The plasma membrane Ca^{2+} pump PMCA4b regulates intracellular Ca^{2+} homeostasis and migratory and metastatic activity of BRAF mutant melanoma cells

PhD Thesis

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Introduction

Calcium is an important second messenger that takes part in the regulation of vital processes from proliferation to cell death. Expression and activity of Ca^{2+} regulatory proteins are often altered in tumor cells. Plasma membrane calcium ATPases (PMCA) maintain the resting low intracellular calcium concentration by pumping out excess calcium from the cytosol. The four plasma membrane Ca^{2+} ATPases (PMCA1-4) are coded by four different genes (ATP2B1-4), from which more than 20 isoforms are generated through alternative splicing. PMCA isoforms differ in their kinetic and regulatory features that define their ability to respond to a variety of incoming Ca^{2+} signals.

Alterations in the expression and the activity of PMCA proteins have been described in several types of cancer. A strong decrease in PMCA4 expression was found in lymph node metastasis of colorectal cancer when compared to normal tissue and adenomas. Similarly, PMCA4b is abundantly expressed in normal breast epithelium but it is present in a very low amount in breast cancer cells. Furthermore, histone deacetylase inhibitor treatment was able to induce PMCA4b expression in gastric and colon cancer cells, and in breast cancer MCF-7 cell line. Spontaneous differentiation of post-confluent Caco-2 colon cancer cell cultures also induced PMCA4b expression. These results suggest that PMCA4b expression strongly correlates with the degree of differentiation of cells.

Malignant melanoma is a highly invasive and metastatic type of cancer with poor prognosis. In melanomas BRAF is the most frequent oncogene up to 50% incidence and that induces the constitutive activation of the RAS-RAF-MEK-ERK signaling cascade. Several specific inhibitors have been developed against the BRAF and MEK kinases that initiated quick and high response rate however, in patients intrinsic resistance and in most of the cases acquired resistance shortly emerged. More recently, very promising results were achieved with immune checkpoint inhibitors - CTLA-4 and PD-1 inhibitors – but still there is a large group of patients who do not respond to either of these therapies or develop resistance quickly. The expression of several Ca^{2+} regulatory proteins was found to be altered in melanoma cells, nevertheless, the role of plasma membrane Ca^{2+} pumps in melanoma progression has not been investigated.

Aims

In this work we wanted to examine how different PMCA isoforms affected the cytosolic Ca^{2+} signal and investigate if PMCA proteins played a role in the migratory and metastatic activity of melanoma cells. In order to study this:

1. We analyzed the differential effects of PMCA isoforms PMCA4b, PMCA4a and PMCA2b on the pattern of the SOCE induced Ca^{2+} signal. Therefore, we expressed mCherry-tagged PMCA variants together with the genetically encoded Ca^{2+} indicator GCaMP2 in Hela cells.
2. We studied the influence of PMCA proteins on Ca^{2+} signaling in both BRAF mutant and BRAF wild type melanoma cells. We determined the expression pattern of the PMCA proteins and analyzed the effect of mutant BRAF inhibitor treatment on the PMCA expression and localization, and on the intracellular Ca^{2+} signal.
3. Since metastatic melanoma cells are highly motile we aimed to analyze the effect of mutant BRAF inhibition on the migratory capacity of melanoma cells.
4. We investigated how PMCAs – PMCA4b in particular - affected the motility of melanoma cells. Therefore, we generated BRAF mutant cell lines overexpressing PMCA4b and tested their migratory characteristics *in vitro,* and their metastatic capacity *in vivo*.
5. Since HDAC inhibitor treatment has been shown to induce PMCA4b expression in breast and colon cancer cells, we treated the melanoma cells with HDAC inhibitors alone or in combination with the mutant BRAF inhibitor. We examined the expression, localization and activity of the PMCA proteins, and determined the migratory activity of the cells in response to the HDAC inhibitor treatments.

Methods

Cell lines

In our experiments we used the HeLa cervix adenocarcinoma cell line and four melanoma lines: the BRAF/NRAS wild type MEWO, the NRAS mutant MJZJ and the two BRAF mutant A375 and A2058 cells. sh-HeLa cell lines were created by stable transfection with PMCA4 shRNA plasmid or with control shRNA plasmid-A.

Transient transfection of Hela cells

HeLa cells were transiently co-transfected with one of the mCherry-PMCA4b, mCherry-PMCA4b-LA, mCherry-PMCA4a or mCherry-PMCA2b constructs and the genetically coded Ca^{2+} indicator GCaMP2. Transient transfections were performed with the transfection reagent FuGENE HD.

