INFLUENCE OF NAADP AND BAFILOMYCINE A1 ON ACTIVITY OF ATPase IN LIVER POSTMITOCHONDRIAL FRACTION

S. Bychkova

Ivan Franko National University of Lviv, 4, Hrushevskyi St., Lviv 79005, Ukraine
e-mail: s.bychkova@gmail.com

Recycling of the endo-lysosomal organelles occur by permanent in the cell. This store is thought to be the acidic store because V-type of H⁺-ATPase is responsible for their acidification. Bafilomycine A1 is a selective inhibitor of vacuolar H⁺ ATPase, and is often used as anticancer drug. However, it is not clear if applying of bafilomycine A1 is safe for normal cells. Endo-lysosomes are important cellular Ca²⁺ storage, sensitive to nicotinic acid adenine dinucleotide phosphate (NAADP). Hepatocytes were shown to have NAADP-sensitive acidic store. But the correlation between acidic store and endoplasmatic reticulum or plasmatic membrane during endo-lysosomal organelles recycling and fusion is still unknown. The main goal of this study was to examine influence of bafilomycine A1 and NAADP on activity of Na⁺/K⁺-ATPase, basal Mg²⁺-ATPase and Ca²⁺-ATPase of plasmatic membrane (PM) and endoplasmatic reticulum (ER) for better understanding of the relationship between acidic organelles and PM/ER. Besides possibility of bafilomycine A1 applying as anticancer drug is examined. All experiments were conducted using rat liver postmitochondrial fraction.

It was shown that SERCA activity is increasing under bafilomycine A1 presence, as well as NAADP. At the same time, changes in PMCA activity was not found. Preincubation of rat liver postmitochondrial fraction with bafilomycine A1 completely prevented NAADP-induced increasing of SERCA activity. Bafilomycine A1 decreased activity of Na⁺/K⁺-ATPase and amplificated NAADP-induced decreasing of this pump. Applying of NAADP caused more intensive decrease in Na⁺/K⁺-ATPase activity after preincubation of this fraction with bafilomycine A1. It was also shown that bafilomycine A1 caused increasing of basal Mg²⁺-ATPase activity and NAADP intensify bafilomycine A1-induced changes of function of this pump. These effects are realized due to changes of pH inside the endo-lysosomal ogranels, acidification of incubation medium, as well as calcium concentration in local contact sites.

A conclusion was made that NAADP-sensitive store is bafilomycine A1-sensitive, which also has direct contact sites to ER, but not to PM. Besides, existing of NAADP-sensitive, but bafilomycine-insensitive store in this fraction is supposed, which is more likely represented by endosomes. So, applying bafilomycine A1 in anticancer therapy may cause a damage of endo-lysosomal organelles recycling in healthy cells.

Keywords: NAADP, bafilomycine A1, H⁺-pump, Na⁺/K⁺-ATPase, SERCA, PMCA, basal Mg²⁺-ATPase, endosomes, lysosomes.
INTRODUCTION

Eukaryotic cells contain lysosomes, with the exception of some highly specialized cells such as mammalian erythrocytes. Lysosomes degrade exogenous and endogenous macromolecules derived from biosynthetic and endocytic pathways, and catabolize cytosolic components that are obtained from the autophagic pathway [22]. Lysosomes, lysosome-related organelles, and endosomes are important Ca\(^2+\) storage cellular compartments with a crucial role in intracellular Ca\(^2+\) signalling. V-type H\(^+\)-ATPases are responsible for acidification of these organelles. Bafilomycin A1, a macrolide antibiotic isolated from the *Streptomyces* species, is an inhibitor of vacuolar H\(^+\)-ATPase (V-ATPase). It binds to the V\(_0\) sector subunit C of the V-ATPase complex and inhibits H\(^+\) translocation, causing an accumulation of H\(^+\) in the cytoplasm of treated cells [2]. The anticancer effects of bafilomycin A1 are considered to be attributable to the intracellular acidosis caused by V-ATPase inhibition. However, it is not clear it applying of bafilomycine A1 as anticancer drug is safe for normal cells.

