Biochemical Pharmacology, Vol. 41, No. 9, pp. 1279–1282, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

CYTOPLASMIC ALKALINIZATION INDUCED BY INSULIN THROUGH AN ACTIVATION OF Na^+–H^+ ANTIPORTER INHIBITS TYROSINE HYDROXYLASE ACTIVITY IN STRIATAL SYNAPTOSOMES

SALVATORE AMOROSO, GIANFRANCO DI RENZO, MAURIZIO TAGLIALATELA, LORELLA M. T. CANZONIERO, EDWARD J. CRAGOE JR* and LUCIO ANNUNZIATO†‡

Department of Pharmacology, 2nd School of Medicine, University of Naples, via S. Pansini, 5, Naples,* P.O. Box 631548 Nacogdoches Tx 75963-1548, and
†Institute of Behavioural and Neurological Sciences, University of Chieti, Italy

(*Received* 14 *May* 1990; *accepted* 1 *October* 1990)

Abstract—Insulin dose-dependently inhibited tyrosine hydroxylase (TH) activity and increased intrasynaptosomal pH (pH_i) in rat striatal nerve endings. Both these effects of insulin on TH and pH_i were prevented by the 5-(*N*-methyl-*N*-(guanidinocarbonylmethyl) amiloride (MGCMA), a putative selective inhibitor of the Na^+–H^+ antiporter. Interestingly when, by changing the extracellular pH (pH_o), the pH_i was increased, from 7.1 up to 7.5, an equivalent inhibition of TH activity occurred. The inhibitory action exerted from insulin on TH activity disappeared when the hormone was added to synaptosomes whose pH_i was lowered to 6.83. Collectively, the results of the present study showed that insulin inhibited TH activity in striatal synaptosomes. This effect seems to involve the activation of the Na^+-H^+ antiporter. This exchange system once activated, may induce an intrasynaptosomal alkalinization, a condition in which TH activity is inhibited.

The possibility of a direct effect of insulin on brain physiology has long been the subject of considerable debate. Previously reported studies demonstrated the presence of insulin [1] and of its specific binding sites [2] in the rat brain. This evidence suggests that insulin may influence neuronal function. In fact it is known that insulin modifies catecholamine turnover [3] and release [3, 4] from central neurons and that it modulates monoamine uptake in cultured neuronal cells [5]. On the other hand it has been reported that insulin modifies the activity of the membrane Na^+–H^+ exchanger, which is involved in the modulation of intracellular pH [6]. Since a variation of the pH_o may modify TH (L-tyrosine 3-monooxygenase; EC 1.14.16.2) activity in synaptosomes of rat corpus striatum [7, 8], a dopaminergic region where receptor binding sites for insulin have been detected [2], we investigated whether insulin could modify TH activity via an action on Na^+–H^+ exchange.

MATERIALS AND METHODS

Porcine or human recombinant insulin (Actrapid) was from Novo Industries, Copenhagen, Denmark. Recombinant insulin-like growth factor-1 (IGF-1) was a kind gift of Drs H. H. Peter and K. Scheibli from Ciba Geigy (Basle, Switzerland) and Chiron Corporation (Emeryville, CA, U.S.A.). 2,7′-Bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM§) was from Molecular Probes (Eugene, OR, U.S.A.). MGCMA was synthesized by previously described methods [9]. All the other drugs were purchased from the Sigma Chemical Co (St Louis, MO, U.S.A.). All chemicals used were from standard commercial sources. L-[1-carboxyl-^{14}C]tyrosine (sp. act. 51 mCi/mmol) was obtained from Amersham International.

Male Wistar rats (200–250 g) were decapitated and the striata were dissected out and placed in polyethylene tubes on ice. The tissue was homogenized in 0.32 M sucrose, and centrifuged at 1000 *g* for 5 min. The supernatant obtained was then centrifuged at 20,000 *g* for 20 min to sediment the P_2 fraction. Most of the TH activity in the P_2 fraction is associated with synaptosomes [10]. The quality of our synaptosomes preparation was checked by using electron microscopy.

TH activity was assayed by measuring the production of $^{14}CO_2$ from L-[1-^{14}C]tyrosine according to [11]. The P_2 pellet was resuspended in glucose 0.32 M 1:1.5 (w/v). Synaptosomes, 250 μg protein assayed by the method of Bradford [12], were incubated in a standard Tris-buffered medium containing the following composition: 3 mM KCl, 131 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 20 mM Tris, 5 mM Na_2HPO_4 and 1 mM ascorbic acid at pH_o 7.4 (final volume 0.5 mL). To obtain the desired pH_o, small aliquots of 1 M Tris or 1 M HCl were added to the media. Synaptosomes were preincubated for 15 min at 37°. Unless otherwise specified the drug examined was added to the incubation medium at the 10th min of preincubation.

