvent (1 h) and again after washout of NAV2729 and of controls. The second KCl-induced contractions following application of solvent/NAV2729 and washout were not significantly different. Thus, KCl-induced contractions following application and washout of DMSO were 173 ± 25% of KCl-induced contractions before application of DMSO in the same samples and 221 ± 36% of KCl-induced contractions before application of NAV2729 following application and washout of NAV2729 in the same samples (tissues from n = 10 prostates, allocated to both groups).
Figure 1. Detection of ARF6 in human prostate tissues and WPMY-1 cells. A, human prostate tissues were subjected to Western blot analysis using antibodies raised for ARF6, for the smooth muscle marker calponin, for cytokeratins as a marker for (glandular) epithelial cells, for PSA, and for β-actin. Shown are blots using prostate tissues from eight patients, with tissues being arranged in the same order for each blot. Positions of marker bands next to the bands of interest are indicated at the left side of each blot (sizes in kDa). B, RT-PCR for ARF6 in human prostate tissues and WPMY-1 cells. Data \(2^{-\Delta\Delta CT}\) values \(2^{-\Delta\Delta CT_{ARF6} - \Delta\Delta CT_{GAPDH}}\) from all analyzed samples, normalized to the mean of each of both groups. C, correlation analysis for ARF6 expression and PSA content, based on intensity of bands in Western blotting (left) or on mRNA content (right). D, correlation analysis for ARF6 expression and calponin and cytokeratins, based on intensity of bands in Western blot analysis. E, WPMY-1 cells were subjected to Western blot analysis using antibodies raised for different monomeric GTPases (ARF6, ARF1, RhoA, and Rac1) and for markers and β-actin. Shown are blots using samples from the same three independent experiments in each blot. Positions of marker bands next to the bands of interest are indicated at the left of each blot (sizes in kDa).
**Effects of NAV2729 on monomeric GTPase activities in prostate tissues**

Human prostate tissues were incubated with NAV2729 (5 μM) or solvent, and activities of monomeric GTPases were subsequently compared between NAV2729-incubated and solvent-incubated (control) tissues by pulldown assay. NAV2729 significantly reduced the content of GTP-ARF6, but not total ARF6 content, reflecting inhibition of ARF6 activity by NAV2729 in prostate tissues (Fig. 3). In contrast, NAV2729 did not inhibit activities of ARF1, Rac1, or RhoA (Fig. 3).

**Effects of brefeldin A on prostate smooth muscle contraction**

Brefeldin A is supposed to inhibit activation ARF1, but not of ARF6 (23). To confirm that inhibition ARF6, but not of ARF1,
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**GTP-ARF6**

**total ARF6**

**β-actin**

**GTP-ARF1**

**total ARF1**

**β-actin**

**GTP-Rac1**

**total Rac1**

**β-actin**

**GTP-RhoA**

**total RhoA**

**β-actin**
Figure 3. Effects of NAV2729 on monomeric GTPase activities in human prostate tissues. Prostate tissues were incubated with NAV2729 (5 μM, 75 min) or solvent (DMSO) and subjected to pulldown assays to assess the content of active GTPases (GTP-ARF6, GTP-ARF1, GTP-Rac1, and GTP-RhoA) and to Western blot analysis to assess the total content of GTPases. Shown are representative experiments (left panels), and quantification from all experiments (n = 2 for ARF6, n = 4 for ARF1, n = 5 for Rac1, n = 4 for RhoA) (means ± S.E. (error bars)) (δ, p < 0.05 for DMSO versus NAV2729). In each single experiment, samples from the same tissue were used for the control and inhibitor group. Positions of marker bands next to the bands of interest are indicated at the left side of each blot (sizes in kDa).

Figure 4. Effects of brefeldin A on contraction of human prostate tissues. A, contractions in an organ bath were induced during preincubation and exposure to brefeldin A (50 μM) or solvent (DMSO) by EFS and noradrenaline. To eliminate heterogeneities, including any individual variations, different degree of BPH, or varying smooth muscle content (compare Fig. 1), tensions have been expressed as percentage of high molar KCl-induced contraction, which was assessed before application of inhibitors or solvent. In each single experiment, samples from the same tissue were used for the control and inhibitor group. Data are means ± S.E. (error bars) from series with tissues from n = 7 patients for EFS and n = 5 for noradrenaline.

Figure 5. Effects of NAV2729 on MYPT1 and MLC phosphorylation in human prostate tissues.

Effects of adrenergic stimulation on ARF6 activity in human prostate tissues

Several Gα_q-coupled receptors may activate ARF6-Gα_q-dependently or -independently in vascular smooth muscle or other cells (24–27). As prostatic α1-adrenoceptors also couple to Gα_q, we examined the effects of α1-adrenergic agonists on ARF6 activity in human prostate tissues by pulldown assays. Neither the stimulation with noradrenaline (30 μM, 15 min) nor the stimulation with methoxamine (30 μM, 10 min, 20 min) resulted in activation of ARF6 (Fig. 5).

Effects of NAV2729 on MYPT1 and MLC phosphorylation in human prostate tissues

Procontractile signaling pathways may finally result in increased myosin light chain (MLC) phosphorylation by phosphorylation of the Myosin Phosphatase-Targeting Subunit 1 (MYPT1), which is an ultimate requirement for smooth muscle contraction in parallel with actin organization (18). Consequently, we incubated human prostate tissues with NAV2729 (5 μM) or solvent and compared contents of phospho-MYPT1 (threonine 696), total MYPT1, phospho-MLC (threonine 18-serine 19), and total MLC between NAV2729-incubated and control (solvent) tissues by Western blotting. NAV2729 did not reduce the content of any of these antigens, suggesting that NAV2729 did not inhibit phosphorylation of MYPT1 or MLCs (Fig. 6A).

Effects of ARF6 stimulation on ARF6 activity in human prostate tissues

The stimulation with methoxamine (30 μM, 10 min, 20 min) resulted in activation of ARF6 (Fig. 5). The stimulation with methoxamine (30 μM, 15 min) resulted in activation of ARF6 (Fig. 5).
Effects of NAV2729 on MYPT1 and MLC phosphorylation in WPMY-1 cells

NAV2729-treated (5 μM, 2 h) WPM-1 cells and solvent-treated controls were subjected to Western blot analysis to compare the contents of phospho-MYPT1 (threonine 696), total MYPT1, phospho-MLC (threonine 18/serine 19), and total MLC. NAV2729 did not reduce the content of these antigens, suggesting that NAV2729 did not inhibit phosphorylation of MYPT1 or MLCs (Fig. 6B).