Compounds

Melanoma cells were treated with mutant BRAF (V600E) inhibitors vemurafenib (PLX4032) and GDC0879, MEK kinase inhibitor selumetinib, HDAC inhibitors valproic acid sodium salt and suberoylanilide hyroxamic acid (SAHA). We used two different PMCA4b inhibitors: $LaCl_3$ and peptide inhibitor caloxin 1c2 to test the functional role of the PMCA.

Ca^{2+} signal measurements

To detect the changes in the intracellular Ca^{2+} concentration the genetically encoded Ca^{2+} indicator GCaMP2 or Fluo-4 AM green fluorescent Ca^{2+} indicator were used. Store operated Ca^{2+} entry was induced in two steps. First the intracellular Ca^{2+} stores were depleted in nominally Ca^{2+} free solution by thapsigargin and ATP. Then store operated Ca^{2+} entry was induced by restoring the external Ca^{2+} concentration to 2 mM. Ca^{2+} signal was also evoked by A23187 in a medium containing 2 mM Ca^{2+}.

Protein analysis

Total protein was precipitated with 6% TCA and pellet was dissolved in a Laemmli-type sample buffer. Samples were analyzed by Western Blotting.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde solution and after permeabilization they were stained with primary antibody against PMCA4b and secondary antibody Alexa Flour 488-conjugated anti-mouse IgG. Images were acquired by an Olympus IX-81 and a Zeiss LSM500 laser scanning confocal microscopes.

Quantitative real-time reverse transcription PCR

Total RNA was isolated and reverse transcription was performed. qPCR was performed either with SYBR Green master mix or with TaqMan assays.

Proliferation assay

Proliferation was analyzed by BrdU incorporation (colorimetric) assay.

Viability assay and cell cycle analysis

Both viability and the ratio of cells in each cell cycle phases were determined by the NucleoCounter NC-3000^{TM} system. For the viability assay cells were stained with Acridine Orange and DAPI. Viability was expressed as total cells - nonviable cells/total cells. The ratio of cells in each cell cycle phases was analyzed based on the DNA content of the cells.

Cell migration assays

Random migration of melanoma cells was measured with either phase contrast videomicroscopy or with fluorescent cell nuclei tracking assay. Directional migration capacity of the cells was assessed by using the modified Boyden chamber assay with fibronectin as chemoattractant in the bottom chamber.

Lung colonization assay

Tail vein injection of A375-GFP and two independently generated A375-GFP-PMCA4b (I and II) cell lines was performed in 11-week old female SCID mice (10 mice / group). Mice were sacrificed 6 weeks later. The lung and the tumor tissue in the chest cavity were taken out and after embedding hematoxylin-eosin stained sections from the tissue blocks were analyzed with TissueFAXS System.

Results

Distinct PMCA isoforms shape intracellular Ca^{2+} transients differentially

PMCA isoforms strongly differ in their kinetic properties. Regarding their activation by Ca^{2+}-calmodulin PMCAs can be categorized as fast or slow pumps. Depending on their inactivation rates PMCAs with short and long lasting activity can be distinguished that is referred to as a memory from earlier Ca^{2+} spikes. We investigated the effect of three PMCA proteins (PMCA4b, PMCA2b, PMCA4a) on the pattern of the intracellular Ca^{2+}signal after store operated calcium entry or ionophore stimulus in HeLa cells. We found that overexpression of a slow pump with memory - such as the PMCA4b variant - strongly altered the shape of the Ca^{2+} transient. The first quick rise in the intracellular Ca^{2+} concentration was pursued by a faster decay phase than in the control HeLa cells and this was followed by periodic baseline oscillations. Transient expression of a fast pump with no memory – such as the PMCA4a - had a very different effect. After a rapid increase, the intracellular Ca^{2+} concentration quickly decreased to an elevated steady-state level instead of returning to the original resting state. Cells expressing a fast pump with long memory – such as the PMCA2b - cleared very quickly the excess Ca^{2+} from the cytosol producing only a single Ca^{2+} spike. Our results showed that PMCA proteins can uniquely shape the incoming Ca^{2+} signal.