Bafilomycin A1 has been used in the study of autophagy as an inhibitor of fusion between autophagosomes and lysosomes and as an inhibitor of lysosomal degradation [26]. Autophagy is a cellular catabolic pathway that is involved in lysosomal degradation and recycling of proteins and organelles, and therefore is considered as an important survival mechanism for both normal cells and cancer cells in response to metabolic stress or chemotherapy. The defects in these processes can result in disease.

Interest to endo-lysosomes as Ca\(^2+\)-storage organelles has intensified with the discovery that NAADP (nicotinic acid adenine dinucleotide phosphate), a Ca\(^2+\)-mobilizing messenger first discovered to evoke Ca\(^2+\) signals in sea urchin eggs [9], does so in many cases by activating Ca\(^2+\)-release mechanisms on acidic stores with characteristics of lysosomes [4]. The fundamental question that remains unanswered is the identity of the NAADP receptor. Transient receptor potential mucolipin (TRPML) and two-pore channels (TPCs) are Ca\(^2+\)-permeable ion channels revealed within the endolysosomal system. Both structures have been proposed as potential targets for NAADP [19]. Besides, it was shown that TPCs are not gated by NAADP [8]. It was identified an endolysosomal ATP-sensitive Na\(^+\) channel (lysoNa\(_{ATP}\)), which is a complex formed by TPCs1 and TPCs2 and mTOR (mammalian target of rapamycin) [8]. Although there is substantial evidence that RyRs are the principal effectors of cADPR-induced Ca\(^2+\) release from the endoplasmic reticulum (ER), a number of studies have also implicated RyRs in NAADP-evoked Ca\(^2+\) release [10; 12].

It was suggesed that the effect of NAADP is dependent on ER luminal calcium in hepatocytes [6]. Besides, it was also assumed that RyRs have been involved in indirect modulation of the NAADP effect via increasing the level of ER luminal calcium [6]. This suggestion predicts presence of the specific membrane contact sites between acidic organelles and the ER. So, relationship between acidic organelles of the endolysosomal system to ER Ca\(^2+\) store remains unclear. The recycling of endosome and fusion of lysosome to autophagosome as results of autophagy may affect this relationship. Thus, different ions transported systems may be also involved in this process.

The main goal of this study was to examine influence of bafilomycine A1 and NAADP on activity of ATPases for better understanding relationship between the acidic organelles and endoplasmic reticulum / plasmatic membrane. At the same time, these results should provide a comprehension of bafilomycine A1 safety as an anticancer drug.
MATERIALS AND METHODS

Isolation of a subcellular fraction. Experiments were conducted on male and female nonlinear white type rats (0.18–0.2 kg). All procedures with animals were in accordance with the “International Convention for working with animals” under approval of the Bioethics Committee of Biological Faculty (Ivan Franko National University of Lviv) (Protocol N 11/15 dated by 10.12.2015). After ether anesthesia rats were decapitated. Isolated rat liver was perfused briefly with homogenizing medium (buffer solution) containing (mmol/L): sucrose – 250.0; EDTA – 1.0; Tris-HCl – 10.0 (pH 7.4 t = 37 °C). Then, chilled tissue was crushed by passing through the press. Next, buffer solution was added to the minced liver Minced tissue was added buffer solution (in a ratio of 1:8) and tissue was homogenized with a Potter-Elvehjem teflon-glass homogenizer at a speed of 300 rev / min. Liver subcellular fractions obtained by differential centrifugation, the essence of which is to conduct a series of successive centrifuging the mixture of organelles and membrane fragments obtained after homogenization of tissue. First of all, homogenates were centrifuged for 10 min at 3000 g (centrifuge PC-6) for the deposition of intact cells and nuclei, mitochondrial fraction precipitated for 10 minutes on a 6500 g and a temperature 0–2 °C. The resulting postmitochondrial supernatant used in the experiment as “subcellular fractions of rat liver”.