Figure 5. Effects of adrenergic stimulation on ARF6 activity in human prostate tissue. Prostate tissues were incubated with noradrenaline (NA) (30 μM, 15 min), methoxamine (MTX) (30 μM, 10 or 20 min), or water (control) and subjected to pulldown assays to assess the content of active ARF6 (GTP-ARF6) and to Western blot analysis to assess the total content of ARF6. Shown are representative experiments (top panels) and quantification from all experiments (n = 6 for noradrenaline, n = 5 for methoxamine 10 min, n = 5 for methoxamine 20 min) (means ± S.E. (error bars)). In each single experiment, samples from the same tissue were used for the control and inhibitor group. Positions of marker bands next to the bands of interest are indicated at the left of each blot (sizes in kDa).

Effects of NAV2729 on viability of WPMY-1 cells

Effects of NAV2729 on viability were assessed by CCK-8 assay. NAV2729 (1, 2.5, and 5 μM) induced concentration- and time-dependent decreases of viability in WPMY-1 cells (Fig. 9A). Significant decreases were observed following incubation periods from 24 to 48 h, ranging from between 44 ± 2.7% from 1 μM and 89 ± 1.6% from 5 μM after 24 h to nearly complete reduction of viability for all three concentrations after 72 h (Fig. 9A). Following short incubation periods resembling those
applied in an organ bath, decreases were not significant, reaching 19 ± 1.1% from 5 μM after 1 h and 21 ± 1.3% after 2 h (Fig. 9A).

Effects of NAV2729 on proliferation of WPMY-1 cells

Effects of NAV2729 (24 h) on proliferation were assessed by different readouts, with results confirming each other (Fig. 9, B–D). In the 5-ethynyl-2′-deoxyuridine (EdU) assay, NAV2729 caused concentration-dependent decreases of the proliferation rate, amounting to 20 ± 2.5% reduction of the proliferation rate for 2.5 μM NAV2729 and to 40 ± 1.9% for 5 μM (Fig. 9B). Similarly, concentration-dependent reduction of proliferation was observed in colony formation, amounting to 58 ± 11.5% reduction of colony formation after 5 μM (Fig. 9C). Again, the mRNA content of the proliferation marker Ki-67 was decreased concentration-dependently by NAV2729 (24 h), amounting to
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GTP-ARF6

|       | DMSO | NAV2729 |
|-------|------|---------|
| 20    |      |         |
| 15    |      |         |
| 10    |      |         |
| 5     |      |         |
| 0     |      |         |

GTP-ARF6 (% of DMSO)

| DMSO | NAV2729 |
|------|---------|
| 100  | 100     |
| 90   | 90      |
| 80   | 80      |
| 70   | 70      |
| 60   | 60      |

β-actin

|       | DMSO | NAV2729 |
|-------|------|---------|
| 20    |      |         |
| 15    |      |         |
| 10    |      |         |
| 5     |      |         |
| 0     |      |         |

GTP-ARF6

|       | DMSO | secinH3 |
|-------|------|---------|
| 20    |      |         |
| 15    |      |         |
| 10    |      |         |
| 5     |      |         |
| 0     |      |         |

GTP-ARF6 (% of DMSO)

| DMSO | secinH3 |
|------|---------|
| 100  | 100     |
| 90   | 90      |
| 80   | 80      |
| 70   | 70      |
| 60   | 60      |

β-actin

|       | DMSO | secinH3 |
|-------|------|---------|
| 20    |      |         |
| 15    |      |         |
| 10    |      |         |
| 5     |      |         |
| 0     |      |         |

GTP-ARF1

|       | DMSO | NAV2729 |
|-------|------|---------|
| 20    |      |         |
| 15    |      |         |
| 10    |      |         |
| 5     |      |         |
| 0     |      |         |

GTP-ARF1 (% of DMSO)

| DMSO | NAV2729 |
|------|---------|
| 100  | 100     |
| 90   | 90      |
| 80   | 80      |
| 70   | 70      |
| 60   | 60      |

β-actin

|       | DMSO | NAV2729 |
|-------|------|---------|
| 20    |      |         |
| 15    |      |         |
| 10    |      |         |
| 5     |      |         |
| 0     |      |         |

GTP-Rac1

|       | DMSO | NAV2729 |
|-------|------|---------|
| 25    |      |         |
| 20    |      |         |
| 15    |      |         |
| 10    |      |         |
| 5     |      |         |

GTP-Rac1 (% of DMSO)

| DMSO | NAV2729 |
|------|---------|
| 100  | 100     |
| 90   | 90      |
| 80   | 80      |
| 70   | 70      |
| 60   | 60      |

β-actin

|       | DMSO | NAV2729 |
|-------|------|---------|
| 20    |      |         |
| 15    |      |         |
| 10    |      |         |
| 5     |      |         |
| 0     |      |         |

GTP-RhoA

|       | DMSO | NAV2729 |
|-------|------|---------|
| 25    |      |         |
| 20    |      |         |
| 15    |      |         |
| 10    |      |         |
| 5     |      |         |

GTP-RhoA (% of DMSO)

| DMSO | NAV2729 |
|------|---------|
| 100  | 100     |
| 90   | 90      |
| 80   | 80      |
| 70   | 70      |
| 60   | 60      |

β-actin

|       | DMSO | NAV2729 |
|-------|------|---------|
| 20    |      |         |
| 15    |      |         |
| 10    |      |         |
| 5     |      |         |
| 0     |      |         |
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Transfection of WPMY-1 cells with ARF6 siRNA resulted in silencing of ARF6 expression, reflected by reduced levels of ARF6 mRNA and weaker ARF6 bands in Western blot analysis compared with WPMY-1 cells transfected with scrambled siRNA or with WT WPMY-1 cells (Fig. 10A and B). Silencing of ARF6 expression mimicked the effects of NAV2729 on viability, proliferation, and actin organization observed in CCK-8 and EdU assays and by phalloidin staining. Thus, in WPMY-1 cells transfected with ARF6 siRNA, viability was reduced by 32 ± 7.1% and 22 ± 9.0% compared with WT cells and cells transfected with scrambled siRNA, respectively (Fig. 10C). The proliferation rate in WPMY-1 cells transfected with ARF6 siRNA was reduced by 34 ± 2.3% compared with WT cells and by 30 ± 1.8% compared with cells transfected with scrambled siRNA (Fig. 10D). Following phalloidin staining, WPMY-1 cells without transfection or transfected with scrambled siRNA showed an actin staining similar to that observed for untreated cells, whereas reduced staining and no filament organization were observed in WPMY-1 cells transfected with ARF6 siRNA (Fig. 10E).

