Biochemical Pharmacology, Vol. 39, No. 12, pp. 1891–1895, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

CONTROL OF [^{3}H]OUABAIN BINDING TO CEREBROMICROVASCULAR ($Na^+ + K^+$)-ATPase BY METAL IONS AND PROTEINS

MARY LOU CASPERS,*† TY M. KWAISER* and PAULA GRAMMAS‡

* Department of Chemistry, University of Detroit, Detroit, MI; and ‡ Department of Pathology, Wayne State University School of Medicine, Detroit, MI, U.S.A.

(*Received* 13 *February* 1989; *accepted* 18 *October* 1989)

Abstract—The ($Na^+ + K^+$)-ATPase is localized to the cerebral endothelium, i.e. the blood–brain barrier, and is important for the maintenance of the brain electrolyte environment. Data from the present study indicate that Pb^{2+} inhibits the binding of [^{3}H]ouabain to the cerebral microvascular ($Na^+ + K^+$)-ATPase in a time- and dose-dependent manner. Pb^{2+}-induced inhibition developed slowly with a maximum obtained after 40 min. Inhibition of [^{3}H]ouabain binding to the enzyme was 48% at 10 μM Pb^{2+} and appeared maximal (89%) at 100 μM Pb^{2+} when compared to [^{3}H]ouabain binding in untreated microvessels at 40 min. In contrast, 100 μM Al^{3+} caused a 55% increase in [^{3}H]ouabain binding to the ($Na^+ + K^+$)-ATPase, relative to untreated microvessels at 40 min. Insulin or bovine serum albumin stimulated [^{3}H]ouabain binding to the enzyme when added at similar concentrations. However, the addition of both insulin and bovine serum albumin did not result in an additive effect. These results show that insulin exerts a nonspecific effect on [^{3}H]ouabain binding to the ($Na^+ + K^+$)-ATPase similar to that evoked by bovine serum albumin. However, the metal ions Pb^{2+} and Al^{3+} provoke selective alterations in the cerebromicrovascular ($Na^+ + K^+$)-ATPase with Pb^{2+} inhibiting and Al^{3+} stimulating [^{3}H]ouabain binding.

Cerebromicrovascular ($Na^+ + K^+$)-ATPase [ATP; Mg^{2+}-dependent, ($Na^+ + K^+$)-activated ATP phosphohydrolase; EC 3.6.1.3], located at the blood–brain barrier, is critical for maintenance of a stable ionic milieu in the CNS [1]. Ouabain, a specific inhibitor of the ($Na^+ + K^+$)-ATPase, binds in 1:1 stoichiometry with phosphorylation sites on the enzyme [2, 3]; therefore, measurement of [^{3}H]ouabain bound can be utilized to quantitate the ($Na^+ + K^+$)-ATPase. The high-affinity ouabain binding isoform of the ($Na^+ + K^+$)-ATPase exists in a variety of tissues including cerebral microvessels [4–6]. This cerebromicrovascular enzyme has been shown to respond differently than the synaptosomal ($Na^+ + K^+$)-ATPase to arachidonic acid metabolites [7]. Recently, it was demonstrated that arachidonic but not palmitic acid stimulates [^{3}H]ouabain binding to the high-affinity ouabain binding isoform of the cerebromicrovascular ($Na^+ + K^+$)-ATPase [8]. Insulin receptors exist on brain capillary cells [9] but a regulatory role for insulin on the cerebromicrovascular ($Na^+ + K^+$)-ATPase has not been addressed. Lytton [10], using rat adipocytes, showed that insulin affects the Na^+ affinity of the high-affinity ouabain binding isoform of the enzyme.

Heavy metal ions, including Pb^{2+}, have been shown to inhibit the ($Na^+ + K^+$)-ATPase activity in rat brain [11, 12]. In lead encephalopathy, severe brain edema may be the result of injury to brain capillaries [13] since more than 50% of the Pb^{2+} present in brain is concentrated in the capillary endothelium [14, 15].

* Address correspondence to: Dr. Mary Lou Caspers, Department of Chemistry, University of Detroit, 4001 West McNichols Rd., Detroit, MI 48221-3090.

Recently, attention has focused on the role of Al^{3+} in the pathogenesis of hemodialysis-induced dementia as well as senile dementia of the Alzheimer's type [16, 17]. Al^{3+} has been shown to induce changes in blood–brain barrier permeability [18] and in carrier-mediated processes in the cerebral endothelium [19]. It has been suggested that Al^{3+} deposition in the CNS occurs via alteration of the blood–brain barrier [20].