Measurement of the ATPase activity. ATPase activity was determined by orthophosphate content released after ATP hydrolysis. At the beginning of the experiment 200 µL of subcellular fraction were transferred to a standard incubation medium which contained (mmol/L) NaCl – 50.0; KCl – 100.0; Tris-HCl – 20.0; MgCl₂ – 3.0; CaCl₂ – 0.01; NaN₃ – 1; ATP – 3.0; pH 7.4 at 37 °C. The reaction was started by adding 3 mM ATP (Sigma) and incubating samples for 15 min at 37 °C at moderate shaking in a water bath. Before the end of incubation 0.4 ml of medium was taken for the determination of protein content by the method of Lowry [15]. For activation NAADP-induced Ca²⁺-releasing we used NAADP (Sigma) at subthreshold concentration 7 µmol/L. Bafilomycine A1 (Sigma) was used as inhibitor V-ATPase at high concentration 1 µmol/L. To compare the impact NAADP against the background of bafilomycine A1 we conducted two parallel incubation samples that contained bafilomycine A1 alone and simultaneous presence bafilomycine A1 and NAADP. Reaction was stopped by adding 5 ml of 10% trichloroacetic acid to samples and incubating them for 10 min followed by 10 min centrifugation at 1600 g. Supernatant obtained was used to determine the content of inorganic phosphorus by the spectrophotometric method of Fiske–Subbarow.

The total ATPase activity of subcellular fraction was calculated as a difference of inorganic phosphorus in the media with different composition (with bafilomycine A1 or NAADP) expressed as micromoles of inorganic phosphorus equivalent to 1 mg of protein per 1 h. Specific Na⁺/K⁺-ATPase activity was calculated as difference of inorganic phosphorus content in medium with or without ouabain (Sigma) at concentration 1 mmol/L. For the determination of Ca²⁺/Mg²⁺-ATPase activity, we quantified the difference between the total Ca²⁺/Mg²⁺- and Na⁺/K⁺-ATPase activity. Thapsigargin (1 mkmol/L) was used to calculate SERCA contribution into the total Ca²⁺/Mg²⁺-ATPase activity. Specific basal Mg²⁺-ATPase activity was determined in incubation medium that contained 1 mmol/L EGTA (Sigma) and lacked ouabain. In all experiments, as a control for the absence of enzymatic ATP hydrolysis was incubation medium with no added tissue.

Data analysis. A significance of differences between different groups was calculated using Wilcoxon-Mann-Whitney test or Student’s t-test. P<0.05 was considered to be statistically significant.
RESULTS AND DISCUSSION

Activity of Ca\(^{2+}\)-ATPases of rat liver postmitochondrial fraction under bafilomycine A1 and NAADP action. Previously we established that NAADP substantial increase common Ca\(^{2+}\)-ATPase activity in liver [24]. But it is still unknown which Ca\(^{2+}\)-pumps realize this effect. Using thapsigargine as selective inhibitor of specific Ca\(^{2+}\)-ATPase of ER (SERCA), we found that NAADP increased its activity on \((142.87\pm18.05)\% \quad (P\leq0.05; \quad n=5)\). It was also revealed that specific SERCA activity increased by 3 fold \((P\leq0.05; \quad n=5)\) under bafilomycine A1 presence (Fig. 1). At the same time no NAADP, nor bafilomycine A1 didn’t cause statistically authentic change of specific activity of plasmatic membranes Ca\(^{2+}\)-ATPase (PMCA) (Fig. 2). Besides it was also found that bafilomycine A1 completely prevented NAADP-induced increasing of specific SERCA activity (Fig. 1).

