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One-step Bioluminescence *ATP*ase Assay for the Evaluation of Neurotoxic Effects of Metal Ions

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Summary. Membrane-bound *ATP*ases, such as Na,K-*ATP*ase and nucleotide triphospho-diphosphohydrolase (*NTPD*ase), being one of the first targets of a toxic action are generally considered as good markers for estimating toxicity. A bioluminescence assay was applied for fast and sensitive evaluation of heavy metals effect on the rat brain synaptosomal membrane *ATP*ase activity. The assay consists of *ATP*-consuming reaction catalyzed by synaptic plasma membrane *ATP*ases coupled to the luminescent firefly luciferase reaction, which consumes residual *ATP* after the course of *ATP*ase reaction. The bioluminescence *ATP*ase assay was applied to study the effect of heavy and transitional metals (Cu^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}) on rat brain *ATP*ase activity after assay optimization. All metals applied inhibited synaptic membrane *ATP*ase activity in a concentration dependent manner. The IC_{50} values ($Hg^{2+} < Cu^{2+} < Cd^{2+} < Pb^{2+}$) obtained with the bioluminescence assay were highly correlated with those obtained by the spectrophotometric method. The fast bioluminescence *ATP*ase assay with small sample and substrate requirements could be adjusted for high-throughput environmental and pharmacological screening.

Keywords. Bioluminescence *ATP*ase assay; Heavy metal ions; Rat brain; Synaptic plasma membranes.

Introduction

Heavy metals present a major hazard to ecosystems and a serious danger to human populations. They are waste products of numerous industrial processes and widespread environmental pollutants which have already caused numerous episodes of intoxication. Toxicological studies revealed that heavy metals cause severe impairment in the central nervous system and damage to other vital organs.

Most of the heavy metals are non-specific neurotoxicants reacting with a wide spectrum of cellular components and disturbing many cellular functions. At molecular level, among other biochemical effects, heavy metals inhibit membrane-bound enzymes altering cerebral metabolism, membrane permeability and energy production [1, 2]. Therefore, the membrane-bound proteins, being one of the first targets of a toxic action are generally considered as good markers for estimating neurotoxicity.

Membrane bound adenosine triphosphatases (*ATP*ases) play an important role in the ionic transfer across the membrane and may also be a target for the effects of metal ions in central nervous system (CNS). The total membrane *ATP*ase activity comprises of Na,K-*ATP*ase and *ecto*-diphosphohydrolase (*ecto-NTPD*ase) activity. Na,K-*ATP*ase is the integral membrane enzyme with the crucial role in the cellular homeostasis. The enzyme transfers chemical energy of adenosine triphosphate (*ATP*) hydrolysis to potential energy of electrochemical gradients of sodium and potassium ions across membrane. *Ecto-NTPD*ase is an *ecto*-enzyme that hydrolyses *ATP* to adenosine di- or monophosphate (*ADP* or *AMP*), thus terminating signaling action of *ATP*, a neurotransmitter and neuromodulator in the synaptic cleft [3].

Toxicological studies revealed that membrane bound *ATP*ases are sensitive to different heavy metals

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that are common environmental pollutants. Inhibitions of *ATP*ase activity by copper, cadmium, mercury and zinc salts *in vivo* [4–6] and copper, cadmium and mercury *in vitro* [4–8] were reported by using the colorimetric determination of inorganic phosphate liberated by hydrolysis of *ATP*.

During the last two decades, the bioluminescence reaction of *ATP* with luciferin-luciferase system has been used increasingly for detection of bacterial pathogens, as well as toxicological, ecological and environmental studies. Firefly luciferase [EC 1.13.12.7] (*Luc*) catalyses the multistep *ATP*-dependent oxidation of luciferin (*D-LH*$_2$), a heterocyclic carboxylic acid, to oxyluciferin with emission of light [9, 10], whereas the number of emitted photons (hν) is directly proportional to the *ATP* concentration in the reaction mixture:

$$Luc + D\text{-}LH_2 + ATP + O_2 \overset{Mg^{2+}}{\longleftrightarrow} Luc + \text{Oxyluciferin} + AMP + PPi + CO_2 + h\nu$$

If luciferase is included in the reaction mixture after the cessation of the *ATP*ase reaction, the number of emitted photons would be oppositely proportional to the rate of *ATP*ase activity. The maximum light emission is obtained in the range between 500 and 600 nm with maximum emission at 562 nm.

