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Effects of insulin-induced hypoglycemia on somatostatin level and binding in rat cerebral cortex and hippocampus

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The effects of severe insulin-induced hypoglycemia on somatostatin level and specific binding in the cerebral cortex and hippocampus were examined using ^{125}I-Tyr^{11}-somatostatin as a ligand. Severe insulin-induced hypoglycemia did not affect the level of somatostatin-like immunoreactivity in the brain areas studied. However, the number (but not the affinity) of specific somatostatin receptors was significantly decreased in membrane preparation from the hippocampus but not in the cerebral cortex at the onset of hypoglycemic coma (5–10 min). Administration of glucose at the onset of hypoglycemic coma brought about extensive recovery of hippocampal somatostatin receptor number. These results suggest that glucose modulates the somatostatin receptor in the rat hippocampus. The physiological significance of these findings remains to be clarified.

INTRODUCTION

Somatostatin (SS), a cyclic tetradecapeptide, was first isolated from sheep hypothalamic tissue[6]. This peptide has also been found in the extrahypothalamic areas of the brain[17] and affects both excitation and inhibition of neuronal firing[23]. The primary event mediating the effect of SS in target tissues is an interaction of the peptide with specific membrane receptors[9].

Reports concerning the effects of extracellular glucose concentration on cerebral SS content are very scarce and contradictory. Agnati et al.[3] have shown a reduction in somatostatin-like immunoreactivity (SLI) in the frontoparietal cortex and dorsal hippocampal formation in rats with insulin-induced hypoglycemia. Berelowitz et al.[5] did not find any change in SLI release from large rat cerebral cortex explants exposed to a drop in extracellular glucose. Regulation of cerebral SS receptors in hypoglycemic animals has not been studied. Since the receptor is an integral component of the mechanism by which SS activates and/or modulates neuronal synaptic activity, a study of SS receptors in hypoglycemic rats may provide further insight into the role of SS in the hypoglycemic process. The present study was designed to measure SLI and specific SS receptor binding in rat cerebral cortex and hippocampus at the onset of hypoglycemic coma. We have also attempted to reverse the observed effects by means of glucose administration.

MATERIALS AND METHODS

Experimental animals

Adult female Sprague–Dawley rats weighing 200–220 g were fasted 24 h but allowed free access to water. Blood was sampled from all animals after amputation of the tips of their tails. Each rat was injected i.p. with either 40 IU/kg of insulin Novo actrapid dissolved in 0.75 ml of Krebs–Henseleit solution before injection or an equal volume of this solution as previously described[3,8].

When the insulin-injected rats lost their righting

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reflex and response to any external stimuli, they were in hypoglycemic coma[18]. At this time a second blood sample was obtained as before, and some of the animals were given 1.0 ml of a 30% (w/v) glucose solution by tail-vein injection and another 0.5 ml of the same solution was given after 15 min. Glucose-treated rats were sacrificed 15 min after the last glucose injection. Control animals were sacrificed 150–180 min after solution injection. Animals treated only with insulin were sacrificed 5–10 min after loss of the righting reflex. After death, the brain was rapidly removed and the cerebral cortex and hippocampus were dissected according to the method of Glowinski and Iversen[13]. Plasma glucose was assayed with the glucose–oxidase (Boehringer) method.