Thus separately bafilomycine A1 and NAADP cause the same effect on SERCA due to local increasing of Ca\(^{2+}\) concentration. It is possible only if there is close position between an acidic store and ER. Our data also confirmed that NAADP-sensitive Ca\(^{2+}\)-store in hepatocytes is at the same time bafilomycine A1-sensitive. That is why bafilomycine A1 completely prevented NAADP’s effect on SERCA. Our finding is made agree with Kilpatrick and other (2013) postulated existing lysosome-ER membrane contact sites [13]. It is important to note that there was no effect of NAADP and bafilomycine A1 on PMCA. This means that acidic store is not associated to PM in this subcellular fraction.

Activity of Na\(^{+}\)/K\(^{+}\)-ATPases of rat liver subcellular fraction under bafilomycine A1 and NAADP action. Besides Na\(^{+}\)/K\(^{+}\)-ATPase is responsible for generating and maintaining transmembrane ionic gradients that are of vital importance for cellular
function and subservient activities such as volume regulation, pH maintenance, and generation of action potentials and secondary active transport as well as calcium content through Na⁺/Ca²⁺-exchangers, respectively [11]. It was shown that Na⁺/K⁺-ATPases is a key player in the regulation of endosomal pH and endocytosed membrane traffic [11]. Presumably, functional Na⁺/K⁺-ATPases are retained within the plasma membrane/endosomal axis as a result of endosome recycling [19]. They reinforce a lumen positive membrane potential and therefore will inhibit H⁺ pumping in the membrane of organelles [19]. So Na⁺/K⁺-ATPase inhibits acidification of endosomes lumen [11]. We found that bafilomycin A1 decreased activity of Na⁺/K⁺-ATPase by (41.67±7.03) % (P≤0.001; n = 6) (Fig. 3). It is probably due to inhibition of H⁺-pump by bafilomycin A1 and alkalization of endosome's lumen. The dependence of Na⁺/K⁺-ATPase activity on pH in the range of 6.0–7.5 is characterized by the bell-shaped curve [14]. Alkalization more than pH 7.5 inhibits this protein as well as acidification lower pH 6.0 [14]. Previously it was also found that NAADP decreased specific activity of Na⁺/K⁺-ATPase [24]. A.J. Morgan et al. estimated that NAADP caused alkalization of endo-lysosomal lumen [18]. Thus, most likely that NAADP, as well as bafilomycin A1, decreased activity of Na⁺/K⁺-ATPase due to a increase in pH.

The TPCs family of endolysosomal proteins was shown to be regulated by NAADP [3]. Based on their sequence similarity to voltage-gated Ca²⁺/Na⁺ channels, TPCs are predicted to comprise two homologous domains each consisting of 6 transmembrane regions with a putative pore-forming domain located between the 5th and 6th membranespanning regions [20]. Recently, it was shown that endolysosomal ATP-sensitive Na⁺ channel (lysoNa(ATP)) is a complex formed by TPC1 and TPC2 [8]. It was also demonstrated that TPC2/NAADP/Ca²⁺ signaling alkalinizes lysosomal pH to specifically inhibit the later stage of basal autophagy progression [16]. Such as TPCs function as Na⁺-selective channels apparently insensitive to NAADP, so it is possible to assume that under NAADP action Na⁺ concentration inside the lumen of endosomal store may changed and this also decreased activity of Na⁺/K⁺-ATPase.

After preincubation of liver subcellular fraction with bafilomycin A1, next applying NAADP caused more intensive decreasing of Na⁺/K⁺-ATPase activity. Such as bafilomycin A1 amplified NAADP’s effect we assumed that it is possible an existing of

![Fig. 3. Influence of bafilomycin A1 and NAADP on Na⁺/K⁺-ATPase activity in postmitochondrial fraction of rat liver (M±m): * – P≤0.05 vs control; # – P≤0.05 vs bafilomycin](image-url)
NAADP-sensitive but bafilomycine-insensitive store in this subcellular fraction. This assumption was made early observing decreasing of stored calcium in permeabilized hepatocytes, which was not sensitive to thapsigargine or nigericine [6]. This store is the most likely represented by endosome, which may possess Na⁺/K⁺-pump as results of endocytosis [11].

