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In Search of Synaptosomal Na^+,K^+-ATPase Regulators

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Contents

Abstract

The arrival of the nerve impulse to the nerve endings leads to a series of events involving the entry of sodium and the exit of potassium. Restoration of ionic equilibria of sodium and potassium through the membrane is carried out by the sodium/potassium pump, that is the enzyme Na^+,K^+-ATPase. This is a particle-bound enzyme that concentrates in the nerve ending or synaptosomal membranes. The activity of Na+,K^+-ATPase is essential for the maintenance of numerous reactions, as demonstrated in the isolated synaptosomes. This lends interest to the knowledge of the possible regulatory mechanisms of Na^+,K^+-ATPase activity in the synaptic region. The aim of this review is to summarize the results obtained in the author's laboratory, that refer to the effect of neurotransmitters and endogenous substances on Na^+,K^+-ATPase activity. Mention is also made of results in the field obtained in other laboratories.

Evidence showing that brain Na^+,K^+-ATPase activity may be modified by certain neurotransmitters and insulin have been presented. The type of change produced by noradrenaline, dopamine, and serotonin on synaptosomal membrane Na^+,K^+-ATPase was found to depend on the presence or absence of a soluble brain fraction. The soluble brain fraction itself was able to stimulate or inhibit the enzyme, an effect that was dependent in turn on the time elapsed between preparation and use of the fraction.

The filtration of soluble brain fraction through Sephadex G-50 allowed the separation of two active subfractions: peaks I and II. Peak I increased Na^+,K^+- and Mg^{2+}-ATPases, and peak II inhibited Na^+,K^+-ATPase. Other membrane enzymes such as acetylcholinesterase and 5'-nucleotidase were unchanged by peaks I or II.

In normotensive anesthetized rats, water and sodium excretion were not modified by peak I but were increased by peak II, thus resembling ouabain effects. ^{3}H-ouabain binding was unchanged by peak I but decreased by peak II in some areas of the CNS assayed by quantitative autoradiography and in synaptosomal membranes assayed by a filtration technique. The effects of peak I and II on Na^+,K^+-ATPase were reversed by catecholamines. The extent of Na^+,K^+-ATPase inhibition by peak II was dependent on K^+ concentration, thus suggesting an interference with the K^+ site of the enzyme. Peak II was able to induce the release of neurotransmitter stored in the synaptic vesicles in a way similar to ouabain. Taking into account that peak II inhibits only Na^+,K^+-ATPase, increases diuresis and natriuresis, blocks high affinity ^{3}H-ouabain binding, and induces neurotransmitter release, it is suggested that it contains an ouabain-like substance.

Index Entries: Na^+,K^+-ATPase regulators; ouabain-like substances; brain endogenous factors; synaptosomal membranes; Na^+,K^+-ATPase inhibitors.

Introduction

It is known that in the resting state, internal neuronal concentration and membrane permeability of K^+ are greater than those of Na^+. Therefore, during the resting condition, small amounts of K^+ are lost, an exit that is greatly increased during neuronal activity, concurrent with Na^+ entry to the cell. In order to recover K^+ and expel Na^+, a pumping mechanism is necessary to move these cations against their concentration gradients (for references, *see* Albers et al., 1989).

The brain has a high oxidative metabolism and consumes great amounts of chemical energy as ATP molecules. A major portion of this energy consumption is required to maintain cellular Na^+ and K^+ gradients (McIlwain, 1969). An adequate balance between K^+ and Na^+ concentration is essential to propagate neuronal impulse, to prevent osmotic cell rupture, and to maintain cation homeostasis (for references, *see* Stahl, 1986; Wu, 1986). In the maintenance of Na/K gradients, the participation of a Mg^{2++}-dependent Na^+,K^+-ATPase (Skou, 1957) is required. It is now well established that Na^+,K^+-ATPase (E.C. 3.6.1.3.) is the enzymatic version of the Na^+/K^+ pump. In this connection, it has been observed that tissues having the highest rate of Na^+ and K^+ fluxes also present the highest Na^+,K^+-ATPase activities (*see* Albers et al., 1989).

Na^+,K^+-ATPase translocates internal Na^+ ions out of and external K^+ ions into the cell in a stoichiometric ratio of 3:2, using one ATP molecule in the cationic exchange, and it is selectively inhibited by cardiac glycosides such as ouabain. The phosphorylation site and the ATP binding site are located on the cytoplasmic side of the α-catalytic subunit of the Na^+,K^+-ATPase, whereas the ouabain binding site is on the extracellular side (Rossier et al., 1987).

Na^+,K^+-ATPase plays a leading role in the normal cell cycle and differentiation of the nervous system, and it seems to modify the influence of nerve growth factor on nerve cells. It has been postulated that Na^+,K^+-ATPase is involved in the development of seizures and in the control of nerve conduction velocity in diabetic nerve (*see* Albers et al., 1989; Stahl, 1986; Wu, 1986; Haglund and Schwartzkroin, 1990).

This enzyme is very active in gray matter and is found in large amounts in synaptic nerve endings, where neurotransmission takes place. It is a membrane-bound enzyme and is especially abundant in nerve ending (synaptosomal) membranes (Rodríguez de Lores Arnaiz et al., 1967). It should be stressed that the inhibition of Na^+,K^+-ATPase activity by ouabain blocks the various functions occurring in isolated synaptosomes: synthesis of low-mol wt substances (ATP, creatine phosphate), macromolecules and neurotransmitters, as well as Na^+ extrusion and K^+ entry (*see* Rodríguez de Lores Arnaiz and De Robertis, 1972).

The existence of multiple Na^+,K^+-ATPase isozymes in the central nervous system (CNS) has been described. They present affinity differences for ATP, Na^+, and cardiac glycosides. The three forms of the catalytic subunit, denominated α_1, α_2, and α_3, derive from different genes. The use of isozyme-specific monoclonal antibodies (MAb) indicates that the three Na^+,K^+-ATPases are differentially localized in the mammalian CNS. Whereas the three isoforms are present in neuronal structures, only α_1 and α_2 are present in glial cells (McGrail et al., 1991). The suggestion that Na^+,K^+-ATPase isozymes might be differentially modulated by endogenous inhibitors has been advanced (for refs, *see* Sweadner, 1989).

