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The interaction between endogenous calcineurin and the plasma membrane calcium-dependent ATPase is isoform specific in breast cancer cells

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Abstract Plasma membrane calcium/calmodulin-dependent ATPases (PMCAs) are high affinity calcium pumps that extrude calcium from the cell. Emerging evidence suggests a novel role for PMCAs as regulators of calcium/calmodulin-dependent signal transduction pathways via interaction with specific partner proteins. In this work, we demonstrate that endogenous human PMCA2 and -4 both interact with the signal transduction phosphatase, calcineurin, whereas, no interaction was detected with PMCA1. The strongest interaction was observed between PMCA2 and calcineurin. The domain of PMCA2 involved in the interaction is equivalent to that reported for PMCA4b. PMCA2–calcineurin interaction results in inhibition of the calcineurin/nuclear factor of activated T-cells signalling pathway.

Keywords: PMCA; Calcineurin; Interaction; MCF-7; Signalling; NFAT

1. Introduction

Plasma membrane calcium P-type ATPases (PMCAs) are high affinity calcium pumps that contribute to the maintenance of intracellular calcium homeostasis by exporting calcium from the cytosol to the extracellular environment [1].

There are four different isoforms of PMCA, numbered 1–4, encoded by four independent genes. PMCA1 and PMCA4 are expressed ubiquitously, whereas, PMCA2 and PMCA3 show a more restricted cell- and tissue-specific pattern of expression [2].

Structurally, PMCAs consist of 10 transmembrane domains, two big intracellular loops, and N- and C-terminal cytoplasmic tails [2].

The intracellular regions of PMCAs have been reported to mediate functional interactions with calcium/calmodulin-dependent enzymes, including nitric-oxide synthase I (nNOS) [3], calcium/calmodulin-dependent serine protein kinase (CASK) [4] and, calcineurin [5]. These interactions suggest that PMCA might act as a regulator of signal transduction pathways by tethering signalling proteins to cellular microenvironments where the concentrations of calcium and calmodulin are maintained at low levels. In this sense, the interaction between PMCA4 and calcineurin results in inhibition of the calcineurin/nuclear factor of activated T-cells (NFAT) signal transduction pathway [5].

The calcineurin/NFAT pathway plays a critical role in the progression of several human pathologies [6]. At present, the only clinically available inhibitors for the calcineurin/NFAT pathway are the immunosuppressant drugs cyclosporine A and FK506, which are unfortunately associated with severe side effects [7]. The recent identification of ectopically expressed PMCA4 as an inhibitor of the calcineurin/NFAT pathway [5] has prompted us to further investigate the interaction between endogenous PMCA and calcineurin, and to extend our previous observations to the analyses of other PMCA isoforms in an attempt to characterise new cellular inhibitors of this pathway.

Here we demonstrate that the interaction between endogenous PMCA and calcineurin is isoform specific in MCF-7 human breast cancer cells. Endogenous PMCA2 and -4 both interact with calcineurin, whereas, no interaction has been detected for PMCA1. The strongest interaction was detected between PMCA2 and calcineurin A. PMCA2 interacts with calcineurin through a domain equivalent to that reported for PMCA4b [5] and also inhibits the calcineurin/NFAT signalling pathway.

2. Materials and methods

2.1. Cell culture

Human embryonic kidney cell (HEK293) and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich) supplemented with 10% Fetal Calf Serum, 1% penicillin/streptomycin and 1% L-Glutamine.

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Abbreviations: PMCA, plasma membrane calcium ATPase; NFAT, nuclear factor of activated T-cells; HEK293, human embryonic kidney cell; RIPA, radioimmunoprecipitation assay; TBS-T, Tris-buffered saline plus Tween 20; PMA, phorbol-12 myristate 13-acetate

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2.2. Plasmids

pFlag-PMCA4b-(428–651) has been described previously [5].

pcDNA3-hPMCA2b contains the human PMCA2b cDNA and was a gift from Prof. Carafoli (University of Padova, Italy).

pcDNA3-hPMCA1b contains the human PMCA1b cDNA cloned into the XhoI and KpnI cloning sites of pcDNA3.1(-) (Invitrogen).