‡ Correspondence to: Prof. Lucio Annunziato, Department of Pharmacology, 2nd School of Medicine, Via S. Pansini, 5, Naples, 80131 Italy.

§ Abbreviations: BCECF, 2,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein; AM, acetoxymethyl ester; pH_o, extracellular pH; pH_i, intrasynaptosomal pH; TH, tyrosine hydroxylase; IGF-1, insulin-like growth factor-1; MGCMA, 5-(*N*-methyl-*N*-(guanidinocarbonyl) amiloride.

Insulin and IGF-1 were added to the incubation medium (pH_o 7.4) in presence of bovine serum albumin (0.1%) plus bacitracin (20 μM), in order to avoid the binding of the insulin to glass tubes and its inactivation, respectively. The reaction was started by adding 6 μL of L-[1-^{14}C]tyrosine (0.3 μCi) to give a final concentration of 10 μM. The tubes were capped with rubber stoppers and contained a center well with 200 μL of hyamine hydroxide absorbed into a strip of Whatman No. 1 paper to trap the evolved $^{14}CO_2$. The incubation was performed for 20 min and the reaction was stopped by injecting 200 μL of 10% trichloroacetic acid into the tubes. After a 2 hr incubation at 37° the radioactivity trapped in the paper plus the center well was counted by liquid scintillation spectrometry. Blank samples incubated in the absence of the tissue were 0.03% (200 dpm) of the total radioactivity added. The basal rate of $^{14}CO_2$ formation ranged from 15 to 20 times more than the blank values. Time course analysis indicated that the release of $^{14}CO_2$ was linear up to 30 min. pH_i was determined by loading synaptosomes with 10 μM of the AM-ester of BCECF for 45 min at 37°, pH_o 7.4. Synaptosomes were then washed twice to remove the extracellular BCECF-AM and resuspended in the incubation medium. Fluorescence (excitation: 500 nm, emission: 530 nm) was recorded in a thermostated quartz cuvette in a Perkin-Elmer LS 5 spectrofluorimeter, equipped with a magnetic stirrer. Calibration of fluorescence signal as a function of pH_i was performed by the method of Thomas *et al.* [13] using the K^+–H^+ ionophore nigericin (0.5 μg/mL) added to synaptosomes suspended in a medium containing 140 mM K^+ and 5 mM NaCl. Unless otherwise specified, insulin was used in a medium pH_o 7.4. The pH_o was changed by adding small aliquots of 1 M Tris or HCl and measured with an electrode inserted into the cuvette. Intracellular fluorescence was detected and plotted as a function of the pH_o.

The data were analysed by means of the analysis of variance followed by Neuman–Keul's and Dunnett's tests.

RESULTS

Dose-dependent inhibition of striatal TH activity by insulin

Insulin, added to the incubation medium in concentrations ranging from 21 to 63 μM inhibited TH activity in striatal synaptosomes in a dose-dependent manner (Fig. 1). This effect was not dependent by the presence of zinc ions in the insulin preparation, since this cation (7, 21, 63 μM), which is present in a 1:3 molar ratio with insulin, did not modify TH activity (data not shown). IGF-1 at all the concentrations used, failed to modify TH activity in striatal synaptosomes (6.6 ± 0.3, 6.6 ± 0.3, 6.7 ± 0.2, 6.3 ± 0.3, 5.8 ± 0.3 picomoles $^{14}CO_2$/min/mg protein in control, 5, 50, 500, 5000 nM IGF-1 groups, respectively).

Effect of MGCMA on insulin-inhibited TH activity in striatal synaptosomes

MGCMA at the concentration of 100 and 300 μM, prevented the inhibitory action exerted by insulin (21 and 63 μM) on TH activity (Table 1).

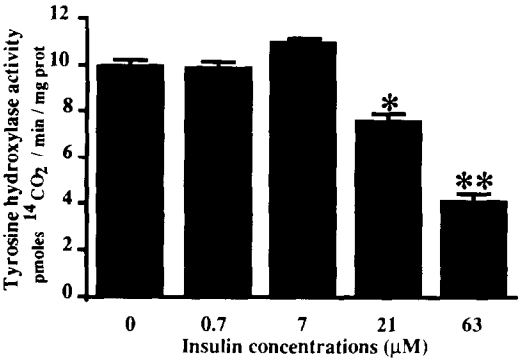

Fig. 1. Effects of different concentrations of porcine insulin on basal TH activity in striatal synaptosomes. Columns represent the mean ± SE of six determinations. *P < 0.01 vs all the other values. **P < 0.01 vs 21 μM insulin value.