The regulatory mechanisms that control this enzyme activity are of special interest for the maintenance of metabolic activity of the synaptic region and in processes directly related to neurotransmission.

In this review, results obtained in the author's laboratory, which indicate the modification of neuronal Na^+,K^+-ATPase activity by neurotransmitters and soluble brain factors, are summarized and discussed in connection with findings reported by diverse investigators.

Effect of Neurotransmitters and Other Endogenous Substances on Na^+,K^+-ATPase Activity

Neurotransmitters and Na^+,K^+-ATPase

The effect of various neurotransmitters on Na^+,K^+-ATPase activity has been studied by several authors (*see* Stahl, 1986; Wu, 1986). In this laboratory, it was observed that such enzyme activity in whole homogenates of rat cerebral cortex is stimulated by noradrenaline (Rodríguez de Lores Arnaiz and Mistrorigo de Pacheco, 1978). However, Na^+,K^+-ATPase activity of synaptosomal membrane fractions, rich in this enzyme (Rodríguez de Lores Arnaiz et al., 1967), is inhibited by noradrenaline (Rodríguez de Lores Arnaiz and Mistrorigo de Pacheco, 1978) and dopamine (Rodríguez de Lores Arnaiz and Antonelli de Gómez de Lima, 1981).

The inhibition achieved by dopamine on Na^+,K^+-ATPase activity of synaptosomal membrane preparations was not prevented by the presence of the D_2 dopamine antagonists haloperidol, droperidol, or spiperone (Rodríguez de Lores Arnaiz, 1987). However, other studies carried out on isolated neostriatal neurons have indicated that Na^+,K^+-ATPase inhibition

by dopamine seems to occur through D_1 and D_2 dopamine receptor synergism (Bertorello et al., 1990).

Insulin and Other Endogenous Substances on Na^+,K^+-ATPase Activity

It is known that insulin is present in brain, and that its concentration in rat brain is about 25 times higher than plasma insulin levels (Havrankova et al., 1978). We have observed that insulin is able to modify Na^+,K^+-ATPase activity of synaptosomal membranes and whole homogenates prepared from rat cerebral cortex; insulin effect, whether stimulatory or inhibitory, seems dependent on the integrity of certain cell membranes (Rodríguez de Lores Arnaiz and Bojorge, 1986; Bojorge and Rodríguez de Lores Arnaiz, 1987). More recently, the addition of insulin *in vitro* has been shown to stimulate the α_2 isozyme, without affecting the α_1 isoform (Brodsky, 1991).

Several other substances present in the brain, such as vanadate and some neurotransmitters *(see above)*, also may inhibit Na^+,K^+-ATPase. Besides, the peptide hystidil-proline diketopiperazine also decreased this enzymatic activity (Battaini and Peterkofsky, 1980).

Factors Present in the Brain that Modify Na^+,K^+-ATPase Activity

The existence of a Na^+,K^+-ATPase inhibitor in the brain was first advanced by Albers et al., (1965) on the basis of subcellular fractionation studies, which showed that Na^+,K^+-ATPase activity in isolated subcellular fractions was tenfold higher than that in the whole brain homogenate. Later on, the inhibition of membrane-bound Na^+,K^+-ATPase activity in brain subcellular fractions by the corresponding soluble fractions was reported (Schaefer et al., 1972).

The brain soluble fraction by itself was able to modify Na^+,K^+-ATPase activity. The observed effect depended on the time elapsing from preparation to use. Thus, the fresh soluble fraction stimulated Na^+,K^+-ATPase activity, and this effect decayed as a function of time to become inhibitory (Rodríguez de Lores Arnaiz and Antonelli de Gómez de Lima, 1988).

Modification of Neurotransmitters Effect on Na^+,K^+-ATPase by a Soluble Brain Fraction

The abovementioned effect of the catecholamines noradrenaline and dopamine on membrane Na^+,K^+-ATPase may be changed by the presence of a soluble brain fraction. In fact, it was demonstrated that noradrenaline was able to inhibit or stimulate Na^+,K^+-ATPase activity, depending on the absence or presence of a soluble fraction (acqueous supernatant after 100,000*g* for 30 min) prepared from rat cerebral cortex. With the membranes alone, noradrenaline produced a significant decrease of Na^+,K^+-ATPase. In the presence of the brain soluble fraction, noradrenaline stimulated Na^+,K^+-ATPase. Mg^{2+}-ATPase activity of the membranes was not modified by noradrenaline alone, and it was stimulated in the presence of the soluble fraction plus noradrenaline (Table 1).

According to this observation, it may be mentioned that the stimulation of Na^+,K^+-ATPase by noradrenaline in some subcellular brain fractions resuspended in their respective soluble fractions was reported (Schaefer et al., 1972). The activation of Na^+,K^+-ATPase by noradrenaline was not reproduced by cyclic AMP (cAMP) and was not antagonized by either α- or β-adrenergic blocking agents. These findings suggested that the stimulation achieved by noradrenaline was a direct effect on the enzyme and not mediated by cAMP or adrenergic receptors (Rodríguez de Lores Arnaiz and Mistrorigo de Pacheco, 1978; Rodríguez de Lores Arnaiz 1983).

