Current Topics in Peptide & Protein Research
Vol. 15, 2014

Original Communication

Kinectics of Na^+, K^+-ATPase inhibition by calcitonin and neurotensin

María Graciela López Ordieres[1,2,*] and Georgina Rodríguez de Lores Arnaiz[1]

[1]Instituto de Biología Celular y Neurociencias "Prof. E. De Robertis", CONICET-UBA, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121-Buenos Aires, Argentina; [2]Cátedra de Farmacología, Facultad de Farmacia y Bioquímica, Junín 956, Universidad de Buenos Aires, 1113-Buenos Aires, Argentina.

ABSTRACT

The kinetics of interactions between the peptides calcitonin or neurotensin, and synaptosomal membrane Na^+, K^+-ATPase activity was characterized in this study. Calcitonin is a 32-amino acid peptide produced by the thyroid gland, whose main role is to prevent bone resorption. Calcitonin produced a non-competitive type of inhibition at varied potassium concentrations whereas in the presence of sodium and ATP, calcitonin produced an uncompetitive and competitive type of inhibition, respectively. Neurotensin is a basic tridecapeptide which also inhibits Na^+, K^+-ATPase activity. Therefore, in the presence of higher sodium concentrations the peptide produced a competitive interaction and it produced a non-competitive type of inhibition at varied potassium and ATP concentrations. In summary, calcitonin and neutotensin behave as enzyme inhibitors but, each one leads to a characteristic type of enzyme inhibition at varied substrate concentrations due to differences in peptide structure and functionality that could be influencing the kinetics of substrate interactions.

KEYWORDS: calcitonin, neurotensin, Na^+, K^+-ATPase

INTRODUCTION

Na^+, K^+-ATPase is a plasma membrane enzyme ubiquitous in animal cells which catalyses the efflux of three Na^+ and influx of two K^+ per ATP hydrolyzed and is involved in the maintenance of membrane potential [1, 2]. Structurally this enzyme is composed of alpha, beta and gamma subunits. Alpha subunit is the catalytic subunit of this enzyme, beta subunit is required for enzyme assembly [1, 3] and gamma subunit belongs to FXYD proteins, a family of small regulatory proteins that modulate enzyme kinetic properties [4].

Modulation of Na^+, K^+-ATPase activity is crucial for neurotransmission, thus it is possible to find natural and synthetic substances that are able to modify the activity of this enzyme [2], such as calcitonin and neurotensin [5].

Calcitonin is a 32-amino acid peptide produced by the thyroid gland, whose main role is to prevent bone resorption. This hormone also reduces food intake, gastric secretion and amphetamine-induced locomotor activity and induces hyperthermia and analgesia through central and peripheral mechanisms [6-9]. Neurotensin is a basic tridecapeptide which can behave as a neurotransmitter or as a neuromodulator [10]. Neurotensin can interact with a group of receptors which have been characterized both in CNS and peripheral tissues. Two of them, NTS1 and NTS2, are membrane receptors coupled to G proteins [11, 12] while the third one belongs to the Vps10p containing domain receptor family [13].

Previous findings from this laboratory indicated that calcitonin and neurotensin inhibit neuronal

*Corresponding author: glopez@ffyb.uba.ar

Na^+, K^+-ATPase activity [14, 15]. Therefore, we found it to be of interest to study this enzyme activity in the presence of calcitonin or neurotensin at varied ATP, sodium and potassium concentrations in order to elucidate the nature of the interaction of these peptides with Na^+, K^+-ATPase.

MATERIALS AND METHODS

Animals and drugs

Adult male Wistar rats weighing 100-120 g were used. All studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health (USA). Animals had access to a standard commercial diet and water *ad libitum* and were kept in a room maintained at 25 °C ± 2 °C with a 12 h light/dark cycle. ATP (grade I, prepared by phosphorylation of adenosine), ouabain, and neurotensin acetate were obtained from Sigma Chemical Co., St. Louis, MO, USA. Salmon calcitonin Peptech, Denmark, was provided by Gramón Millet, Argentina. Peptide solutions in redistilled water were freshly prepared for each experiment. Reagents were of analytical grade.