To assess possible limitations regarding the specificity of NAV2729, effects of NAV2729 on viability and proliferation were assessed in WPMY-1 cells with silenced ARF6 expression. In contrast to WT cells (see Fig. 9A), no effects on viability were observed in WPMY-1 cells with silenced ARF6 expression following exposure with 1 or 2.5 μM NAV2729 for 24 h (Fig. 10F). Exposure with 5 μM for 24 h reduced viability by 58 ± 0.3% (Fig. 10F), but not nearly completely as observed in WT cells (Fig. 9A). In the EdU assay, no significant effect of 2.5 μM NAV2729 on proliferation was observed, whereas 5 μM NAV2729 still reduced the proliferation rate by 12 ± 2.8% in WPMY-1 cells with silenced ARF6 expression (Fig. 10G).

Discussion

A role of ARF6 for smooth muscle contraction has been recently supposed for the first time but has to the best of our knowledge never been directly addressed (22). A small molecule inhibitor with assumed ARF6 specificity, NAV2729, is now available (21, 24). Here, we examined effects of NAV2729 on contraction of human prostate tissues and on growth of prostate stromal cells, which are important factors for pathogenesis of LUTS suggestive of BPH (1). Our findings demonstrate that NAV2729 inhibits α₁-adrenergic and neurogenic contractions of human prostate tissues and proliferation and actin organization in stromal cells. Molecular analysis suggested up-regulation of prostatic ARF6 expression with increasing degree of BPH. Silencing of ARF6 expression confirmed an involvement of ARF6 in proliferation and viability of prostate stromal cells and suggested that most parts of the NAV2729 effects are in fact caused by inhibition of ARF6, but not by off-target inhibition. Together, this may point to a possible role of ARF6 in promoting α₁-adrenergic smooth muscle contraction and stromal growth in the hyperplastic human prostate.

Our findings may be interesting from a bifocal perspective. First, from the view of general pharmacology and physiology, these results may suggest a role of ARF6 for smooth muscle contraction, occurring in the prostate but not necessarily being limited to it. Thus, a function of ARF6 for promotion of smooth muscle contraction may also appear in the cardiovascular system, airways, gastrointestinal tract, or bladder, as this would be similar to the role of RhoA that is shared by smooth muscle in different organs (12, 13, 28–30). Second, specifically in the context of BPH and from the clinical view, our findings point to connections between smooth muscle contraction and growth in the hyperplastic prostate and indicate a divergent regulation of α₁-adrenergic and nonadrenergic smooth muscle contraction in the prostate.

Figure 7. Effects of NAV2729 and secinH3 on monomeric GTPase activities in WPMY-1 cells. Cells were incubated with NAV2729 (5 μM, 2 h), secinH3 (30 μM, 2 h), or solvent (DMSO) and subjected to pulldown assays to assess the content of active GTPases (GTP-ARF6, GTP-ARF1, GTP-Rac1, and GTP-RhoA) and to Western blot analysis to assess the total content of GTPases. Shown are representative experiments (left panels) and quantification from all experiments (n = 7 for ARF6, n = 6 for ARF1, n = 5 for Rac1, n = 4 for RhoA using NAV2729 and n = 6 for ARF6 using secinH3) (means ± S.E. (error bars) (#, p < 0.05 for DMSO versus NAV2729). In each single experiment, samples from the same tissue were used for the control and inhibitor group. Positions of marker bands next to the bands of interest are indicated at the left of each blot (sizes in kDa).

Figure 8. Effects of NAV2729 on actin filaments. WPMY-1 cells were exposed to NAV2729 (5 μM) for 2 h (A) or 24 h (B) and subjected to phalloidin staining to visualize polymerized actin. Shown are representative pictures from n = 5 independent experiments for each setting, including separate controls for each incubation period, and quantification of all experiments (means ± S.E. (error bars)) (#, p < 0.05 for DMSO versus NAV2729).

Effects of ARF6 silencing in WPMY-1 cells

Transfection of WPMY-1 cells with ARF6 siRNA resulted in silencing of ARF6 expression, reflected by reduced levels of ARF6 mRNA and weaker ARF6 bands in Western blot analysis compared with WPMY-1 cells transfected with scrambled siRNA or with WT WPMY-1 cells (Fig. 10, A and B).
Figure 9. Effects of NAV2729 on viability and proliferation of WPMY-1 cells. A, WPMY-1 cells were exposed to NAV2729 in different concentrations and different periods as indicated and assessed for viability using a CCK-8 assay. B, WPMY-1 cells were exposed to NAV2729 in different concentrations for 24 h and assessed for proliferation using an EdU assay. C, WPMY-1 cells were exposed to NAV2729 in different concentrations during a plate colony assay. D, WPMY-1 cells were exposed to NAV2729 in different concentrations for 24 h and subjected to RT-PCR for mRNA content of the proliferation marker Ki-67. In all series, controls (DMSO) were exposed for the same periods as corresponding inhibitor groups. Shown are means ± S.E. (error bars) from n = 5 independent experiments in all series (#, p < 0.05 versus control).
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A

ARF6, 20 kDa
β-actin, 42 kDa

B

ARF6, % of wildtype

C

absorbance (OD)

D

% proliferating cells

E

stained actin (% of area)

F

absorbance (OD)

G

% proliferating cells

control 2.5 µM 5 µM

NAV2729 2.5 µM 5 µM

wildtype scramble ARF6 siRNA

wildtype scramble ARF6 siRNA

wildtype scramble ARF6 siRNA

wildtype scramble ARF6 siRNA
Our findings from RT-PCR, Western blotting, and correlation analyses suggest an up-regulation of ARF6 expression with increasing degree of BPH, which occurs at the transcriptional level. Up-regulation of ARF6 expression in BPH may contribute to increased smooth muscle tone and prostate enlargement and thus to LUTS suggestive of BPH. Prostate samples in our studies may be considered as hyperplastic tissues, as they show diverging content of PSA, which is known to increase with the degree of BPH (31, 32). Finally, correlation of presumed ARF6 bands with calponin and cytokeratin content confirms expression in smooth muscle cells and glandular epithelial cells of the prostate.