A similar pattern of effects was found when dopamine was used instead of noradrenaline (Rodríguez de Lores Arnaiz and Antonelli de Gómez de Lima, 1981; Antonelli de Gómez de Lima and Rodríguez de Lores Arnaiz, 1981). The

Table 1
Activities of Na^+,K^+- andMg^{2+}-ATPases of Synaptosomal Membranes in the Absence and Presence of Brain Soluble Fraction and Noradrenaline

	Soluble fraction	Without noradrenaline, μmol Pi. mg $prot^{-1}$. h^{-1}	With noradrenaline, μmol Pi. mg $prot^{-1}$. h^{-1} %
Na^+,K^+-ATPase	–	17.9 ± 2.7	10.2 ± 2.1 –43[a]
	+	19.2 ± 3.0	46.4 ± 11.3 +142[a]
Mg^{2+}-ATPase	–	4.8 ± 1.5	4.2 ± 2.5 –13
	+	6.5 ± 0.6	12.1 ± 3.1 +86[a]

Synaptosomal membranes were prepared from rat cerebral cortex homogenates by differential centrifugation and sucrose gradient centrifugation (Rodríguez de Lores Arnaiz et al., 1967). The soluble fraction was prepared by homogenization of rat cerebral cortex in water and centrifugation at 100,000*g* for 30 min; the supernatant was used 30 min later. Membrane samples were preincubated (37°C, 10 min) with (or without) the soluble fraction in the absence or presence of $10^{-4}M$ noradrenaline. Samples of these preparations were used to assay ATPases (Albers et al., 1965). Results are means of six experiments ±SD. [a]$p < 0.01$. Original data from Rodríguez de Lores Arnaiz and Mistrorigo de Pacheco, 1978.

idea of Na^+,K^+-ATPase regulation by endogenous catecholamines is also supported by the observation of an increase in this enzyme activity after in vivo stimulation of noradrenergic neurotransmission (Swann, 1984a,b, 1986, 1988; Viola et al., 1989). Serotonin also stimulated ATPases in the presence of brain soluble fraction but produced no changes in the absence of soluble fraction. Histamine, acetylcholine, GABA, and glutamate produced no changes in ATPase activities (Rodríguez de Lores Arnaiz and Antonelli de Gómez de Lima, 1981).

Endogenous Factors that Modify Na^+,K^+-ATPase Activity

Isolation of CNS Endogenous Factors that Inhibit Na^+,K^+-ATPase

The isolation of Na^+,K^+-ATPase inhibitors from the CNS through acetone or water extraction was reported by several authors. The effect of these extracts on enzyme activity and/or ouabain binding was analyzed. From guinea-pig brain, Fishman (1979) obtained an acetone-HCl extract, partially purified later in Sephadex G-10 and desalting. This extract mimics digitalis glycosides by competing with ouabain for binding to Na^+,K^+-ATPase and by inhibiting ^{86}Rb uptake into erythrocytes. The factor thus obtained has a low mol wt and resists acid hydrolysis.

Starting from bovine hypothalamus, Haupert and Sancho (1979) have prepared an extract in acid-acetone partially purified by gel filtration in Sephadex G-25 and ion-exchange chromatography. This extract inhibits sodium transport, ouabain binding, and kidney Na^+,K^+-ATPase activity. The factor involved has a mol wt lower than 2500, resists acid hydrolysis, and is a high-affinity reversible inhibitor of Na^+,K^+-ATPase. Lichtstein and Samuelov (1980) have obtained an acid-acetone extract from whole rat brain that inhibits Na^+,K^+-ATPase activity and ouabain binding of synaptosomal membranes. The factor is resistant to proteolytic enzymes.

Whitmer et al. (1982) have separated brain Na^+,K^+-ATPase inhibitors by extracting the tissue in different conditions: air-acid-acetone, nitrogen-acid-acetone, and nitrogen-water-acetone. With the last condition, a factor that slightly (–11%) but specifically inhibits Na^+,K^+-ATPase was separated. The authors pointed out that extraction procedures involving the use of air and/or acid may produce nonspecific inhibitors.

Shimoni et al. (1984) have prepared an acid-acetone extract from sheep brain purified by Sephadex G-25 chromatography. The factor is nonpeptidic, inhibits Na^+,K^+-ATPase and ouabain binding, and increases the force of contraction in cardiac muscle. Akagawa et al. (1984) have obtained from bovine hypothalamus by acid-acetone extraction a factor that inhibits 80% Na^+,K^+-ATPase and that seems to be a peptide.

Na^+,K^+-ATPase Inhibitors in Plasma and Other Body Fluids

Several investigators have reported the presence of circulating factors that inhibit Na^+,K^+-ATPase activity, some peptidic and others nonpeptidic in nature. A plasma factor (endoxin) that inhibits Na^+,K^+-ATPase and crossreacts with digoxin antibodies has been separated (Gruber et al., 1980) and seems to be peptidic in nature (Gruber et al., 1983).

A Na^+,K^+-ATPase inhibitor in normal human (de Wardener et al., 1981) and rat plasma, as well as in acetone extracts of rat hypothalamus (Alaghband-Zadeh et al., 1983) has been reported. The suggestion that both plasma and hypothalamic activities may be caused by the same ouabain-like factor was advanced. Nevertheless, it fails to exhibit immunoreactivity for serum digoxin (Millet et al., 1987). Endogenous digitalis-like substances that inhibit ^{86}Rb uptake were also partially purified from human neonate and adult plasma (Balzan et al., 1991).

An endogenous sodium pump inhibitor was purified from human plasma; the factor is a highly potent selective inhibitor of ion transport, receptor, and hydrolytic functions of the sodium pump and seems to be nonpeptidic in nature (Hamlyn et al., 1989). Its relationship with the sodium transport inhibitor detected in the plasma of volume-sensitive forms of hypertension (Hamlyn et al., 1982) was suggested. In this regard, a circulating factor presenting ouabain-like immunoreactivity in patients with aldosteronism was described (Masugi et al., 1986). The level of this factor in plasma of such patients was more than twice that of normotensive subjects.

Starting from bovine plasma, a factor that displaces ^3H-ouabain from brain synaptosomes and inhibits erythrocyte Na^+,K^+-ATPase, but not Mg^{2+}- or Ca^{2+}-ATPase activities, was purified (Tal et al., 1986). Some Na^+,K^+-ATPase inhibitors found in plasma are lipidic in nature (Tamura et al., 1985,1987; Kelly et al., 1986), the major inhibitory activity being associated with nonesterified fatty acids (Vasdev et al., 1989), and others were identified as a mixture of three nonesterified fatty acids (mainly oleic acid) and three saturated hydrocarbons (Tal et al., 1989). Another lipidic factor, unlike free fatty acids or lysophosphatidylcholine, that inhibits Na^+,K^+-ATPase in competition with K^+ has been purified from plasma (Masugi et al., 1988).