A fragment of hPMCA2b encoding amino acids 462–684 was generated by 35 cycles of PCR (the conditions were denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min) using oligonucleotides EcoPMCA2sense4 (5′-TCTTCCggAATTCTCCCgTCACCATCTCgTTgg-3′) and BamPMCA2anti684 (5′-CTTggCggATCCTCACTCCgggCTgCTggggAAgTC-3′) and plasmid pcDNA3-hPMCA2b as a template.

A fragment of hPMCA2b cDNA encoding amino acids 1143–1243 was amplified by 35 cycles of PCR (the conditions were denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min) using oligonucleotides HindPMCA2sense1143 (5′-TCTTCCCAAgCTTgTCgTgAAggCgTTCCgTAg-3′) and BamPMCA2anti1243 (5′-CTTCgCggATCCTCAAAgCgACgTCTCCAggCTgT-3′) and plasmid pcDNA3-hPMCA2b as a template.

A fragment of hPMCA2b cDNA encoding amino acids 535–609 was amplified by 35 cycles of PCR (the conditions were denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min 30 s) using oligonucleotides HindPMCA2sense535 (5′-TCTTCCCAAgCTTATggAgCTgCTgATCAATgCC-3′) and BamPMCA2anti609 (5′-CTTCgCggATCCTCACATggACTTgCgCACggAgTTg-3′) and plasmid pcDNA3-hPMCA2b as a template.

Amplified fragments 462–684, 535–609 and 1143–1243 were digested with EcoRI and BamHI, and HindIII and BamHI, respectively, and cloned into the corresponding sites of p3xFlag-CMV7.1 (Sigma–Aldrich). The resulting plasmids, pFlag-PMCA2b (462–684), pFlag-PMCA2b (535–609) and pFlag-PMCA2b (1143–1243), encode flag-tagged PMCA2b truncated proteins containing amino acids 462–684, 535–609 and 1143–1243, respectively, (numbering according to Genbank^{NM} Accession No. NM_001683).

A fragment of hPMCA1b cDNA encoding amino acids 511–585 was amplified by 35 cycles of PCR (the conditions were denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min 30 s) using oligonucleotides EcoPMCA1sense511 (5′-TCTTCCggAATTCTTTgTCCTATCTTgTAACAggAA-3′) and BamPMCA1anti585 (5′-CTTCgCggATCCTCACATggACTTCCTAACAgAATTg-3′) and plasmid pcDNA3-hPMCA1b as a template.

Amplified fragment 511–585 was digested with EcoRI and BamHI, and cloned into the corresponding sites of p3xFlag-CMV7.1 (Sigma–Aldrich). The resulting plasmid, pFlag-PMCA1b (511–585), encodes a flag-tagged PMCA1b truncated protein containing amino acids 511–585 (numbering according to Genbank^{NM} Accession No. J04027).

The fidelity of all amplified products was confirmed by sequencing.

2.3. Transient transfections

For immunoprecipitation experiments, HEK 293 cells were plated in 100×20-mm tissue culture dishes (4.5×10^6 cells/plate) the day before transfection. Cells were transfected with 10 μg of the indicated expression plasmids by using LipofectAMINE 2000 reagent (Invitrogen) as previously described [8].

For luciferase assays, HEK293 cells were plated in 6-well tissue culture plates (0.5×10^6 cells/well) the day before transfection. Cells were transfected with 10 μg of an expression vector encoding human PMCA2b (pcDNA3-hPMCA2b) or the corresponding empty vector (pcDNA3) and 5 μg of the luciferase reporter pNFAT-TA-Luc (Clontech) by using LipofectAMINE2000 reagent (Invitrogen) as previously described [8]. Cells were stimulated with phorbol-12 myristate 13-acetate (PMA) (20 ng/ml) and the calcium ionophore A23187 (1 μM) for 16 h and luciferase activity determined as described [8]. Transfection efficiency was normalised by co-transfection of plasmid pEF-LacZ and determination of β-galactosidase activity [9].