BRAF inhibitor treatment increases PMCA4b expression and enhances Ca^{2+} clearance in BRAF mutant melanoma cells

We treated two BRAF (V600E) mutant (A375, A2058), one NRAS mutant (MZJZ) and one BRAF and NRAS wildtype (MEWO) melanoma cell lines with mutant BRAF specific or MEK inhibitors. We found that BRAF inhibitor treatment selectively increased the expression of PMCA4b in BRAF mutant cells, while MEK inhibition increased PMCA4b abundance both in the BRAF mutant cell lines and the NRAS mutant cells. We showed that the elevated PMCA4b protein expression was coupled with an increased PMCA4b abundance in the plasma membrane. We also investigated the effect of increased PMCA4b abundance on intracellular Ca^{2+} signaling and found that Ca^{2+}clearence was increased in BRAF inhibitor treated cells both after SOCE and Ca^{2+} ionophore stimulus. Importantly, inhibition of PMCA4b activity with $LaCl_3$ or the PMCA4 specific inhibitor caloxin1c2 resulted in a slower decay phase of the Ca^{2+}signal similar to that seen in the

control cells. These results indicate that the up-regulated PMCA4b in BRAF mutant cells was responsible for the faster Ca^{2+} clearance after stimulation.

Elevated PMCA4b expression decreases the migratory and metastatic capacity of A375 melanoma cells

We analyzed the effect of BRAF inhibitor treatment on the migratory capacity of two BRAF mutant and a BRAF wild type melanoma cell lines. We performed time-lapse video microscopy to follow the movement of individual cells during the 3-day-long treatment period. We found that after two days cell migration was strongly reduced by BRAF inhibition in the BRAF mutant cell lines. The inhibitory effect on migration corresponded to the increased PMCA4b expression that was most substantial after 48 hours. In order to investigate the effect of PMCA4b abundance on the migratory capacity of melanoma cells we stably transfected PMCA4b in the BRAF mutant A375 and the BRAF wild type MEWO cell lines. We found a strong decrease in the motility of two independently generated A375-GFP-4b cell lines compared to the control GFP expressing cells while the motility of the MEWO cells were unaffected. Furthermore, the morphology of the PMCA4b expressing cells was also strongly altered. While the control cells had an elongated shape with long outgrowths, the PMCA4b overexpressing cells were more epithelial like with a rounded, cobble-stone shape. We also compared the proliferation rate of the original, the GFP-tagged and the two PMCA4b-expressing A375 cell lines and found that proliferation didn´t differ significantly among them. In order to compare the metastatic capacity of the control and the PMCA4b-expressing A375 cells, we performed a lung colonization assay in mice. We found that in the control group tumor cells established large tumors in the lung parenchyma and some tumor cells invaded the lung tissue, while in the two groups of animals injected with PMCA4b expressing cells, either no tumors or only small tumors were formed on the surface or in the connective tissue of the lung. Our data demonstrates that PMCA4b regulates the migratory and metastatic capacity of the BRAF mutant A375 cells.

HDAC inhibitor treatment increases the expression of PMCA4b and inhibits the migration of melanoma cells independent of ERK

Since it was demonstrated earlier that histone deacetylase inhibitor treatment can induce the expression of PMCA4b in cancer cells, we investigated the effect of two HDAC inhibitors, SAHA and valproate, on the expression of PMCA4b in melanoma cells. We found that these treatments also caused an increase in the expression of PMCA4b in both the BRAF mutant and BRAF wild type melanoma cell lines. We also analyzed the expression of PMCA4b after HDAC inhibitor and BRAF inhibitor combination treatment but we found no additive effect. Interestingly, contrary to vemurafenib treatment, HDAC inhibition did not decrease ERK activation indicating that the effect of HDAC inhibitors on PMCA expression was independent of ERK activation in these cell lines. We showed that after HDAC inhibitor treatment the abundance of PMCA4b in the plasma membrane was increased and this was coupled with an enhanced Ca^{2+} clearance from the cells. Furthermore, PMCA4b specific inhibitor treatment was able to significantly reverse the increase in Ca^{2+} decay underlining the importance of PMCA4b in shaping the Ca^{2+} signal. We also analyzed how HDAC inhibitor treatment alone or in combination with vemurafenib influenced cell viability and cell cycle progression. We found that both the viability and proliferation of the melanoma cells were only moderately affected by HDAC inhibitor treatments and this effect was slightly increased by the combination treatments specifically in the BRAF mutant cell lines. Since valproate treatment increased PMCA4b expression in A375 cells in a similar extent as BRAF inhibition did, we wanted to investigate the effect of valproate on the migratory capacity of the cells. We found that both random and directed migration of A375 cells were decreased by valproate treatment. Furthermore, addition of the PMCA4b specific inhibitor caloxin 1c2 could reverse this effect further supporting that PMCA4b plays an important role in the regulation of A375 cell migration.