Na^+,K^+-ATPase inhibitory activity and/or digoxin-like immunoreactivity have also been detected in urine, and a Na^+ pump inhibitor has been purified from human urine (Crabos et al., 1987; *see also* Hamlyn et al., 1989, for refs). Ouabain-like activity in human cerebrospinal fluid (CSF) was reported (Halperin et al., 1983; Lichtstein et al., 1985). This factor exhibits properties of a small peptide and mimics digitalis glycosides on Na^+,K^+-pump, Na^+,K^+ATPase activity, and ^3H-ouabain binding to the red cell receptor. The inhibitor blocks the stimulation of Na^+,K^+-pump by extracellular K^+ (Halperin, 1989).

Na^+,K^+-ATPase Inhibitors in Tissues Other than Brain

Endogenous Na^+,K^+-ATPase inhibitors have been found in heart (Fagoo and Godfraind, 1985), skin (Shimoni et al., 1984; Flier, 1978), and adrenal gland (Tamura et al., 1988), as well as in intestine (Araki et al., 1989), whose factor is peptidic in nature and inhibits Na^+,K^+-ATPase competitively against Na^+, whereas ouabain is competitive with K^+ (Araki et al., 1989).

Lignans are found in certain plants but have recently been identified in animals and humans (Hirano et al., 1989). Some of these, such as enterolactone, seem to act at the digitalis receptor site of the Na^+,K^+-pump (Fagoo et al., 1986; Braquet

et al., 1986). Mammalian lignans and their derivatives have been reported to crossreact with antidigoxin antibody, to inhibit dog kidney Na^+,K^+-ATPase, and to displace ouabain (Hirano et al., 1989).

Separation of the Two Brain Soluble Fractions that Modify Na^+,K^+-ATPase Activity

Peak I (Stimulator) and Peak II (Inhibitor)

As mentioned previously, in this laboratory, it was demonstrated that a brain soluble fraction is essential to achieve stimulation of synaptosomal membrane Na^+,K^+-ATPase by catecholamines. Filtration of the brain soluble fraction through a Sephadex G-50 column allowed separation of two subfractions, peaks I and II (Fig. 1), which modified ATPase activities. Peak I stimulated both Na^+,K^+- and Mg^{2+}-ATPases, and peak II inhibited Na^+,K^+-ATPase alone (Rodríguez de Lores Arnaiz and Antonelli de Gómez de Lima, 1986).

In order to determine whether peaks I and II were able to modify other membrane-bound enzymes, their effects were tested on acetylcholinesterase and 5'-nucleotidase activities of the synaptosomal membranes. Neither peak I or II produced any effect on these two enzymes, suggesting specific activity for both ATPases (Fig. 2).

The activating effect of peak I on Na^+,K^+-ATPase and on Mg^{2+}-ATPase activities decreased with dilution within a 1:1 to 1:100 range. The inhibitory effect of peak II on Na^+,K^+-ATPase diminished with dilution within a 1:1 to 1:50,000 range (Rodríguez de Lores Arnaiz et al., 1988).

The effect of peaks I and II on ATPase activities was diversely modified according to storage pH. The activating effect of peak I on Na^+,K^+- and Mg^{2+}-ATPases was stable for 24 d when stored at neutral, acid, or alkaline pH, whereas the inhibitory effect of peak II was only preserved at acid pH for 24 d but lost when stored at neutral or alkaline pH (Fig. 3).

The effects of peak I and II fractions on ATPase activity were reversed by catecholamines (Rodríguez de Lores Arnaiz et al., 1988). In the case of peak I, ATPase activity stimulation was not observed in the presence of catecholamines (Fig. 4). The fact that the addition of catecholamines to the membranes alone resulted in enzyme inhibition seems to indicate that peak I and catecholamines are antagonistic *inter se*.

The inhibitory effect of peak II on ATPase activity was unchanged or only partially reversed by catecholamines, depending on the amount of peak II present during the enzyme assay. Interestingly, although both peak II and catecholamines separately inhibit ATPase activity, when added together there is no enhanced inhibition but rather some degree of antagonism (Fig. 4).

Partial Characterization of Endogenous Factors that Modulate Synaptosomal Na^+,K^+-ATPase

In order to determine the properties of the inhibitory factor present in peak II, the fraction was studied in several conditions, and the results obtained support the participation of a polypeptide in the inhibitory effect (Antonelli de Gómez de Lima and Rodríguez de Lores Arnaiz, 1988). It was observed that the inhibitory effect of peak II on Na^+,K^+-ATPase was destroyed by heating in acid pH and that it was sensitive to digestion with carboxypeptidase-A, which cleaves carboxy terminals of peptides (Table 2). Peak II is ninhy-drin-positive, and its subfractionation by high-pressure liquid chromatography (HPLC) in a C-18 column indicated that the inhibitory factor is highly hydrophylic (Rodríguez de Lores Arnaiz and Peña, 1992). In this regard, it may be mentioned that the soluble factor that modulates catecholamines effect on ATPase was partially sensitive to trypsin digestion (Antonelli de Gómez de Lima and Rodríguez de Lores Arnaiz, 1986).