In the present study a simple, fast, and sensitive bioluminescence method was designed for assaying *ATP*ase activity in synaptic plasma membrane (SPM) preparation obtained from whole rat brain. The assay consists of *ATP*-consuming reaction catalyzed by membrane *ATP*ases, paired to luminescent firefly luciferase reaction, which consumes residual *ATP* after the course of *ATP*ase reaction. This assay was applied for the evaluation of the effect of heavy metal ions on total synaptic membrane *ATP*ase activity. Compared to classical spectrophotometric assay, the applied bioluminescence assay offers a more practical method for studying the membrane bound *ATP*ase inhibition by different ions and toxic compounds.

Results and Discussion

Assay Optimization

For all experiments *ATP* standard curves were run in the range of 10^{-10} to 10^{-5} *M*. The standard curves were linear (Fig. 1) with the correlation coefficient of 0.996 or higher.

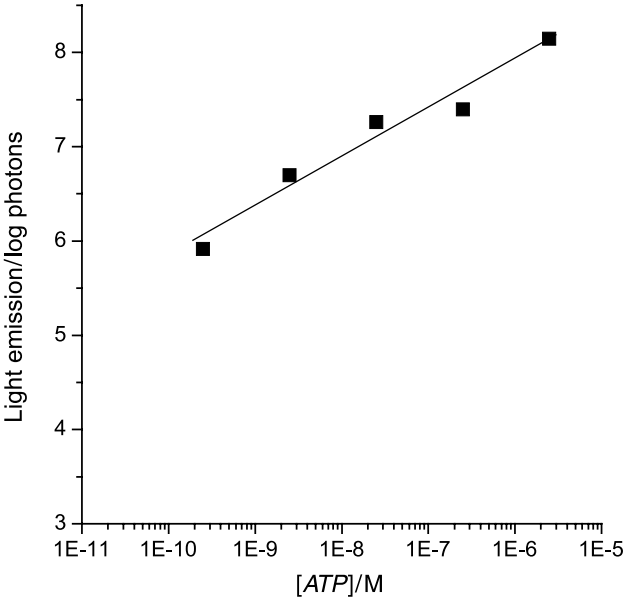

Fig. 1. Representative *ATP* standard curve. Light emission as function of *ATP* concentration in the range of 1×10^{-10} to 1×10^{-5} *M*. The standard curves were generated for each set of samples assayed, using 10-fold serial dilutions of the 1×10^{-3} *M ATP* stock solution in the assay buffer. A standard curve was created by a computer generated four parameter logistic curve fit (Origin 7.0)

Optimum conditions for the bioluminescence *ATP*ase assay concerning the amount of SPM protein (0.5–10 μg), *ATP* content (5×10^{-7}, 5×10^{-6}, 5×10^{-5} *M*), and duration of enzymatic reaction (1–25 min) were further assessed. Figure 2A shows the light emission as a function of the SPM protein amount in the presence of three different *ATP* concentrations after 5 min incubation time. At 5×10^{-5} *M ATP*, almost constant light emission was obtained, while at 5×10^{-6} *M ATP*, light emission slowly decreased as a function of increasing SPM protein amount. At 5×10^{-7} *M ATP*, photon emission exponentially decreased, with a linear decay in the 0.5–2 μg range of SPM proteins. Figure 2B illustrates time-dependent light emission (1–25 min). The light emission was oppositely proportional to the incubation time, with angular coefficient of 0.04 between 5 and 15 min. In compliance to the data presented in Fig. 2, for further experiments 5×10^{-7} *M ATP*, 1 μg of membrane proteins, and 10 min of incubation were chosen.