Conclusions

1. We found that due to their distinct kinetic features PMCA isoforms influence the pattern of the SOCE-mediated Ca^{2+} signal differently.

2. We showed that inhibition of the BRAF/MEK/ERK pathway selectively upregulated the expression of PMCA4b in BRAF mutant melanoma cells and this effect was coupled with an enhanced Ca^{2+} clearance from the cells.

3. Expression of PMCA4b profoundly changed the morphology, decreased the migratory capacity and reduced the metastatic activity of BRAF mutant A375 cells. Interestingly, despite its strong anti-migratory effect PMCA4b did not alter the proliferation of the cells, which is a typical feature of metastasis suppressors.

4. We found that histone deacetylase inhibitor treatment also induced PMCA4b expression in melanoma cells but in contrast to vemurafenib treatment this effect was independent of ERK activation. HDAC inhibition enhanced Ca^{2+} clearance and decreased the motility of the cells in a PMCA4b dependent manner. These results confirm our findings that PMCA4b plays an important role in the regulation of melanoma cell migration.

Thesis is based on the following publications:

1. **Hegedüs L**, Padányi R, Molnár J, Pászty K, Varga K, Kenessey I, Sárközy E, Wolf M, Grusch m, Hegyi Z, Homolya L, Aigner C, Garay T, Hegedüs B, Tímár J, Kállay E, Enyedi A. " Histone Deacetylase Inhibitor Treatment Increases the Expression of the Plasma Membrane Ca^{2+}Pump PMCA4b and Inhibits the Migration of Melanoma Cells Independent of ERK. " *Front. Oncol.*, 24 May 2017, https://doi.org/10.3389/fonc.2017.00095

2. **Hegedűs L**, Garay T, Molnar E, Varga K, Bilecz A, Torok S, Padanyi R , Paszty K , Wolf M, Grusch M, Kallay E, Dome B, Berger W, Hegedus B, Enyedi A." The plasma membrane Ca^{2+} pump PMCA4b inhibits the migratory and metastatic activity of BRAF mutant melanoma cells. " *Int J Cancer.* 2017 Jun 15;140(12):2758-2770. Epub 2016 Nov 17.

3. Pászty K, Caride AJ, Bajzer Ž, Offord CP, Padányi R, **Hegedűs L**, Varga K, Strehler EE, Enyedi A. "Plasma membrane Ca2+-ATPases can shape the pattern of Ca2+ transients induced by store-operated Ca2+ entry. " *Sci Signal.* 2015 Feb;8(364):ra19.

Publications related to the thesis:

Padányi R, Pászty K, **Hegedűs L**, Varga K, Papp B, Penniston JT, Enyedi Á. "Multifaceted plasma membrane Ca2+ pumps: From structure to intracellular Ca^{2+} handling and cancer. " *Biochim. Biophys. Acta.* 2016 Jun;1863(6 Pt B):1351-63. doi: 10.1016/j.bbamcr.2015.12.011. Epub 2015 Dec 17. Review

Varga K, Pászty K, Padányi R, **Hegedűs L**, Brouland JP, Papp B, Enyedi A. "Histone deacetylase inhibitor- and PMA-induced upregulation of PMCA4b enhances Ca2+ clearance from MCF-7 breast cancer cells. " *Cell Calcium*. 2014 Feb;55(2):78-92.

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Publications unrelated to the thesis:

Apáti Á, Pászty K, **Hegedűs L**, Kolacsek O, Orbán TI, Erdei Z, Szebényi K, Péntek A, Enyedi Á, Sarkadi B. "Characterization of calcium signals in human embryonic stem cells and in their differentiated offspring by a stably integrated calcium indicator protein." *Cell Signal*. 2013 Apr;25(4):752-9.

Arbabian A, Brouland JP, Apáti A, Pászyt K, **Hegedűs L**, Enyedi Á, Chomienne C, Papp B. "Modulation of endoplasmic reticulum calcium pump expression during lung cancer cell differentiation." *FEBS Journal* 280 (2013) 5408–5418

Hegedus L, Hyojin C, Xian X, Eliceiri G. (2008). "Additional MDA-MB-231 Breast Cancer Cell Matrix Metalloproteinases Promote Invasiveness." *J Cell Physiol*. 216: 480–485.

Paszty K, Antalffy G, **Hegedus L**, Padanyi R, Penheiter AR, Filoteo AG, Penniston JT , Enyedi A. (2007). "Cleavage of the plasma membrane Ca+ATPase during apoptosis." *Ann N Y Acad Sci.* 1099: 440-450.