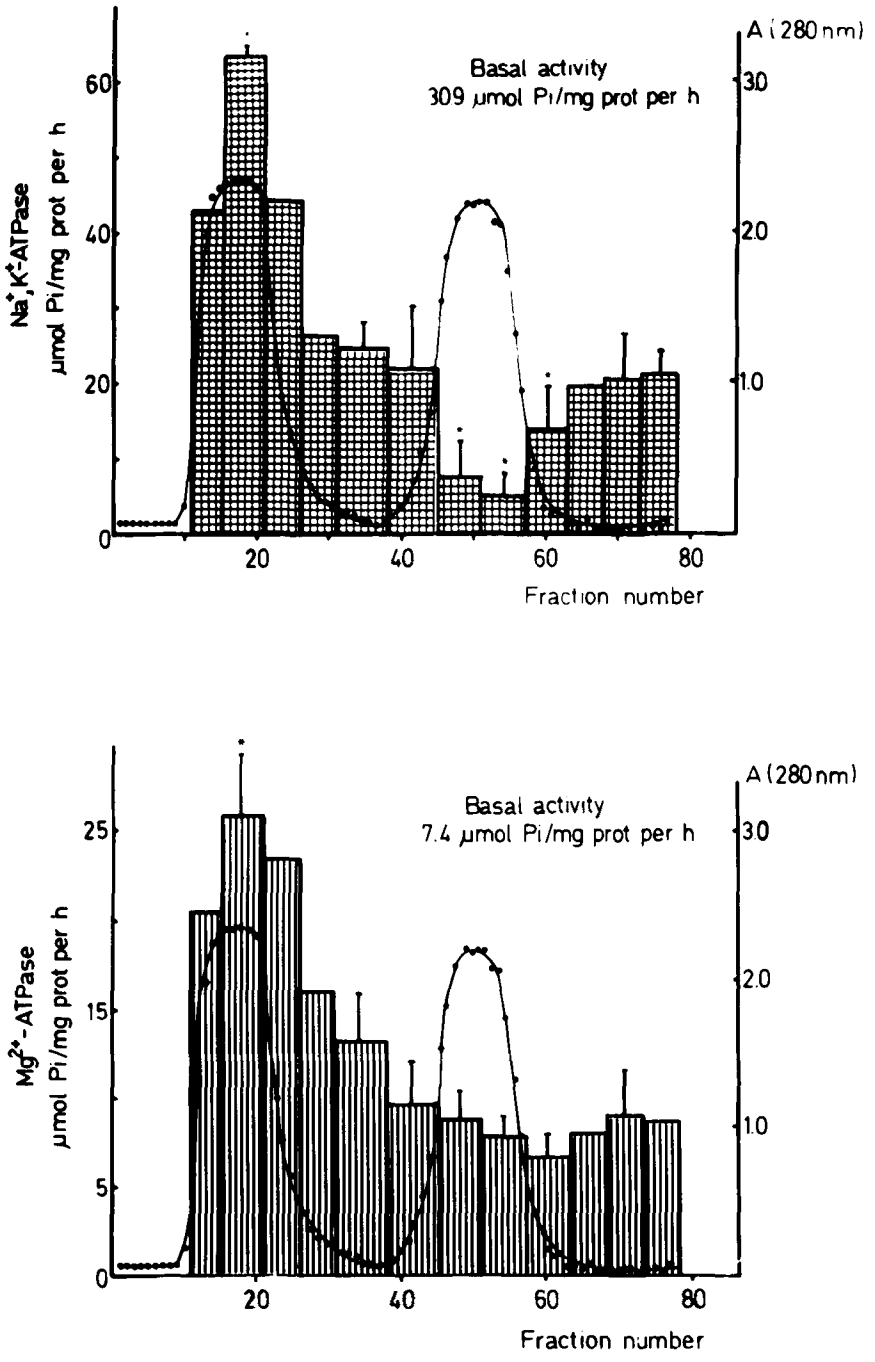

Fig. 1. Activities of Na^+,K^+- and Mg^{2+}-ATPases of synaptosomal membranes in the presence of Sephadex G-50 fractions. Membrane samples were preincubated with each of 12 pooled fractions; samples of these preparations were used to assay ATPases. Closed circles represent UV absorbance of each fraction; bars represent ATPase activities in the presence of each group of fractions and are the mean values obtained in 2–7 experiments. When SD is indicated, the results of at least three experiments were averaged. $^*p < 0.05$. From Rodríguez de Lores Arnaiz et al., 1988.

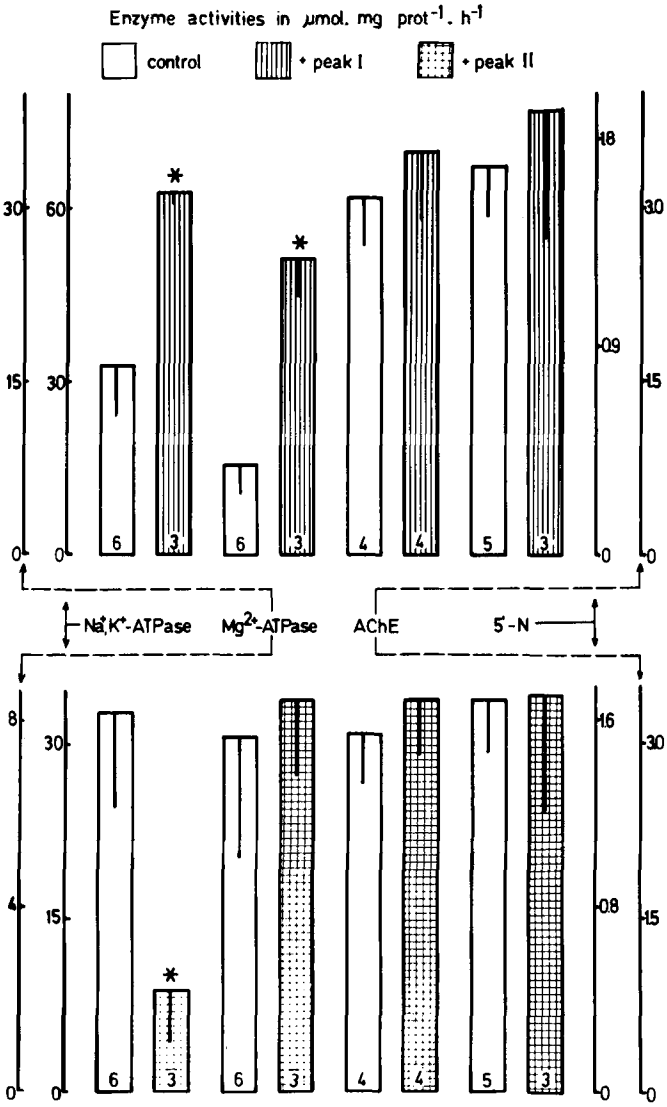

Fig. 2. Effect of peak I and II fractions prepared from rat cerebral cortex soluble fraction on synaptosomal membrane enzymes. AChE, acetylcholinesterase; 5'-N, 5'-nucleotidase. The SD of mean values and the number of experiments are indicated within bars. *$p < 0.05$. From Rodríguez de Lores Arnaiz et al., 1988.

