Phosphorylation of carbonic anhydrase IX controls its ability to mediate extracellular acidification in hypoxic tumors

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Running title: Hypoxia-induced PKA-mediated phosphorylation of CA IX

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Abstract

In the hypoxic regions of a tumor, carbonic anhydrase IX (CA IX) is an important transmembrane component of the pH regulatory machinery that participates in bicarbonate transport. Since tumor pH has implications for growth, invasion and therapy, determining the basis for the contributions of CA IX to the hypoxic tumor microenvironment could lead to new fundamental and practical insights. Here we report that Thr443 phosphorylation at the intracellular domain of CA IX by protein kinase A (PKA) is critical for its activation in hypoxic cells, with the fullest activity of CA IX also requiring dephosphorylation of Ser448. PKA is activated by cAMP, which is elevated by hypoxia, and we found that attenuating PKA in cells disrupted CA IX-mediated extracellular acidification. Moreover, following hypoxia induction, CA IX co-localized with the sodium-bicarbonate cotransporter as well as other PKA substrates in the leading edge membranes of migrating tumor cells, in support of the concept that bicarbonate metabolism is spatially regulated at cell surface sites with high local ion transport and pH control. Using chimeric CA IX proteins containing heterologous catalytic domains derived from related CA enzymes, we demonstrated that CA IX activity was modulated chiefly by the intracellular domain where Thr443 is located. Our findings indicate, that CA IX is a pivotal mediator of the hypoxia-cAMP-PKA axis, which regulates pH in the hypoxic tumor microenvironment.

Key words: carbonic anhydrase, hypoxia, phosphorylation, protein kinase A, cancer
Introduction

CA IX is a highly active α-carbonic anhydrase isoform expressed in a broad range of solid tumors, where it protects tumor cells from hypoxia and acidosis in the tumor microenvironment (1, 2). CA IX functions as an important component of a pH-regulating machinery that is activated in response to metabolic pathways triggered by hypoxia and/or oncogenic alterations (3). It participates in CO₂ diffusion and bicarbonate import as the extracellular constituent of a metabolon: a functional and spatially oriented complex with bicarbonate transporters, such as the anion exchanger and sodium-bicarbonate co-transporter (4, 5). In the metabolon, CA IX catalyzes hydration of pericellular CO₂ and thus ensures high local production of bicarbonate ions that are available for direct influx by the transporters. This results in intracellular neutralization, which is required for tumor cell survival. At the same time, protons as by-products of this CO₂ hydration reaction remain in the pericellular space, acidifying the microenvironment, and thereby supporting tumor cell invasiveness.

In line with this concept, CA IX is expressed and activated in cells exposed to chronic hypoxia, which is inherently linked to acidosis. Transcription of the CA IX-encoding gene is strongly activated by the hypoxia-inducible HIF-1 transcription factor that binds an HRE element localized in the proximal promoter region next to the transcription initiation site (6). Hypoxia and/or acidosis also induce the catalytic activity of CA IX thus providing an effective adaptive cellular response to microenvironmental stresses, including pH regulation and cell migration/invasion (7-10). Since hypoxia is associated with poor cancer prognosis and worse response to treatment, it is obvious that CA IX as its surrogate marker and functional component possesses clinical potential as a prognostic/predictive factor and target for anticancer treatment (11).

Functional inhibition of CA IX has been proposed as an attractive option for the therapeutic targeting of various hypoxic tumors (12). In this regard, major efforts are being directed towards inhibition of the catalytic activity of CA IX by sulfonamides and related compounds that bind to the enzyme’s active site (13). However, our recent studies have revealed that the catalytic domain is not the only determinant of CA IX activity and that the flanking regions, including the N-terminal PG
domain as well as the C-terminal intracellular (IC) tail, play a role in CA IX performance. Particularly an intact IC tail has been shown to be critical for the proper functioning of CA IX as mutations in its juxtamembrane region abolish the CA IX-mediated extracellular acidification in hypoxia (14). However, molecular events underlying this inside-out signaling have remained unexplored. Here we demonstrate for the first time that phosphorylation of the IC tail is involved in the control of CA IX ability to regulate pH, and that the Thr443 residue and cAMP-dependent protein kinase A are key players in this phenomenon.

Materials and methods

Cell culture

MDCK, HT-29, A549, HeLa and HEK293T/17 cells were obtained from the ATCC and grown in DMEM with 10% FCS (BioWhittaker). Cell lines were passaged for a maximum of 3 months, after which fresh seed stocks were thawed for experimental use. They were evaluated routinely for morphology and expression of CA IX. The cells (4x10^5 in 1.5 ml of medium) were plated in 3 cm dishes 24 h prior to 24-48 h incubation in hypoxia (2% O_2, 2% H_2, 5% CO_2, 91% N_2, anaerobic workstation, Ruskinn Technology). Parallel normoxic dishes were maintained in a cell culture incubator with 5% CO_2. At the end of each experiment, pH of the culture medium was measured immediately, the medium harvested for lactic acid determination with the Lactate Reagent kit (Trinity Biotech), and the cells were counted and processed for further analyses.

Inhibitor treatment of cells

The fluorescent carbonic anhydrase inhibitor (FITC-CAI) was obtained from homosulfanilamide and fluorescein isothiocyanate as described (7), dissolved in PBS containing 10% DMSO to 100 mM and diluted in culture medium just before addition to cells at 0.1 mM final concentration. The cells were incubated in hypoxia or normoxia for 44 hr, with the addition of FITC-CAI for the final 4 h. Its binding to live cells was imaged using a Leica DM4500B epifluorescence microscope.
Cloning of CA IX phosphorylation mutants and transfection

In vitro mutagenesis of Thr443 and Ser448 to Gly and/or Asp was performed by inverse PCR using the pSG5C-MN/CA9 expression plasmid containing the full-length human CA9 cDNA as a template (15, GenBank no. X66839). PCR amplification was done with Phusion polymerase (Finnzymes) as described in Supplementary information using primers listed in Supplementary Tab. 1. PCR products were gel-purified, cleaved by EcoRI and SacI and ligated with T4 DNA ligase (Invitrogen). All constructs were verified by sequencing. MDCK cell lines constitutively expressing the wild type CA IX protein or its mutants were obtained by co-transfection of plasmids containing CA9 cDNA or its mutated versions with pSV2neo plasmid in a 10:1 ratio using NeoFectin™ transfection reagent (Mid-Atlantic Biolabs). Transfected cells were selected in 500 μg/ml G418, cloned, screened for CA IX expression by flow cytometry and expanded. Clonal cell lines expressing similar levels of each CA IX mutant were chosen for further analysis. The cells co-transfected with empty pSG5C and pSV2 neo were used as mock controls. All transfected cell lines were frozen as seed stock at early passage.