The purpose of this study was to investigate the abilities of Pb^{2+}, Al^{3+}, insulin and bovine serum albumin to alter [^{3}H]ouabain binding to the high-affinity ouabain binding isoform of the cerebromicrovascular ($Na^+ + K^+$)-ATPase.

MATERIALS AND METHODS

Vanadium-free Mg^{2+}-ATP, ouabain, insulin, Fraction V and fatty acid-free bovine serum albumin were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). New England Nuclear (Boston, MA, U.S.A.) was the source of [^{3}H]ouabain (16.8 Ci/mmol).

A microvessel preparation consisting primarily of capillary segments was isolated from the cerebral cortices of male Sprague–Dawley rats as previously described [21]. Briefly, the brains (devoid of cerebella) were placed in cold Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS). The pial membrane and white matter were removed, and the cerebral cortices were minced and homogenized using twenty up-and-down strokes in a glass homogenizer fitted with a serrated Teflon pestle (0.13 to 0.18 mm clearance) driven at 350 rpm. The homogenate was centrifuged at 2000 *g* for 15 min, and the pellet was resuspended in 15% dextran containing 5% fetal

calf serum (Gibco, Grand Island, NY, U.S.A.) and centrifuged at 3000 *g* for 20 min. The supernatant fraction was discarded, and the pellet was resuspended in HBSS and filtered through a 150-μm nylon mesh sieve and glass bead columns. The capillaries were washed off the glass beads and centrifuged. The purity and capillary nature of the preparation have been characterized previously by scanning electron microscopy, immunofluorescent staining for myosin and γ-glutamyltranspeptidase activity [21, 22]. The final microvessel pellet was resuspended in Dulbecco's modified essential medium containing 10% fetal calf serum supplemented with 20% dimethyl sulfoxide and stored in liquid nitrogen until used. Prior to assay, microvessels were quick-thawed, resuspended in HBSS, and washed three times. Aliquots were taken for protein determination according to the method of Lowry *et al.* [23] using bovine serum albumin as a standard. Microvessels were then centrifuged and resuspended in the assay buffer (pH 7.4) containing 100 mM Tris–HCl, 200 mM NaCl, and 10 mM $MgCl_2$ unless otherwise noted.

Microvessels (70–100 μg protein in 100 μL assay buffer) were incubated with 5 mM ATP, 40 nM [^{3}H]ouabain and $Pb(NO_3)_2$, $Al(NO_3)_3$, bovine serum albumin, insulin or other proteins in a total volume of 250 μL for 15 min at 37° [8, 24]. In studies using $Al(NO_3)_3$ or $Pb(NO_3)_2$, preincubation of microvessels with the metal for 0–60 min preceded addition of ATP and [^{3}H]ouabain. After incubation, the assay was terminated by the addition of ice-cold wash buffer consisting of 50 mM Tris–HCl (pH 7.4), 15 mM KCl and 5 mM $MgCl_2$, and the contents were filtered over Whatman GF/C glass fiber filters under vacuum. The tubes were washed once and the filters twice more with 4 mL of ice-cold wash buffer. The filters were air dried, and the radioactivity was measured in a Searle liquid scintillation counter. Nonspecific binding was determined in the absence of ATP or in the presence of 50 μM ouabain and, in either case, represented less than 2% of total binding. Unless otherwise indicated, all values are expressed as the mean ± SE of three separate experiments performed in triplicate. Statistical significance was determined using Student's *t*-test.

RESULTS

Experiments where microvessels were preincubated with Pb^{2+} indicated that the inhibition of [^{3}H]ouabain binding to the ($Na^+ + K^+$)-ATPase by Pb^{2+} developed slowly (Fig. 1) and reached a maximum after 40 min. Because there was a nonspecific decrease in the amount of [^{3}H]ouabain bound during the preincubation (56% after 10 min), the specific inhibition of Pb^{2+} was calculated relative to the basal values at each time point (Fig. 1, inset). Thus, Pb^{2+} inhibited 34% of the remaining activity at 40 min. Preliminary experiments using sonicated microvessels indicated that sonication did not alter the time course of Pb^{2+} inhibition (data not shown). To test the possibility that the slowly developing inhibition represented an irreversible reaction, dithiothreitol, a potent chelator of Pb^{2+}, was added to the reaction mixture in 1000-fold molar excess.