Reproducibility of the assay was assessed by calculating mean photon emission from all measurements ($N = 24$) and total, within-run and between-

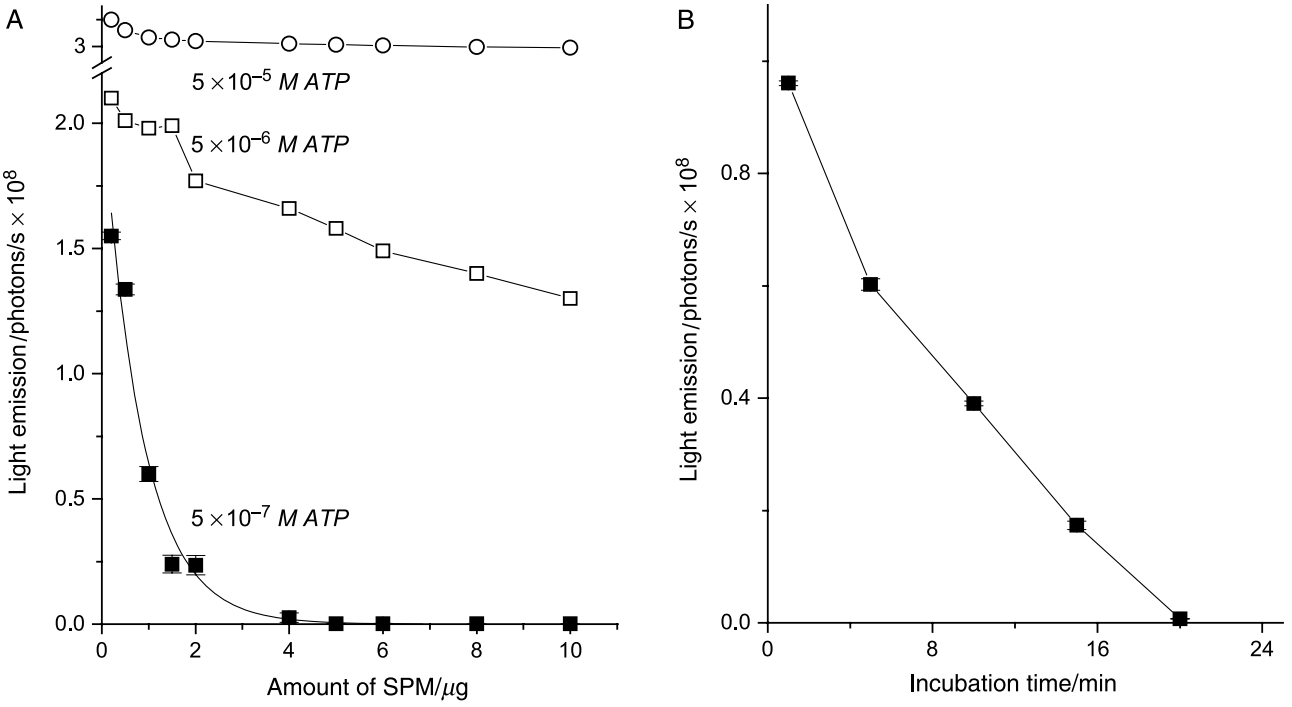

Fig. 2. Bioluminescence *ATP*ase assay optimization. A) Light emission as a function of the SPM protein amount in the presence of three different *ATP* concentrations (5×10^{-7}, 5×10^{-6} and $5\times10^{-5}\,M$) during 5 min incubation time. B) Dependence of light emission from the incubation time in the presence of 1 μg of sample protein and $5\times10^{-7}\,M$ *ATP*. Symbols present mean photon emission $\pm$*S.E.M.* obtained from 3 independent experiments performed in quadruplicate