Cloning of chimeric CA IX with heterologous catalytic domains

Chimeric CA IX was generated by replacing the original catalytic domain (aa 137-390) with corresponding regions of either CA II (aa 2-259) or CA XII (aa 29-289). Relevant regions were amplified from cDNA of the human cell line Caki-1 (kindly provided by Prof. Parkkila, Institute of Medical Technology, Tampere, Finland) incubated at 2% O2 for 24 hours. The amplification was performed with chimeric primers matching the intended junctions between carbonic anhydrase IX, and CAII or XII. pBluescriptII-KS-MN/CA9 vector containing full-length CA9 cDNA (15) served as template for the generation of the CA IX N-terminal (aa 1-136) and C-terminal (aa 391-459) fragments using specific internal primers and vector-specific sequencing primers (Supplementary Tab. 1). These fragments were joined with CAII or CAXII, in two rounds of PCR to create fusion products. The chimeras were cloned into the eukaryotic expression vector pSG5C and resulting constructs were verified by sequencing.
ELISA

To determine surface versus total CA IX levels, transfected cells were plated in parallel in two microplates at a density of $10^4$ cells per well, each cell type in quadruplicates. The cells in one plate were first fixed in methanol, the cells in the second plate were assayed without fixation. The cells were then incubated at 37°C for 30 min with a biotinylated M75 monoclonal antibody diluted 1:5000 (200 ng/ml) in PBS. The amount of bound antibody was determined after 1 h incubation with the peroxidase-conjugated streptavidin (Pierce).

Immunoblotting

The cells were harvested in ice-cold RIPA buffer supplemented with inhibitors of proteases (Complete, Roche) and phosphatases (Phosphatase inhibitor cocktail 1 and 2, Sigma-Aldrich). Protein concentration was quantified using the BCA kit (Pierce). The proteins (50 μg/lane) were resolved in 10% SDS–PAGE under reducing and non-reducing conditions and transferred to PVDF membrane. Active PKA was detected by Phospho-PKA C-(Thr197) antibody, total PKA by PKA C-Alpha antibody, and PKA phosphorylated proteins were detected by Phospho-PKA Substrate (RRXS*/T*) antibody (all from Cell Signaling Technology, at 1:100 dilution). Total CA IX was detected by M75 antibody (15), CA IX phosphorylated at Thr443 by P-T443 CA IX antibody (1:5000, GenScript) and actin as a housekeeping protein was visualized by Actin (C11) antibody (Santa Cruz Biotechnology).

Immunoprecipitation of Phospho-Thr443-CA IX

The cells plated at 4x$10^6$ in 10 cm dishes were incubated in hypoxia or normoxia for 32 h. They were subsequently harvested in RIPA buffer supplemented with inhibitors of proteases and phosphatases. The lysates were precleared by centrifugation and with Protein A Sepharose CL-4B beads (GE Healthcare Bio-Sciences AB). In the meantime, 1 μg of phospho-Thr443 antibody was bound onto 50 μl of beads per sample. Beads with bound antibody were added to the precleared lysates and incubated O/N at 4 °C. Immunocomplexes were separated by SDS-PAGE and transferred
to PVDF membrane. Phosphorylated CA IX was detected by a peroxidase-conjugated M75 monoclonal antibody.

**In vitro phosphorylation of GST-tagged IC tail of CA IX by PKA**

A portion of CA IX cDNA representing the IC tail (nt 1345-1419) was cloned into pGEX-4T1 plasmid (GE Healthcare Bio-Sciences AB) via an EcoRI site from pSG5C-MN/CA9 or its mutated variant pSG5C-T→A, in which Thr443 was substituted with Ala. The fusion proteins GST-ICwt and GST-T→A were produced in Bl-21 competent cells and purified. Concentration of the purified fusion proteins was estimated on SDS-PAGE and the amount of proteins was adjusted to 1 μg per reaction using Glutathione Sepharose 4B beads (GE Healthcare Bio-Sciences AB) in equal volumes of samples. The beads were then washed three times with kinase buffer (20 mM magnesium acetate, 40 mM Tris-HCl, pH 7.4) and the reaction was started by addition of 30 μl of the kinase buffer supplemented with 1 mM DTT, 1μl of CS-PKA (Promega), 0.2 mM ATP (Sigma-Aldrich) and 1 μl of [γ-32P]ATP. The samples were incubated at 30 °C for 30 min and reaction was stopped by an equal volume of 2x Laemmli sample buffer. Finally, the 32P signal was assessed by SDS-PAGE and autoradiography. A similar procedure was applied to CA IX protein immunoprecipitated by M75 MAb from extracts of transfected MDCK cells.

**In vitro dephosphorylation assay**

For in vitro dephosphorylation assay, purified protein phosphatase 2 (PP2A) was used in PP2A A/C heterodimer (Upstate) or A/C/B55 heterotrimer form (prepared in-house). The assay was performed in phosphatase buffer (150 mM NaCl, 0.25% Nonidet P-40, proteases inhibitors, 50 mM Tris-HCl) containing 0.2 unit of PP2A dimer or trimer and 1 μg in vitro phosphorylated GST-ICwt protein at 30°C for 30 min. Termination of the dephosphorylation reaction and assessment of the 32P signal were performed analogously to the CS-PKA in vitro phosphorylation assay.