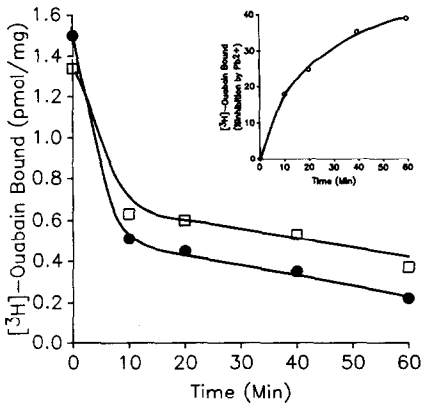

Fig. 1. Preincubation of Pb^{2+} with cerebromicrovascular ($Na^+ + K^+$)-ATPase. Microvessels were incubated at 37° for 0–60 min in the presence (●—●) or absence (□—□) of 10 μM $Pb(NO_3)_2$. ATP and [^{3}H]ouabain were added to the mixture followed by a 15-min incubation at 37°. Data are the means of 3 experiments performed in triplicate. Inset: Percent specific inhibition of [^{3}H]ouabain bound by Pb^{2+} when compared to untreated microvessels at each time point.

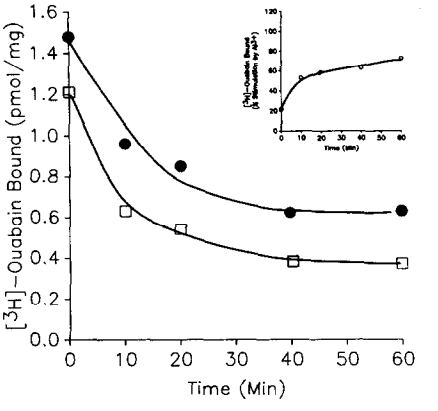

Fig. 2. Preincubations of Al^{3+} with cerebromicrovascular ($Na^+ + K^+$)-ATPase. Microvessels were incubated at 37° for 0–60 min in the presence (●—●) or absence (□—□) of 10 μM $Al(NO_3)_3$. ATP and [^{3}H]ouabain were added to the mixture followed by a 15-min incubation at 37°. Data are the means of 3 experiments performed in triplicate. Inset: Percent increase in [^{3}H]ouabain bound by Al^{3+} when compared to untreated microvessels at each time interval.

When added at the same time as Pb^{2+}, this compound prevented Pb^{2+} inhibition of [^{3}H]ouabain binding to the ($Na^+ + K^+$)-ATPase, whereas if dithiothreitol was added after the 40-min preincubation, it was able to reverse the Pb^{2+} effect. Addition of Al^{3+} caused a slight, but not significant, stimulation (20%) in [^{3}H]ouabain binding to the ($Na^+ + K^+$)-ATPase with no preincubation (Fig. 2). Despite a nonspecific decrease in [^{3}H]ouabain bound with time in the absence of Al^{3+} (i.e. 48% after 10 min), the presence of Al^{3+} caused an increase in binding relative to basal values at each time point. This increase also

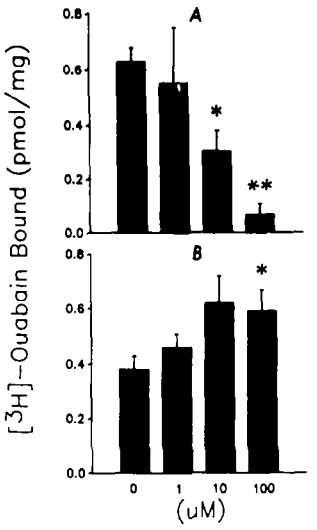

Fig. 3. Effect of increasing metal ion concentration (0–100 μM) on [^{3}H]ouabain bound to cerebromicrovascular ($Na^+ + K^+$)-ATPase. Microvessels were incubated with either $Pb(NO_3)_2$ (A) or $Al(NO_3)_3$ (B) for 40 min at 37° prior to addition of ATP and [^{3}H]ouabain. Data are the means of 3 experiments performed in triplicate. Key: (*) significant change from control ($P < 0.05$), and (**) significant change from control ($P < 0.001$).