ARF6 may be activated by a panel of different guanosine exchange factors (GEFs), depending on cell type (20, 21). NAV2729 binds directly to ARF6 and inhibits ARF6 by preventing its GEF-induced and spontaneous activation, which is independent from nucleotide binding to ARF6 (21, 24). NAV2729 showed IC_{50} values of 1 and 3.4 μM for ARF6 inhibition in fluorometric and orthogonal radiometric nucleotide exchange assays but did not inhibit other ARFs or other GTPases (24). Even at concentrations of 50 μM, NAV2729 was still presumed to act with high specificity and selectivity of ARF6 (24). Here, we applied NAV2726 in a concentration of 5 μM to prostate tissues. Using pulldown assays, we show that NAV2729 inhibits ARF6 activity in prostate tissues and in cultured stromal cells, whereas ARF1, Rac1, and RhoA were not inhibited, which is in line with previous findings (24). Finally, the inhibition of ARF6 by the cytohesin inhibitor secinH3, which has recently been shown in prostate tissues (22), has been shown here again using WPMY-1 cells.

Recently, the concept of ARF6 specificity of NAV2729 has been challenged by the suggestion that it may inhibit ARF1 in addition to ARF6, by using 25 μM of NAV2729 in a fluorescence kinetics–based assay (i.e. exceeding our concentration 5-fold) (23). To confirm that the effect of NAV2729 in our organ bath experiments was mediated by ARF6 but not ARF1, we studied effects of brefeldin A, which may inhibit ARF1, but not ARF6 (23). Brefeldin A did not inhibit EF- or noradrenaline-induced prostate smooth muscle contractions. This may confirm our finding from pulldown assays, where we did not observe ARF1 inhibition by NAV2729 in prostate tissues or WPMY-1 cells. Together, these lines of evidence may suggest a high degree of specificity of NAV2729 for ARF6 at least at 5 μM and under our conditions. This conclusion is further supported by our findings from WPMY-1 cells, where silencing of ARF6 expression suggested that off-target effects of NAV2729 are not large enough to account fully for the effects of NAV2729. Future studies may include knockout models in animals, to confirm the procontractile role of ARF6 in smooth muscle of the prostate or even in other organs.

Prostate smooth muscle contractions may be induced by α_{1}-adrenoceptor activation resulting from adrenergic neurotransmission or by nonadrenergic mediators (probably non-neurogenically) including endothelin-1 and thromboxane A_{2} (1, 10). We observed that NAV2729 inhibited neurogenic contractions of human prostate tissues as well as contractions induced by α_{1}-adrenoceptor agonists. To substantiate the reproducibility of this result, we confirmed the inhibition with three different α_{1}-adrenoceptor agonists (i.e. noradrenaline, phenylephrine, and methoxamine). Together with our findings from pulldown assays and from WPMY-1 cells with silenced ARF6 expression, both suggesting a high degree of specificity of NAV2729 for ARF6, this raises the idea that ARF6 exerts a procontractile function in prostate smooth muscle. As we did not observe effects of NAV2729 on high molar KCl-induced contractions or on viability of cultured stromal cells following incubation periods similar to that in the organ bath, we exclude the possibility that the inhibition of contraction resulted from reduced viability of smooth muscle cells or from cytotoxic effects.

In contrast to neurogenic and α_{1}-adrenergic contractions, NAV2729 did not inhibit contractions induced by endothelin-1 or by the thromboxane A_{2} analog U46619. Notably, this may point to a divergent regulation of adrenergic and nonadrenergic (i.e. thromboxane A_{2}– and endothelin–induced) prostate smooth muscle contraction, which is imparted by ARF6. Obviously, ARF6 or at least a NAV2729-sensitive mechanism may selectively promote α_{1}-adrenergic, but not endothelin- or thromboxane-induced, contractions in the hyperplastic prostate. Despite the presumably high clinical relevance, differences between α_{1}-adrenergic and nonadrenergic smooth muscle contractions in the prostate are still unknown. Unlike the well-known procontractile signaling pathways, which are shared by different contractile receptors and include inositol 1,4,5-triphosphate/calcium, diacylglycerol/protein kinase C, and RhoA/Rho kinase, the NAV2729-sensitive contraction mechanism is apparently not associated with all receptors and imparts divergent regulation of adrenergic and nonadrenergic contraction in the prostate. It is assumed that nonadrenergic mediators contribute to prostate smooth muscle tone in parallel with α_{1}-adrenoceptors and may maintain urethral obstruction and LUTS despite therapy with α_{1}-blockers (1, 10, 11). Probably, this accounts for the low efficacy of α_{1}-blockers, which will improve urinary flow and symptom scores by not more than 50% (1, 3, 10, 11). Recently, it became increasingly obvious that nonadrenergic smooth muscle contractions in the prostate merit large attention and need to be considered for development of novel options for LUTS therapy with higher efficacy than available medications (10). Certainly, improved understanding of adrenergic and nonadrenergic contractions, including their divergent regulation, represents a prerequisite to develop such medications. In fact, novel options for medical therapy of male LUTS with higher efficacy than current options are in high demand, so that exploring mechanisms of contrac-