**Inhibition of PKA activity by dominant-negative PKA**
Inhibition of PKA activity was achieved using the pcDNA3.1+dnPKA plasmid encoding the murine dominant-negative mutant of PKA (DN-PKA) regulatory subunit I. The plasmid was obtained by recloning the dnPKA sequence from M7 pdnPKA-GFP (16716 Addgene plasmid, 16) to pcDNA3.1+ plasmid to remove GFP that would interfere with imaging of the FITC-labeled inhibitor of CA IX. MDCK-CA IX cells were transiently transfected with pcDNA3.1+dnPKA and an empty vector similarly as described above. After 24 hours the cells were subjected to normoxic or hypoxic treatment for 44 hours. Then 100 μl aliquots of growth media were used to measure pH. Next, 0.1 mM FITC-CAI was added to cells followed by cultivation for additional 4 hours. Finally, accumulation of inhibitor was imaged by fluorescence microscopy.

**Co-transfection of plasmids encoding CS-PKA, DN-PKA and CA IX into HEK293T/17 cells**

HEK293T/17 cells were co-transfected with various combinations of the following plasmids: pCalpha EV PKA encoding CS-PKA (pCo, Catalytic subunit C alpha, 15310 Addgene plasmid, 17), pcDNA3.1+dnPKA encoding DN-PKA (pdn), pSG5-C-MN/CA9 encoding CA IX (pCA9) and respective empty plasmids by NeoFectin™ in the ratio 10:1, 4 μg of plasmid DNA in total.

cAMP direct immunoassay and 8-Br-cAMP treatment

MDCK-CA IX cells were incubated either in normoxia or hypoxia for 32 hours. Then they were lysed in 0.1 M HCl and protein levels were determined. The assay was performed with 200 μg of proteins per sample using the cAMP Direct Immunoassay Kit (Biovision Research Products). To stimulate PKA activity in normoxia, MDCK-CA IX cells were grown for 48 h in normoxia, treated with 0.5 mM 8-Br-cAMP (Sigma) for 1 h, lysed and analyzed for CA IX Thr443 phosphorylation by immunoprecipitation and immunoblotting as described above.

**Immunofluorescence and confocal microscopy**

A549 cells grown on glass coverslips in a sparse monolayer were exposed to hypoxia for 24 h to induce CA IX expression and subsequently fixed in ice-cold methanol at −20 °C for 5 min. To co-

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localize CA IX with Na⁺/HCO₃⁻ co-transporter 1 (NBC1, SLC4A4), the fixed cells were first incubated with rabbit anti-NBC1 polyclonal antibody raised against aa 338-391 (1:100, AB 3212 Millipore) and then with mouse M75 monoclonal antibody against CA IX. Similar procedure was used to co-localize CA IX with phospho-PKA substrates.

Anti-mouse and anti-rabbit antibodies conjugated with Alexa Fluor 594 and Alexa Fluor 488 (1:2000, Invitrogen), respectively, were used as secondary antibodies. Nuclei were stained with DAPI (1:36 000, Sigma-Aldrich). Samples stained only with secondary antibodies and DAPI were used as negative control. All samples were analyzed by Zeiss LSM 510 Meta confocal microscope. The acquired images were processed in ImageJ using colocalization highlighter plugins.

**Migration assays**

For the wound-healing assay, MDCK cells transfected with pSG5C-MN/CA9, pSG5C-T→D/S→G, pSG5C-T→G/S→D plasmids were grown to confluent monolayers, wounded with a pipette tip, and allowed to migrate in presence of hepatocyte growth factor (HGF, 20ng/ml) in serum-free medium. The wound was photographed at the start of the healing process and after 10 hr. The diameter of the wound was measured and the closure was calculated in %.

For the scatter assay, cell clusters were obtained by rotation of suspended MDCK transfectants on an orbital shaker for 16 hr. The cell clusters were allowed to attach and spread on the bottom of dishes for 24 hr. The colonies (at least 20 for each cell type) were photographed at the beginning and at the end of the spreading period. The diameter of the scattered colonies was measured and their size was statistically analyzed using Student’s t test.

**Results**

**Mutations of potential phosphorylation sites Thr443 and Ser448 modulate CA IX-mediated extracellular acidification**

As described earlier, mutations of basic amino acids within the 436-444 region of the IC tail abolish CA IX ability to acidify extracellular pH (pHe) and bind the CA IX-selective sulfonamide
inhibitor in hypoxia (14). In order to shed light on the underlying mechanism(s), we investigated the role of phosphorylation. Cytoplasmic portion of the CA IX molecule contains three possible phosphorylation sites: Thr443, Ser448 and Tyr449. Previous data suggest that phosphorylated Tyr449 signals to the Akt/PI3K pathway (18), but no information is available on Thr443 and Ser448. Indeed, in silico analysis (NetPhosK 1.0) identified Thr443 and Ser448 as potential phosphorylation sites for several kinases.

To examine the biological role of these putative phosphorylation sites, we generated CA IX mutants with Thr443 or Ser448 individually substituted by glycine, a non-phosphorylatable residue. The corresponding T443→G and S448→G mutants were transfected into MDCK cells, an established functional model for CA IX-mediated extracellular acidification in hypoxia (7) and examined with respect to proper oligomerization and plasma membrane localization. The mutations had no effect on these properties since both mutants were indistinguishable from wild-type CA IX (Fig. 1A-C). However, the T443→G mutant was totally impaired in its ability to acidify pH in hypoxia. In contrast, the S448→G mutation resulted in acidosis comparable to the wild type protein (Fig. 1D). These results were corroborated by the accumulation of the FITC-labeled CA inhibitor (Fig. 1E), which in this cellular context binds only to the active enzyme (7). This data indicated that Thr443 is instructive to the activation of CA IX.

Cross-talk between phosphorylation of Thr443 and Ser448 regulates CA IX function, with dominant positive role of p-Thr443 and modifying negative role of p-Ser448

We then generated a series of CA IX double mutants at positions Thr443 and Ser448, either mimicking phosphorylation (by substitution with Asp) or dephosphorylation (by substitution with Gly) (Fig. 2A). None of the double mutants showed altered oligomerization ability or cell surface localization (not shown). However, pH measurements showed striking differences in the enzymatic activities of the various CA IX mutants.

