UDC 577.352:616-006.44:542.978 https://doi.org/10.26641/2307-0404.2022.3.265768

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THE ROLE OF BAFILOMYCIN AS A THERAPEUTIC AGENT IN THE MODULATION OF ENDO-LYSOSOMAL STORE OF RAT HEPATOCYTES

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Цитування: Медичні перспективи. 2022. Т. 27, № 3. С. 22-26
Cited: Medicni perspektivi. 2022;27(3):22-26

Key words: *autophagy, hepatocytes, bafilomycin A1, ATPases, NAAD, Ca^{2+}-store*
Ключові слова: *автофагія, гепатоцити, бафіломіцин А1, АТФази, НААДФ, Ca^{2+}-депо*

Abstract. The role of bafilomycin as a therapeutic agent in the modulation of endo-lysosomal store of rat hepatocytes. Bychkova S.V., Stasyshyn A.R., Bychkov M.A. *Endo-lysosomal system through the process of autophagy is involved in the pathogenesis of many diseases. Acidification of these organelles is carried out by V-type H+-ATPases, which is inhibited by bafilomycin A1. Endosomes and lysosomes are also important Ca^{2+}-storage in a cell. Nicotinic acid adenine dinucleotide phosphate (NAADP) releases Ca^{2+} from endo-lysosomes. The main purpose of the study was to found out the effect of bafilomycin A1 and NAADP on stored Ca^{2+} and on the ATPase activity of rat hepatocytes. The stored Ca^{2+} was estimated using chlorotetracycline in permeabilized hepatocytes of rats. ATPase activity was determined by level of orthophosphate spectrophotometrically. It was found that bafilomycin A1 reduces stored Ca^{2+} in permeabilized hepatocytes of rats in the micromolar range of concentration (20 and 0.04 mkM) and averted the effect of NAADP on calcium content. Lower concentrations of bafilomycin A1 (0.001 mkM) did not alter the content of stored calcium, but prevented the influence of NAADP in permeabilized hepatocytes of rats. In the subcellular fraction of rat liver bafilomycin A1 (0.001 mkM) increased Ca^{2+}-ATPase and basal Mg^{2+}-ATPase activities and reduced Na^{+}/K^{+}-ATPase activity. Preincubation of the subcellular fraction with bafilomycin A1 completely averts any changes in the activity of estimated ATPases by means of NAADP. It was concluded that the bafilomycin-sensitive store in hepatocytes of rats is NAADP-sensitive endo-lysosomal Ca^{2+}-store. Using of bafilomycin A1 may be useful in treating autophagy-depended diseases.*

Реферат. Роль бафіломіцину як терапевтичного агента в модуляції ендолізосомального депо гепатоцитів щурів. Бичкова С.В., Стасишин А.Р., Бичков М.А. *Ендолізосомальна система через процес автофагії бере участь у патогенезі багатьох захворювань. H^{+}-АТФази V-типу відповідають за закислення цих органел. Бафіломіцин А1 є інгібітором вакуолярних H^{+}-АТФаз. Ендолізосомальна система також має важливе значення як депо Ca^{2+} в клітині. Нікотинової кислоти аденіндинуклеотид фосфат (НААДФ) вивільнює Ca^{2+} з ендолізосом. Головним завданням роботи було встановити дію бафіломіцину А1 та НААДФ на рівень Ca^{2+} всередині внутрішньоклітинних органел пермеабілізованих гепатоцитів щурів та на активність АТФаз. Вміст Ca^{2+} у гепатоцитах щурів контролювали за допомогою хлоротетрацикліну. АТФазну активність досліджували спектрофотометрично, визначаючи вміст ортофосфату, який виділявся після гідролізу АТФ субклітинною фракцією печінки щурів. Було встановлено, що бафіломіцин А1 знижує вміст Ca^{2+} у пермеабілізованих гепатоцитах щурів за дії в мікромолярному діапазоні концентрацій (20 та 0,04 мкмоль/л) і повністю запобігає індукованому НААДФ вивільненню депонованого Ca^{2+}. Більш низькі концентрації бафіломіцину А1 (0.001 мкмоль/л) не змінювали вміст депонованого кальцію, але також запобігали впливу НААДФ. Показано, що бафіломіцин А1 (0.001 мкмоль/л) підвищує активність Ca^{2+}-АТФази, а також базальної Mg^{2+}-АТФази та знижує активність Na^{+}/K^{+}-АТФази субклітинної фракції печінки щурів. Попередня інкубація з бафіломіцином А1 повністю запобігає будь-яким змінам активності оцінюваних АТФаз за дії НААДФ у субклітинній фракції печінки щурів. Було зроблено висновок, що чутливе до бафіломіцину депо гепатоцитів щурів є одночасно чутливим до НААДФ ендолізосомним Ca^{2+}-депо. Застосування бафіломіцину А1 у такому разі може бути доцільним для лікування захворювань, що залежать від автофагії.*