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**Figure 10. Silencing of ARF6 expression by siRNA in WPMY-1 cells.** A and B, cells were transfected with scrambled siRNA or ARF6 siRNA, and ARF6 expression was compared with cells not being transfected (WT) by Western blotting (A) and RT-PCR (B). Viability was compared by CCK-8 (C), proliferation by EdU assay (D), and actin organization by phallolidin staining (E) between WT cells and cells transfected with scrambled siRNA or ARF6 siRNA. Effects of NAV2729 (24 h) in the indicated concentrations on viability and proliferation were assessed in cells transfected with ARF6 siRNA by CCK-8 (F) and EdU assay (G). Shown are representative experiments (A and D–F) and means ± S.E. (error bars) from n = 5 independent experiments in all series (#, p < 0.05 versus WT and scramble in A–E; #, p < 0.05 versus control in F and G). Positions of marker bands next to the bands of interest are indicated at the left of each blot (sizes in kDa).
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...tion and discovery of novel targets in the hyperplastic prostate is an active research field with clinical relevance. Whereas the clinical relevance of the divergent regulation of \(\alpha_1\)-adrenergic and nonadrenergic prostate smooth muscle contraction imparted by ARF6 is obvious, the precise molecular mechanisms underlying the promotion of \(\alpha_1\)-adrenergic contraction by the NAV2729-sensitive mechanism still remain unclear. In line with previous studies, this points out again that the mechanisms of prostate smooth muscle contraction are still incompletely understood (1). Nevertheless, several conclusions are possible. Based on our analyses of MYPT1 and MLC phosphorylation, a mechanism of contraction inhibition taking place by inhibition of MLC phosphorylation appears unlikely. Phosphorylation of MYPT1 and MLC are increased by contraction pathways (calcium, protein kinase C, and Rho kinase), which couple to \(G_{\alpha_q}\)-coupled receptors in smooth muscle cells (18, 33). As prostate \(\alpha_1\)-adrenoceptors also couple to \(G_{\alpha_q}\) and because ARF6 was reported to be activated by \(G_{\alpha_q}\)-coupled receptors in several cell types (1, 24–27), we examined the possible activation of ARF6 by \(\alpha_1\)-adrenoceptor agonists in prostate tissues. In line with the lacking effect of NAV2729 on MYPT1 and MLC phosphorylation, we observed that ARF6 is not activated by \(\alpha_1\)-adrenoceptors in human prostate tissue. In addition to MLC phosphorylation, smooth muscle contraction depends on actin organization. Phalloidin stainings of WPMY-1 cells suggested that the actin organization remains unaffected by NAV2729 if exposure periods are similar to those applied in the organ bath (i.e., 1–2 h). In conclusion, ARF6 is not coupled to \(\alpha_1\)-adrenoceptors and is not a downstream effector in prostate smooth muscle. Rather, it selectively regulates a procontractile pathway, which is specific for \(\alpha_1\)-adrenoceptors and works without MLC phosphorylation or actin organization.

Following longer exposure periods (24 h), a breakdown of actin organization by NAV2729 was observed by phalloidin staining in WPMY-1 cells. In contrast to organ experiments, it appears possible that this breakdown of actin organization takes place if NAV2729 is applied in vivo, where exposure to the drug would be longer than in our organ bath experiments. In vivo, this may result in inhibition of nonadrenergic prostate contractions, in addition to inhibition of adrenergic contractions and of prostate growth, as actin organization is an essential requirement for smooth muscle contraction. Thereby, the efficacy of NAV2729 in reducing experimentally induced or even clinical LUTS may exceed that of \(\alpha_1\)-blockers. Whether or not NAV2729 induces urodynamic effects, improvements of LUTS, or reduction of BPH may be explored by in vivo studies, including animal models with experimentally induced LUTS or BPH. In mouse models, NAV2729 showed no toxicity (24). Together, NAV2729 may be a promising compound to be studied in vivo in animal models of BPH and LUTS, but nevertheless, side effects may appear and may limit clinical application.

In WPMY-1 cells, NAV2729 inhibited proliferation, as evidenced by reduced numbers of proliferating cells in the EdU assay, reduced content of Ki-67, and reduced colony formation. This may be connected with reduced survival, which was observed in viability assays. Silencing of ARF6 expression mimicked the effects of NAV2729 on proliferation and viability, confirming a role of ARF6 in growth of prostate stromal cells. Compared with WT cells, effects of NAV2729 were attenuated by silencing of ARF6 expression, pointing to a high degree of specificity of NAV2729 under our conditions. The small remaining effects of NAV2729 in cells with silenced ARF6 expression may be explained (a) by residual ARF6 expression (silencing reduced ARF6 expression by \(\sim 50\%\)) or (b) by off-target effects. Although it remains speculative which of the two options applies for the remaining effects, it becomes clear that the extent of possible nonspecific effects is insufficient to account completely for the large effects of NAV2729 in WT cells. Thus, off-target effects are low even at 5 \(\mu\)M, so that most parts of the effects are caused by inhibition of ARF6 but not by off-target effects.

Our findings showing inhibition of proliferation and viability of WPMY-1 cells by NAV2729 and suggesting a role of ARF6 in these processes are in line with previous studies reporting a role of ARF6 in proliferation of several malignant and nonmalignant cell types, including vascular smooth muscle cells (19). Accordingly, ARF6 is involved in the progression of several types of cancer, including melanoma, prostate cancer, and others (24, 34–36). WPMY-1 cells are obtained from the prostate stroma, where smooth muscle cells are the predominant cell type, and they show increased proliferation and contribute to prostate growth in BPH (3, 37). Consequently, WPMY-1 cells show typical characteristics of prostate smooth muscle cells or may be considered as such, as shown here and in previous studies (17).

Our findings suggest a dual, simultaneous role of ARF6 for contraction and growth in prostate smooth muscle. Such connections have been recently suggested by similar observations, when inhibitors for Rac GTPases, Src family kinases, or Rho kinase inhibited prostate smooth muscle contraction and proliferation of stromal cells (17, 32, 38). Contrary to what has been assumed for decades, prostate contraction and growth in BPH are not separate processes and do not randomly contribute at the same time to urethral obstruction in BPH; rather, they are closely linked with each other. Obviously, they are both at least partially mediated by shared molecular mechanisms, including a NAV2729-sensitive mechanism. At present, combination therapies are applied to target \(\alpha_1\)-adrenergic contraction and prostate enlargement in patients with LUTS suggestive of BPH (4). Identification of molecular mechanisms with dual functions in contraction and growth in BPH may offer novel perspectives to develop single compounds targeting both at once.