The effect of peak II was partially or totally reversed by the chelators EGTA and EDTA, respectively. This finding could indicate the coparticipation of an ionic component in the effect of peak II on membrane Na^+,K^+-ATPase (Antonelli de Gómez de Lima and Rodríguez de Lores Arnaiz, 1988).

Inhibitory Effect of Peak II Not Attributable to Endogenous Vanadium

It is known that vanadium is present in brain (Underwood, 1977) and that it is able to inhibit Na^+,K^+-ATPase activity, an inhibition that is blocked by catecholamines and enhanced by increasing Mg^{2+} concentration (Josephson and Cantley, 1977). Therefore, it could be possible that the stimulation achieved by these amines was the result of a reversion of an endogenous vanadate effect. However, two facts should be mentioned: The inhibition of Na^+,K^+-ATPase produced by peak II remained almost unchanged by the enhancement of Mg^{2+} concentration (Antonelli de Gómez de Lima and Rodríguez de Lores Arnaiz, 1988), and the assay of vanadium content by emission spectroscopy indicated that it was not detected in peak II fraction (D. Batistoni, CNEA, personal communication). These findings fail to support the hypothesis that endogenous vanadium could be responsible for the inhibition of Na^+,K^+-ATPase by brain peak II fraction.

Tissue-Specificity of Catecholamines and Endogenous Factors Effects on ATPases

As mentioned in the first part of this article, to observe the stimulation of synaptosomal membrane Na^+,K^+-ATPase by catecholamines, the presence of the brain soluble fraction was essential. The participation of the soluble fraction in the stimulatory effect of catecholamines was not exclusive to the nervous tissue, since other soluble fractions (from kidney or liver) were also effective. On the other hand, dopamine effects were tissue-specific with respect to the enzyme source (Antonelli de Gómez de Lima and Rodríguez de Lores Arnaiz, 1981).

Starting with kidney tissue, peak I and II fractions homologous to those from brain were separated. As observed with brain peak I, kidney peak

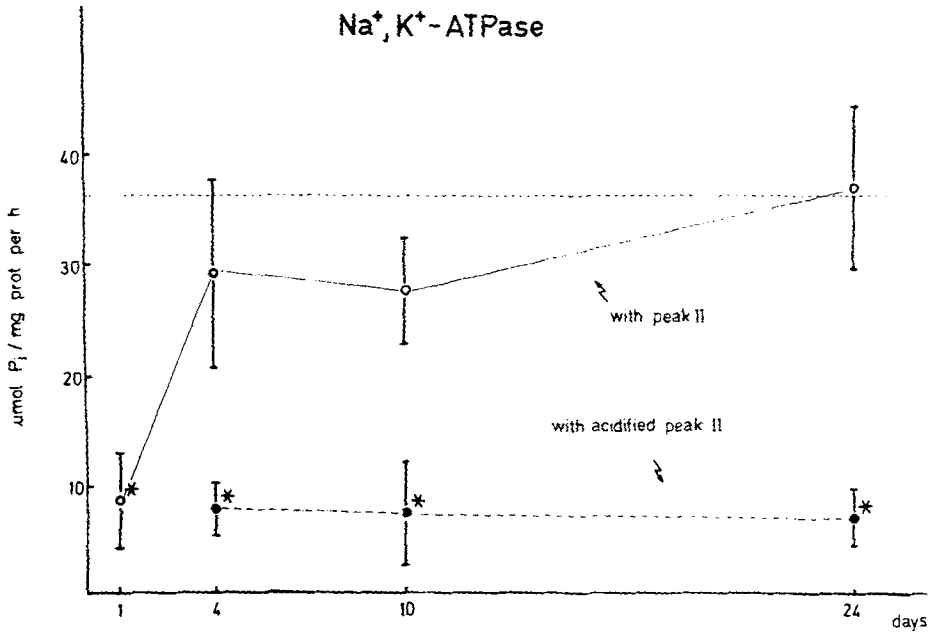

Fig. 3. Na^+,K^+-ATPase activity of synaptosomal membranes in the presence of brain peak II fraction stored at pH 2 or 7 for 24 d. Results presented are mean values ±SD of 3–8 experiments. $^*p < 0.05$ with respect to the control without peak II. Original data from Rodríguez de Lores Arnaiz and Antonelli de Gómez de Lima, 1986.

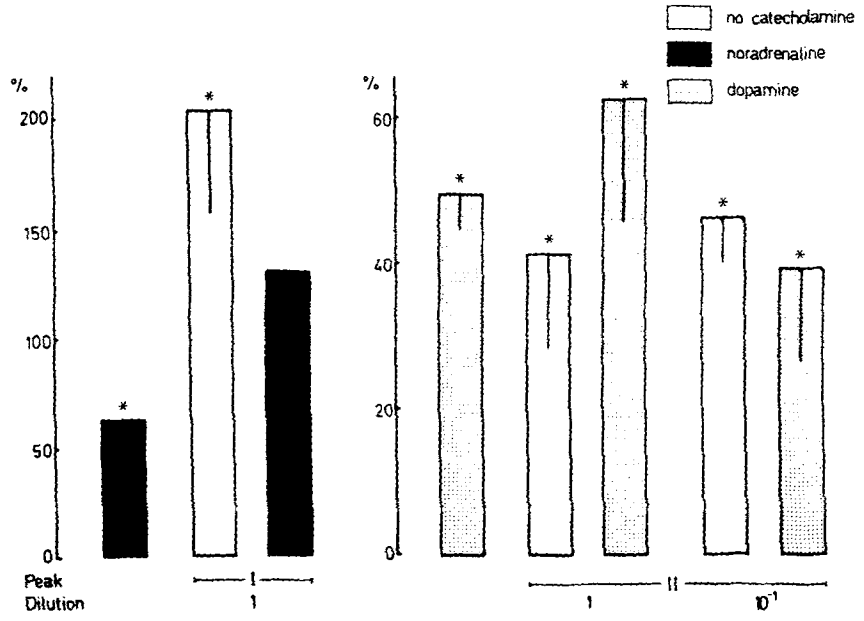

Fig. 4. Effect of soluble brain fractions and catecholamines on synaptosomal membrane ATPase activity. The experimental procedure was similar to that described in Table 1, except that the membranes were mixed with undiluted peak I, peak II (1 and 10^{-1} dilution), and 10^{-4}M catecholamines. Values are given as means ±SD; $n = 3–8$. $^*p < 0.05$ with respect to its control without additions.