The endosomes and lysosomes or the so-called cell acid store, is a heterogeneous term that includes organoids having an acidic content. These include lysosomes (primary and secondary), autophagosomes, secretory granules, endosomes. The acidic content of these organoids is provided by the vacuolar H^+-pump [8], which is inhibited by bafilomycin A1 [7]. This macrolide antibiotic causes the accumulation of protons inside the cells, inhibiting the V_0 subunit of the pump. It causes cellular acidosis and is therefore potent to use in anti-cancer therapy [3, 6]. In addition, bafilomycin A1 is used as an inhibitor of autophagy, since it inhibits the infusion between lysosomes and autophagosomes, and as an inhibitor of lysosomal degradation. Autophagy involves lysosomal degradation and recycling of organelles and is thus considered an important mechanism for the survival of both normal and cancer cells. Therefore, the cell constantly converts acidic organoids, which are both Ca^{2+}-containing cell store. The NAADP releases calcium from this store. NAADP/Ca^{2+} signalling mechanism may serve as a potential target for T cell- or cardiomyocyte-related diseases such as multiple sclerosis or arrhythmia [9].

It was shown that bafilomycin A1 (1 mkM) averted NAADP induced Ca^{2+} signaling in permeabilized rat hepatocytes [2]. Such as bafilomicine A1 is potent chemotherapeutic agent for cancer treatment [3, 6] it is important to establish the effect of bafilomycin A1 in different concentration on stored Ca^{2+} and ATPase activity in rat liver and its role in NAADP/ Ca^{2+} signalling mechanism.

By bafilomycin A1 we estimated the H^+ pump localization in rat hepatocytes and their correlation to other ATPases in plasmatic membrane (PM) and endoplasmatic reticulum (EPR). The practical significance of these results will be to understand the influence of bafilomycin A1 on healthy liver cells when used in anticancer therapy.