In conclusion, a NAV2729-sensitive mechanism promotes adrenergic contraction and stromal cell growth in the human prostate. This is probably ARF6-mediated. This mechanism imparts a molecular connection between regulation of smooth muscle contraction and stromal growth in the hyperplastic prostate and a divergent regulation of adrenergic and nonadrenergic contraction. Urodynamic effects of NAV2729 as well as a reduction of prostate size appear possible in vivo.

Experimental procedures

Human prostate tissues

Human prostate tissues were obtained from patients \((n = 115)\) undergoing radical prostatectomy for prostate cancer. Patients who underwent previous transurethral resection of the
prostate were excluded. This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and has been approved by the ethics committee of the Ludwig-Maximilians University (Munich, Germany). Informed consent was obtained from all patients. Samples and data were collected and analyzed anonymously. Samples were taken immediately after prostatectomy, following macroscopic examination by a uro-pathologist. All tissues were taken from the periurethral zone, considering that most prostate cancers arise in the peripheral zone (39, 40). Upon pathologic evaluation, only tissue samples that did not exhibit histological signs of neoplasia, cancer, or inflammation were collected. BPH is present in 80–83% of patients with prostate cancer (41, 42). For macroscopic examination and sampling, the prostate was opened by a single longitudinal cut from the capsule to the urethra. Subsequently, both intersections were checked macroscopically for any obvious tumor infiltration. Because tumors are usually located in the peripheral zone, tumor infiltration in the periurethral zone (where sampling was performed) was very rare (found in less than 1% of prostate). Prostates showing tumors in the periurethral zone on macroscopic inspection were not subjected to sampling and were not included in this study. Organ bath studies and stimulation experiments for pull-down assay and phosphorylation analyses were performed immediately after sampling, whereas samples for molecular analyses were shock-frozen in liquid nitrogen and stored at −80 °C.

**RT-PCR**

RNA from frozen prostate tissues or cells was isolated using the RNaseasy Mini kit (Qiagen, Hilden, Germany). For isolation from tissues, 30 mg of tissue were homogenized using the FastPrep®-24 system with matrix A (MP Biomedicals, Illkirch, France). RNA concentrations were measured spectrophotometrically. Reverse transcription to cDNA was performed with 1 μg of isolated RNA using the Reverse Transcription System (Promega, Madison, WI). RT-PCR was performed with a Roche Light Cycler (Roche, Basel, Switzerland) using primers provided by Qiagen (Hilden, Germany) as ready-to-use mixes, based on the RefSeq accession numbers NM_001663 for ARF6 (catalogue no. PPH101866A), NM_011030047 for KLK3 (syonymous prostate-specific antigen (PSA)) (PPH100322B), NM_001154966 for Ki-67 (PPH101214E), and NM_001256799 for GAPDH (PPH01150F). PCRs were performed in a volume of 25 μl containing 5 μl of LightCycler® FastStart DNA MasterPlus SYBR Green I (Roche), 1 μl of template, 1 μl of primer, and 18 μl of water. Denaturation was performed for 10 min at 95 °C and amplification with 45 cycles of 15 s at 95 °C followed by 60 s at 60 °C. The specificity of primers and amplification was demonstrated by subsequent analysis of melting points, which revealed single peaks for each target. Results were expressed using the ΔΔCt method, where the number of cycles (Ct) at which the fluorescence signal exceeded a defined threshold for GAPDH was subtracted from Ct values for target (CtTarget − CtGAPDH = ΔCt), and values were calculated as 2−ΔΔCt and normalized to each other. Spearman’s correlation analysis was performed using Ct values for ARF6 and PSA from human prostate samples.

**Western blot analysis**

Frozen prostate tissues were homogenized in a buffer containing 25 mM Tris/HCl, 10 μM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 10 μg/ml leupeptin hemisulfate, using the FastPrep®-24 system with matrix A (MP Biomedicals, Illkirch, France). After centrifugation (20,000 × g, 4 min), supernatants were assayed for protein concentration using the DC-Assay kit (Bio-Rad, Munich, Germany) and boiled for 10 min with SDS sample buffer (Rotth, Karlsruhe, Germany). Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer and removed from flasks after 15 min of incubation on ice. Cell debris was removed by centrifugation (10,000 × g, 10 min, 4 °C), and supernatants were assayed for protein concentration and boiled for 10 min with SDS sample buffer.

Samples (20 μg of protein/lane) were subjected to SDS-PAGE, and proteins were blotted on Protran® nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membranes were blocked with PBS containing 5% milk powder (Roth) overnight and incubated with following primary antibodies (if not otherwise stated, diluted 1:500 in PBS containing 5% milk powder): mouse monoclonal anti ARF6 (sc-7971) (1:200), mouse monoclonal anti-pan-cytokeratin (sc-8018), mouse monoclonal anti-calponin 1/2/3 (sc-136987), mouse monoclonal anti-PSA (sc-7316) (1:800), and mouse monoclonal anti-β-actin antibody (sc-47778) (all from Santa Cruz Biotechnology, Inc.) and rabbit anti-phospho-myosin light chain 2 (Thr-18/Ser-19) (catalogue no. 3674) (1:1000), rabbit anti-myosin light chain 2 (catalogue no. 8505) (1:1000) (Cell Signaling), rabbit anti-phospho-MYPT1 (Thr-696) (1:1000), and rabbit anti-MYPT1 (catalogue no. 8574) (1:1000) (all from Cell Signaling Technology, Danvers, MA). Detection in pulldown assays was performed using the antibodies of the kits (see below). Primary antibodies were diluted in PBS containing 0.1% Tween 20 (PBS-T) and 5% milk powder. Subsequently, detection was continued using secondary biotinylated horse anti-mouse or horse anti-goat IgG (BA-1000, BA-2000, and BA-9500) (Vector Laboratories, Burlingame, CA), followed by incubation with avidin and biotinylated horseradish peroxidase from the “Vectastain ABC kit” (Vector Laboratories, Burlingame, CA), both diluted 1:600 in PBS. Membranes were washed with PBS-T after any incubation with primary or secondary antibodies, or biotin-horseradish peroxidase. Finally, blots were developed with enhanced chemiluminescence (ECL) using ECL Hyperfilm (GE Healthcare, Freiburg, Germany). Intensities of the resulting bands were quantified densitometrically using ImageJ (National Institutes of Health, Bethesda, MD). For correlation analysis, values (arbitrary units) for targets (ARF6, PSA, calponin, and cytokeratin) were divided by corresponding values for β-actin and subsequently plotted against each other and subjected to Spearman’s correlation analysis. For semiquantitative calculation of pulldown assays and of phosphorylation experiments, the value of each sample was normalized to the mean value of the corresponding control group (solvent, without inhibitor), and data were expressed as mean ± S.E. of all samples for each group.
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Tension measurements