Table 1. Reproducibility of the bioluminescent *ATP*ase assay

	Mean photon emission / photons × 10^7	Total *CV* / %	Within-run *CV*/%	Between-day *CV*/%
Control sample	1.59	12.58	10.21	12.92

Light emission was measured during 60 s acquisition time in the presence of 1 μg of SPM preparation, $5\times10^{-7}\,M$ *ATP* during 10 min incubation time in a coupled *ATP*ase-luciferase assay (as described in *Experimental*). The mean value was calculated arithmetically, and intra-assay variations were calculated from four experiments in quadruplicate for each of 6 different determinations carried out during 2-week period

day *CV* (%) from 6 different measurement (every three days) carried out over 2-week period, performed four times in quadruplicate. The results are presented in Table 1.

Effect of Metal Ions on ATPase Activity

Figure 3 shows the effect of raising concentrations (1×10^{-10} to $1\times10^{-3}\,M$) of Cu^{2+} (Fig. 3A), Hg^{2+} (Fig. 3B), Cd^{2+} (Fig. 3C), and Pb^{2+} (Fig. 3D) on total *ATP*ase activity. The effects were assessed by using spectrophotometric and bioluminescence assays. Since luciferase reaction was the monitoring system for the effect of different metal cations on *ATP*ase activity, all metal ions were first screened for their ability to interfere with the luciferase-catalyzed photon emission. The influence of metal cations on luciferase activity was assessed in the absence of SPM and results were presented at the corresponding figures. As shown, luciferase activity was not affected by raising concentrations of Cu^{2+} (Fig. 3A), Cd^{2+} (Fig. 3C), and Pb^{2+} (Fig. 3D). However, luciferase activity was significantly inhibited by mercurial ion (Fig. 3B) at concentrations higher than $10^{-7}\,M$ $HgCl_2$. Half-maximum luciferase inhibition was achieved in the presence of $1.07\pm0.01\times10^{-5}\,M$ $HgCl_2$. Potent inhibition of firefly luciferase activity was also previously reported [11, 12]. It was shown that Hg^{2+} and Pb^{2+} inhibit protein function by acting on protein-SH groups. Luciferase protein possesses two SH-groups at luciferin binding site [13] that could be target for inhibitory action of these ions.

