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Membrane androgen receptor sensitive Na^+/H^+ exchanger activity in prostate cancer cells

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ABSTRACT

Membrane androgen receptors (mAR) are expressed in several tumors. mAR activation by testosterone albumin conjugates (TAC) suppresses tumor growth and migration. mAR signaling involves phosphoinositide-3-kinase (PI3K) and Rho-associated protein kinase (ROCK). PI3K stimulates serum- and glucocorticoid-inducible kinase SGK1, which in turn activates Na^+/H^+-exchangers (NHE). In prostate cancer cells cytosolic pH (pH_i) was determined utilizing 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-fluorescence and NHE-activity utilizing Na^+-dependent cytosolic realkalinization following an ammonium pulse. TAC (100 nM) significantly increased pH_i and NHE-activity, effects abrogated by NHE1-inhibitor cariporide (10 μM), SGK1-inhibitors EMD638683 (50 μM) and GSK650349 (10 μM) and ROCK-inhibitors Y-27632 (10 μM) and fasudil (100 μM). TAC treatment rapidly and significantly increased cell volume and actin polymerization, effects abolished in the presence of cariporide. Thus, mAR-activation activates cariporide-sensitive Na^+/H^+-exchangers, an effect requiring SGK1 and ROCK activity.

1. Introduction

Membrane androgen receptors (mAR) are expressed in a variety of tumor cells including prostate [1,2], breast [3,4] and colon [5] cancer cells. Expression and function of mAR are up-regulated by the serum & glucocorticoid inducible kinase SGK1 [6]. Activation of mAR receptors by specific testosterone albumin conjugates (TAC) in prostate cancer cells triggers the non-genomic FAK/PI3K/Akt/Rac1 and RhoA/B/ROCK/LimK/Cofilin signaling pathways [1,2] leading to rapid actin reorganization, while late downregulation of these signaling pathways [1,2] was shown to regulate various long-term mAR-governed cell responses such as inhibition of cell migration and invasiveness as well as induction of apoptosis [1,7–10]. Along those lines activation of mAR counteracts tumor growth *in vivo* [5,11,12] and mAR stimulators are considered for the treatment of malignancy [10,13].

Carriers participating in the regulation of cell volume [14–17], migration [14,18–20] and cell survival [21] include the Na^+/H^+ exchanger. Signaling regulating its activity includes Rho/ROCK [22] and SGK1 [23]. However, whether mAR activation leads to altered Na^+/H^+ exchanger activity, remained elusive.

The present study thus explored the influence of mAR activation on Na^+/H^+ exchanger activity. To this end experiments have been performed in prostate cancer cells prior to and following stimulation with testosterone albumin conjugates (TAC).

2. Materials and methods

2.1. Cell culture

DU145 and LNCaP prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and were studied exactly as previously described [1,2,8] ensuring cell authentication. Both cell lines were cultured in RPMI-1640 medium, containing 10% fetal calf serum (FCS), 1% antibiotic/antimycotic solution and 2.05 mM glutamine. For the experiments 10^6 cells were seeded in 35 mm culture dish or chamber slides (BD Biosciences).

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2.2. Treatments

Before each experiment testosterone-3-(O-carboxymethyl) oxime-BSA (Sigma), named testosterone–albumin conjugate (TAC), was dissolved in serum-free culture medium to a final concentration of 10 μM. This stock solution was incubated for 30 min at room temperature with 0.3% charcoal and 0.03% dextran, centrifuged at 3000×g and passed through a 0.45 μm filter to remove any potential contamination with free steroid. The TAC solution was used in a final concentration of 100 nM throughout all studies. Where indicated, the cells were further exposed to Na^+/H^+ exchanger inhibitor cariporide (10 μM, kind gift from Aventis), Rho-associated kinase (ROCK) inhibitors Y-27632 (10 μM, Selleckchem) or fasudil (100 μM Selleckchem) and SGK1 inhibitors EMD638683 (50 μM, kind gift from Merck Darmstadt) or GSK650349 (10 μM).

2.3. Determination of cytosolic pH

For digital imaging of cytosolic pH (pH_i), the cells were incubated in a HEPES-buffered Ringer solution containing 10 μM bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)-AM (Molecular Probes, Leiden, The Netherlands) for 15 min at 37 °C [24]. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epifluorescence mode with a 40 × oil immersion