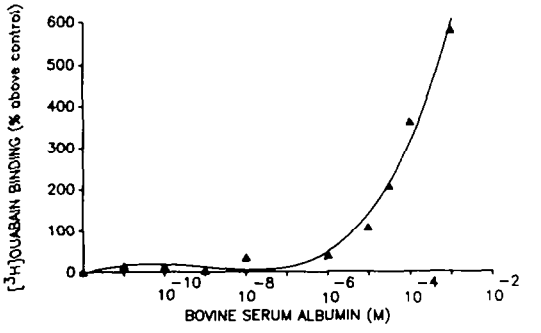

Fig. 4. Effect of bovine serum albumin on [^{3}H]ouabain bound to cerebromicrovascular ($Na^+ + K^+$)-ATPase. Microvessels (70–90 μg total protein) were incubated with 36 nM [^{3}H]ouabain in the presence of 0–1 mM bovine serum albumin at 37° for 15 min. Data are expressed as percent increase in binding over the control level (2.85 pmol/mg). Each point represents the mean of triplicate experiments.

Table 1. Enhancement of [^{3}H]ouabain bound to ($Na^+ + K^+$)-ATPase with insulin and bovine serum albumin (BSA)

Protein (10 μM)	[^{3}H]Ouabain bound (pmol/mg)
	1.28 ± 0.05
BSA	5.79 ± 0.39*
Insulin	6.03 ± 0.49*
Insulin + BSA	6.84 ± 0.07*

Microvessels (70–90 μg protein) were incubated for 15 min at 37°. Data are the means ± SE of triplicate experiments.

* Significant change from control ($P < 0.001$).

appeared to be time dependent and reached a plateau after 20 min (Fig. 2, inset).

The effects of 0–100 μM Pb^{2+} or Al^{3+} on [^{3}H]ouabain binding to the ($Na^+ + K^+$)-ATPase are shown in Fig. 3A and B. Preincubation of microvessels with Pb^{2+} for 40 min prior to assay resulted in a significant inhibition of [^{3}H]ouabain binding of 48% at 10 μM ($P < 0.05$) and 89% at 100 μM Pb^{2+} ($P < 0.001$) (Fig. 3A). With no preincubation, Pb^{2+} did not inhibit the enzyme until present at 1 mM (data not shown). While a slight, but not significant, increase in [^{3}H]ouabain binding was noted with 1–10 μM Al^{3+}, a 55% increase ($P < 0.05$) was observed at 100 μM Al^{3+} (Fig. 3B).

In the presence of bovine serum albumin (Fraction V), [^{3}H]ouabain binding to cerebromicrovascular ($Na^+ + K^+$)-ATPase increased in a dose-dependent manner from 38% at 1 μM to 574% at 1 mM (Fig. 4). Similar results were obtained using fatty acid-free bovine serum albumin. Addition of 10 μM insulin caused a 370% increase in [^{3}H]ouabain binding over basal levels. However, this stimulation was comparable to that evoked by the same concentration of bovine serum albumin. Addition of both these proteins did not result in an additive effect (Table 1). Experiments to optimize the conditions for insulin action on the cerebral endothelium, including preincubation of microvessels with 10 nM insulin for 0–60 min at 25° or 37°, and use of Krebs–Ringer buffer or freshly isolated microvessels, did not potentiate the response to insulin.

DISCUSSION

The results of this study indicate that Pb^{2+} and Al^{3+} cause an inhibition and increase, respectively, of [^{3}H]ouabain binding to the ($Na^+ + K^+$)-ATPase of the cerebromicrovasculature when compared to untreated microvessels under the same conditions. The reversible Pb^{2+}-induced inhibition developed slowly. One possible explanation for this phenomenon is that Pb^{2+} must be transported across the cell membrane since Pb^{2+} is thought to bind to the enzyme near the Na^+ site which is oriented towards the cytoplasmic side of the membrane [25]. However, data from experiments with sonicated microvessels, where the transport of Pb^{2+} is not rate limiting, tend to argue against this notion because Pb^{2+}-induced inhibition of [^{3}H]ouabain binding was still time dependent. The time dependency may also reflect the binding of Pb^{2+} initially to nonspecific sites on the membrane, which presumably are more numerous than the ($Na^+ + K^+$)-ATPase molecules, or the interaction of Pb^{2+} with another protein that then indirectly affects the ($Na^+ + K^+$)-ATPase [12].