Preparation of synaptosomal membrane fractions

Synaptosomal membranes from rat cerebral cortex were isolated by differential and sucrose gradient centrifugation as previously described by Rodríguez de Lores Arnaiz *et al.* [16]. In each experiment, lots of five rats were processed. Cerebral cortices were dissected, homogenized and subjected to differential centrifugation to separate the crude mitochondrial pellet, which was resuspended in redistilled water for the osmotic shock. A pellet containing the synaptosomal membranes was separated by centrifugation (20,000 x g for 30 min), then resuspended in 0.32 M sucrose, layered on top of a sucrose gradient (0.8, 0.9, 1.0, and 1.2 M) and ultracentrifuged at 50,000 x g for two hours. The fraction at the level of 1.0 M sucrose was collected and spun down (100,000 x g for 30 min) to obtain the synaptosomal membrane fraction. Pellets were stored at -70 °C and, prior to enzyme assay, resuspended by brief homogenization in redistilled water, stored frozen, and used for three weeks without appreciable change in enzyme activities.

Enzyme assays

ATPase activity was measured as described by Albers *et al.* [17]. Total ATPase activity was assayed in a medium containing 3.1-100 mM NaCl, 1.25-40 mM KCl, 3 mM $MgCl_2$, 0.20 M Tris-HCl buffer (pH 7.4) and 2-8 mM ATP. Mg^{2+}-ATPase activity was determined in a similar medium with no Na^+ and K^+ added, and containing 1 mM ouabain. Na^+, K^+-ATPase activity was calculated by the difference between these two enzyme assays. Before performing the ATPase assay, samples of synaptosomal membrane fractions were pre-incubated with 0.20 M Tris-HCl buffer (pH = 7.4) or 1.0×10^{-5} M calcitonin or 3.0×10^{-6} M neurotensin at 37 ºC for 10 min; incubation volume (µl) was 35:5 for buffer and membranes. Aliquots of pre-incubated fractions (3 µl) were distributed in two series of microtubes containing the respective medium (40 µl) for the assay of total- and Mg^{2+}-ATPase activities, and incubated at 37 ºC for 30 min. The reaction was stopped with 30% trichloroacetic acid solution. ATPase activity was monitored by colorimetric determination of orthophosphate released [18]. In all enzyme assays, tubes containing enzyme preparations and assay media maintained at 0 ºC throughout the incubation period were used as blanks.

Protein measurement

Protein content in the membrane fractions was determined by the method of Lowry *et al.* [19], using bovine serum albumin as standard.

Statistical analysis

For saturation assays, non-linear regression of the data was processed using the program Graph Pad Prism 4.0 (2003). Data for Na^+, K^+-ATPase activity was expressed as µmol of inorganic phosphate released per mg protein per hour, (mean ± SD).

RESULTS AND DISCUSSION

The kinetics of the interactions between the peptides calcitonin or neurotensin, and synaptosomal membrane Na^+, K^+-ATPase was characterized in this study. Na^+, K^+-ATPase is located at the plasma membrane where it regulates the efflux of three Na^+ and influx of two K^+ per molecule of ATP hydrolysed. This enzyme is involved in the

normal cell cycle, osmotic balance and the maintenance and restoration of the resting membrane potential [20].

Most of the biochemical reactions take place thanks to the help of enzymes since they usually lower the activation energy of a reaction, enabling the reaction to occur much faster than it would in the absence of enzymes. Factors that regulate the enzyme activity are: temperature, pH, the substrate concentration and the presence of inhibitors. Competitive inhibitors occupy the active sites and compete with the substrate for them, whereas uncompetitive inhibitors bind only to the enzyme-substrate complex without affinity for the free enzyme; this requires the prior formation of the enzyme-substrate complex for binding and inhibition. Finally, a non-competitive inhibitor is one that displays binding affinity for both the free enzyme and the enzyme-substrate complex, emphasizing that a non-competitive inhibition cannot be overcome by high concentrations of the substrate [21].