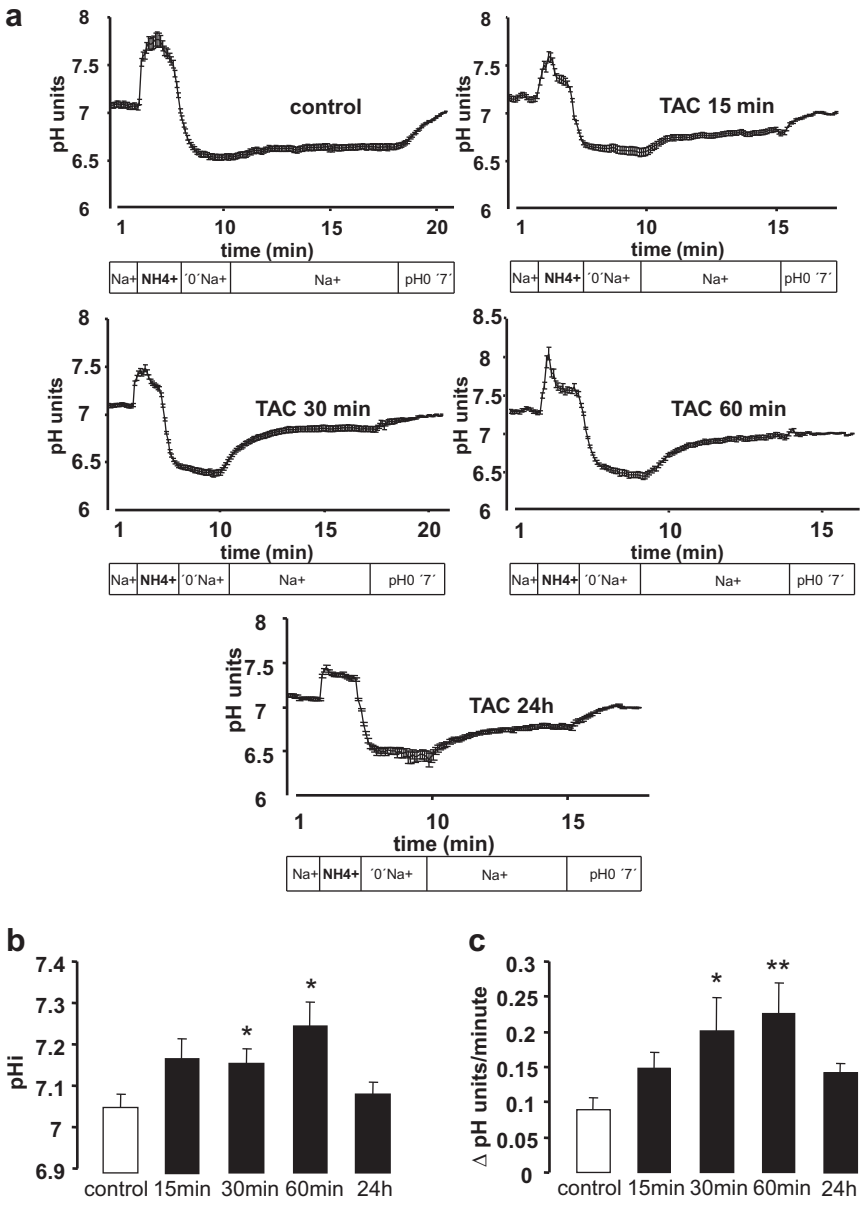

Fig. 1. Effect of mAR activation by TAC on cytosolic pH and Na^+/H^+ exchanger activity in DU145 prostate cancer cells. Alterations of cytosolic pH (pH_i) in prostate cancer cells following an ammonium pulse without or with prior treatment with testosterone albumin conjugates (TAC). (a) Time dependent changes (±S.E.M.s) of cytosolic pH in typical experiments prior to (control) and following a 15, 30, 60 min and 24 h treatment with 100 nM testosterone albumin conjugates (TAC). (b) Arithmetic means ± S.E.M. ($n = 8$ independent experiments) of cytosolic pH prior to the ammonium pulse (pH_i) in prostate cancer cells without (white bars) or with (black bars) prior treament with 100 nM testosterone albumin conjugates (TAC) for the indicated time periods. *($P < 0.05$) indicates statistically significant difference from absence of TAC. (c) Arithmetic means ± S.E.M. ($n = 8$ independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) in prostate cancer cells without (white bars) or with (black bars) prior treament with 100 nM testosterone albumin conjugates (TAC) for the indicated time periods. *($P < 0.05$) and **($P < 0.01$) indicates statistically significant difference from absence of TAC.

objective (Zeiss Neoplan, Germany). BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA) [25]. Between 10 and 20 cells were outlined and monitored during the course of the measurements [26]. The results from each cell were averaged and taken for final analysis. Intensity ratio (490/440) data were converted into pH_i values using the high-K^+/nigericin calibration technique [27]. To this end, the cells were perfused at the end of each experiment for 5 min with standard high-K^+/nigericin (10 μg/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the r_{max}, r_{min}, pK_a values previously generated from calibration experiments to generate a non-linear standard curve (pH range 5–8.5) [25].

For acid loading, cells were transiently exposed to a solution containing 20 mM NH_4Cl leading to initial alkalinization of cytosolic pH (pH_i) due to entry of NH_3 and binding of H^+ to form NH_4^+ [25,28]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (ß) of the