Effect of insulin on pH_i

Human recombinant insulin produced a rise in pH_i from 7.14 to 7.28 in striatal synaptosomes (Fig. 2), in a dose-dependent manner (EC_{50} 11 μM). Insulin-induced alkalinization was not dependent by the presence of zinc ions since this cation at the concentration of 63 μM did not cause any variation in pH_i (data not shown). MGCMA (100 μM) prevented the increase in pH_i evoked by insulin (Fig. 3).

Effect of different pH_i values, on TH activity

When striatal synaptosomes were exposed to various external pH values, a change in pH_i occurred and the rate of TH activity was modified (Fig. 4). The alkalinization of pH_i, obtained by elevating pH_o, to values of 7.3 and 7.38, inhibited TH activity. On the contrary the acidification of pH_i to value of 6.26 stimulated TH activity. The inhibitory effect of insulin on TH activity occurred only when the pH_i was at a steady state value of 7.14, whereas, when pH_i value was 6.83, the hormone did not inhibit TH activity, although it was able to increase pH_i (Fig. 4).

DISCUSSION

The results of the present paper showed that insulin inhibited TH activity in striatal synaptosomes in a dose-dependent manner. This effect was not dependent by the presence of zinc ions in the insulin preparation, since this cation (7–63 μM), which is present in a 1:3 molar ratio with insulin, did not modify TH activity in striatal synaptosomes (data not shown). Since it has been reported that insulin may act through an interaction with IGF-1 receptors [14], whose presence in rat brain has been recently detected [15], we examined the possibility that the inhibition of TH activity evoked by insulin could be

Table 1. Effect of MGCMA on the inhibition of TH activity induced by different concentrations of insulin

Treatment	TH activity (pmol $^{14}CO_2$/min/mg protein)
Control	9.7 ± 0.3
Insulin (21 μM)	7.7 ± 0.9*
Insulin (21 μM) + MGCMA (100 μM)	10.6 ± 0.4†
Insulin (21 μM) + MGCMA (300 μM)	10.4 ± 0.3†
Control	8.9 ± 0.9
Insulin (63 μM)	4.2 ± 0.8*
Insulin (63 μM) + MGCMA (100 μM)	5.6 ± 0.2†
Insulin (63 μM) + MGCMA (300 μM)	6.5 ± 0.4‡

Each value is the mean ± SE of six values.
* $P < 0.01$ vs all the other values of each experimental group.
† $P < 0.05$ vs the respective insulin value.
‡ $P < 0.01$ vs the respective 63 μM insulin value and vs 63 μM insulin + 100 μM MGCMA.

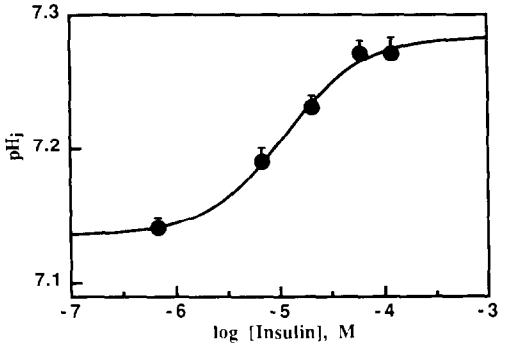

Fig. 2. Effect of different human recombinant insulin concentrations on pH_i in striatal synaptosomes. Each point represents the mean of four independent experiments. pH_i values were obtained as described in Materials and Methods.

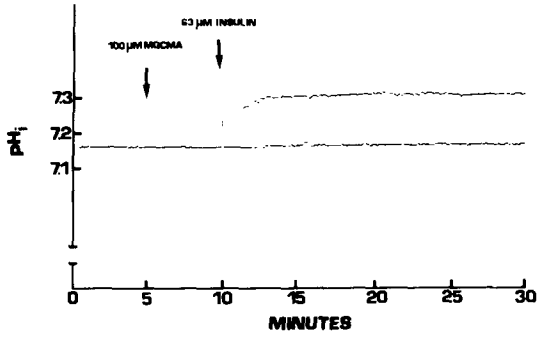

Fig. 3. Effects of porcine insulin and MGCMA on pH_i in striatal synaptosomes. The upper trace represents the effect of insulin (63 μM) on pH_i values, when used alone, whereas the lower trace represents the effect of MGCMA (100 μM) plus insulin (63 μM). The arrows indicate when the drugs were added to the incubation medium. Traces are representative of six experiments.