As expected, mimicking phosphorylation at Thr443 while simultaneously preventing phosphorylation at Ser448 (T→D/S→G mutant) did not significantly alter enzymatic activity of CA IX under hypoxic conditions (Fig. 2B). However, this mutant was constitutively active and unlike
wild-type CA IX led to acidification of pHe in normoxia. This may indicate that phosphorylation at Ser448 negatively controls enzymatic activation in normoxia. The T→G/S→D mutant, mimicking phosphorylation at Ser448 and dephosphorylation at Thr448 had completely lost its acidification capacity in hypoxia. Similar observations were made with the T→G/S→G mutant non-phosphorylatable at both sites, suggesting that the release of the negative control provided by Ser448 is insufficient for hypoxic CA IX activation. Finally, the T→D/S→D mutant mimicking phosphorylation at both Thr443 and Ser448 demonstrated lower acidification activity in hypoxia than the wild-type CA IX. Altogether, these data enlightened the importance of Thr443 and Ser448 in regulating the catalytic activity of CA IX and suggested that phosphorylation of each of these residues may have opposing effects on the functional status of CA IX.

Our mutational analysis indicated that phosphorylation of Thr443 positively affected CA IX activity. Thus, we investigated whether this residue was indeed phosphorylated in vivo. To this aim, CA IX was immunoprecipitated from both MDCK-CA IX and HT-29 cells, cultured in normoxia or hypoxia, and analyzed by immunoblotting using an antibody specific to phosphorylated Thr443 (p-T443). As shown in Fig. 2C, phosphorylation of Thr443 was increased under hypoxic conditions in both cell types. Because hypoxia also activates CA IX enzymatic activity, this observation supported the view that phosphorylation of Thr443 might participate in the activation of CA IX in hypoxia and prompted us to search for the responsible kinase.

**Thr443 is a substrate of c-AMP-dependent protein kinase A**

The sequence around Thr443 perfectly matches the canonical recognition motif of c-AMP-dependent protein kinase A (PKA), i.e. RRXS*/T* (19). To test whether Thr443 is a bona fide phosphorylation site for PKA, we generated GST-fusion proteins of the 25-aa intracellular (IC) fragment of wt or T443→A mutant CA IX. These fusion proteins were subjected to an in vitro kinase assay in the presence of recombinant catalytic subunit of PKA (CS-PKA) and γ[^32P]-ATP (Fig. 3A). The IC domain of wild-type CA IX was very efficiently phosphorylated by CS-PKA (Fig. 3A). In contrast, the T443→A mutant was very poorly phosphorylated under the same conditions thus identifying Thr443 as the major PKA site in the IC domain of CA IX.
PP2A is the primary PKA-opposing phosphatase (20). We thus tested whether it could dephosphorylate PKA-phosphorylated Thr443. To this aim, we performed an in vitro dephosphorylation assay of PKA-phosphorylated GST-CA IX using recombinant PP2A. As shown in Fig. 3B, an active PP2A core enzyme efficiently dephosphorylated phospho-Thr443 of CA IX in vitro, suggesting that it might be involved in balancing the PKA-mediated CA IX activation and thus negatively regulating CA IX function.

In the next step, we used a “cold” PKA kinase assay followed by Western blotting with the p-T443 antibody to confirm that the antibody recognized the PKA-modified phospho-Thr443 in CA IX (Fig. 3C). The relationship between CS-PKA and phosphorylation of CA IX at Thr443 was demonstrated via co-transfection of plasmids encoding wt-CA IX (pCA9) and either CS-PKA (pCα) or the dominant negative mutant DN-PKA (pdn). For this purpose we used HEK293T/17 cells, which allow for high efficiency of transient transfection. Western blotting analysis of the transfected cell extracts was performed simultaneously with antibodies specific for the activated form of CS-PKA phosphorylated at Thr197, for phosphorylated Thr443 of CA IX and for a collection of PKA substrate proteins (Fig. 3D). The results revealed a clear Thr443-related phosphorylation signal only in cells co-transfected with pCA9 in combination with pCα, but not with the pdn plasmid encoding DN-PKA or with the empty plasmid. This corresponded well with the activation status of the CS-PKA protein, which was strongly phosphorylated in cells expressing CS-PKA but not DN-PKA. Weak endogenous CS-PKA phosphorylation that could be seen also in the mock-transfected cells was probably insufficient for the detection threshold of CA IX phosphorylated at Thr443. Accordingly, phosphorylation of the PKA substrates was much stronger in cells transfected with 3.64 μg of the pCα plasmid compared to ten times less plasmid and was negligible in the presence of pdn (Fig. 3D).

**PKA activation in hypoxia is associated with CA IX-mediated extracellular acidification**

To establish a functional link between PKA, hypoxia and CA IX activation, we examined the phosphorylation of Thr197 of CS-PKA, which correlates with the activity of the kinase. Levels of active CS-PKA were considerably higher in hypoxic A549 and HeLa carcinoma cells compared to
normoxic cells (Fig. 4A). Similar observations were made in MDCK-CA IX cells (Fig. 4A), where hypoxia-induced PKA activity was linked to significantly higher levels of cAMP in hypoxia versus normoxia (Fig. 4B). We then used a membrane-permeable cAMP analog 8-Br-cAMP to demonstrate that activation of PKA can lead to CA IX phosphorylation in normoxia, thus supporting the view that PKA is essential for the activation of CA IX (Fig. 4C).

To assess the functional relevance of CA IX regulation by PKA, we evaluated the role of PKA in CA IX-dependent extracellular acidification. No difference in pHe values was found between cells transiently transfected with DN-PKA versus mock-transfected cells under normoxia. However, in the presence of DN-PKA, hypoxic cells showed a significantly lower ability to acidify extracellular pH (Fig. 4D). DN-PKA expression also decreased accumulation of the FITC-conjugated CA inhibitor in hypoxia when compared to cells with an intact endogenous PKA pathway (Fig. 4E,F). These findings supported the idea that down-regulation of PKA activity by DN-PKA could perturb CA IX function.