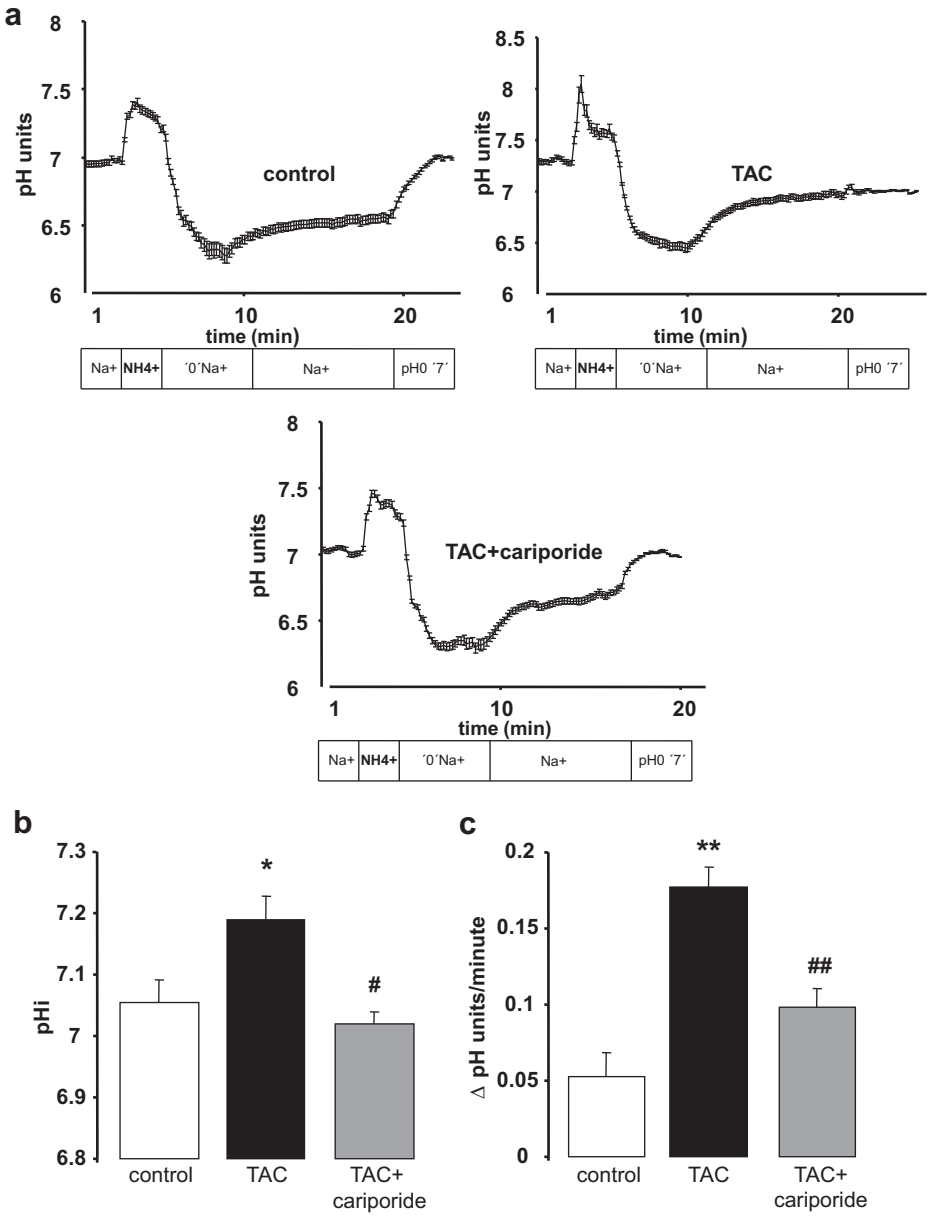

Fig. 2. Influence of NHE1 inhibitor cariporide on the effect of TAC on cytosolic pH and Na^+/H^+ exchanger activity in prostate cancer cells. Alterations of cytosolic pH (pH_i) in prostate cancer cells following an ammonium pulse without or with prior treatment with testosterone albumin conjugates (TAC) in the absence and presence of NHE1 inhibitor cariporide (10 μM). (a) Time dependent changes (±S.E.M.s) of cytosolic pH in typical experiments following a 60 min treatment without (control) or with 100 nM testosterone albumin conjugates alone (TAC) or together with NHE1 inhibitor cariporide (TAC + cariporide). (b) Arithmetic means ± S.E.M. ($n = 6$ independent experiments) of cytosolic pH prior to the ammonium pulse (pH_i) in prostate cancer cells without (white bar) or with prior treatment with 100 nM testosterone albumin conjugates (TAC) for 60 min in the absence (black bar) and presence (light grey bar) of NHE1 inhibitor cariporide (10 μM). $^*(P < 0.05)$ indicates statistically significant difference from absence of TAC. $^\#(P < 0.05)$ indicates statistically significant difference from TAC in the absence of cariporide. (c) Arithmetic means ± S.E.M. ($n = 6$ independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) in prostate cancer cells without (white bar) or with prior treatment with 100 nM testosterone albumin conjugates (TAC) for 60 min in the absence (black bar) and presence (light grey bar) of NHE1 inhibitor cariporide (10 μM). $^{**}(P < 0.01)$ indicates statistically significant difference from absence of TAC. $^{\#\#}(P < 0.01)$ indicates statistically significant difference from TAC in the absence of cariporide.

Table 1
Cytosolic pH (ΔpH_i), sodium independent and sodium dependent pH recovery in prostate cancer cells prior to (control) and following treatment with Na^+/H^+ exchanger NHE1 inhibitor cariporide (10 μM), SGK1 inhibitor EMD638683 (50 μM) and GSK650349 (10 μM), ROCK inhibitor Y-27632 (10 μM) and fasudil (100 μM) and mAR activator TAC (100 nM). All the values of NHE1, SGK1 and ROCK inhibitors are representative of DU145 cells for 1 h incubation.

	Intracellular pH (units)	Sodium independent pH recovery (Δ pH units/min)	Sodium dependent pH recovery (NHE activity, Δ pH units/min)	Number of cells
Control $_{DU145}$	7.23 ± 0.05	−0.02 ± 0.01	0.07 ± 0.01	158
Control $_{LNCaP}$	7.25 ± 0.06	−0.013 ± 0.005	0.05 ± 0.02	74
Cariporide	7.06 ± 0.01	−0.03 ± 0.01	0.10 ± 0.01	118
EMD638683	7.11 ± 0.01	−0.02 ± 0.01	0.09 ± 0.005	96
GSK650349	7.24. ± 0.05	−0.03 ± 0.004	0.11 ± 0.008	99
Y-27632	7.12 ± 0.03	−0.02 ± 0.01	0.08 ± 0.01	124
Fasudil	7.21 ± 0.08	−0.016 ± 0.003	0.07 ± 0.01	135
TAC 1hr $_{DU145}$	7.42 ± 0.05*	−0.02 ± 0.007	0.18 ± 0.01**	174
TAC 1hr$_{LNCaP}$	7.46 ± 0.05*	−0.03 ± 0.01	0.15 ± 0.007**	121

* ($P < 0.05$) Indicates significant difference from control.
** ($P < 0.01$) Indicates significant difference from control.

cells [28,29]. Assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3:

$$\beta = \Delta[NH_4^+]_i/\Delta pH_i,$$

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[NH_4^+]_i$ is the decrease of cytosolic NH_4^+ concentration, which is identical to the concentration of $[NH_4^+]_i$ immediately before the removal of ammonia. The pK for NH_4^+/NH_3 is 8.9 [30,31] and at an extracellular pH (pH_o) of 7.4 the NH_4^+ concentration in extracellular fluid ($[NH_4^+]_o$) is 19.37 [20/(1 + 10^{pHo-pK})]. The intracellular NH_4^+ concentration ($[NH_4]_i$) was calculated from:

$$[NH_4]_i = 19.37 \cdot 10^{pHo-pHi}.$$

The calculation of the buffer capacity required that NH_4^+ exits completely. After the initial decline, pH_i indeed showed little further change in the absence of Na^+, indicating that there was no relevant further exit of NH_4^+.