MATERIALS AND METHODS OF RESEARCH

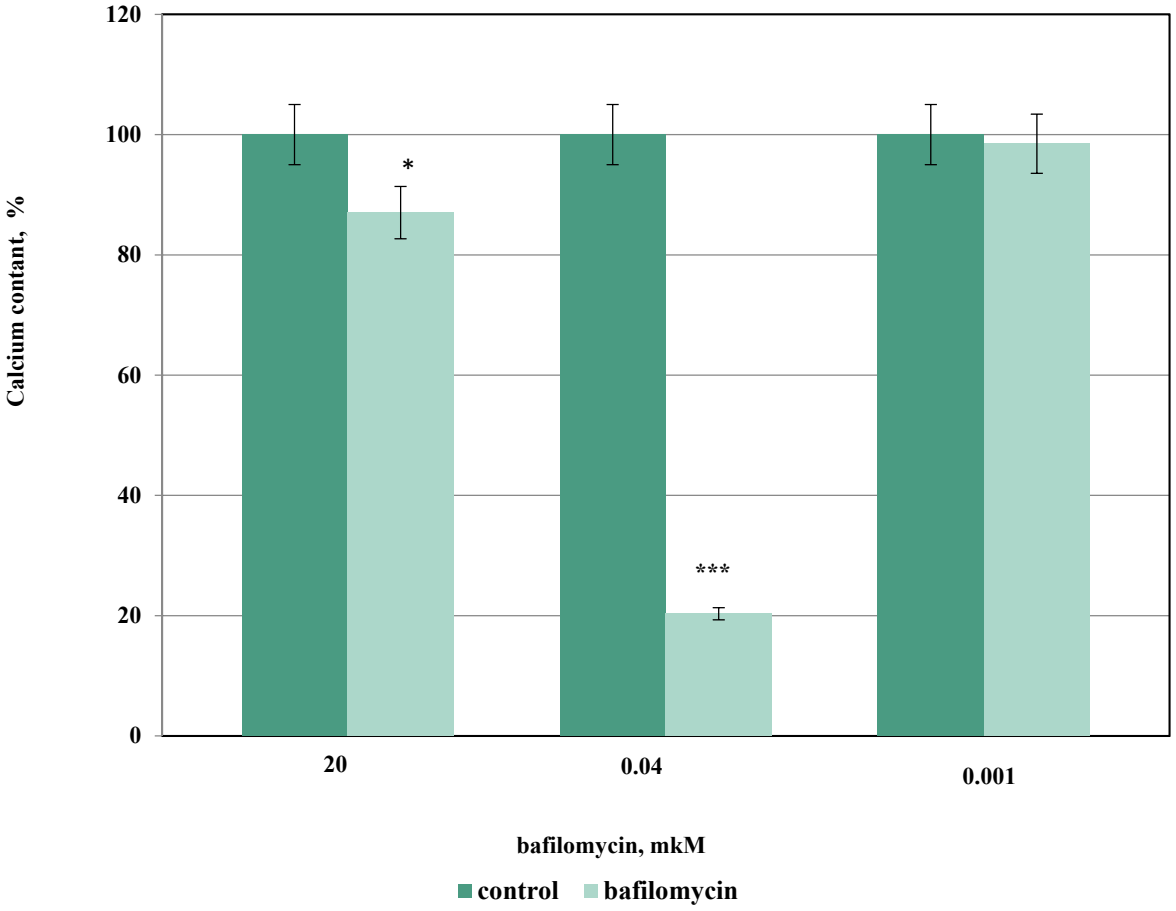
All procedures with animals were performed in accordance with the "International Convention for Working with Animals" under approval of the Bioethics Committee of Biological Faculty at the Ivan Franko National University of Lviv, protocol No. 10/12, 2019. Nonlinear type male rats (180-200 g) were anaesthetized with an inhalation of chloroform and subsequently decapitated. The liver was isolated and fermented with the "two step" collagenase II type (Sigma, USA) perfusion method as described early for the preparation of isolated hepatocytes [2]. Stored calcium was measured by fluorescence of Ca^{2+}- chlorotetracycline complex. This Ca^{2+}-sensitive is used to monitor the stored Ca^{2+} concentration [2] in the lumen of organelle. Isolation of subcellular postmitochondrial fraction of the rat liver was made as described previously [1, 3, 5]. Assay of ATPase activity was carried out basing on determining the content of inorganic phosphorus by the spectrophotometric method. Statistical processing of information was performed using the Software SPSS Statistics 17.0. Wilcoxon-Mann-Whitney test [4] was used in the ATPase activity analysis when a data distribution was not normal, while Student's t-test was used when a data distribution was normal. $p \leq 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Firstly we estimated the influence of bafilomycin and NAADP on calcium content in permeabilized rat hepatocytes. It was found that bafilomycin A1 (0.04 mkM) caused a significant decrease of stored calcium by (77.95±1.44)% ($p \geq 0.001$), at a higher concentration (20 mkM) only by (9.66±4.65) % ($p \geq 0.01$), and no changes of stored calcium in rat's permeabilizing hepatocytes was observed by applying lower concentration of bafilomycin A1 (0.001 mkM) (Fig. 1). We think that the decreasing of stored calcium by the bafilomycin (20 and 0.04 mkM) reflects a change of its content mainly in the EPR and/or endosomes. As it was shown [2], CTC is used for monitoring the Ca^{2+}-signal from the EPR (pH 7.2), as well as from weakly acidic organelles presented by endosomes with a pH between 6 and 6.4. Lysosomes are known to have much lower pH values in the range of 4.5 to 5 [8]. No changes of calcium content by bafilomycin (0.001 mkM) may explain that its effect at this concentration is limited only by lysosomes and does not involve other Ca^{2+}-stores, and therefore does not change the fluorescence intensity of the Ca^{2+}-CTC complex.

We also examined effect of bafilomycine A1 on NAADP-induced calcium release in permeabilized rat hepatocytes to determine the role of acid store. It is known that NAADP is able to release Ca^{2+} from acid store [4], which has been confirmed for various tissues, including hepatocytes [1].

The combined action of bafilomycinA1 (20 and 0.04 mkM) and NAADP (7 mkM), did not change the calcium content of permeabilized hepatocytes as compared to the medium with bafilomycin A1. Simultaneous effect of lower concentration of bafilomycin (0.001 mkM) and NAADP (7 mkM) caused a statistically significant increase in stored calcium by (40.23±3.47)%, ($p \geq 0.001$), which we believe is due to its accumulation in the EPR (Fig. 2). This is possible because in the series the "nominally calcium-free" cell incubation medium was used, to which no calcium salts were added, but no chelators were used.