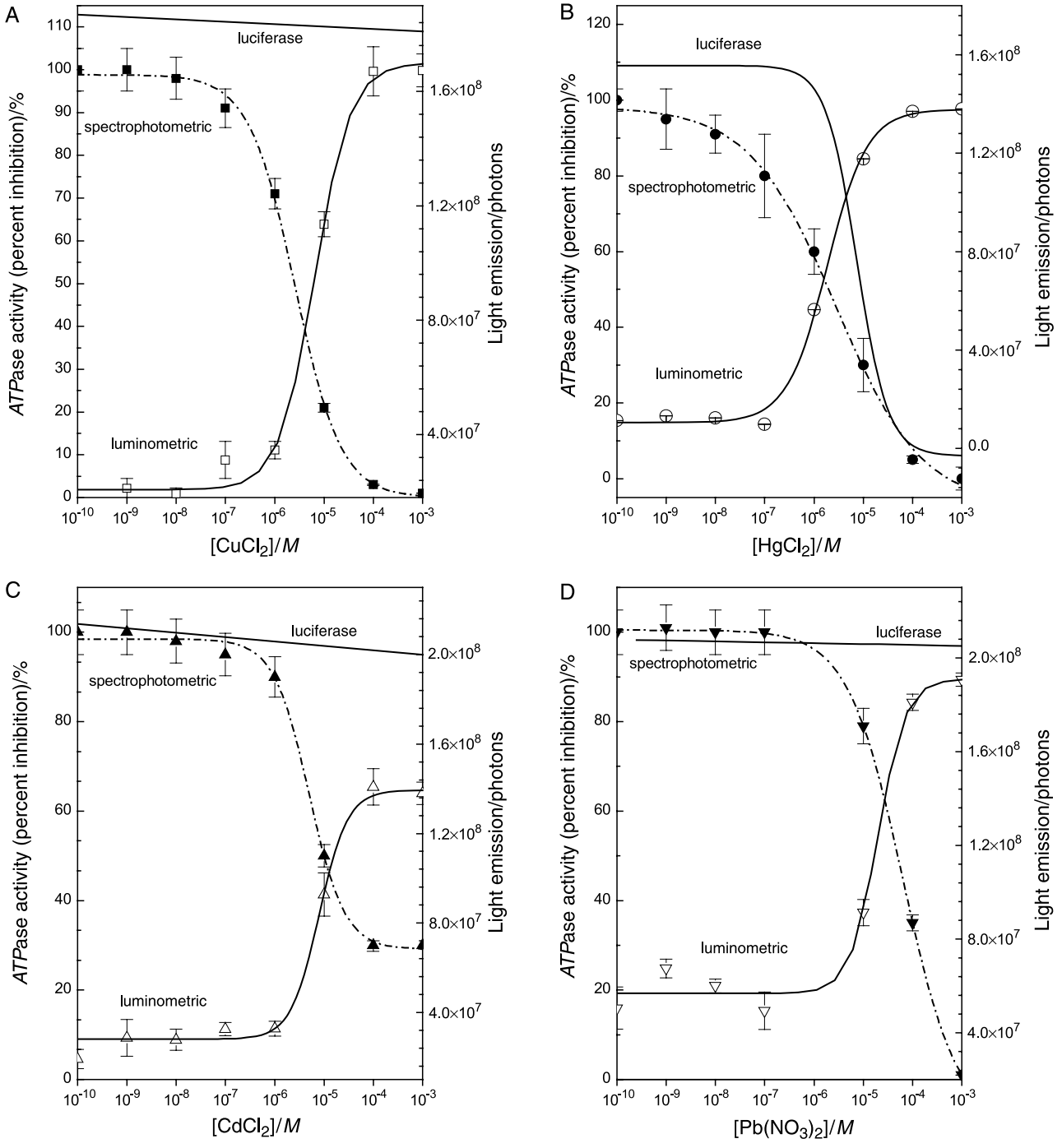

Fig. 3. Effect of different metal ions in the concentration range 1×10^{-10}–1×10^{-3} *M* on luciferase activity (lines) and *ATP*ase activity determined by the bioluminescence assay (open symbols) and by spectrophotometric assay (solid symbols). A) Effect of $CuCl_2$; B) Effect of $HgCl_2$; C) Effect of $CdCl_2$; D) Effect of $Pb(NO_3)_2$. Symbols present mean photon emission $\pm$*S.E.M.* obtained from $\geq$3 independent experiments performed in quadruplicate

It could be expected that both Hg^{2+} and Pb^{2+} induce inhibition of light emission. Since Pb^{2+} does not affect luciferase activity it is possible that Hg^{2+}-induced luciferase inhibition is mediated by another mechanism. It was previously shown that *EDTA*, divalent ion chelator with high affinity for Hg^{2+}, significantly potentiates Hg-induced inhibition of brain tubulin guanosine triphosphate (*GTP*) bind-

ing activity [14, 15]. The inhibition occurred in a concentration- and time-dependent manner and was specific for Hg*EDTA* complex. Since the monitoring system we used contains *EDTA*, it could be speculated that Hg^{2+}-induced inhibition of luciferin-luciferase light emission is due to Hg*EDTA* interference with luciferase *ATP* binding activity. *Brunker* [11] and *Wen et al.* [12] reported significantly reduced light emission from the *ATP* bioluminescence reaction in the presence of Hg^{2+} at concentrations higher than $10^{-5}\,M$. In our experiments inhibition was detected at Hg^{2+} concentration of two magnitudes lower ($10^{-7}\,M$) which may be a consequence of Hg*EDTA* inhibition.