To calculate the ΔpH/min during re-alkalinization, a manual linear fit was placed over a narrow pH range (pH 6.7–6.9) which could be applied to all measured cells.

The solutions were composed of (in mM): standard HEPES: 115 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4 10 glucose, 32.2 HEPES; sodium free HEPES: 132.8 NMDG Cl, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 KH_2PO_4, 32.2 HEPES, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl); high K^+ for calibration 105 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 32.2 HEPES, 10 mannitol, 5 μM nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37 °C.

2.4. Forward scatter estimation

Forward scatter in prostate cancer cells was determined using flow cytometry. Briefly, 2×10^5 cells were taken in a culture dish and treated with TAC ± cariporide as described above. After the treatment, cells were collected and were centrifuged at 1200 rpm for 2–5 min and the pellet was washed twice with ice-cold PBS. The pellet was then resuspended in FACS buffer and the forward scatter was analysed with a flow cytometer (FACS-calibur from Becton Dickinson; Heidelberg, Germany).

2.5. Quantitative RT-PCR

To determine the gene expression of the four NHE members (NHE1–4) DU145 cells were seeded in a 35 mm dish at a density of 2×10^5 and cultured for 48 h in a complete medium (Gibco RPMI 1640, with 10% FBS and 1% penicillin/streptomycin.) under standard conditions. After washing twice with PBS cells were lysed with 1 ml TriFast Reagent (Peqlab, Erlangen, Germany). The RNA was isolated according to the manufacturer's protocol. 2,5 μg of the RNA were transcribed to cDNA using the GoScript™ Reverse Transcription System (Promega Corporation, Madison, USA) and random hexamer primers. Quantitative real-time PCR of the respective genes were performed on the CFX96 cycler (Bio-Rad, Hercules, CA) in a total volume of 20 μl using 2 μl of cDNA, 500 nM forward and reverse primer and 2× GoTaq® qPCR Master Mix (Promega Corporation, Madison, USA). Cycling conditions were as described: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s.

For the amplification the following primers were used (5′ → 3′orientation):

NHE1 forward primer: ACCTGGTTCATCAACAAGTTCCG
NHE1 forward reverse primer: TTCACAGCCAACAGGTCTACCA
NHE2 forward primer: TCCTGCTGCTGTGTTTCCTC
NHE2 forward reverse primer: AGTTGTGACCCCAATGTCCAC
NHE3 forward primer: AACGAGGTCCTGTTCATCATCG
NHE3 forward reverse primer: TCGATGATACGCACATGCTTG
NHE4 forward primer: ACGAGCAGCTCTACATGATGATC
NHE4 forward reverse primer: ACATGAAGACGATGAGTGGCTC
GAPDH forward primer: TGAGTACGTCGTGGAGTCCACTG
GAPDH reverse primer: CACCACCAACTGCTTAGCACC

Relative quantification of the gene expression was achieved using the ΔΔCt method and GAPDH as housekeeping gene.

2.6. G-Total actin ratio measurements

The Triton X-100 soluble G-actin- and total-actin-containing fractions of cells exposed to TAC ± cariporide as described above were analyzed by Western blotting as previously described [32]. Briefly, cells treated as indicated were incubated in 500 μl of Triton extraction buffer (0.3% Triton X-100, 5 mM Tris HCl, 2 mM EGTA, 300 mM sucrose, 400 μM PMSF, 10 μM Leupeptin, 2 μM phalloidin, pH 7.4) for 5 min on ice. The Triton soluble fractions (G-actin) were precipitated with equal volumes of 6% perchloric acid (PCA). The Triton insoluble fractions (F-actin) were precipitated with 1 ml 3% PCA. Equal volumes from each fraction were subjected to SDS electrophoresis and proteins were transferred to nitrocellulose membrane. Membranes blocked with 5% non-fat dry milk were then incubated with monoclonal anti-actin antibody, followed by incubation with the appropriate labelled secondary antibody. Blots were developed using the enhanced chemiluminescence (ECL) western blot kit. Nitrocellulose blots were exposed for variable

lengths of time. Bands were quantified using PC-based image analysis (Image analysis Inc, Ontario, Canada). A decrease of the triton-soluble (G-) to the total (T-) actin ratio is indicative of actin polymerization.

2.7. Statistics

Data are provided as means ± S.E.M., *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test or ANOVA and results with $P < 0.05$ were considered statistically significant.

3. Results

The present study explored whether activation of the membrane androgen receptor (mAR) modified cytosolic pH (pH_i) and Na^+/H^+ exchanger activity in DU145 prostate cancer cells. To this end, BCECF fluorescence was determined. Activation of mAR by