Summarizing these data, we assume that Na⁺/K⁺-ATPase may be active in the endosomal organels and bafilomycine A1, as well as NAADP decreased pump’s activity due to alkalinization of endosomal lumen.

Influence of bafilomycine A1 and NAADP on basal Mg²⁺-ATPase activity in rat liver subcellular fraction. It should to note that activity of basal Mg²⁺-ATPase is coupled to H⁺-translocation in plasma membrane [17; 23] as well as in endosomal fraction [21]. Also in hepatocytes Mg²⁺-ATPase is considered as markers of canalicular membrane [1]. It was observed that bafilomycine A1 caused increasing of basal Mg²⁺-ATPase activity by (60.13±11.68) % (P≤0.05; n = 15) (Fig. 4). This effect doesn’t associated with the calcium concentration increasing because of EGTA presence in incubation medium. The most likely that bafilomycine’s effect is due to pH changing. Previously NAADP-induced decreasing of basal Mg²⁺-ATPase activity was found in subcellular fraction of rat liver, which has been also explained by NAADP-induced alkalinization of endo-lysosomal lumen [24].

Our results are in agreement with kinetic interpretation of the original pH-dependence of enzymatic activity of basal Mg²⁺-ATPase of the smooth muscle sarcolemma [14]. S. O. Kosterin et al. [14] showed that a cause of linear pH-dependence of enzymatic activity of the basal Mg²⁺-ATPase is that H⁺ is a competitive inhibitor of given enzyme: the increase of protons concentration leads to a decrease of the affinity of Mg²⁺-ATP substrate for the enzyme. Our data suggest that bafilomycine A1, as well as NAADP, decrease basal Mg²⁺-ATPase activity due to alkalinization of endo-lysosomal lumen.

Simultaneous presence at incubation medium of bafilomycine A1 and NAADP caused an increase of basal Mg²⁺-ATPase activity by 2 folds (P≤0.05; n = 15) compared to control (Fig. 4). So, NAADP intensify the effect of bafilomycine A1 which confirm presence of the additional NAADP-sensitive store in this subcellular fraction.

This supports our hypothesis that NAADP and bafilomycine A1 cause changes of pH inside the endo-lysosomal organels, as well as in the incubation medium.
CONCLUSIONS

It was shown that SERCA activity increased under bafilomycine A1 presence as well as NAADP in rat liver postmitochondrial fraction. At the same time, changes in PM Ca^{2+}-ATPase activity were not found. These effects are explained by an existing of lysosome-ER membrane contact sites with close apposition between NAADP-sensitive Ca^{2+}-channels of acidic store and SERCA. Previously, preincubation of rat liver postmitochondrial fraction with bafilomycine A1 completely prevented to NAADP-induced increasing of SERCA activity. It was made conclusion that NAADP-sensitive store is at the same time bafilomycine A1-sensitive, which has direct contact sites to ER, but not to PM.

It was found that bafilomycine A1 decreased activity of Na^{+}/K^{+}-ATPase and amplified NAADP-induced decreasing of Na^{+}/K^{+}-ATPase activity. After preincubation of liver subcellular fraction with bafilomycine A1 next applying NAADP caused more intensive decreasing of Na^{+}/K^{+}-ATPase activity, and bafilomycine A1 amplified NAADP’s effect. We assumed that Na^{+}/K^{+}-ATPase may be active in endosomal organelles and bafilomycine A1, as well as NAADP decreased its activity due to alkalization of their lumen. Besides, existing of NAADP-sensitive but bafilomycine-insensitive store in this subcellular fraction is supposed, which is more likely represented by the endosomes. It was also observed that bafilomycine A1 caused an increase in basal Mg^{2+}-ATPase activity, and NAADP intensify bafilomycine A1 -induced changes of this pump function. These effects are realized due to changes of pH inside the endo-lysosomal organelles.

Thus, close association between bafilomycine-sensitive store and ER is postulated for rat liver postmitochondrial fraction. Bafilomycine A1 applying changes activity of active ion-transport systems which may disturb as trasformation of the endolysosomal systems organelles due to pH changes.