Table 2
Effect of Brain Peak II
Following a Carboxypeptidase Digestion on Na^+,K^+-ATPase Activity

Membranes preincubated	Na^+,K^+-ATPase, μmol Pi. mg $prot^{-1}$. h^{-1}	%	Mg^{2+}-ATPase, μmol Pi. mg $prot^{-1}$. h^{-1}	%
Tris buffer	28.9 ± 5.2 (6)	—	8.5 ± 3.4 (6)	—
Peak II	10.8 ± 4.2 (4)	−63[a]	10.3 ± 1.6 (4)	+20
CP digested peak II	22.6 ± 3.7 (5)	−22	8.9 ± 3.3 (5)	+ 5
Peak II + denatured CP	9.2 ± 2.5 (3)	−68[a]	11.5 ± 2.0 (3)	+35

Synaptosomal membranes were preincubated with buffer, brain peak II (at 0°C, for 60 min), carboxypeptidase A (CP) digested peak II (190 U/mL Sigma CP A, at 37°C for 60 min), or peak II + denatured CP. Samples of these preparations were used to assay ATPases. Values are mean ±SD; the number of experiments is indicated between brackets, and columns indicating percentage change are included. [a]$p < 0.05$. Data from Antonelli de Gómez de Lima and Rodríguez de Lores Arnaiz, 1988.

Table 3
Effect of Brain Peak I and II Fractions on Dog Kidney Na^+,K^+-ATPase

	Mg^{2+},Na^+,K^+-ATPase, μmol Pi. mg $prot^{-1}$. h^{-1}	%	Na^+,K^+-ATPase, μmol Pi. mg $prot^{-1}$. h^{-1}	%
Control	12.4 ± 1.3	—	10.4 ± 0.4	—
Peak I	15.3 ± 0.6[b]	123	12.1 ± 1.2[a]	116
Peak II	15.3 ± 1.1[b]	123	12.5 ± 0.4[b]	120

Samples of commercial dog kidney Na^+,K^+-ATPases were assayed in the absence or presence of brain peak I and II fractions. Results are means of three experiments (±SD). [a]$p < 0.05$; [b]$p < 0.025$. Data from Rodríguez de Lores Arnaiz, 1990.

I also stimulated synaptosomal membrane Mg^{2+}- and Na^+,K^+-ATPase activities (Rodríguez de Lores Arnaiz 1990a). In support, an activator of brain ATPases also has been described (Das et al., 1989) and found to occur in cytosolic fractions of tissues such as the brain and kidney, thus resembling our peak I fraction.

Kidney peak II fraction inhibited brain Na^+,K^+-ATPase, as does brain peak II. These results indicate that the modulatory factors of ATPase activities are not tissue-specific. On the other hand, ATPase activity of a crude kidney micro-somal fraction was unchanged by kidney peak I or brain peak II and slightly increased by kidney peak II or brain peak I. ATPase purified from kid-ney was barely increased by brain peak I and II fractions (Table 3). On comparison with the data presented in Fig. 1, such findings suggest that there is tissue specificity with respect to the enzyme source (Rodríguez de Lores Arnaiz, 1990a), perhaps as a result of the fact that the isoform in kidney is α_1, but neurons contain the three isoforms.

Ouabain-Like Factors

Criteria to Identify Ouabain-Like Endogenous Factors

Certain endogenous substances have been reported to present properties similar to those of digitalis, such as inhibition of Na^+,K^+-ATPase activity (Hamlyn et al., 1982; Gruber et al., 1983) and ouabain-binding (Kelly et al., 1986), as well as crossreaction with antidigoxin antibodies (Gruber et al., 1980). A very high correlation has been observed between the ^{3}H-ouabain receptor measurement and the direct test that assays Na^+,K^+-ATPase inhibition by measurement of inorganic phosphate production (Vasdev et al., 1987).

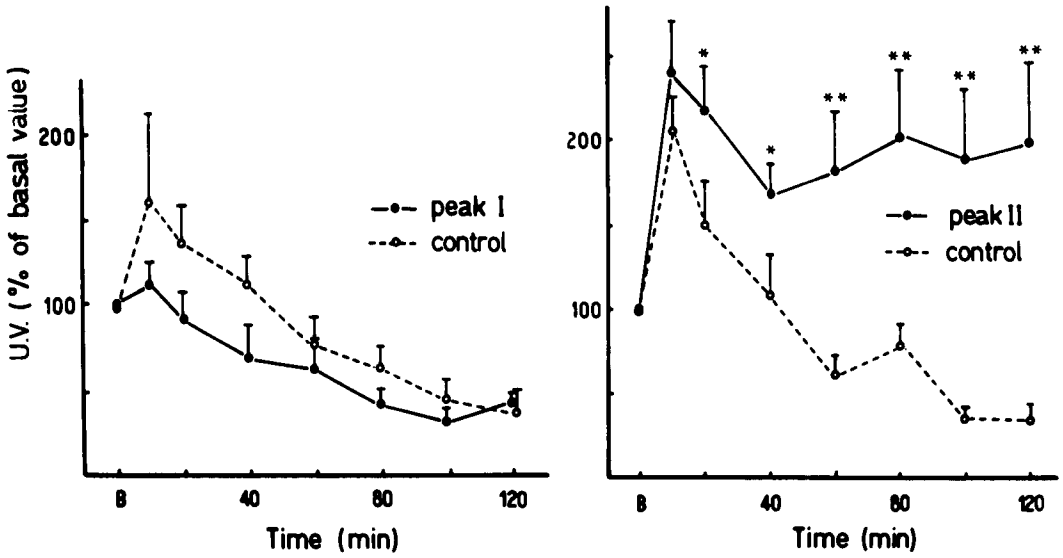

Fig. 5. Changes in urine vol (UV) as a function of time. Brain peak I or II fractions were injected intraarterially in anesthetized rats; as controls, albumine solution (2.7 mg/mL) or saline solution (0.9% NaCl) were injected, respectively. The UV is expressed as a percentage of corresponding basal values (B). Results are ±SEM of 6–8 rats. $^{*}p < 0.05$; $^{**}p < 0.01$. From Nowicki et al., 1990.