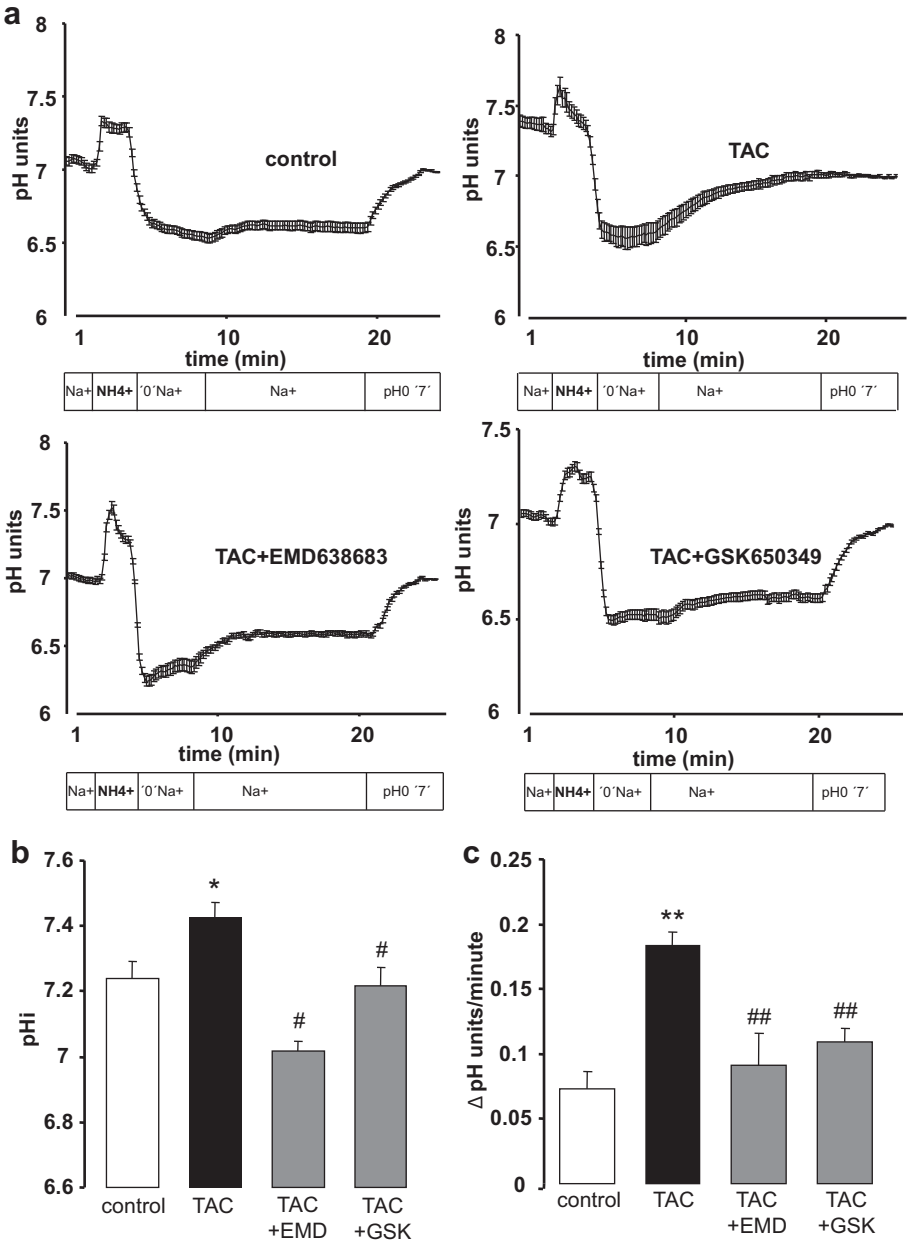

Fig. 3. Influence of SGK1 inhibitors EMD638683 and GSK65034 on the effect of TAC on cytosolic pH and NHE activity in prostate cancer cells. Alterations of cytosolic pH (pH_i) in prostate cancer cells following an ammonium pulse without or with prior treatment with testosterone albumin conjugates (TAC) in the absence and presence of SGK1 inhibitors EMD638683 (50 μM) and GSK650349 (10 μM). (a) Time dependent changes (±S.E.M.s) of cytosolic pH in typical experiments prior to (control) and following a 60 min treatment with 100 nM testosterone albumin conjugates (TAC) alone (TAC) or together with SGK1 inhibitor EMD638683 (TAC + EMD638683) and GSK650349 (TAC + GSK650349). (b) Arithmetic means ± S.E.M. ($n = 7$ independent experiments) of cytosolic pH prior to the ammonium pulse (pH_i) in prostate cancer cells without (white bar) or with prior treatment with 100 nM testosterone albumin conjugates (TAC) for 60 min in the absence (black bar) and presence (light grey bar) of SGK1 inhibitor EMD638683 (50 μM) and GSK650349 (10 μM). $^*(P < 0.05)$ indicates statistically significant difference from absence of TAC. $^\#(P < 0.05)$ indicates statistically significant difference fromTAC in the absence of EMD638683 or GSK650349. (c) Arithmetic means ± S.E.M. ($n = 7$ independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) in prostate cancer cells without (white bar) or with prior treatment with 100 nM testosterone albumin conjugates (TAC) for 60 min in the absence (black bar) and presence (light grey bar) of SGK1 inhibitor EMD638683 (50 μM) and GSK650349 (10 μM). $^{**}(P < 0.01)$ and $^{\#\#}$ $(P < 0.01)$ indicates statistically significant difference from absence of TAC. $^\#(0.05)$ indicates statistically significant difference from TAC in the absence of EMD638683 or GSK650349.

100 nM testosterone albumin conjugates (TAC) increased pH_i, an effect reaching statistical significance within 30–60 min (Figs. 1b and 2b). Similar results were obtained in LNCaP prostate cancer cells (Suppl. Fig. 1).