Many substances can act as Na^+, K^+-ATPase inhibitors, but the best known are the cardiac glycosides [22]. Moreover, compounds with a peptide structure can exert modulatory effects on Na^+, K^+-ATPase activity, as it occurs in the cases of calcitonin [14] and neurotensin [15]. These peptides behave as enzyme inhibitors but each one produces a characteristic inhibition of Na^+, K^+-ATPase and K^+-*p*-nitrophenylphosphatase (K^+-*p*-NPPase) activities. This is an enzyme which is considered to be involved in K^+-dependent hydrolysis of the phosphoenzyme in the reaction cycle of Na^+, K^+-ATPase. Previous work performed in cortical synaptosomal membranes disclosed that calcitonin decreased Na^+, K^+-ATPase and K^+-*p*-NPPase activities by 20-40% and 15-25%, respectively. Na^+, K^+-ATPase activity is also inhibited by neurotensin and K^+-*p*-NPPase activity is likewise inhibited but only under strict experimental conditions [23].

In order to study Michelis-Menten kinetics of Na^+, K^+-ATPase inhibition, we considered Vmax as the theoretical maximum rate of the reaction and Km as numerically equal to the substrate concentration at which the reaction rate is half its maximal value. Km is an important characteristic of an enzyme-catalyzed reaction and is significant for its biological function. The results were plotted according to Hanes-Woolf plot that represents [Substrate]/velocity *versus* [Substrate]. Herein X intercept is equal to –Km and the slope is equal to 1/Vmax.

Synaptosomal membranes were evaluated in the presence of varied Na^+ concentrations, the extent of enzyme inhibition remained unaltered over the 3.1-100 mM range in the presence of 10^{-5} M salmon calcitonin (Fig. 1) and 3.0 x 10^{-6} M neurotensin (Fig. 2). Calcitonin produced an uncompetitive type of inhibition determined by a reduction in the Km and Vmax values (Fig. 1). This occurs when calcitonin binds only to the complex formed between Na^+, K^+-ATPase and sodium ions. However, in the presence of 3.0 x 10^{-6} M neurotensin (Fig. 2) the slope of the plot S/v versus S did not change, indicating a competitive type of inhibition.

Neurotensin can interact with Na^+, K^+-ATPase either directly or through the neurotensin NTS1 receptor [15]. The binding affinity of neurotensin to the NTS1 receptor is decreased in the presence of sodium ions which could prevent the inhibitory effect of neurotensin on Na^+, K^+-ATPase activity. For this reason, to avoid a decrease in Na^+, K^+-ATPase activity, the highest sodium concentrations used was 100 mM NaCl [24].

At varied K^+ concentrations, calcitonin and neurotensin inhibited enzyme activity over the 2.5-40 mM range (Fig. 3 and Fig. 4). Salmon calcitonin at a 10^{-5} M concentration decreased the Vmax values from 61 to 49 µmol Pi x mg $protein^{-1}$ x h^{-1}, while the Km values remained unchanged in the presence of this peptide. Calcitonin inhibited this enzyme activity over the 1-8 mM range in the presence of higher ATP concentrations. This peptide produced a competitive type of inhibition due to an increase in the Km values (4.8 to 16.3) (Fig. 5). Neurotensin 3.0 x 10^{-6} M exhibited a reduction in Vmax values from 45.1 to 42.1 µmol Pi x mg $protein^{-1}$ x h^{-1} and the Km values remained unchanged at higher potassium concentrations (Fig. 4).

Previous results indicated that neurotensin decreases [^{3}H]-ouabain binding to cerebral cortex membranes