2.4. Immunoprecipitation

MCF-7 or transfected HEK293 cells were lysed as described [5]. One milliliter extracts were incubated overnight with the corresponding immunoprecipitating antibodies and 40 μl of protein A-agarose beads (Roche), at 4 °C with shaking. Beads were recovered by centrifugation at 3000 rpm and washed three times with 500 μl of radioimmunoprecipitation assay (RIPA) buffer. Washed beads were resuspended in 60 μl of $2 \times$ SDS–PAGE loading buffer (Invitrogen) and analyzed by Western blot.

2.5. Western blot

Samples were boiled and resolved as previously described [5]. Western blot membranes were incubated at room temperature with a 0.1% (v/v) solution of anti-Flag M2 peroxidase-conjugated monoclonal antibody (Sigma–Aldrich) in Tris-buffered saline plus Tween 20 (TBS-T) (for Flag-epitope detection) or with a 0.25% (v/v) solution of isoform specific rabbit polyclonal anti-PMCA antibody (Swant) for detection of the corresponding isoform of PMCA for 3 h. Bound antibodies were detected by ECL as described [5].

3. Results

3.1. Co-precipitation of endogenous human PMCA and calcineurin A in human breast adenocarcinoma cells

To investigate the interaction between endogenous PMCA isoforms 1, 2 or 4 and calcineurin A in human breast adenocarcinoma cells, protein extracts were immunoprecipitated with a monoclonal anti-calcineurin A antibody (Sigma) and precipitated proteins were probed by western blot using isoform specific anti-PMCA antibodies (Swant).

A very faint but reproducible band corresponding to PMCA4 was detected in samples probed with a rabbit anti-PMCA4 antibody (Fig. 1, upper panel) suggesting a weak interaction between endogenous calcineurin A and human PMCA4 in MCF-7 cells. Control immunoprecipitations carried out with the 5F10 anti-PMCA monoclonal antibody revealed the presence of high levels of the PMCA4 isoform in MCF-7 cells (Fig. 1, upper panel) indicating that the weak band detected was not attributable to low expression levels of PMCA4 or low affinity of the antibody.

Western blot with an anti-PMCA2 antibody showed high levels of co-precipitated PMCA2 (Fig. 1, middle panel) suggesting a strong interaction between endogenous calcineurin A and human PMCA2.

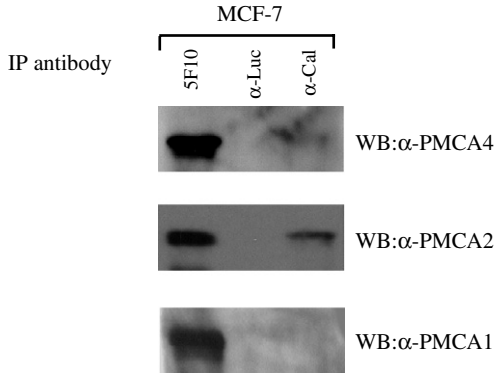

Fig. 1. The interaction between endogenous PMCA and calcineurin A in human breast adenocarcinoma MCF-7 cells is isoform specific. Co-precipitation of PMCA2 or PMCA4 (but not PMCA1) and calcineurin using an antibody against calcineurin A demonstrates the physical interaction between the two proteins in MCF-7 cells. (α-CnA) anti-calcineurin A monoclonal antibody, (5F10) anti-PMCA monoclonal antibody, (α-luc) anti-luciferase antibody. Western blots of immunoprecipitated proteins were probed with antibodies specific for PMCA1, PMCA2, or PMCA4 (Swant) to detect PMCA1 (WB: PMCA1), PMCA2 (WB: PMCA2) and PMCA4 (WB: PMCA4), respectively.

Immunoprecipitation of calcineurin A failed to co-precipitate PMCA1 in MCF-7 cells as evidenced by the absence of PMCA1 detection when the precipitated proteins were probed with an antibody recognising specifically PMCA1 (Fig. 1, lower panel). This finding suggests a lack of interaction between endogenous calcineurin A and PMCA1 in human MCF-7 cells. Immunoprecipitation performed with the 5F10 anti-PMCA antibody precipitated high levels of PMCA1 protein (Fig. 1, lower panel) ruling out low expression levels of PMCA1 as the cause for lack of interaction with calcineurin A.