Na^+/H^+ exchanger activity in both prostate cancer cell lines was subsequently estimated with the ammonium pulse technique. Loading of the cells with H^+ was accomplished by transient exposure to 20 mM NH_4Cl. As illustrated in Figs. 1 and Suppl. Fig. 1, the addition of NH_4Cl in the extracellular bath was followed by a sharp cytosolic alkalinization due to NH_3 entry into the cells followed by binding of H^+ to form NH_4^+. The subsequent removal of NH_4Cl was followed by sharp cytosolic acidification due to NH_3 exit and cellular H^+ retention (Fig. 1 and Suppl. Fig. 1). Neither in the absence nor in the presence of TAC the acidification was followed by significant realkalinization as long as Na^+ was absent (Table 1). Thus, the cells did not express appreciable Na^+-independent H^+ extruding transport in the absence or presence of TAC. The addition of Na^+ was followed by a rapid cytosolic realkalinization pointing

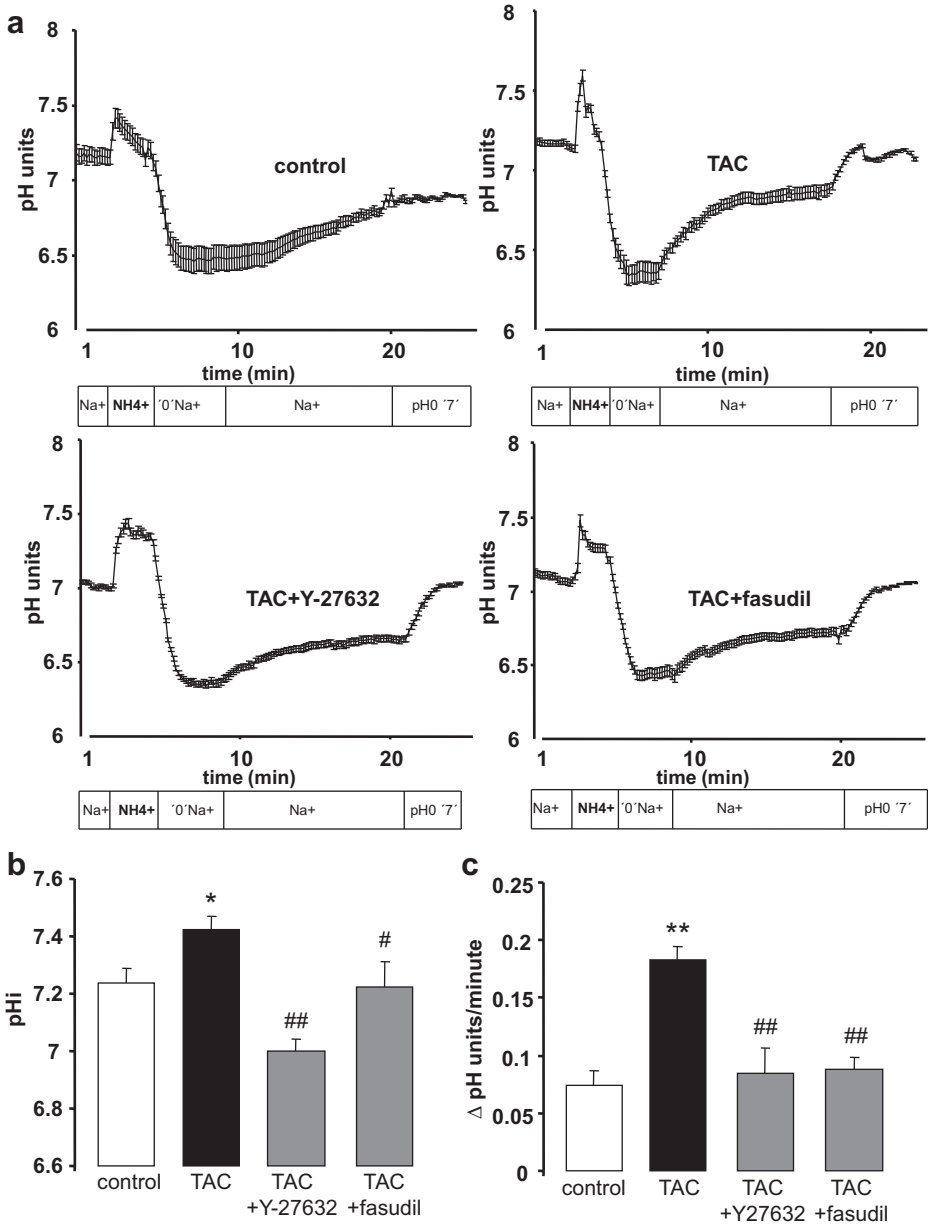

Fig. 4. Influence of ROCK inhibitor Y-27632 and fasudil (HA-1077) on the effect of TAC on cytosolic pH and NHE activity in prostate cancer cells. Alterations of cytosolic pH (pH_i) in prostate cancer cells following an ammonium pulse without or with prior treatment with testosterone albumin conjugates (TAC) in the absence and presence of ROCK inhibitors Y-27632 (10 μM) and fasudil (100 μM). (a) Time dependent changes (±S.E.M.s) of cytosolic pH in typical experiments following a 60 min treatment without (control) or with 100 nM testosterone albumin conjugates in the absence (TAC) and presence (TAC + Y-27632) of ROCK inhibitor Y-27632 and fasudil (TAC + fasudil). (b) Arithmetic means ± S.E.M. ($n = 7$ independent experiments) of cytosolic pH prior to the ammonium pulse (pH_i) in prostate cancer cells without (white bar) or with prior treatment with 100 nM testosterone albumin conjugates (TAC) for 60 min in the absence (black bar) and presence (light grey bar) of ROCK inhibitor Y-27632 (10 μM) and fasudil (100 μM). $^*(P < 0.05)$ indicates statistically significant difference from absence of TAC. $^{\#\#}(P < 0.01)$ and $^{\#}(P < 0.05)$ indicates statistically significant difference from TAC in the absence of Y-27632 and fasudil respectively. (c) Arithmetic means ± S.E.M. ($n = 7$ independent experiments) of Na^+-dependent recovery of cytosolic pH (ΔpH/min) in prostate cancer cells without (white bar) or with prior treatment with 100 nM testosterone albumin conjugates (TAC) for 60 min in the absence (black bar) and presence (light grey bar) of ROCK inhibitor Y-27632 (10 μM) and fasudil (100 μM). $^{**}(P < 0.01)$ indicates statistically significant difference from absence of TAC. $^{\#\#}(p < 0.01)$ indicates statistically significant difference from TAC in the absence of Y-27632 and fasudil, respectively.

to Na^+/H^+ exchanger activity. As illustrated in Fig. 1, the pH_i recovery following addition of Na^+ was accelerated by prior exposure to TAC for 15–60 min. Thus, TAC enhanced Na^+ dependent realkalinization reflecting Na^+/H^+ exchanger activity. Interestingly, after long-term (24 h) TAC treatment of DU145 and LNCaP prostate cancer cells, pHi increase and Na^+/H^+ exchanger activity returned to control levels (Fig. 1b and Suppl. Fig. 1), indicating a transient TAC-effect.