*** – p≤0.001 vs control; * – p≤0.05 vs control.

Fig. 1. Bafilomycin *(20; 0,04; 0,001 mkM)* effect on calcium content in ermeabilized rat hepatocytes

In order to control the calcium content of the incubation medium more effectively, we conducted the following series of experiments, setting different concentrations of free calcium in the incubation medium using EGTA-Ca^{2+} buffers.

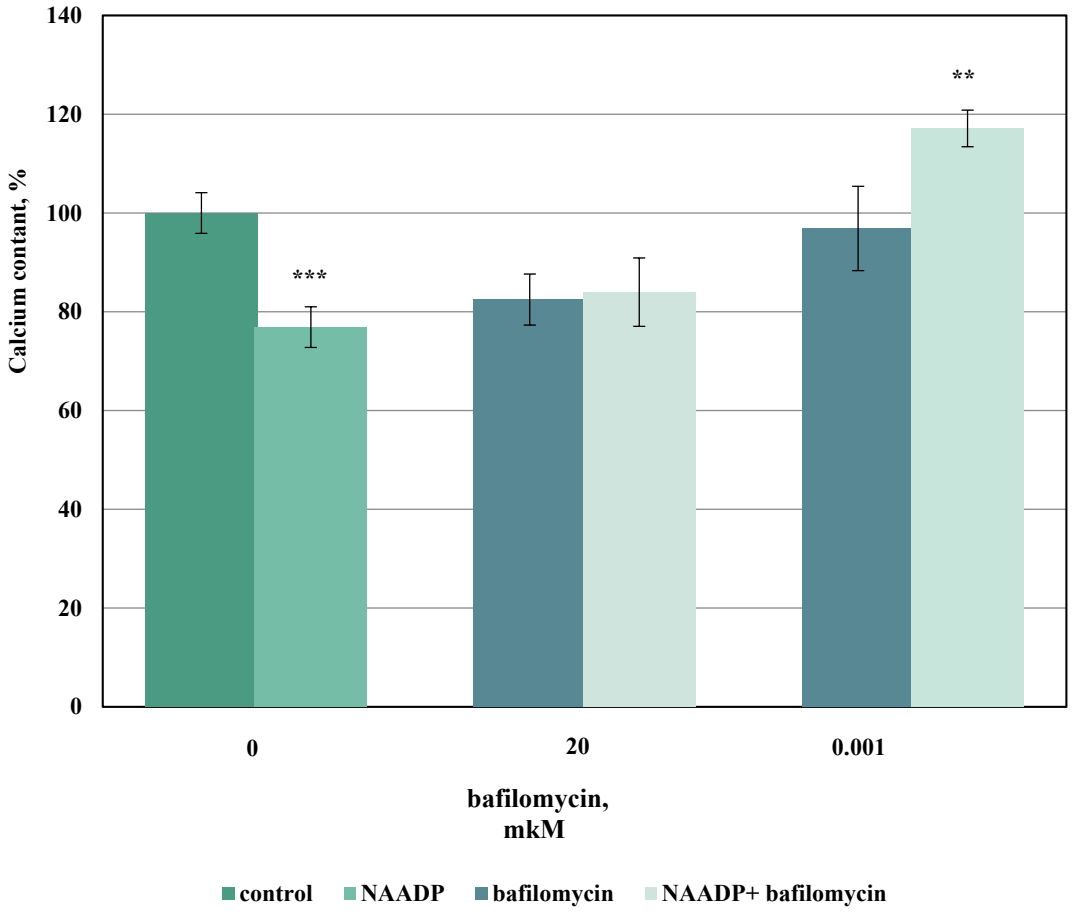
Medium A contained high concentrations of EGTA (0.100 mM) and Ca^{2+} salts (0.050 mM), while *medium B* contained a lower concentrations of EGTA (0.05 mM) and Ca^{2+} salts (0.025 mM). In both cases, the concentration of free calcium was maintained by EGTA-Ca^{2+} buffer solution at the level of 240 nM, which is physiological level at rest.

Bafilomycin A1 (0.001 mkM) did not alter the stored calcium content of rat hepatocytes at any mediums. NAADP (7 mkM) caused a decrease stored calcium only in *medium B* with low EGTA content by (33.21±4.16)%, (p≥0.001). Combined influence of bafilomycin A1 (0.001 mkM) and NAADP (7 mkM) causes an increase in calcium content by (41.36±3.92)%, (p≥0.001) versus NAADP alone. However, this effect is not statistically significant as compared to control or bafilomycin A1. Thus we established that bafilomycin A1 (0.001 mkM) prevents the effects of NAADP.