The catalytic function of CA IX is linked to its cooperation with bicarbonate transporters, including sodium-bicarbonate cotransporter 1 (NBC1), forming a bicarbonate transport metabolon protecting cells from acidosis (21, 2). Thus, we examined whether CA IX was spatially associated with NBC1 and with PKA in tumor cells to enable coupling of the hypoxia-stimulated PKA activation with the NBC1-CA IX metabolon. We therefore used A549 lung carcinoma cells exposed to hypoxia to co-localize these proteins by immunofluorescence. All cells showed diffuse intracellular staining of the studied proteins, which most probably represented their internalized forms resulting from HGF-induced perturbation of cell-cell contacts followed by endocytosis of plasma membrane proteins including CA IX as described earlier (22, 23). Nevertheless, we could demonstrate that CA IX accumulated and colocalized with NBC1 in protruding membranes of hypoxic A549 cells stimulated by HGF to migration as well as with phosphorylated PKA substrates that indirectly indicated the positions at which activated PKA was operating (Figs. 5A,B and Supplementary Figs. 1,2). These data were fully compatible with the concept of the PKA-driven phosphorylation-mediated functional activation of CA IX in the context of the bicarbonate metabolon and with its role in modulating a cellular response to the hypoxic microenvironment.
We then examined whether CA IX phosphorylation could affect cell migration, which critically depends on tight pH regulation (involving CA IX catalytic activity) at protruding cell membranes (23). MDCK cells transfected either with the wild type CA IX or with its mutated variants T→D/S→G and T→G/S→D, were induced to motility in wound healing and scatter assays. In line with the proposed activating role of Thr443 phosphorylation, results of both assays clearly showed a significantly increased motility of cell expressing the T→D/S→G mutant and decreased motility of cells expressing the T→G/S→D mutant (Fig. 5 C,D). These data suggest that phosphorylation of CA IX is functionally relevant to cell phenotype.

**Hypoxia modulates activity of CAII and CA XII inserted into the CA IX backbone**

To bring additional evidence for the critical role of the intracellular region as the mediator of the hypoxia-driven activation of the extracellular catalytic domain of CA IX, we generated chimeric CA IX molecules containing heterologous CA domains. Two chimeras were obtained by an insertion of either the CA II or CA XII catalytic domains, in between the N-terminal proteoglycan-like (PG) segment and the C-terminal segment containing the transmembrane and intracellular regions of CA IX (Fig. 6A). Both CA II and CA XII are active CA isoenzymes expressed in many normal cell types and tissues as well as in some tumors and are known to function and bind sulfonamide inhibitors also under normoxic conditions (24, 13). However, when either CA II or CA XII were expressed in the context of the CA IX backbone, they behaved similarly to the original catalytic domain of CA IX. Both chimeric constructs were ectopically expressed in MDCK cells and showed cell surface localization like the wild-type CA IX (data not shown). Analogously to CA IX, the chimeric proteins were capable of binding the FITC-labeled CA inhibitor only in hypoxia, but not in normoxia (Fig. 6B). This finding suggests that the enzymatic activity of the chimeric proteins is not determined by the CA domains originating from the different isoforms, but rather by the flanking regions originating from CA IX. Since we previously showed that the deletion of the PG domain has no effect on the hypoxia-related binding of the FITC-labeled CA inhibitor (7), the observed effect can be attributed to the intracellular region.
Discussion

Protein kinase A has been implicated in a broad range of cellular processes, including cell proliferation, survival, differentiation, metabolism, transcription etc. It acts at a crossroad of many signal transduction cascades via communication with other regulatory proteins and orchestration of different molecular pathways in response to second messenger signaling. Accordingly, PKA has a spectrum of substrates that involves hormone receptors, Raf-1 kinase and RhoA GTP binding protein, CREB and NFκB transcription factors, histones, metabolic enzymes such tyrosine hydroxylase and pyruvate kinase etc. (19). PKA also regulates water and ion transport in various physiological conditions. Although it is a ubiquitous protein working in virtually all differentiated cell types, its increased expression/activation has been associated with tumors derived from breast, colon, kidney and other tissues. PKA-regulated processes directly contribute to tumor biology, particularly to invasion, treatment resistance and adaptation to stress (25). Interestingly, PKA cooperates with the hypoxic machinery by phosphorylation of HIF-1α, a key regulator of molecular responses to hypoxia (26), and thereby influences a whole panel of molecules induced by hypoxia, some of them also via direct phosphorylation.

In this study, we present evidence that PKA controls the CA IX-mediated pH regulation in hypoxic cells. It occurs via phosphorylation of the Thr443 residue within the PKA recognition site RRGT, which conforms to the RRXS*/T* consensus sequence (19). Mutation of this residue into a non-phosphorylatable amino acid eliminates the CA IX-related extracellular acidosis produced in cells exposed to chronic hypoxia. Since the PKA consensus site is embedded in the juxtamembrane basic amino acid motif, our finding offers an explanation for an earlier observation linking mutations of this motif with abolished functioning of CA IX (14). It is quite conceivable that conversion of the basic motif to a highly acidic amino acid stretch changes the local charge and/or architecture, thus precluding the recognition by PKA and phosphorylation of Thr443. As noticed previously, the basic amino acid motif is conserved in the cytoplasmic domains of CA IX proteins expressed in different species, including human, mouse, rat and dog, as well as in the other two transmembrane CA isoforms, namely CA XII and CA XIV (14). However, the threonine residue is present within this
motif only in CA IX, but neither in CA XII nor in CA XIV, suggesting that CA IX is the only transmembrane isoform regulated by PKA and that this regulation provides CA IX with the ability to respond to physiological stimuli transmitted via the PKA pathway.