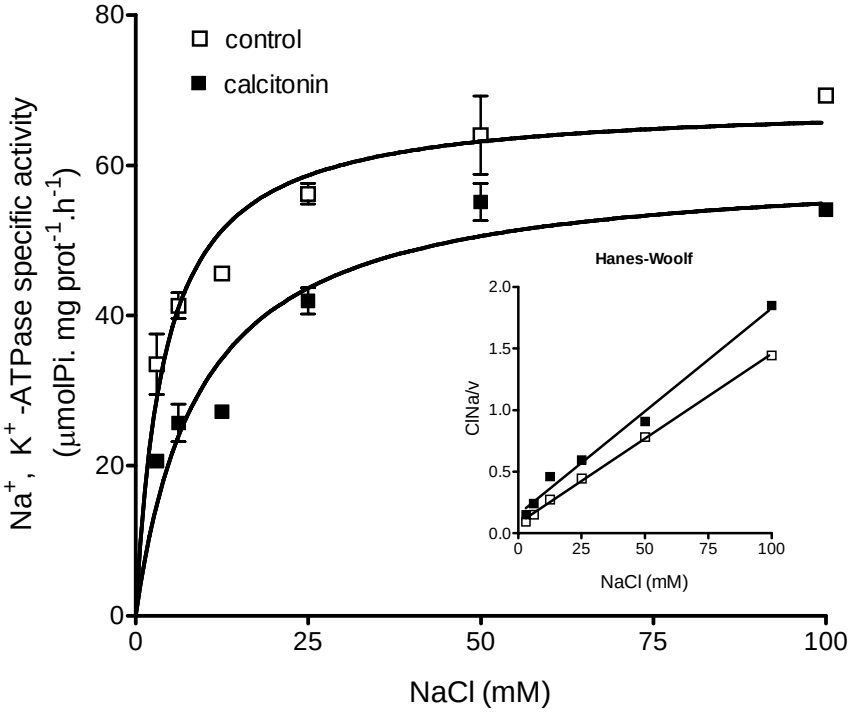

Fig. 1. Na^+, K^+-ATPase activity of cortical synaptosomal membranes as a function of NaCl concentration in the absence and presence of 1 x 10^{-5} M calcitonin. Results of three experiments are presented as µmol of Pi released per mg protein and as Hanes-Woolf plot (inset).

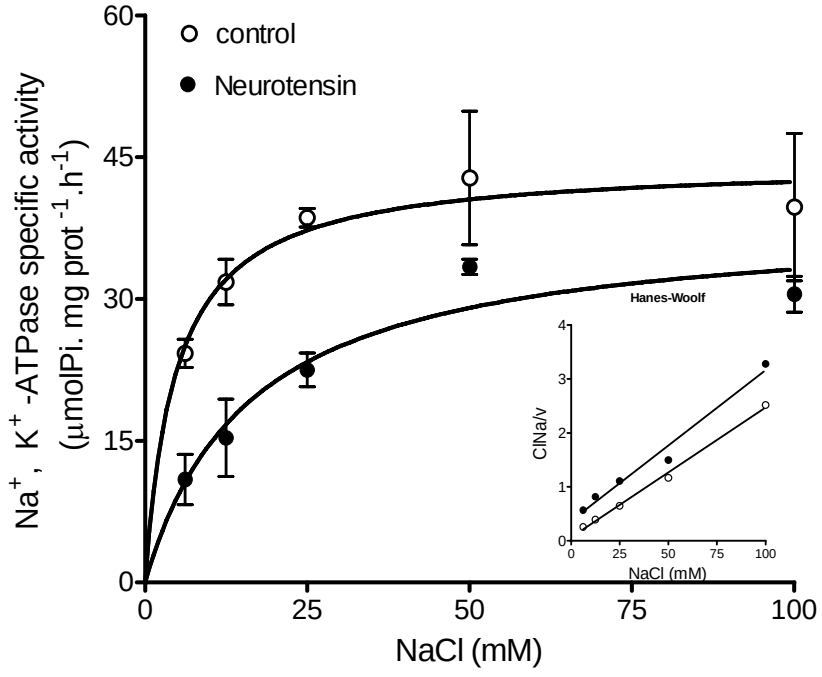

Fig. 2. Na^+, K^+-ATPase activity of cortical synaptosomal membranes as a function of NaCl concentration in the absence and presence of 3 x 10^{-6} M neurotensin. Results of three experiments are presented as µmol of Pi released per mg protein and as Hanes-Woolf plot (inset).