Prostate strips (6 × 3 × 3 mm) were mounted in 10-ml aerated (95% O2 and 5% CO2) tissue baths (Danish Myotechnology, Aarhus, Denmark), containing Krebs–Henseleit solution (37 °C, pH 7.4) with the following composition: 118 mM NaCl, 4.7 mM KCl, 2.55 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 7.5 mM glucose. Preparations were stretched to 4.9 millinewtons and left to equilibrate for 45 min. In the initial phase of the equilibration period, spontaneous decreases in tone are usually observed. Therefore, tension was adjusted three times during the equilibration period, until a stable resting tone of 4.9 millinewtons was attained. After the equilibration period, maximum contraction induced by 80 mM KCl was assessed. Subsequently, chambers were washed three times with Krebs–Henseleit solution for a total of 30 min. Cumulative concentration–response curves for noradrenaline, phenylephrine, methoxamine, endothelin-1, and U46619 or frequency–response curves induced by EFS were created 30 min after the addition of NAV2729 (5 μM), brefeldin A (50 μM), or DMSO for controls. Application of EFS simulates action potentials, resulting in the release of endogenous neurotransmitters, including noradrenaline. For EFS, tissue strips were placed between two parallel platinum electrodes connected to a Grass S48 stimulator (Danish Myo Technology, Aarhus, Denmark). Square pulses with durations of 1 ms were applied with a voltage of 20 V for a train duration of 10 s and using a delay of 1 ms between single pulses. EFS-induced contractile responses were studied at frequencies of 2, 4, 8, 16, and 32 Hz, with train intervals of 30 s between stimulations. For calculation of agonist- or EFS-induced contractions, tensions (peak height in EFS-induced contractions and maximum contractions following agonist-exposure) were expressed as percentage of KCl-induced contractions, as this may correct different stromal/epithelial ratios, different smooth muscle content, varying degree of BPH, or any other heterogeneity between prostate samples and patients. Only one curve was recorded with each sample (agonist or EFS). Tissues were incubated with NAV2729 (80 mM), followed by washout (three times, 30 min) after full contraction, resulting in return to baseline tone. Next, NAV2729 or DMSO (controls) was applied for 60 min, followed by washout (three times, 60 min). Finally, tissues were again contracted by high molar KCl (80 mM). For calculation of results, the tension of the second KCl-induced contraction was calculated as a percentage of the first KCl-induced contraction. Due to the washout of NAV2729 before the second KCl-induced contraction, the second KCl-induced contraction could only be modified by irreversible effects, such as cell death resulting from reduced viability or cytotoxicity, which should resist even after washout.

Pulldown assays

Tissues from each prostate included were cut into several small strips (6 × 1 × 1 mm), which were allocated to two samples using 6-well plates filled with Custodial solution, in which tissues were incubated with NAV2729 (5 μM) or DMSO for 90 min at 37 °C under continuous shaking or with noradrenaline (30 μM, 15 min), methoxamine (30 μM, 10 or 20 min), or water (corresponding periods) under the same conditions. Following incubations, tissues were shock-frozen with liquid nitrogen, and homogenization, centrifugation, and protein determination were subsequently performed as described for Western blot analysis. For experiments with WPMY-1 cells, cells were grown in T75 flasks. After 48 h, NAV2729 (final concentration 5 μM), secinH3 (final concentration 30 μM), or solvent (DMSO) was added. After 2 h, cells were lysed with RIPA buffer and removed from flasks after 15 min of incubation on ice. Cell debris was removed by centrifugation (10,000 × g, 10 min, 4 °C), and supernatants were subjected to protein determination and subsequent pulldown assays. Aliquots containing 300 μg of protein were subjected to pulldown assays for ARF6, ARF1, Rac1, and RhoA. Assays were performed using pulldown activation assay kits BK033, BK032, BK035, and BK036 obtained from Cytoskeleton (Denver, CO), according to the manufacturer’s instructions, and included Western blot analysis as described above. Series for each pulldown were repeated in at least five independent experiments using five different prostates or cells from five independent experiments, with the exception for two series using prostate tissues and one series of WMPY-1 cells, where the series were discontinued after four independent experiments because it was obvious that no effect can be expected. Densitometric quantification and calculation of results were performed as described above for Western blot analysis.

Cell culture

WPMY-1 cells are an immortalized cell line obtained from human prostate stroma without prostate cancer. Cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and kept in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37 °C with 5% CO2. Before the addition of NAV2729 (5 μM), secinH3 (30 μM), or DMSO, the medium was changed to an FCS-free medium. For Western blot analysis, cells were lysed using RIPA buffer (Sigma-Aldrich) and removed from flasks after 15 min of incubation on ice. Cell debris was removed by centrifugation (10,000 × g, 10 min, 4 °C), and different aliquots of supernatants were either subjected to protein determination or boiled with SDS sample buffer.

Silencing of ARF6 expression

WPMY-1 cells were transfected with scrambled siRNA (Silencer Select scrambled negative control siRNA duplex, 4390843) or ARF6 siRNA duplex (4390824, ID s1566, sequence AGACGGUGACUUACAAAAAtt) (both from the Ambion Silencer Select Library, Life Technologies, Inc.). The ARF6 and scrambled siRNAs were diluted in Opti-MEM (110 pmol of siRNA in every 200 μl, providing final concentrations of siRNA in the cell culture medium of 50 nM). Subsequently, Stromal
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Phalloidin staining

For fluorescence staining with phalloidin, cells were grown on Lab-Tek chamber slides (Thermo Fisher Scientific) with inhibitors or solvent. Staining was performed using 100 μM FITC-labeled phalloidin (Sigma-Aldrich, Munich, Germany), according to the manufacturer’s instructions. Labeled cells were analyzed using a laser-scanning microscope (Leica SP2, Wetzlar, Germany). Finally, stainings were quantified using ImageJ (National Institutes of Health).