In order to further define the mechanism accomplishing Na^+ dependent realkalinization, experiments were performed in the presence of Na^+/H^+ exchanger 1 (NHE1) inhibitor cariporide. Prior to TAC treatment, cariporide did not significantly modify pH_i or Na^+-dependent realkalinization (Table 1). As illustrated in Fig. 2, cariporide significantly blunted the stimulation of Na^+-dependent cytosolic realkalinization by TAC and virtually abrogated the TAC-induced increase of pH_i.

A further series of experiments was performed to gain insight into the signaling underlying the stimulating effect of TAC on Na^+/H^+ exchanger activity. The involvement of SGK1 was tested by performing experiments in the absence and presence of the SGK inhibitors EMD638683 (50 μM) and GSK650349 (10 μM). Prior to TAC treatment, inhibitors did not significantly modify pH_i or Na^+-dependent realkalinization (Table 1). As illustrated in Fig. 3, EMD638683 and GSK650349 significantly blunted the increase of pH_i and Na^+/H^+ exchanger activity following TAC treatment.

The involvement of Rho-associated protein kinase (ROCK) was tested utilizing the ROCK inhibitors Y-27632 (10 μM) and fasudil (100 μM). Prior to TAC treatment, Y-27632 did not significantly modify pH_i or Na^+-dependent realkalinization (Table 1). As illustrated in Fig. 4, Y-27632 and fasudil (100 μM) significantly blunted the increase of pH_i and Na^+ dependent realkalinization following TAC treatment for 60 min.

RT-PCR was employed to define the expression of the various NHE isoforms. As illustrated in Suppl. Fig. 2 DR145 cells predominantly express NHE1 and to a lesser extent NHE2.

Since NHEs are known to be involved in cell volume regulation [33] we further addressed the role of TAC in modifying DU145 cell volume in the presence and absence of cariporide. As shown in Fig. 5a and b, TAC treatment induced a rapid and significant cell volume increase, an effect abolished in the presence of cariporide.

Actin cytoskeleton reorganization, which represents a main regulatory step in cell volume regulation [34–36], has been shown to regulate as well NHE exchanger activity [37]. On the other hand TAC induces potent actin polymerization in DU145 cells [1]. Accordingly, we further tested the effect of cariporide in actin restructuring upon TAC treatment. In line with the previous reports [1], TAC generated early and significant actin polymerization (Fig. 5d). This effect was abrogated to insignificant levels in the presence of cariporide (Fig. 5c), suggesting involvement of NHE activity in TAC-induced actin reorganization.

4. Discussion

The present study reveals that TAC acutely activates the cariporide sensitive Na^+/H^+ exchanger in DU145 and LNCaP prostate