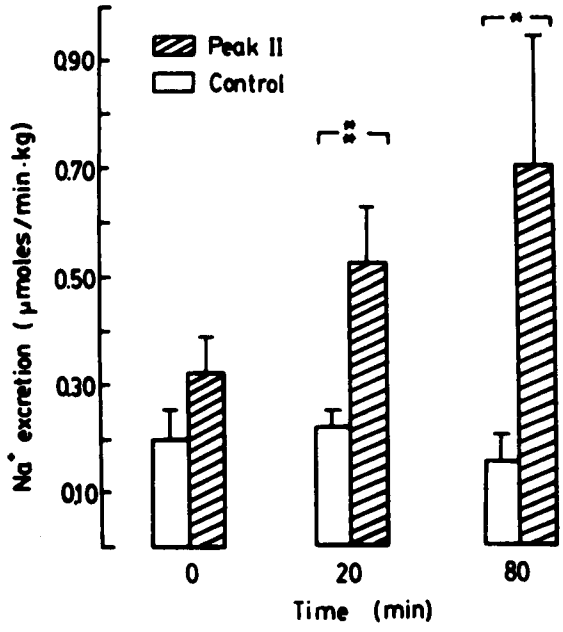

Fig. 6. Increase in sodium excretion by brain peak II. Peak II fraction or control solution (0.9% NaCl) was injected in anesthetized rats. Results are ±SEM from 7 rats. $^{*}p < 0.05$; $^{**}p < 0.025$. From Nowicki et al., 1990.

The close agreement between inmunological and biological assays, the latter implying ouabain binding and ^{86}Rb influx, suggests a single factor inhibiting binding to receptor and antibody, as well as depressing sodium pump activity (Clerico et al., 1988). However, immunoreactivity with antidigoxin antibodies failed to correlate at all times with the ability of mammalian tissue factors to inhibit Na^+,K^+-ATPase activity (Hamlym et al., 1982; Fagoo and Godfraind, 1985; Kelly et al., 1985; Clerico et al., 1987). Besides, it has been demonstrated that major substances detected in rat plasma by digoxin-like immunoreactivity are entirely different from those inhibiting Na^+,K^+-ATPase activity Yamada et al., 1988).

Ouabain-Like Properties of Brain Peak II Fraction

After intraarterial administration of brain peak I and II fractions to normotensive rats, it was observed that heart rate and arterial pressure were not significantly modified by either fraction. On the other hand, water and sodium excretion were unchanged by peak I but significantly increased by peak II (Figs. 5,6) (Nowicki et al.,

1990). In this connection, it is worthwhile mentioning that the increase in sodium excretion by ouabain has already been described (Hook, 1969).

High-affinity ^{3}H-ouabain binding in rat brain and rabbit cerebral cortex was determined by quantitative autoradiography in the presence of brain peak I and II fractions. It was observed that peak I failed to modify ^{3}H-ouabain binding. However, peak II was able to block ^{3}H-ouabain binding in rat cerebral cortex (areas 3 and 4), dentate gyrus, stria terminalis, thalamic nuclei, and basal ganglia, and in rabbit cerebral cortex, as well as to inhibit ^{3}H-ouabain binding to synaptosomal membranes isolated from rat cerebral cortex assayed by a filtration technique. Since peak I has no effect on ouabain binding but stimulates Na^+,K^+-ATPase, the responsible factor may be regulating the internal active site. On the other hand, peak II, which inhibits both ouabain binding and ATPase activity, which may be acting on the external side as an ouabain-like substance (Antonelli de Gómez de Lima, Casillas, and Rodríguez de Lores Arnaiz, 1991).

Inhibition of Na^+,K^+-ATPase has been described to induce neurotransmitter release in several experimental models (*see* Vizi, 1978). Brain peak II fraction was able to decrease a newly formed neurotransmitter pool but not another preexisting pool stored in pineal nerve synaptic vesicles (Rodríguez de Lores Arnaiz and Pellegrino de Iraldi, 1989,1991). Interestingly, a peak II fraction inactivated by storing in neutral pH (Fig. 3), which fails to inhibit Na^+,K^+-ATPase (Rodríguez de Lores Arnaiz and Antonelli de Gómez de Lima, 1986), had no inhibitory effect on neurotransmitter content of synaptic vesicles. Such releasing effect of peak II was similar to that induced by ouabain on the neurotransmitter stored in monoaminergic synaptic vesicles (Pellegrino de Iraldi and Rodríguez de Lores Arnaiz, 1989). Measurement of Na^+,K^+-ATPase activity of synaptosomal membranes with vari-able K^+ concentration has shown that enzyme activation produced by K^+ was lower in the presence of peak II, a result suggesting that peak II interferes with the K^+ site of this enzyme (unpublished).

In summary, the fact that brain peak II fraction only inhibits Na^+,K^+-ATPase but fails to change other membrane-bound enzymes points to its enzyme specificity. Furthermore, on the basis of findings that peak II is able to induce diuresis and natriuresis, to block ^{3}H-ouabain binding, and to induce neurotransmitter release much like ouabain, it may be speculated that the responsible factor, probably a peptide, behaves as an ouabain-like substance (Rodríguez de Lores Arnaiz, 1990b).

Conclusions

During the last few years, several investigators have been engaged in research on endogenous modulators of Na^+,K^+-ATPase activity. In this laboratory, it was demonstrated that a soluble fraction prepared from brain was able to modify the effect of the neurotransmitters noradrenaline, dopamine, and serotonin on Na^+,K^+ ATPase activity. Actually, these neurotransmitters might well regulate neuronal excitability through their action on the Na^+ pump, a process involving the participation of endogenous soluble factors.

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