It would be of interest to know if the cerebromicrovascular ($Na^+ + K^+$)-ATPase is inhibited in lead intoxication. Toews *et al.* [15] subjected rats to Pb^{2+} and determined that cerebral capillaries contain 1 mmol Pb^{2+}/mg protein. In our experiments, significant inhibition of [^{3}H]ouabain binding to the

$(Na^+ + K^+)$-ATPase occurred at 10 μM Pb^{2+}. This represents approximately 25 mmol Pb^{2+}/mg protein (100 μg protein in a 250-μL reaction volume) and is well within range for uptake of Pb^{2+} into rat brain capillaries. Thus, Pb^{2+}-induced inhibition of the cerebromicrovascular $(Na^+ + K^+)$-ATPase may contribute, at least in part, to ion imbalances in the edema of lead encephalopathy.

In the present study, 100 μM Al^{3+} increased [^{3}H]ouabain binding to the cerebromicrovascular $(Na^+ + K^+)$-ATPase, although a specific interaction with the enzyme remains to be elucidated. Evidence from the literature suggests that Al^{3+} does selectively alter the blood–brain barrier, i.e. by increasing its permeability to neuropeptides [26]. Alternatively, the increase of [^{3}H]ouabain binding by Al^{3+} may occur via a nonspecific mechanism because Al^{3+} is known to displace cations from membrane surfaces [27] which could affect the function of the $(Na^+ + K^+)$-ATPase.

The results of the present study indicate that similar concentrations of fatty acid-free bovine serum albumin or insulin evoke comparable stimulation of [^{3}H]ouabain binding and that addition of both proteins does not produce any additive or synergistic effects. The action of insulin on the $(Na^+ + K^+)$-ATPase, as measured by [^{3}H]ouabain binding, appears to be a nonspecific effect of protein rather than a hormone-mediated interaction. Interestingly, Resh *et al.* [28] show that, in the presence of bovine serum albumin, insulin exerts a selective effect on the ion transport properties of the $(Na^+ + K^+)$-ATPase but not on the phosphorylated form of the enzyme as detected by [^{3}H]ouabain binding.

In summary, the results of these experiments demonstrate that [^{3}H]ouabain binding to the $(Na^+ + K^+)$-ATPase is affected by metal ions and proteins. While insulin enhanced [^{3}H]ouabain binding, this stimulation appeared to be nonspecific because bovine serum albumin produced comparable results. Considering the wide use of bovine serum albumin in a variety of experimental protocols, care must be taken to avoid misinterpretation of results by use of appropriate controls. Our data with metal ions, showing inhibition of [^{3}H]ouabain binding by Pb^{2+} and an Al^{3+}-induced increase of [^{3}H]ouabain binding, indicate that these metal ions can selectively regulate the cerebromicrovascular $(Na^+ + K^+)$-ATPase. These latter results suggest that alteration of the $(Na^+ + K^+)$-ATPase function may play a role in the molecular mechanism of metal toxicity in the CNS.

Acknowledgements—The authors gratefully acknowledge the technical assistance of Denise Bessert and Sharon Johnson, the expert typing of Jane Schley and the helpful discussions with Drs F. Giacomelli and J. Wiener. This work was partially supported by United States Public Health Services Research Grant HL 23603 from The Heart, Lung and Blood Institute, National Institutes of Health, Ernest Voliwiler grant from Alzheimer's Disease and Related Disorders Assoc., American Health Assistance Foundation, a faculty development award from the University of Detroit and a gift from J. D. Rose.