In all cases, control immunoprecipitations carried out with an irrelevant antibody (anti-luciferase) precipitated no protein at all, confirming the selectivity of the interactions (Fig. 1).

In summary, these results indicate that the interaction between endogenous calcineurin A and PMCAs is isoform specific and suggest that calcineurin A interacts very strongly with endogenous PMCA2 but very weakly with PMCA4 in human breast adenocarcinoma cells.

3.2. The region 462–684 of PMCA2b is essential for interaction with calcineurin A

We have previously reported that the region encompassing amino acids 428–651 of human PMCA4b is involved in the interaction with calcineurin A [5]. Comparison of the sequence for the equivalent region in PMCA2b revealed a high degree (76%) of amino acid homology suggesting that this region might be involved in the interaction with calcineurin A. To test this hypothesis, we generated FLAG-tagged fusion proteins containing the region spanning amino acids 462–684, or 1143–1243 of PMCA2b (Fig. 2A).

HEK293 cells were transfected with plasmids pF-PMCA2b-(462–684) or -(1143–1243). Commercially available calcineurin A (Sigma) was added to the protein lysates to a final concentration 40 nM. Protein lysates were then immunoprecipitated by overnight incubation with monoclonal anti-calcineurin A antibody, and, subsequently, probed by western blot. FLAG-PMCA2b-(462–684) co-precipitated with calcineurin A (Fig. 2B, left upper panel). No precipitation was observed for FLAG-PMCA2b-(1143–1243) demonstrating the selectivity of the interaction (Fig. 2B, left upper panel). Cells transfected with pF-PMCA4b-(428–651) were used as a positive control (Fig. 2B, left upper panel) [5]. These results demonstrate that the region 462–684 of the catalytic large intracellular loop of PMCA2b is implicated in the interaction with calcineurin A.

Our previous observations for human PMCA4b suggested that the fragment 501–575 of PMCA4b is essential for the interaction of this protein with calcineurin [5]. To test the ability of the equivalent region in hPMCA2b (fragment 535–609) to interact with calcineurin we generated a Flag-tagged fusion protein containing this region (Fig. 2A). Flag-hPMCA2b(535–609) co-precipitated with calcineurin (Fig. 2B, right upper