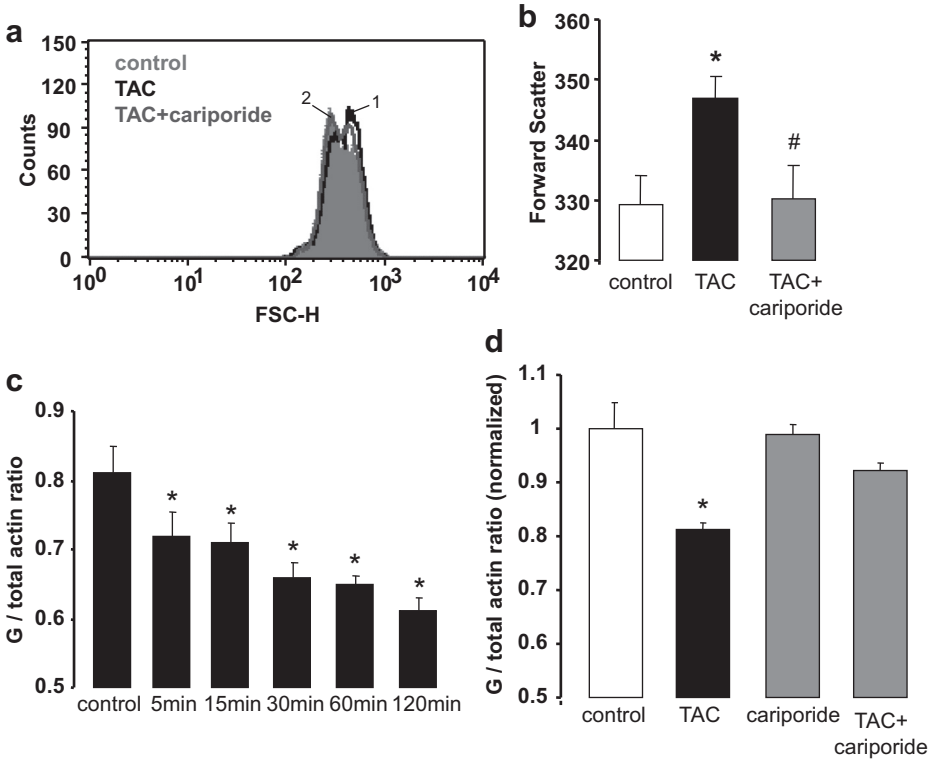

Fig. 5. Effect of NHE1 inhibitor cariporide on the effect of TAC on forward scatter and actin polymerization in prostate cancer cells. (a) Representative FACS histograms depicting the forward scatter of prostate cancer cells prior to (control, grey shadow) and following a 60 min treatment with 100 nM testosterone albumin conjugates alone (1, TAC, black line) or together with NHE1 inhibitor cariporide (2, TAC + cariporide, grey line). (b) Arithmetic means ± S.E.M. ($n = 6$ independent experiments) of forward scatter in prostate cancer cells without (white bar) or with prior treament with 100 nM testosterone albumin conjugates (TAC) for 60 min in the absence (black bar) and presence (light grey bar) of NHE1 inhibitor cariporide (10 μM). *($P < 0.05$) indicates statistically significant difference from absence of TAC. #($P < 0.05$) indicates statistically significant difference from TAC in the presence of cariporide (TAC + Cariporide). (c) Time course of the arithmetic means ± S.E.M. ($n = 8$) of G/Total actin ratio in DU145 prostate cancer cells treated with 100 nM TAC for the indicated time. *($P < 0.05$) indicates statistically significant difference to control value. (d) Arithmetic means ± S.E.M. ($n = 6$ independent experiments) of G/Total actin ratio (normalized) in prostate cancer cells without (white bar) or with prior treament with 100 nM testosterone albumin conjugates (TAC) for 60 min in the absence (black bar) and presence of NHE1 inhibitor cariporide (10 μM, grey bars). *($P < 0.05$) indicates statistically significant difference from absence of TAC.

cancer cells. The effect was rapid, implying a non-genomic membrane androgen effect. As a matter of fact DU145 cells fail to respond to transcriptional androgen regulation either due to the expression of non-functional intracellular androgen receptors (iAR) [38], or due to complete lack of iAR [39]. On the other hand DU145 cells express functional membrane androgen receptors that may induce multiple non-genomic androgen actions [1,12].

Interestingly, after long-term (24 h) TAC treatment of DU145 and LNCaP prostate cancer cells pHi increase and Na^+/H^+ exchanger activity returned to control levels (Fig. 1b and Suppl. Fig. 1), indicating that this TAC-effect is a transient phenomenon. This transient effect may also account for the seeming paradox that TAC stimulates the sodium hydrogen exchangers which is known to participate in the machinery accomplishing cell migration [40] and yet inhibits cell migration as previously reported [7].

According to previous studies [41], Na^+/H^+ exchanger activity is modified by phosphatidylinositide (PI) 3 kinase dependent signaling and may be disrupted by PI3K inhibitors wortmannin and LY294002. The effect of PI3 kinase signaling may be partially mediated by Akt and SGK isoforms. Both, Akt2 [42] and SGK1 [23] have previously been shown to upregulate Na^+/H^+ exchanger activity. SGK1 may further be effective by upregulating mAR [6]. According to the present observations the SGK specific inhibitors EMD638683 and GSK650349 completely inhibited the increase of Na^+/H^+ exchanger activity and cytosolic alkalinization following TAC treatment.

Similarly, exposure of prostate cancer cells to ROCK inhibitors Y-27632 and fasudil fully abrogated the increase of Na^+/H^+ exchanger activity following TAC treatment indicating that stimulation of ROCK is required for the stimulation of Na^+/H^+ exchanger activity by mAR. This result is in line with previously reported findings that the Na^+/H^+ exchanger may act downstream of RhoA and participate in the integrin-induced cytoskeletal reorganization [22]. Since mAR activation in DU145 cells triggers actin reorganization by early stimulation of RhoA/B/ROCK signaling [1], our results imply that Na^+/H^+ exchanger activation by TAC may contribute to actin signaling that controls late cellular outcomes in prostate cancer cells [7,8]. Although further experiments are needed to address this hypothesis, our finding showing inhibition of TAC-induced actin polymerization in the presence of cariporide (Fig. 5c) provides experimental evidence for a possible involvement of NHE activation in mAR-governed actin reorganization.

TAC treatment induced as well rapid and significant cell volume increase in DU145 cells an effect abolished in the presence of cariporide (Fig 5a and b). Na^+/H^+ exchanger activity is known to impact on cell volume, which is regulated in a multitude of cells by parallel activity of Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger [16,17]. The carriers mediate the entry of Na^+ and Cl^- in exchange for H^+ and HCO_3^-, respectively. H^+ and HCO_3^- are replenished from CO_2 and are thus not osmotically relevant [16,17]. The mAR-associated effect fully supports the regulatory role of NHE exchanger in cell volume control [33] and is in line with the inhibition of TAC-induced actin polymerization by cariporide.

RT-PCR analysis (Suppl. Fig. 2) revealed that NHE1- and to a lesser extent NHE2-isoforms are predominantly expressed in DU145 cells, indicating that TAC may stimulate further NHE-isoforms. This assumption may be supported as well by the observation that cariporide treatment did not seem to completely abolish the TAC-induced NHE stimulation (Fig. 2).

At least in theory, TAC sensitive Na^+/H^+ exchanger activity may impact on several cellular functions. Cytosolic pH modifies reactive oxygen species (ROS) formation [43,44], which may thus be regulated by Na^+/H^+ exchanger activity. Conversely, Na^+/H^+ exchanger activity is stimulated by ROS [41,45]. Cytosolic pH contributes to the regulation of diverse further cellular functions [46–52], which may thus be modified by mAR sensitive Na^+/H^+ exchanger activity.

5. Conclusion

The present study demonstrates that TAC activates the cariporide sensitive Na^+/H^+ exchanger. The effect requires both SGK and Rho/ROCK signaling.

Conflict of interest

The authors declare that they do not have any conflicts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.02.040.

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