Although we propose that Thr443 plays a dominant role in CA IX regulation as an acceptor of the phosphorylation signal from PKA, this conclusion does not exclude the participation of additional two phosphorylation sites in the modulation of CA IX function. Based on the results of the in vitro mutagenesis approach, phosphorylation of Ser448 may represent a negative regulatory event diminishing the ability of CA IX to acidify pHe. In this respect, dephosphorylation of Ser448 would be necessary, but not sufficient for full CA IX performance. Our in vitro experiment indicates that Ser448 is very unlikely to be a PKA phosphorylation site. In addition, it is localized within a different amino acid context that does not match the PKA consensus. At this moment it is not clear whether Ser448 is actually phosphorylated in vivo and if so, which kinase modifies this residue. In contrast, Tyr449 phosphorylation was shown to occur in kidney cancer cells and was linked to downstream activation of the PI3K/Akt pathway (18). However, this phenomenon was not reproduced in breast cancer cells (9), which may indicate that it is cell-type specific. Moreover, information on the pertinent kinase and whether phosphorylation of Tyr449 affects CA IX function is still missing. We can only deduce that the close proximity of Ser448 and Tyr449 possibly prevents their simultaneous modification, but the space constrains and/or induced allosteric changes may also affect the interplay of PKA with other kinases and determine the hierarchy of phosphorylation signaling to the CA IX molecule. Undoubtedly, many open questions remain to be solved via further extensive experimentation to achieve a better understanding of CA IX regulation.

One important question emerging from this study is how the CA IX phosphorylation via PKA fits in the concept of the bicarbonate metabolon operating in hypoxic cells. To provide a rationale, we have to take into account several facts. [A] The metabolon paradigm involves spatial and functional cooperation of CA IX with bicarbonate transporters, such as the sodium-bicarbonate co-transporter NBC1 and anion exchanger AE2 (4, 5). [B] NBC1 and AE2 are PKA substrates functionally implicated in pH regulation and migration phenomena in cancer cells and/or regulated by the VHL/HIF pathway (23, 27). [C] PKA-mediated phosphorylation of NBC1 at Ser982 or Ser1026
permits the negatively charged aspartic acid residues in the C terminus of NBC1 to interact electrostatically with the bicarbonate ion binding site on the transporter, resulting in a transport stoichiometry favorable for bicarbonate import (28). This mode is compatible with intracellular neutralization activated in cells exposed to hypoxia/acidosis (5). [D] PKA works in a compartmentalized manner conducive to spatial coordination of its substrates, such as CA IX and NBC1. [E] Hypoxia increases intracellular cAMP concentration and level of activated PKA and thus creates a mechanism feeding the phosphorylation signaling via PKA (24, and also this work), [F] Hypoxia increases the catalytic activity of CA IX and its ability to acidify pH (7, 9).

In favor of the above concept, we found that CA IX co-localized with NBC1 as well as with phosphorylated PKA substrates (indicating the presence of activated PKA) in the leading edge membranes of hypoxic A549 lung carcinoma cells stimulated to migration by HGF (Fig. 5A,B, Supplementary Figs 1, 2). Noteworthy, co-expression of CA IX and PKA substrates is visible also in parallel tissue areas of serial renal cell carcinoma sections (Supplementary Fig. 3), suggesting that the crosstalk between CA IX and PKA might also occur in vivo. Thus, on the basis of available data it is possible to propose a model, in which hypoxia leads to increased levels of cAMP, which activates PKA. PKA then phosphorylates both CA IX and NBC1, thereby stimulating import of bicarbonate ions generated by activated CA IX (Fig. 7). Based on other examples (such as EGFR, 29) it is imaginable that phosphorylation affects CA IX in terms of folding (e.g. by an allosteric change and opening of the active site) and/or in terms of dimerization (e.g. by releasing the S-S bonds facilitated by the reducing hypoxic microenvironment and re-orienting the active sites towards the bicarbonate transporter “entrance”). This could result in an efficient coupling of bicarbonate production and transport. Such scenario is fully compatible with the definition of metabolons as complexes that form transiently to enhance channeling of constituents (30).

Indeed, CA IX phosphorylation at Thr443 is functionally relevant to cell behavior, since it significantly affects cell migration (Fig. 5). This appears to be directly connected to the regulation of CA IX catalytic activity, which is needed for the intense bicarbonate ion transport and proper pH regulation across plasma membrane areas that represent the leading edges of moving cells. Such phenomenon is particularly meaningful to hypoxic tumor cells, which tend to escape from the hostile
tumor microenvironment to survive and thus gain a migratory phenotype that involves the assembly and activation of the pH-regulatory machinery including the bicarbonate metabolon (23). This view is in agreement with the studies that present CA IX as a dissociation factor destabilizing E-cadherin mediated cell-cell contacts and a protein regulating cell migration and invasion (22, 10, Witarski et al, submitted).

These findings strongly imply that the hypoxia-induced activation of PKA leading to phosphorylation of CA IX at Thr443 in the intracellular tail is an important determinant of the enzymatic activity of CA IX. This is supported by the data obtained with chimeric proteins composed of the CA IX backbone and heterologous catalytic domains corresponding to CA II and CA XII. Although all three CA isoforms differ from each other by their catalytic activity and affinity to inhibitors, they behave very similarly when expressed in the context of a CA IX-derived intracellular tail. Thus, it appears that the intracellular region of CA IX (the only one among other transmembrane CAs, which contains Thr443 that can accept signals from PKA) is principally responsible for the functional regulation of the catalytic activity of CA IX.