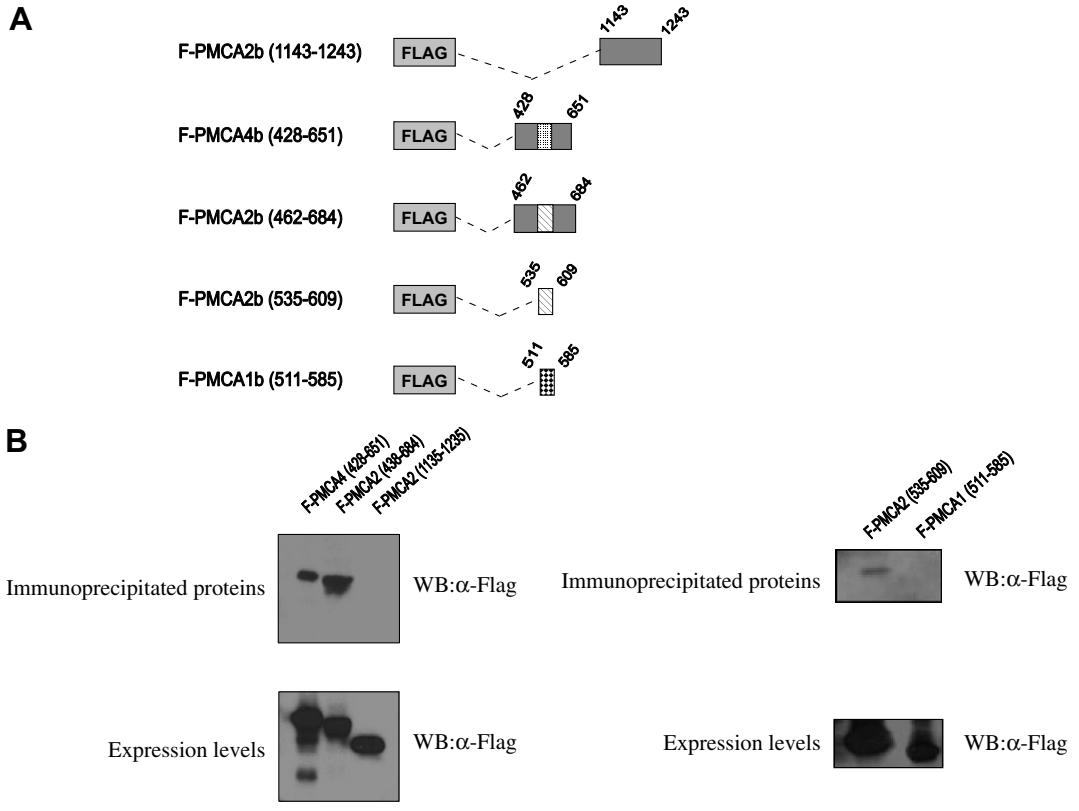

Fig. 2. The region 462–684 of human PMCA2b interacts with calcineurin A. (A) Schematic representation of FLAG-tagged proteins containing; the C-terminal (amino acids 1143–1243) intracellular region of human PMCA2b, the region 462–684 of its catalytic domain or the equivalent region (428–651) in the PMCA4b catalytic domain, and the small interaction domain 539–609 of hPMCA2b or the equivalent region (511–585) in hPMCA1b. (B) Immunoprecipitation analysis of the interaction between calcineurin A and the FLAG–tagged proteins described above. The region 462–684, located within the catalytic big intracellular loop of PMCA2b, specifically interacts with calcineurin A. A small fragment encompassing amino acids 539–609 of hPMCA2b still interacts with calcineurin, however, the equivalent sequence of hPMCA1b (511–585) failed to co-precipitate with calcineurin.

panel) although to a lesser degree than that observed with the full fragment (462–684) suggesting that adjacent sequences might contribute to the interaction. This result indicates that the region 535–609 of PMCA2b interacts with calcineurin.

A Flag-tagged fusion protein containing the equivalent sequence for hPMCA1b, Flag-hPMCA1b(511–585) (Fig. 2A), failed to co-precipitate with calcineurin (Fig. 2B, right upper panel), confirming the lack of interaction observed between endogenous PMCA1 and calcineurin.

Western blot analysis of the protein lysates prior to immunoprecipitation showed expression of the corresponding Flag-tagged fusion proteins, disproving that lack of expression may have led to a lack of interaction (Fig. 2B, lower panels).

3.3. PMCA2b significantly inhibits NFAT transcriptional activity

Previous investigations have reported that PMCA4b negatively regulates the activity of endogenous calcineurin A in mammalian cells [5]. We speculated that PMCA2 could also function as an inhibitor of the calcineurin/NFAT pathway.

HEK293 cells were co-transfected with pcDNA3 (empty plasmid) and the NFAT-dependent luciferase reporter vector pNFAT-TA-Luc (Clontech) and then stimulated with PMA and the calcium ionophore A23987 (Io) to induce calcineurin A activation and consequent NFAT transcriptional activity. Co-transfection of pNFAT-TA-Luc with pcDNA3-hPMCA2b, an expression plasmid encoding hPMCA2b, significantly reduced (32% inhibition, $P \leq 0.05$) PMA plus Io-dependent activation of the luciferase reported vector (Fig. 3). These results suggest that PMCA2b negatively regulates the activity of endogenous calcineurin A in mammalian cells.