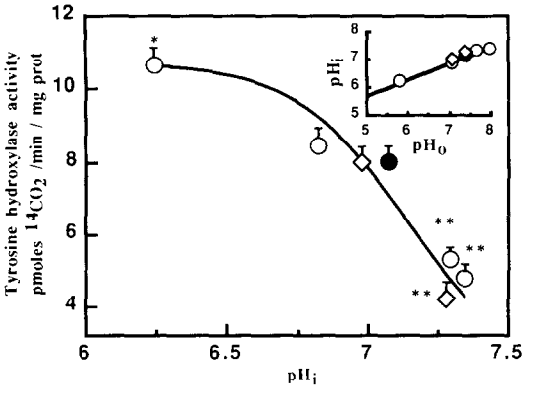

Fig. 4. Effects of different pH_i values on TH activity. Synaptosomes were exposed to different pH_o values 15 min before the addition of L[1-^{14}C]tyrosine to the incubation medium and then incubated for further 20 min at the chosen pH_o values. Each point represents the mean of six determinations after 20 min of incubation. The inset indicates the pH_i values corresponding to indicated pH_o values. The black circles indicate respectively the pH_i value and TH activity level obtained at pH_o value of 7.4. The rhomb symbols represent respectively the pH_i value and TH activity level obtained at pH_o values of 7.1 and 7.4 in presence of 63 μM insulin. *$P < 0.01$ vs all the other values. **$P < 0.01$ vs the values obtained at the pH_o 7.4.

ascribed to a stimulation of IGF-1 receptors. However this possibility can be ruled out since IGF-1 was devoid of any inhibitory effect on striatal TH activity.

It has been reported that insulin effects in skeletal muscle [6, 16, 17] and in quiescent fibroblasts [18] involve an activation of the Na^+–H^+ membrane antiporter. This cation exchanger, whose biochemical properties have been described in rat brain synaptosomes [19], is involved in the regulation of pH_i [6]. Our results showed that, when the Na^+–H^+ antiporter was blocked by MGCMA, an amiloride derivative which lacks in inhibitory properties on other Na^+ membrane transporting systems (Cragoe,

personal communication), the inhibitory action of insulin on TH activity was prevented. Therefore an activation of Na^+–H^+ antiporter seems to be involved in the inhibitory effect exerted by insulin on TH activity. The hypothesis that insulin inhibited TH activity via an activation of the Na^+–H^+ antiporter, which led to a cytoplasmic alkalinization, appears to be also validated by the observation that insulin produced a dose-dependent elevation of pH_i and that this increase was prevented by the Na^+–H^+ antiporter blocker MGCMA. On this regard it should be noted that MGCMA by itself did not lower pH_i, however it should be considered that also in cultured cardiac cells [20], when the pH_i value is in a steady-state basal condition, the inhibition of the Na^+–H^+ antiporter does not produce a lowering of pH_i therefore suggesting that pH_i regulation is not due exclusively to the Na^+–H^+ antiporter. The hypothesis that intracellular alkalinization induced by insulin causes an inhibition of TH activity was further supported by the data showing that when pH_i value was increased to values of 7.3 and 7.38, obtained by elevating pH_o, an inhibition of TH activity was observed. On the other hand the inhibitory effect of insulin on TH activity can occur only when the pH_i reaches the basal steady-state value of 7.14, whereas insulin, when added to synaptosomes, whose pH_i was 6.83, did not inhibit TH activity, although it was able to increase pH_i.

However, since 100 μM MGCMA, completely prevented insulin-induced alkalinization (Fig. 3) whereas both 100 and 300 μM MGCMA were unable to completely reverse the inhibiting action of insulin on TH activity (Table 1), we cannot exclude that other mechanisms than changes in pH_i may also be involved in insulin action on TH activity. This consideration may also explain the little difference between the half maximal effects induced by insulin on TH activity and on pH_i.

Finally, it should be considered that, since at the concentrations used, insulin may partially exist in a dimeric form, the possibility that insulin effects may be due to a portion of the hormone present in a dimeric form cannot be ruled out. However, the fact that, in other biological systems, insulin modulates the activity of the Na^+–H^+ antiporter at the concentrations lower (i.e. in a monomeric form) [16, 17] than those utilized in the present study, would suggest that insulin should act in a monomeric form.