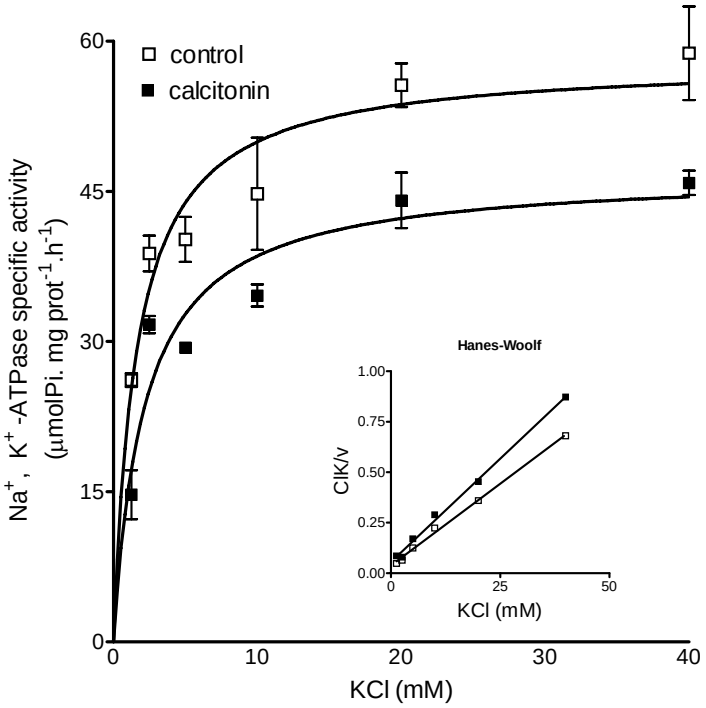

Fig. 3. Na^+, K^+-ATPase activity of cortical synaptosomal membranes as a function of KCl concentration in the absence and presence of 1 x 10^{-5} M calcitonin. Results of three experiments are presented as µmol of Pi released per mg protein and as Hanes-Woolf plot (inset).

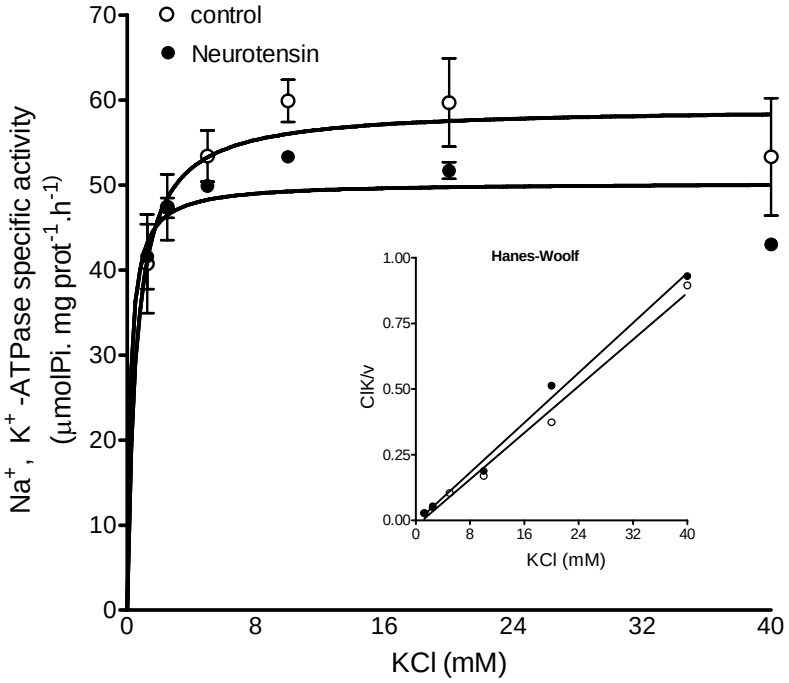

Fig. 4. Na^+, K^+-ATPase activity of cortical synaptosomal membranes as a function of KCl concentration in the absence and presence of 3 x 10^{-6} M neurotensin. Results of three experiments are presented as µmol of Pi released per mg protein and as Hanes-Woolf plot (inset).