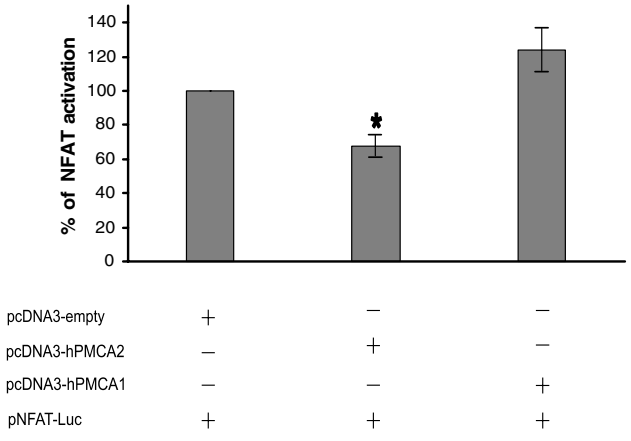

Fig. 3. PMCA2b significantly inhibits NFAT transcriptional activity. PMCA2b inhibits the activation of an NFAT-dependent luciferase reporter vector in response to PMA plus Calcium ionophore (Io) A23187. 10 μg of pcDNA3-hPMCA2b, pcDNA3-hPMCA1b or control vector pcDNA3, and 5 μg of the NFAT-dependent luciferase reporter vector pNFAT-TA-Luc were co-transfected in HEK293 cells. Cells were stimulated with PMA (20 ng/ml) plus Io (1 μM) for 16 h. Induction after PMA/Io stimulation of reporter vector luciferase activity in the presence of co-transfected control plasmid pcDNA3 was taken as reference (100%). Co-expression of human PMCA2b resulted in a significant reduction (32% inhibition) of luciferase induction. However, co-expression of human PMCA1b did not result in inhibition of the luciferase reporter vector. *, statistically significant ($P \leqslant 0.05$, according to Student's *t*-test). Means ± S.E. of three independent experiments are shown.

In agreement with the lack of interaction observed between endogenous hPMCA1b and calcineurin, co-transfection of pNFAT-TA-Luc with pcDNA3-hPMCA1b, an expression plasmid encoding human PMCA1b, did not result in inhibition of the luciferase reporter vector (Fig. 3).

4. Discussion

In this investigation we have shown that the interaction between endogenous PMCA and calcineurin is isoform specific in MCF-7 breast adenocarcinoma cells. A strong interaction was detected between PMCA2 and calcineurin A. Expression of PMCA2 leads to inhibition of the calcineurin/NFAT pathway.

Emerging evidence indicates a major role for PMCA2 in mammary gland physiology; PMCA2b is highly expressed in lactating mammary tissues [10], mice homozygous for a null-mutation of the *pmca2* gene produced milk with 60% less calcium than wild type animals [11] and expression of PMCA2 is up-regulated in breast cancer cell lines [12]. These findings suggest that deregulation of PMCA might contribute to breast tumorigenesis.

In this work, we demonstrate that PMCA2 inhibits the calcineurin/NFAT pathway. Increased PMCA2 expression in breast cancer is, therefore, expected to inhibit the calcineurin/NFAT pathway. High calcineurin activity leads to apoptosis [13] thereby, PMCA2-mediated down-regulation of calcineurin activity might result in inhibition of apoptosis and promotion of tumorigenesis in cancer cells. In agreement with this hypothesis, Padma et al. [14] have shown that calcineurin activity is down-regulated in cervical carcinoma. It would be interesting to analyse the level of PMCA2 expression in these cells and determine if it regulates calcineurin activity and, therefore, apoptosis in cervical carcinomas.

PMCA2-mediated inhibition of calcineurin and, therefore, calcineurin-mediated apoptosis, may also be involved in the hearing deficits observed in PMCA2-deficient mice [15]. High levels of PMCA2 have been found in the outer hair cells of the cochlea [16]. Impairment by mutation of PMCA2 function in deafwaddler mice (dfw/dfw) results in apoptotic hair cell death [17]. Lack of functional PMCA2 in the deafwaddler mouse might lead to an increase in calcineurin activity that would result in calcineurin-mediated apoptosis of outer hair cells. In accordance with this hypothesis, heightened activity of calcineurin has been shown to increase apoptosis of outer hair cells and promote cochlear acoustic injury [18]. Moreover, Minami et al. [19] have reported that the calcineurin inhibitors, FK506 and cyclosporine A, reduce noise-induced hair cell death. Therefore, it is highly probable that the interaction between PMCA and calcineurin is a major regulator of apoptosis in hair cells.

In conclusion, our results demonstrate that the interaction between endogenous PMCA and calcineurin is isoform-specific in MCF-7 adenocarcinoma cells and suggest that this interaction might play an important role in the regulation of breast tumorigenesis and hearing loss.

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