The synaptosomal membrane bound *ATP*ase activity was inhibited in a concentration-dependent manner by all metal cations tested (Fig. 3). The inhibitory effect is assigned to the metal ions since preliminary experiments demonstrated that different anionic moieties did not affect the pattern of inhibition (not shown). Similar patterns of inhibition were obtained in the presence of raising Cu^{2+} (Fig. 3A), Hg^{2+} (Fig. 3B), and Pb^{2+} (Fig. 3D) concentrations, as determined by spectrophotometric (solid figures) and bioluminescence (open figures) assay. Total *ATP*ase inhibition was achieved in the presence of $10^{-4}\,M$ $HgCl_2$ and $10^{-3}\,M$ $CuCl_2$ and $Pb(NO_3)_2$, when number of photons emitted in *ATP*ase-luciferase coupled assay was equal to luciferase catalyzed photon emission, indicative of complete inhibition of *ATP*ase activity. IC_{50} values obtained by bioluminescence and spectrophotometric assay are summarized in Table 2.

Table 2. IC_{50} values calculated from the inhibition curves obtained from bioluminescence and spectrophotometric assay

Metal salt	$IC_{50}/10^{-6}\,M$	
	Bioluminescence assay	Spectrophotometric assay
$CuCl_2$	5.97 ± 0.76	2.55 ± 1.26
$HgCl_2$	1.35 ± 0.21	2.14 ± 0.81
$CdCl_2$	7.91 ± 0.19	9.17 ± 1.55
$Pb(NO_3)_2$	61.2 ± 7.52	62.6 ± 2.63

The IC_{50} values determined from the plots presented in Fig. 3. The response curves were obtained by fitting the data by a nonlinear regression analysis to the four-parameter sigmoidal function, and the IC_{50} values were calculated by a computer-assisted software package (Origin 7.0)

Figure 3C describes the effect of Cd^{2+} on *ATP*ase activity, determined by spectrophotometric (solid triangles) and bioluminescence (open triangles) assay. The inhibition is dose-dependent, however only 70% inhibition of the enzyme activity was obtained in the presence of $10^{-3}\,M$ $CdCl_2$.

Over the last few years there have been a number of publications concerning the effect of different pollutants on brain *ATP*ase activity [10, 16–23]. Possible outcomes of Na,K-*ATP*ase and *ecto-NTPD*ase inhibition are of interest considering their pharmacological relevance [24–26] and role in the brain development and plasticity [3, 27, 28].

Determination of *ATP*ase activity has been traditionally based on the spectrophotometric measurement of inorganic phosphate liberated during hydrolysis of *ATP* [29–31]. In recent years, because of their rapidity and high sensitivity, different bioluminescent enzyme assays have been successfully developed and applied in the field of environmental and toxicological analysis. We have developed a simple bioluminescence *ATP*ase assay that offered several advantages over the spectrophotometric methods and represents slight improvement of the existing *ATP*ase bioluminescence techniques [32–34]. The assay is fast, because no extraction or centrifugation steps are required. The measuring time is very short (about a minute) and is essentially that required for the instrument to measure the number of photons emitted after the course of *ATP*ase reaction. Even a simple portable instrument, such as a small luminometer can be used for rapid analysis. Second, the assay requires a small sample amount, which makes it excellent for fast routine kinetic analysis, particularly when minute sample amounts are available. Further the sensitivity of the assay is in the picomolar range of *ATP* concentration (detection limit from 5×10^{-11} to $1 \times 10^{-8}\,M$) and the number of photons emitted is directly proportional to the *ATP* concentration left after the course of *ATP*ase reaction. The quantum yield of this reaction is almost 100% and flow of photons at *ATP* concentration of $10^{-9}\,M$ is about 5×10^7 photons/s. The assay shows acceptable performance as assessed by calculating total, within-run, and between-day *CV*s.