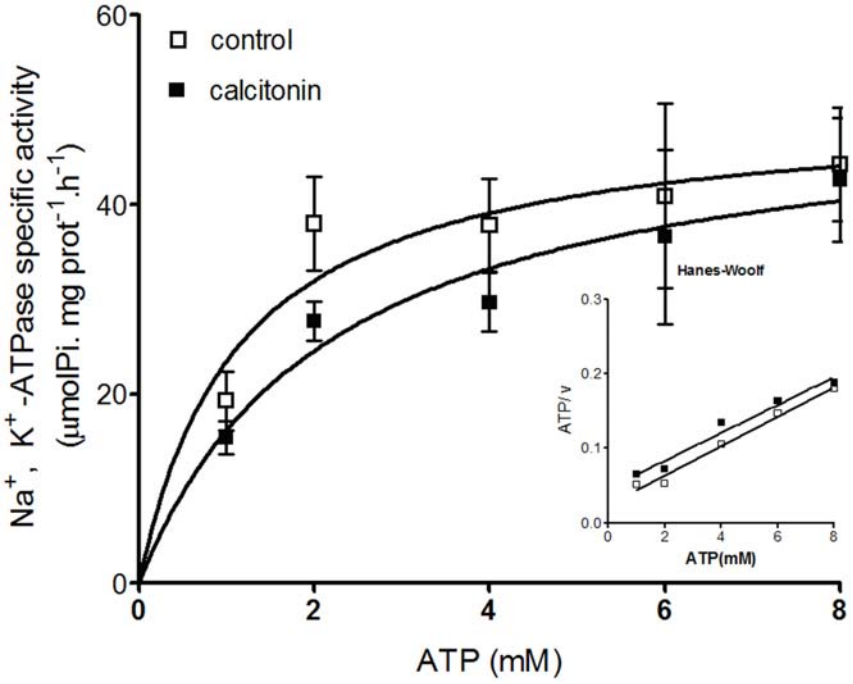

Fig. 5. Na^+, K^+-ATPase activity of cortical synaptosomal membranes as a function of ATP concentration in the absence and presence of 1 x 10^{-5} M calcitonin. Results of three experiments are presented as µmol of Pi released per mg protein and as Hanes-Woolf plot (inset).

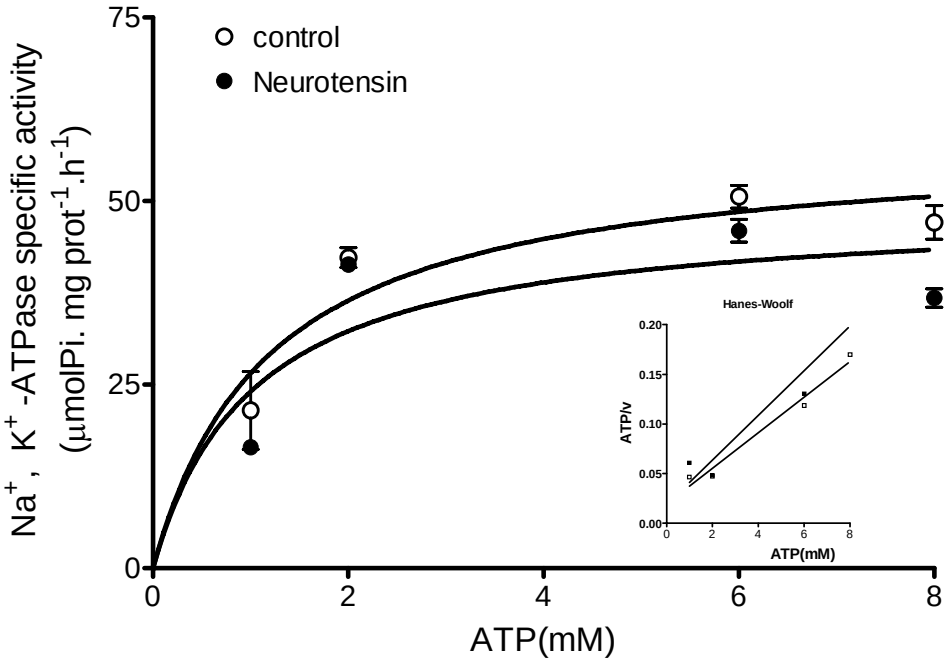

Fig. 6. Na^+, K^+-ATPase activity of cortical synaptosomal membranes as a function of ATP concentration in the absence and presence of 3 x 10^{-6} M neurotensin. Results of three experiments are presented as µmol of Pi released per mg protein and as Hanes-Woolf plot (inset).

and produces a competitive type inhibition of [^{3}H]-ouabain binding, with an increase of Kd value [25]. These results suggest that the peptide also interacts with Na^+, K^+-ATPase at the K^+ site. Then, if this site was occupied by high potassium concentrations, neurotensin could bind to the free enzyme or the enzyme substrate complex, producing a non-competitive inhibition. Similarly, a non-competitive type of interaction was observed in the presence of varied ATP concentrations since neurotensin led to a reduction of the Vmax values (58.2 to 48.9) and no changes in Km values (Fig. 6).

In summary, calcitonin and neurotensin behave as enzyme inhibitors, but each one leads to a characteristic enzyme type of inhibition at varied substrate concentrations due to their peptide structure and functionality that may be influencing the kinetics of substrate interactions.