Acknowledgements—We are grateful to Dr Giuseppe Galizia for his collaboration in the computerized statistical analysis of the data and Mr L. Scognamiglio and V. Grillo for their technical assistance. This study was supported by the C.N.R. Target Project on Biotechnology and Bioinstrumentation.

REFERENCES

1. Havrankova J, Schemechel D, Roth J and Brownstein M, Identification of insulin in rat brain. *Proc Natl Acad Sci USA* **75**: 5737–5741, 1978.
2. Havrankova J, Roth J and Brownstein M, Insulin receptors are widely distributed in the central nervous system of the rat. *Nature* **272**: 827–829, 1978.
3. Sauter A, Goldstein M, Engel J and Ueta K, Effect of insulin on central catecholamines. *Brain Res* **260**: 330–333, 1983.
4. Amoroso S, Taglialatela M, Canzoniero LMT, Cragoe EG Jr, Di Renzo GF and Annunziato L, Possible involvement of Ca^{2+} ions, protein kinase C and Na^+–H^+ antiporter in insulin-induced endogenous dopamine release from tuberoinfundibular neurons. *Life Sci* **46**: 885–894, 1990.
5. Boyd FT, Clarke DW, Muther TF and Raizada MK, Insulin receptors and insulin modulation of norepinephrine uptake in neuronal cultures from rat brain. *J Biol Chem* **260**: 15880–15884, 1985.
6. Moore RD, Effects of insulin upon ion transport. *Biochim Biophys Acta* **737**: 1–49, 1983.
7. Boarder MR and Fillenz M, Synaptosomal tyrosine hydroxylation in the rat brain: comparison of activity from hippocampus and hypothalamus with activity from striatum. *J Neurochem* **31**: 1419–1426, 1978.
8. Patrick RL and Rendel MT, pH-induced alterations in dopamine synthesis regulation in rat brain striatal synaptosomes. *J Neurochem* **34**: 1506–1513, 1980.
9. Cragoe EJ Jr, Woltersdorf OW Jr, Bicking JB, Kwong SF and Jones JH, Pyrazine Diuretics. II. *N*-Amidino-3-amino-5-Substituted-6-Halopyrazinecarboxamides. *J Med Chem* **10**: 66–75, 1967.
10. Coyle JT, Tyrosine hydroxylase in rat brain. Cofactor requirements, regional and subcellular distribution. *Biochem Pharmacol* **21**: 1935–1944, 1972.
11. Waymire JC, Bjur A and Weimer N, Assay of tyrosine hydroxylase by coupled decarboxylation of dopa formed from 1-^{14}C-L-tyrosine. *Anal Biochem* **43**: 588–600, 1971.
12. Bradford MMA, A rapid, and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
13. Thomas JA, Buchsbaum AN, Zimniak A and Racker E, Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. *Biochemistry* **18**: 2210–2218, 1979.
14. Goodyear CG, De Stephano L, Lai WH, Guyda HJ and Posner BI, Characterization of insulin-like growth factor receptors in rat anterior pituitary, hypothalamus and brain. *Endocrinology* **114**: 1187–1195, 1984
15. Arajo DM, Apchak L, Collier B, Chabot IG and Quiron R, Insulin growth factor 1 (somatomedin C) receptors in the rat brain: distribution and interaction with the hippocampal cholinergic system. *Brain Res* **484**: 130–137, 1989.
16. Klip A, Ramlal T and Cragoe EJ Jr, Insulin-induced cytoplasmic alkalinization and glucose transport in muscle cells. *Am J Physiol* **250**: C720–C728, 1986.
17. Fidelman ML, Seeholzer SH, Walsh KB and Moore RD, Intracellular pH mediates action of insulin on glycolysis in frog skeletal muscle. *Am J Physiol* **242**: C87–C93, 1982.
18. Paris S and Pouysségur J, Growth factors activate the Na^+–H^+ antiporter in quiescent fibroblasts by increasing its affinity for intracellular H^+. *J Biol Chem* **259**: 10989–10994, 1984.
19. Jean T, Frelin C, Vigne P, Barbry P and Lazdunski M, Biochemical properties of the Na^+–H^+ exchange system in rat brain synaptosomes. *J Biol Chem* **260**: 9678–9684, 1985.
20. Frelin C, Vigne P and Lazdunski M, The role of the Na^+–H^+ exchange system in the regulation of the internal pH in cultured cardiac cells. *Eur J Biochem* **148**: 1–4, 1985.