This is indirectly evidenced by the fact that the combined action of bafilomycin A1 and NAADP prevents NAADP-induced release of calcium from the acidic store. This is also a confirmation that the NAADP-sensitive hepatocyte store contains a bafilomycin-sensitive H^+- pump, and thus being an acid store corresponding to the endo-lysosomal cell system.

We observed that bafilomycin A1 (0.001 mkM) significantly increased total activity of Ca^{2+}-ATPases in subcellular fraction of rat liver, which was due to 3-fold increased EPR Ca^{2+}-pump activity (p≤0.05). However, it did not change PM Ca^{2+}-ATPase activity. We assume the close co-localization of the bafilomycin-sensitive acid store to the EPR, but not to PM, because bafilomycin A1 caused an increasing activity of EPR Ca^{2+}-ATPase activity but not PM Ca^{2+}-ATPase. The bafilomycinA1 (0.001 mkM) significantly reduced the Na^+/K^+-ATPase activity. We explained the effect of bafilomycin A1 on Na^+/K^+-ATPase by its presence in the membranes of the endo-

lysosomal store, which may enter the endosomes as a result of PM invagination. We found that bafilomycin A1 (0.001 mkM) increased basal Mg^{2+}-ATPase more than 2 folds ($p \leq 0.05$). Most likely, the influence of bafilomycin A1 on these two ATPases is realized through a change in pH.



*** – $p \leq 0.001$ vs control; ** – $p \leq 0.01$ vs NAADP.

Fig. 2. Simultaneous effect of bafilomycin *(20; 0,001mkM)* and NAADP on calcium content in permeabilized rat hepatocytes

The combined influence of bafilomycin A1 and NAADP did not change activity of Ca^{2+}-ATPases in subcellular fraction of rat liver, but caused even more potent inhibition on the activity of Na^+/K^+-pumps and completely prevented any changes of Mg^{2+}-ATPase basal activity as compared to NAADP influence alone. Although it was previously established that NAADP caused a significant increase in Ca^{2+}-ATPase activity in the subcellular fraction of rat liver [3] due to the increase in Ca^{2+}-ATPase of EPR. The combined influence of bafilomycin A1 and NAADP inhibited the H^+-pump of the acid store, prevented the H^+-gradient on its membranes, which is the driving force for the transport of Ca^{2+} ions, and therefore NAADP did not release calcium and therefore did not change Ca^{2+} activity of the EPR pump. This confirms that NAADP-sensitive receptors localized in the endo-lysosomal store are closely associated with the EPR. Apparently, a certain morphological contact is formed between the EPR and the endo-lysosomal store, which persists even after receiving the subcellular fraction after centrifugation.

It has been previously found that NAADP decreased the activity of the basal Mg^{2+}-ATPase and the activity of Na^+/K^+-ATPase [3]. The combined effect of bafilomycin A1 and NAADP caused even more potent inhibition on the activity of Na^+/K^+-pumps and completely prevented any changes in basal Mg^{2+}-ATPase activity as compared to NAADP. Therefore, NAADP, like bafilomycin A1, exerts a unidirectional effect on this system of active ion transport, apparently altering the pH and / possibly the concentration

of sodium in the medium. Therefore, our assumption is correct: the effect of NAADP and bafilomycin A1 on the activity of basal Mg^{2+}-ATPase is realized through a change in pH.

CONCLUSIONS

Bafilomycin A1 was found to effectively reduce the stored calcium, preventing effect of NAADP on this finding. Bafilomycin A1 increases the EPR Ca^{2+}-ATPase and basal Mg^{2+}-ATPase activities and reduces Na^{+}/K^{+}-ATPase activity in subcellular fraction of rat liver. The combined action of bafilomycin A1 and NAADP completely prevents any changes of Ca^{2+}-ATPase and basal Mg^{2+}-ATPase activities as compared to the medium with NAADP. Therefore, the bafilomycin-sensitive store has a close functional and physical contact with the EPR Ca^{2+}-ATPases and at the same time is NAADP-sensitive. In addition, some fraction of bafilomycin-sensitive store, which may be represented by endosomes, contains Na^{+}/K^{+}-ATPase. We have confirmed that the in hepatocytes Ca^{2+}-store is simultaneously sensitive to NAADP and bafilomycin A1 and is represented by the endo-lysosomal cell system.

Contributors:

Bychkova S.V. – conceptualization, investigation, writing – original draft;

Stasyshyn A. R. – formal analysis, resources;

Bychkov M.A. – conceptualization, supervision, writing - review & editing.

Funding. This research received no external funding.

Conflict of interests. The authors declare no conflict of interest.

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Стаття надійшла до редакції
22.01.2021