ВЛИЯНИЕ БАФИЛОМИЦИНА И НААДФ НА АКТИВНОСТЬ АТФ-аз ПОСТМИТОХОНДРИАЛЬНОЙ ФРАКЦИИ ПЕЧЕНИ КРЫС

С. В. Бычкова
Львовский национальный университет имени Ивана Франко
ул. Грушевского, 4, Львов 79005, Украина
e-mail: s.bychkova@gmail.com

В клетке происходит постоянное рециклювание органоидов эндо-лигосомальной системы, принадлежащих к так называемому "кислотному депо" клетки.
Н+-АТФ-аза создает трансмембранный протонный градиент на этих мембранах, закисляя их изнутри. Селективный ингибитор Н+-помпы бафиломицин используют как противопухолевой препарат, однако мало изученной является его безопасность для нормальных клеток. Кислотные депо являются одновременно Са2+-содержащими органоидами, которые могут ослабевать кальций при действии никотиновой кислоты и нуклеотидфосфата (НААДФ). Для клеток печени показано, что кислотные депо являются НААДФ-чувствительными. Однако не изучена взаимосвязь между системами активного транспорта ионов и кислым депо гепатоцитов, которая может отображать состояние рециклингирования органоидов эндо-лизосомальной системы в клетке. Целью работы было исследовать влияние бафиломицина и НААДФ на активность Na+, K+-АТФ-азы, базальной Mg2+-АТФ-азы и Са2+-АТФ-азы плазматической мембраны (ПМ) и эндоплазматического ретикулума (ЭПР) для понимания возможных взаимосвязей между органоидами эндо-лизосомального депо и ЭПР/ПМ и оценить возможность использования бафиломицина как противоопухолевого препарата. Опыты проведены на безъядерной и безмитохондриальной фракции печени крыс. Определяли активность АТФ-аз, измеряя содержание неорганического фосфора методом Фиске–Субароу.

Показано, что бафиломицин вызывает рост активности Ca2+-помпы ЭПР и не влияет на Ca2+-помпу ПМ. При действии НААДФ также возрастает активность Ca2+-помпы ЭПР и нет изменений в работе Ca2+-помпы ПМ. После предварительной инкубации субклеточной фракции печени крыш с бафиломицином мы не обнаружили влияния НААДФ ни на Ca2+-помпу ЭПР, ни на Ca2+-помпу ПМ. Выявлено, что при действии бафиломицина снижается активность Na+, K+-АТФ-азы. Инкубация с бафиломицином еще больше усиливалась вызванное НААДФ подавление активности этой помпы. Установлено, что при действии бафиломицина возрастает активность базальной Mg2+-АТФ-азы. При инкубации субклеточной фракции печени с НААДФ усиливаются повыше базальной Mg2+-АТФ-азной активности при воздействии бафиломицина. Эти эффекты, по нашему мнению, связаны с бафиломицином-индуцированным изменением рН, а также реализуются через локальные сайты с повышенной концентрацией кальция.

Сделан вывод об активной рециклингации органоидов эндо-лизосомального кислого депо в клетках печени. Мы считаем, что НААДФ-чувствительное депо гепатоцитов является бафиломицин-чувствительным и имеет функциональный и физический контакт с мембранами ЭПР. А также мы предполагаем существование определенной доли эндо-лизосомального депо, которое не чувствительно к бафиломицину, но чувствительно к НААДФ и, очевидно, представлено эндосомами. Таким образом, применение бафиломицина как противоопухолевого препарата может влиять на здоровые клетки печени, проявляясь в нарушении внутриклеточной рециклизации.

**Ключевые слова:** кислое депо, бафиломицин, Н+-помпа, эндо-лизосомальная система клеток, АТФ-азная активность, НААДФ, Na+, K+-помпа, Са2+-помпа ПМ, Са2+-помпа ЭПР.

Одержано: 18.10.2015