REFERENCES

1. Betz AL and Goldstein GW, Specialized properties and solute transport in brain capillaries. *Annu Rev Physiol* **48**: 241–250, 1986.
2. Erdmann E and Schoner W, Ouabain–receptor interactions in $(Na^+ + K^+)$-ATPase preparations from different tissues and species. Determination of kinetic constants and dissociation constants. *Biochim Biophys Acta* **307**: 386–398, 1973.
3. Anner BM, The receptor function of the Na^+,K^+-activated adenosine triphosphatase system. *Biochem J* **227**: 1–11, 1985.
4. Marks MJ and Seeds NW, A heterogeneous ouabain–ATPase interaction in mouse brain. *Life Sci* **23**: 2735–2744, 1978.
5. Sweadner KJ, Two molecular forms of $(Na^+ + K^+)$-stimulated ATPase in brain: Separation and difference in affinity for strophanthidin. *J Biol Chem* **254**: 6060–6067, 1979.
6. Harik SI, Doull GH and Dick APK, Specific ouabain binding to brain microvessels and choroid plexus. *J Cereb Blood Flow Metab* **5**: 156–160, 1985.
7. Koide T, Asano T, Matsushita H and Takakura K, Enhancement of ATPase activity by a lipid peroxide of arachidonic acid in rat brain microvessels. *J Neurochem* **46**: 235–242, 1986.
8. Caspers ML and Grammas P, Effect of fatty acids on [^{3}H]ouabain binding to cerebromicrovascular $(Na^+ + K^+)$-ATPase. *J Neurochem* **50**: 1215–1219, 1988.
9. Pardridge WM, Eisenberg J and Yang J, Human blood–brain barrier insulin receptor. *J Neurochem* **44**: 1771–1778, 1985.
10. Lytton J, Insulin affects the sodium affinity of the rat adipocyte $(Na^+ + K^+)$-ATPase. *J Biol Chem* **260**: 10075–10080, 1985.
11. Siegel GJ and Fogt SK, Effects of lead ion on brain microsomes: Inhibition of cation transport ATPase and stimulation of phosphorylation. *Trans Am Neurol Assoc* **101**: 1–3, 1976.
12. Bertoni JM and Sprenkle PM, Inhibition of brain cation pump enzyme by *in vitro* level ion: Effects of low level [Pb] and modulation by homogenate. *Toxicol Appl Pharmacol* **93**: 101–107, 1988.
13. Joo F, The blood–brain barrier *in vitro*: Ten years of research on microvessels isolated from brain. *Neurochem Int* **7**: 1–25, 1985.
14. Thomas JA, Dallenbach FD and Thomas M, The distribution of radioactive lead (^{210}Pb) in the cerebellum of developing rats. *J Pathol* **109**: 45–50, 1973.
15. Toews AD, Kolber A, Hayward J, Krigman MR and Morelli P, Experimental lead encephalopathy in the suckling rat: Concentration of lead in cellular fractions enriched in brain capillaries. *Brain Res* **147**: 131–138, 1978.
16. Perl DP, Relationship of aluminum to Alzheimer's disease. *Environ Health Perspect* **63**: 149–153, 1985.
17. Goyer RA, Toxic effects of metals. In: *Casarett and Doull's Toxicology, the Basic Science of Poisons* (Eds. Klaaseen CD, Amdur MO and Doull J), 3rd Edn, pp. 582–635. Macmillan, New York, 1986.
18. Kim YS, Lee MH and Wisniewski HM, Aluminum induced reversible change in permeability of the blood–brain barrier to [^{14}C]sucrose. *Brain Res* **377**: 286–291, 1986.
19. Banks WA and Kastin AJ, Aluminum alters the permeability of the blood–brain barrier to some non-peptides. *Neuropharmacology* **24**: 407–412, 1985.
20. Vinters HV and Pardridge WM, The blood–brain barrier in Alzheimer's disease. *Can J Neurol Sci* **13**: 446–448, 1986.
21. Diglio CA, Grammas P, Giacomelli F and Weiner J, Primary culture of rat cerebral microvascular endothelial cells. Isolation, growth and characterization. *Lab Invest* **46**: 554–563, 1982.

22. Grammas P, Diglio CA, Marks BH, Giacomelli F and Weiner J, Identification of muscarinic receptors in rat cerebral cortical microvessels. *J Neurochem* **40**: 645–651, 1983.
23. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
24. Hauger R, Luu HMD, Meyer DK, Goodwin FK and Paul SM, Characterization of "high-affinity" [^{3}H]ouabain binding in the rat central nervous system. *J Neurochem* **44**: 1709–1715, 1985.
25. Siegel GJ, Fogt SK and Iyengar S, Characteristics of lead ion-stimulated phosphorylation of *Electrophorus electricus* electroplax ($Na^+ + K^+$)-adenosine triphosphatase and inhibition of ATP–ADP exchange. *J Biol Chem* **253**: 7207–7211, 1978.
26. Banks WA and Kastin AJ, Aluminum increases permeability of the blood–brain barrier to labelled DSIP and β-endorphin: Possible implications for senile and dialysis dementia. *Lancet* **ii**: 1227–1229, 1983.
27. DeLeers M, Cationic atmosphere and cation competition binding at negatively charged membranes: pathological implications of aluminium. *Res Commun Chem Path Pharmacol* **49**: 277–292, 1985.
28. Resh MD, Nemenoff RA and Guidotti G, Insulin stimulation of ($Na^+ + K^+$)-adenosine triphosphatase-dependent $^{86}Rb^+$ uptake in rat adipocytes. *J Biol Chem* **255**: 10938–10945, 1980.