Phosphorylation assessments

Tissues from each prostate included were cut into several small strips (6 × 1 × 1 mm), which were allocated to two samples using 6-well plates filled with Custodiol solution, in which tissues were incubated with NAV2729 (5 μM) or DMSO for 90 min at 37 °C under continuous shaking. Following incubations, tissues were shock-frozen with liquid nitrogen, and homogenization, centrifugation, and protein determination were subsequently performed as described for Western blot analysis. For experiments with WPMY-1 cells, cells were grown in T75 flasks. After 48 h, NAV2729 (final concentration 5 μM), secinH3 (final concentration 30 μM), or solvent (DMSO) was added. After 2 h, cells were lysed with RIPA buffer and removed from flasks after 15 min of incubation on ice. Cell debris was removed by centrifugation (10,000 × g, 10 min, 4 °C), and supernatants were subjected to protein determination and subsequent Western blotting analyses. Finally, all samples were subjected to Western blot analysis for phospho-MYPT1, MYPT1, phospho-MLC, MLC, and β-actin and to densitometric quantification and calculation of results, which were performed as described above for Western blot analysis.

Cytotoxicity assay

Viability of cells was assessed using the Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich). Cells were grown in 96-well plates (5,000 cells/well) for 24 h before NAV2729 or solvent were added in the indicated concentrations. Subsequently, cells were grown for different periods (1, 2, 48, and 72 h). Separate controls were performed for each period. At the end of this period, 10 μl of [2-(2-methoxy-4-nitrophenyl)-3-(4-nitroph- enyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) from CCK-8 was added, and absorbance in each well was measured at 450 nm after incubation for 2 h at 37 °C.

EdU proliferation assay

WPMY-1 cells were plated with cell line–specific densities (50,000/well) on 16-well chambered coverslips (Thermo Fisher Scientific). After 24 h, cells were treated with NAV2729 (final concentration 5 μM), secinH3 (30 μM), or solvent. After a further 24 h, the medium was changed to a 10 mM Edu solution in FCS-free medium containing NAV2729 or solvent. 20 h later, cells were fixed with 3.7% formaldehyde. Edu incorporation was determined using the “EdU-Click 555” cell proliferation assay (Baseclick, Tutzung, Germany) according to the manufacturer’s instructions. In this assay, incorporation of Edu into DNA is assessed by detection with fluorescing 5-carboxytetramethylrhodamine. Counterstaining of all nuclei was performed with 4’,6-diamidino-2-phenylindole. Cells were analyzed by fluorescence microscopy (excitation, 546 nm; emission, 479 nm).

Colony formation assay

About 100 cells were placed into each well of a 6-well culture plate and were treated with NAV2729 in the indicated concentrations. Cells were incubated at 37 °C for 14 days and then washed twice with phosphate-buffered saline and fixed by 2 ml of 10% trichloroacetic acid overnight (4 °C). Subsequently, all plates were washed five times with cold water and stained with 0.4% sulforhodamine B solution (diluted in 1% acetic acid) at room temperature for 30 min. Before taking photographs, all plates were labeled and washed five times with 1% acetic acid. The number of colonies containing 50 cells or more was counted under a microscope.

Drugs and nomenclature

NAV2729 (3-(4-chlorophenyl)-5-(4-nitrophenyl)-2-(phenylmethyl)pyrazolo[1,5-a]pyrimidin-7-(4H)-one) is an ARF inhibitor with assumed selectivity for ARF6 as described above. secinH3 (N-[4-[5-(1,3-benzodioxol-5-yl)-3-methoxy-1H-1,2,4-triazol-1-yl]phenyl]-2-(phenylthio)acetamide) is a cytohesin family–specific inhibitor, showing IC50 values of 2.4, 5.4, 5.6, 5.6, 65, and >100 μM for human cytohesin-2, human cytohesin-1, mouse cytohesin-3, human cytohesin-3, Drosophila stepkpe, yGea2-S7, and hEFA6-S7, respectively, as described recently. Stock solutions (10 mM) were prepared in DMSO and stored at −20 °C until use. Phenylephrine ([R]-3-[1-hydroxy-2-(methylamino)ethyl]phenol) and methoxamine (α-1-aminoethyl)-2,5-dimethoxybenzyl alcohol) are selective agonists for α1-adrenoceptors. U46619 ([Z]-7-[(1S,4R,5R,6S)-5-[(E,3S)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enonic acid) is an analogue of thromboxane A2 and frequently used as an agonist for thromboxane A2 receptors. Aqueous stock solutions of phenylephrine and noradrenaline (10 mM) were freshly prepared before each experiment. Stock solutions of U46619 were prepared in ethanol and stored at −80 °C until use. Aqueous stock solutions of endothelin-1 were stored at −20 °C until use. NAV2729, secinH3, and U46619 were obtained from Tocris (Bristol, UK); phenylephrine, methoxamine, and noradrenaline were obtained from Sigma (Munich, Germany);
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and endothelin-1 was from Enzo Life Sciences (Lörrach, Germany).

Statistical analysis

Data are presented as mean ± S.E. with the indicated number (n) of independent experiments. Multivariate analysis of variance and two-way analysis of variance were used for unpaired observations and were performed using SPSS® version 20 (IBM SPSS Statistics, IBM Corp., Armonk, NY). p values <0.05 were considered statistically significant. Spearman’s correlation analysis was performed using GraphPad Prism 6 (Statcon, Wittenhausen, Germany). All groups included in the statistical analyses were based on five or more independent experiments and included tissues from five or more patients in each group in the case of experiments performed with human tissues. For some series (endothelin-1–induced contractions, pulldown assays for ARF1 and RhoA, MYPT1/MLC-detection in WMPY-1 cells), it was obvious after less than five experiments that no effect could be expected. Therefore, these series were stopped after three or four independent experiments, and no statistical tests were applied to the results. Thus, the minimum group size subjected to statistical tests was n = 5. Moreover, all groups being compared with each other by statistical tests showed identical group sizes; consequently, any statistical comparisons between groups of different sample sizes or between groups composed of tissues from different patients were not performed.

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