The present bioluminescence assay is based on a single enzyme step, which is coupled to the bioluminescence *ATP* monitoring reaction. No interference with the assay was observed by any of the studied metal cations except by mercurial ion at con-

centrations higher than $10^{-7}\,M$. Thus, it is necessary to check the system for every new compound that might be added in the reaction mixture.

We have applied an endpoint assay, in which *ATP* detection reagents, containing luciferase and luciferin were added at the end of the *ATP*ase reaction. This approach is more suitable for a detection of compounds and agents that inhibit *ATP*ase activity, since they increase luminescence by reversing the enzyme-dependent loss of signal. The assay also enables easy differentiation between *ATP*ase and luciferase inhibitors, since the latter reduce the luminescence.

Data from the present study confirm that heavy metals are potent inhibitors of synaptic membrane *ATP*ase activity, which is in agreement with earlier reports. Exposure to heavy and transitional metals, such as Cu^{2+}, Hg^{2+}, Cd^{2+}, Pb^{2+}, Fe^{2+}, and Zn^{2+} induce many complex processes, among which membrane disturbances are one of the key biological effects. These metal ions are well-known potent reagents for thiol groups and react with functional groups of the lipid bilayer before being complexed with the enzyme protein. For example, Cd^{2+} and Pb^{2+} binding to sulfhydryl groups have been implicated in both Na,K-*ATP*ase inhibition [21, 35] and physiological effects [36].

All metal cations tested inhibited *ATP*ase activity in a dose-dependent manner. IC_{50} values obtained with use of the bioluminescent assay were in the same rank of order as obtained from the spectrophotometric determination in this and other studies [4, 7, 8, 16, 21]. The order of IC_{50} values for synaptosomal *ATP*ase inhibition ($Hg^{2+} < Cu^{2+} < Cd^{2+} < Pb^{2+}$) is in general agreement with the literature [21, 22, 37, 38].

In conclusion, the bioluminescence *ATP*ase assay can be used for simple and fast evaluation of metal ions-induced *ATP*ase inhibition. The assay can be adjusted for the evaluation of other agents that inhibit *ATP*ase, such as pharmacological agents or environmental pollutants. Using a CCD camera and 384-well black polystyrene microtitre plates, 384 different samples can be analyzed simultaneously or in an automated system, it could enable high-throughput screening of more than 5000 samples per hour.

Experimental

Chemicals and Material

All chemicals used were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO). *ATP* monitoring reagent (containing $1.6\,\mu g/cm^3$ luciferase from *Photinus Pyralis*, $7 \times 10^{-4}\,M$ *D*-luciferin, $0.02\,M$ $MgCl_2$, $4 \times 10^{-3}\,M$ *EDTA*, $3.6 \times 10^{-4}\,M$ dithiothreitol, $3 \times 10^{-4}\,M$ *AMP*, $0.04\,M$ HEPES) was purchased from Boehringer–Mannheim (Mannheim, Germany). Lyophilized reagents were dissolved with double distilled water, according to the manufacture instructions.

Rat brain synaptic plasma membrane (SPM) was prepared according to the method of *Towle* and *Sze* [39], as described elsewhere [40]. The final pellet was resuspended in $5 \times 10^{-3}\,M$ *Tris*-buffer *pH* 7.4 and stored at $-80°$ until used. Before each experiment synaptic plasma membranes were resuspended in *Tris*-buffer to the concentration of $1\,mg/cm^3$. The protein content was determined by the method of *Markwell et al.* [41].