In conclusion, this work allowed us to make significant progress in elucidating important aspects of the signal transduction that underlies functional regulation of CA IX and its impact on cell phenotype. Moreover, we reinforced our understanding that hypoxia induces CA IX at the level of transcription as well as at the level of enzymatic activation. This secondary control mechanism might be important for maximizing the catalytic performance of CA IX and also for its dynamic, microenvironmentally-driven regulation in the background of high CA IX protein stability (31). The knowledge obtained here will serve for further investigation towards elucidation of relevant molecular mechanisms and for the development of new approaches aiming at blocking CA IX function from the “inside” and thus reducing survival of cancer cells in therapeutic settings.
References


Figure Legends

**Figure 1.** Oligomerization, localization and functional assays of CA IX variants with single inactivating mutations in phosphorylation sites Thr443 (T443→G) and Ser448 (S448→G). (A) Immunoblotting analysis of the mutants separated under non-reducing conditions (without β2-mercaptoethanol, -ME) and reducing conditions (+ME) demonstrates their correct molecular weight and oligomerization. Blotted proteins were visualized with M75 Mab. The figure shows a representative blot of three independent experiments. (B) Immunofluorescence analysis with FITC-conjugated M75 MAb shows plasma membrane localization of both mutants. (C) Cell surface fraction of CA IX assessed by ELISA was calculated as a ratio of absorbance values obtained from live cells (corresponding to cell surface CA IX only) and fixed cells (corresponding to both surface and intracellular CA IX). The data shows that the mutations did not affect cell surface localization of CA IX. (D) Effect of mutations on pH was measured in transfected MDCK cells incubated in normoxia and hypoxia for 48 h. The graph shows differences in extracellular pH values (ΔpH), T443→G mutant was clearly unable to acidify pH, whereas S448→G mutant displayed significant acidification comparable to the wt CA IX. Data correspond to mean values from 10 independent experiments (**p<0.01), bars represent standard deviations. (E) Accordingly, accumulation of FITC-CAI occurred in hypoxic cells expressing wild-type CA IX and the S448→G mutant, whereas it was diminished in mock-transfected cells and in cells expressing the T443→G mutant.

**Figure 2.** Effects of double mutations of CA IX at Thr443 and Ser448, and phosphorylation of Thr443 in hypoxic cells. (A) Domain composition of CA IX and amino acid sequences of the cytoplasmic portion and its mutated variants. SP – signal peptide, PG – proteoglycan-like region, CA – carbonic anhydrase domain, TM – transmembrane domain, IC – intracellular tail. IC mutations are indicated by boxes (dark grey for pseudophosphorylated residues and light grey for dephosphorylated residues). Empty box with solid line frame encloses the consensus binding site for protein kinase A, empty box with dotted line frame encloses the basic amino acids, mutation of which was shown to
disturb the function of CA IX (14). (B) Effect of double mutations on pHε in transfected MDCK cells incubated for 48 h in normoxia or hypoxia. The graph shows differences between mean pH values (ΔpH) +/- SD from 10 independent experiments (**p<0.01) and indicates that phosphorylation of Thr443 is of critical importance to CA IX-mediated acidification in hypoxia, whereas phosphorylation of Ser448 negatively modulates CA IX function. (C) Phosphorylated subpopulation of CA IX molecules was immunoprecipitated from cell extracts using the pT443 antibody to CA IX with phospho-Thr443, blotted and visualized using the peroxidase-conjugated M75. Both MDCK-CA IX cells with constitutive expression of wtCA IX and HT-29 with natural expression of CA IX revealed a stronger phosphorylation signal in hypoxia compared to normoxic control. The figure represents one of five independent experiments with similar results.

**Figure 3.** In vitro phosphorylation and dephosphorylation of Thr443 by PKA and PP2A, respectively, and CA IX phosphorylation in transfected cells in the presence of the catalytic subunit of PKA (CS PKA) or PKA dominant negative mutant (DN). (A) In vitro kinase assay was performed with the catalytic subunit of PKA on GST fusion proteins containing the IC domain of CA IX in the wild type version and in the mutated version with Thr443 replaced by Ala (T443→A). GST alone was used as a negative control. The upper part of the figure represents a CBB-stained gel (loading control), the lower part represents autoradiograph of the proteins phosphorylated in the presence of γ[32P]-ATP. (B) In vitro phosphatase assay was performed with heterodimeric and heterotrimeric forms of PP2A on PKA-phosphorylated GST-tagged wt-IC fragment of CA IX. The upper part of the figure represents a gel with CBB-stained substrate proteins (loading control), the lower part represents autoradiograph of the proteins after dephosphorylation with PP2A. The data shows Thr443 is a substrate residue for PKA and PP2A. The experiments (in A,B) were repeated twice and representative results are shown here. (C) CA IX was immunoprecipitated by the M75 antibody from cell extracts, in vitro phosphorylated by CS PKA, subjected to PAGE, blotted and incubated with the P-T443 CA IX antibody. In vitro phosphorylated GST-tagged wtIC domain of CA IX protein (GST-IC) was used as a positive control. P-T443 CA IX antibody reacts only with CA IX treated with CS-
PKA. (D) All three blots contain PAGE-resolved extracts from HEK293T/17 cells transfected with different combinations of the following plasmids: pcDNA3.1 (p1), pSG5C (p2), pSG5C-MN/CA9 (pCA9), pcDNA3.1-CSPKA (pCα), pcDNA3.1dnPKA (pdn). The plasmids were co-transfected in a ratio of 10:1. The blots were incubated with antibodies as indicated on the left side. The data demonstrates that phosphorylation of CA IX, activation of PKA and phosphorylation of PKA substrates occur only in the presence of CS PKA. The experiment was repeated four times and results of the representative one are presented here.

**Figure 4.** Relationship between hypoxia, cAMP, PKA, CA IX phosphorylation and pH acidification. (A) Immunoblotting analysis of activation (phosphorylation) of PKA Thr197 in hypoxia. The experiment was repeated three times and representative blot is shown here. (B) Intracellular cAMP levels in hypoxic and normoxic MDCK-CA IX cells evaluated using a direct immunoassay. Hypoxia was found to significantly increase cAMP levels in three independent experiments (*p<0.05). (C) Normoxic MDCK cells (mock- and CA IX-transfected) were treated with 0.5 mM 8-BR-cAMP to activate PKA. PKA activation induced CA IX Thr443 phosphorylation as demonstrated by immunoprecipitation with the pT443-specific antibody, blotting and immunodetection by M75 MAb conjugated with peroxidase. The experiment was repeated twice with similar results. (D) Extracellular pH was measured in MDCK-CA IX cells transiently transfected with DN PKA versus mock-transfected control. DN PKA was able to significantly reduce extracellular acidification mediated by CA IX in the hypoxic cell culture (**p<0.01), as verified in three independent experiments. (E) Binding of the FITC-CAI inhibitor to hypoxic MDCK-CA IX cells was reduced after transient transfection of DN PKA as visible from the reduced fluorescent signal. (F) Relative intensity of the fluorescence signal was evaluated using ImageJ software. The results confirmed that expression of DN-PKA decreased the binding of the FITC-CAI inhibitor to CA IX.