CONCLUSION

In the present work we studied the interaction of calcitonin and neurotensin with Na, K-ATPase at varied sodium, potassium or ATP concentrations. Calcitonin produced a type of inhibition at varied potassium concentrations whereas in the presence of sodium and ATP produced uncompetitive and competitive types of inhibition, respectively. Neurotensin produced a competitive interaction in the presence of higher concentrations of sodium and a non-competitive type of inhibition at varied potassium and ATP concentrations.

ACKNOWLEDGMENTS

G. R. de L. A. is the chief investigator from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). The authors are indebted to CONICET and Universidad de Buenos Aires, Argentina, for their financial support.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

REFERENCES

1. Albers, R. W. and Siegel, G. J. 2012, Basic Neurochemistry: Principles of Molecular, Cellular and Medical Neurobiology, 8th Edn., S. T. Brady, G. J. Siegel, R. W. Albers and D. Price (Eds.), Elsevier Academic Press, Massachusetts, USA, 41.
2. Rodríguez de Lores Arnaiz, G. 2007, Neural Membranes and Transport, Handbook of Neurochemistry and Molecular Neurobiology, A. Lajtha (Ed.), Springer-Verlag, Berlin, Heidelberg, Germany, Vol. 11, 209.
3. Peng, L., Martin-Vasallo, P. and Sweadner, K. J. 1997, J. Neurosci., 15, 3488.
4. Feschenko, M. S., Donnet, C., Wetzel, R. K. Asinovski, N. K., Jones, L. R. and Sweadner, K. J. 2003, J. Neurosci., 15, 2161.
5. Rodríguez de Lores Arnaiz, G. and López Ordieres, M. G. 2003, Current Topics in Peptide and Protein Research, 5, 75.
6. Freed, W. J., Perlow, M. J. and Wyatt, R. J. 1979, Science, 206, 850.
7. Morley, J. E., Levine, A. S. and Silvis, S. E. 1981, Science, 214, 671.
8. Pecile, A., Ferri, S., Braga, P. C. and Olgiati, V. R. 1975, Experientia, 31, 332.
9. Sellami, S. and de Beaurepaire, R. 1993, Brain Res., 616, 307.
10. Kitabgi, P. and Nemeroff, C. B. 1992, The Neurobiology of Neurotensin, Ann. New York Acad. Sci., New York, USA.
11. Vincent, J. P., Mazella, J. and Kitabgi, P. 1999, Trends Pharmacol. Sci., 20, 302.
12. Dobner, P. R. 2005, Cell. Mol. Life Sci., 62, 1946.
13. Mazella, J., Béraud-Dufour, S., Devader, C., Massa, F. and Coppola, T. 2012, Front. Endocrinol. (Lausanne), 3, 143.
14. Rodríguez de Lores Arnaiz, G. and López Ordieres, M. G. 1997, Peptides, 18, 613.
15. López Ordieres, M. G. and Rodríguez de Lores Arnaiz, G. 2000, Peptides, 21, 571.
16. Rodríguez de Lores Arnaiz, G., Alberici, M. and De Robertis, E. 1967, J. Neurochem., 14, 215.
17. Albers, R. W., Rodrıguez de Lores Arnaiz, G. and De Robertis, E. 1965, Proc. Natl. Acad. Sci. USA, 53, 557.
18. Lowry, O. H. and López, J. A. 1946, J. Biol. Chem., 162, 8.
19. Lowry, O. H., Rosebrough, N., Farr, A. L. and Randall, R. J. 1951, J. Biol. Chem., 193, 265.
20. Skou, J. 1957, Biochim. Biophys. Acta, 23, 394.

21. Copeland, R. 2013, Evaluating of Enzyme Inhibiton in Drug Discovery. A Guide for Medicinal Chemist and Pharmacologist, 2nd Edn., W. R. Briggs and J. L. Spudich (Eds.) Wiley, Hoboken, NJ, 572.
22. Lingrel, J. B. 2010, Annu. Rev. Physiol., 72, 395.
23. López Ordieres, M. G. and Rodríguez de Lores Arnaiz, G. 2001, Regul. Pept., 101, 183.
24. Martin, S., Botto, J. M., Vincent, J. P. and Mazella, J. 1999, Mol. Pharmacol., 55, 210.
25. Rosin, C., López Ordieres, M. G. and Rodríguez de Lores Arnaiz, G. 2011, Regul. Pept., 172, 35.