Spectrophotometric ATPase Assay

Typical incubation mixture for the *ATP*ase activity measurement contained: $0.04\,M$ *Tris*-buffer, *pH* 7.4, $0.1\,M$ NaCl, $0.02\,M$ KCl, $5 \times 10^{-3}\,M$ $MgCl_2$, $20\,\mu g$ of SPM proteins, and $2 \times 10^{-3}\,M$ *ATP* in a final volume of $0.2\,cm^3$. The reaction mixtures in the absence of *ATP* were preincubated for 15 min at 37°C in the presence or absence of metal salt (concentration range 10^{-10} to $10^{-3}\,M$). After preincubation, the enzyme reaction was started by the addition of *ATP*, allowed to proceed for 10 min, and stopped by the addition of $22\,mm^3$ ice cold $3\,M$ trichloroacetic acid and by immediate cooling on ice for 15 min. Samples were proceeded for spectrophotometric assay of released inorganic ortho-phosphate (*Pi*) liberated from *ATP* by isobutanol-benzene extraction procedure according to *Pennial* [29].

Bioluminescence ATPase Assay

The assay was carried out in black polystyrene microtitre wells (Dynatech Laboratories, Chantilly, VA). The reaction mixture comprised of $0.04\,M$ *Tris*-buffer, *pH* 7.4, $0.1\,M$ NaCl, $0.02\,M$ KCl, $5 \times 10^{-3}\,M$ $MgCl_2$, and 1–10 μg of SPM proteins, $2\,mm^3$ *ATP* (final concentration as indicated) in the total volume of $20\,mm^3$. The bioluminescence procedure included preincubation for 5–25 min at room temperature and addition of $100\,mm^3$ of *ATP* monitoring agent (Boehringer–Mannheim) in each well for immediate photon emission measurement at 560 nm. For toxicity measurements, $1\,\mu g$ of SPM was preincubated for 15 min with metal salt (concentration as indicated) afterwards the total *ATP*ase was assayed for 10 min in the presence of $5 \times 10^{-7}\,M$ *ATP* as described. Metal salts tested (concentration range 10^{-10} to $10^{-3}\,M$) were: $CuCl_2$, $HgCl_2$, $CdCl_2$, and $Pb(NO_3)_2$.

Assay Optimization

For all experiments *ATP* standard curves were run in the range of 10^{-10} to $10^{-5}\,M$ (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, $10^{-5}\,M$ *ATP*). Calibration solutions were prepared by diluting a $10^{-3}\,M$ *ATP* stock solution with freshly quartz distilled water immediately before measurement. To each well containing $20\,mm^3$ of *ATP* solution and $20\,mm^3$ of incubation mixture, $0.1\,cm^3$ of *ATP* monitoring agent was added for immediate photon emission measurement.

Optimum conditions for the bioluminescence *ATP*ase assay concerning the amount of sample proteins (0.5–10 μg), *ATP* content (5×10^{-7}, 5×10^{-6}, 5×10^{-5} *M*), and duration of enzymatic reaction (1–25 min) were assessed.

Reproducibility of the assay was assessed by calculating total, within-run, and between-day variations over a 2-week period (during 6 days each repeated four times in quadruplicate). The assay was carried out in the same manner as described and light emission was measured following 10 min incubation time in the presence of 5×10^{-7} *M ATP* and 1 μg of SPM proteins.

Since luciferase reaction is the monitoring system for the effect of different metal agents on total membrane *ATP*ase activity, all metal ions were screened for their ability to interfere with the luciferase-catalysed photon emission. Thus, control measurements were carried out, in order to determine the interference of metal ions with luciferase activity. The assay was performed in the same manner as described, with SPM lacking from the incubation mixture.

Data Analysis

The emitted light (photons/well) was recorded after 60 s acquisition time using a multichannel light emission counter (Wallac, VICTOR2 1420, Perkin Elmer, Turku, Finland) with injector. All measurements were run in quadruplicate in $\geq$3 separate determinations. Points on figures present mean number of photons emitted $\pm$*S.E.M*. The response curves were obtained by fitting the data by a nonlinear regression analysis to the four-parameter sigmoidal function ($R>0.998$). IC_{50} values were calculated by a computer-assisted software package (Origin 7.0) as the parameter which represents the metal concentration at which the half-maximum inhibition was achieved (half way between initial and final values at the dose-response curves).

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