**Figure 5.** Co-localization of CA IX with NBC1 and PKA substrates and effect of CA IX phosphorylation on cell migration. (A,B) A549 lung carcinoma cells were incubated in hypoxia for 48 h to induce expression of CA IX. Then they were treated with 20 ng/ml of HGF for 15 min to recruit...
migration-related pH-regulating machinery to leading membrane edges. After fixation with methanol, the cells were double stained with antibodies specific to CA IX, phospho-PKA substrates and NBC1 and analyzed by confocal microscopy (at original magnification 400x). Co-localization of CA IX with the examined proteins is clearly visible at the leading edges of cells stimulated to migration. (C) Wound healing assay on MDCK cells expressing wtCA IX and double mutants in position 443 and 448. Confluent cell monolayers were starved overnight, then wounded and allowed to migrate as described in Materials and methods. Wound width was measured in 16 positions immediately after wounding and 10 h later, and wound closure in % was calculated. Cells expressing T→D/S→G mutant showed significantly improved (**p<0.01) healing capacity. (D) Cell islands were generated by plating the pre-formed cell aggregates. Spreading and outward migration were allowed to proceed for 24 h. Significant increase (**p<0.01) was observed in areas of MDCK cells transfected with T→D/S→G mutant.

**Figure 6.** Construction of chimeric CA IX variants and their effects on binding of FITC-CAI to transfected MDCK cells. (A) Schematic drawing of chimeric constructs with the CA domain of CA IX replaced by CAII and CAXII. Structural domains of CA IX are designated as in Figure 2A. (B) Binding of the CA IX-selective FITC-labeled inhibitor (FITC-CAI) to normoxic and hypoxic cells expressing the chimeric proteins.

**Figure 7.** Model of PKA regulation of the CA IX. In normoxic cells, cAMP levels are relatively low, catalytic subunit of PKA [C] is kept inactive by interaction with the regulatory subunit of PKA [R], and PP2A is presumably associated with the IC tail of CA IX. Thus, CA IX remains unphosphorylated at Thr443 (but might be phosphorylated by another kinase at Ser448) and its activity is low. At the same time, NBC1 is unphosphorylated at indicated Ser residues and the bicarbonate import does not occur. In hypoxia, cAMP levels rise, cAMP binds to R PKA leading to release of C PKA, which can phosphorylate Thr443 of CA IX as well as Ser982 and Ser1026 residues of NBC1. This results in a spatially coordinated and functionally coupled activation of CA IX-catalyzed production of bicarbonate ions as well as in activation of NBC1-mediated bicarbonate
transport across the membrane. Intracellular bicarbonate can then consume intracellular protons to form CO₂, which leaves the cells by diffusion. This contributes to intracellular neutralization. On the other hand protons generated at the outer side of the membrane via the CA IX-catalyzed reaction contribute to extracellular acidification. Since this model counts on the presence of CA IX in normoxia, it is mostly relevant to reoxygenated cells, which contain CA IX induced by preceding hypoxia.

Legends for supplementary figures

**Supplementary figure 1.** CA IX co-localization with PKA-phosphorylated substrates in A549 cells is presented in the form of Z-stack corresponding to Figure 6A. Overlapping staining signals are clearly visible in individual cell layers. Immunofluorescence and confocal analysis were done as described in Materials and methods. Original magnification was 400x.

**Supplementary figure 2.** CA IX co-localization with NBC1 in A549 cells is presented in the form of Z-stack corresponding to Figure 6B. Overlapping staining signals are clearly visible in individual cell layers. Immunofluorescence and confocal analysis were done as described in Materials and methods. Original magnification was 400x.

**Supplementary figure 3.** Immunohistochemical analysis of the parallel expression of CA IX and PKA substrates. The staining with M75 MAb and Phospho-PKA Substrate antibody was done on serial tissue sections from clear cell renal cell carcinoma (kindly provided by Prof. Jan Breza, Department of Urology, Faculty of Medicine, Comenius University, Bratislava). Immunohistochemistry was performed as described elsewhere (Pastorekova e al, Gastroenterology 1997;112:398-408). Staining signals of CA IX and PKA substrates were present in corresponding tumor areas.
Figure 3

A

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PKA substrate

pT197-PKA

pT443-CA IX
**Figure 4**

(A) Western blots showing pT197-PKA and total PKA levels in A549 and HeLa cells under normoxia (No) and hypoxia (Hy) conditions.

(B) Bar graph showing cAMP levels (fmol/μg of protein) in MDCK-mock and MDCK-CA IX cells under normoxia (No) and hypoxia (Hy) conditions. Significance indicated by *.

(C) Western blots showing pT443-CA IX and PKA substrates levels in mock and MDCK-CA IX cells treated with 8-Br-cAMP under normoxia and hypoxia conditions.

(D) Bar graph showing ΔpH levels (mV) in mock and DN PKA cells under normoxia and hypoxia conditions. Significance indicated by **.

(E) Fluorescence images showing intensity of fluorescence in normoxia and hypoxia conditions for mock and DN PKA.

(F) Bar graph showing intensity of fluorescence for mock and DN PKA under normoxia and hypoxia conditions.
Figure 5

A. PKA substrates + CA IX

B. NBC + CA IX

C. Open wound width (%)

- wt CA IX
- T>D/S>G
- T>G/S>D

**

D. Area at t₀

- 10 h
- 24 h

**
Phosphorylation of carbonic anhydrase IX controls its ability to mediate extracellular acidification in hypoxic tumors

Peter Ditte, Franck Dequiedt, Eliska Svastova, et al.

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