Tissue extraction and SS radioimmunoassay

For SLI measurements, the cerebral cortex and hippocampus were rapidly homogenized using a Brinkman polytron (setting 5, 30 s), in 1 ml 2 M acetic acid. Extracts were boiled for 5 min in a water bath, chilled in ice, and aliquots (100 μl) were removed for protein determination[19]. Subsequently homogenates were centrifuged at 15,000 *g* for 15 min at 4 °C, and the supernatant was neutralized with 2 M NaOH. Just prior to assay, extracts were stored at −70 °C until assay. SS level was determined in tissue extracts by a modified radioimmunoassay method[21], with a sensitivity limit of 10 pg/ml. The possibility that substances present in the tissue extracts might interfere with antibody–antigen binding and give rise to erroneous results was discarded by performing serial dilutions of selected extracts in the assay and comparing the resulting changes in hormonal immunoreactivity with those of the diluted standards. In addition, known standard amounts of the hormone were added to varying amounts of the extracts and serial dilutions again assayed in order to determine if this exogenously added hormonal immunoreactivity could be reliably measured in the presence of tissue extracts. Incubation tubes prepared in duplicate contained 100 μl samples of unknown or standard solutions of 0–500 pg cyclic SS tetradecapeptide diluted in phosphate buffer (0.05 M, pH 7.2 containing 0.3% bovine serum albumin, 0.01 M EDTA); 200 μl appropriately diluted anti-SS serum (final dilution usually 1:20,000); 100 μl freshly prepared [^{125}I]Tyr^{11}-SS diluted in buffer to give 6000 cpm (equivalent to 5–10 pg); and enough additional buffer to reach a final volume of 0.8 ml. All reagents and the assay tubes were kept chilled on ice, before incubating for 48 h at 4 °C. Separation of bound and free hormone was accomplished by the addition of 1 ml dextran-coated charcoal (dextran, 0.2% w/v, Pharmacia T70, Uppsala, Sweden; charcoal, Norit A, 2% w/v, Serva, Feinbiochemica, Heidelberg, F.R.G.). Dilution curves for each brain area were parallel to the standard curve. The intra-assay and inter-assay variation coefficients were 6.5 and 8.1%, respectively.

Binding assay on membrane preparations

Synaptosomal membranes from cerebral cortex and hippocampus were prepared as described by Reubi et al.[26]. Cerebral cortex and hippocampus were homogenized in 10 mM HEPES-KOH buffer pH 7.6 (10 w/v) with a Brinkmann polytron homogenizer (setting 5, 15 s). The homogenate was spun at 600 *g* for 5 min at 4 °C and the supernatant centrifuged at 48,000 *g* for 30 min at 4 °C, and the resulting pellet was suspended in 10 mM HEPES-KOH pH 7.6 (10 w/v) and centrifuged as before. The resultant pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5). Samples were stored at −70 °C until assay. Protein was determined by the method of Lowry et al.[19].

Specific SS binding was measured according to the modified method of Czernik and Petrack[10]. Brain membranes (about 1.5 mg protein/ml) were incubated in 250 μl of a medium containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.2% (w/v) bovine serum albumin and 0.1 mg/ml bacitracin with 250 pM [^{125}I]Tyr^{11}-SS either in the absence or presence of 0.01–10 nM unlabeled SS. After 60-min incubation at 30 °C, the free radioligand was separated from the bound radioligand by centrifugation at 12,000 *g* (Beckman microcentrifuge) for 1.5 min and the resultant pellet was counted in a Beckman gamma counter. Non-specific binding, i.e. binding occurring in the presence of a high concentration (10^{-7} M) of unlabeled SS, represented about 20% of the binding observed in the absence of native peptide and was subtracted from the total bound radioactivity in order to obtain the corresponding specific binding. The inactivation of [^{125}I]Tyr^{11}-SS in

the incubation medium after exposure to membranes was studied by observing the ability of the peptide to rebind to fresh membranes[4].

Statistical analysis

Results were given in all cases as mean ± S.E.M. The Student's *t*-test for unpaired variables was employed to assess differences between control and experimental groups, as indicated in the figures. The number of receptors and affinity constant in Scatchard plots[27] were calculated with linear regression analysis on a Hewlett-Packard device.

Chemicals

Synthetic Tyr^{11}-SS and SS tetradecapeptide were purchased from Universal Biologicals Ltd. (Cambridge, U.K.); insulin Novo actrapid from Novo Industri AB, (Denmark); bacitracin and bovine serum albumin (fraction V) from Sigma (St. Louis, MO, U.S.A.); and carrier-free Na ^{125}I (IMS 30, 100 mCi/ml) from the Radiochemical Centre (Amersham, U.K.). Tyr^{11}-SS was radioiodinated by the chloramine-T method[15]. The tracer was purified in a Sephadex G-25 coarse column (1 × 100) which had been equilibrated with 0.1 M acetic acid containing bovine serum albumin 0.1% (w/v). The specific radioactivity of tracer was about 350 Ci/g. The rabbit antibody used in the radioimmunoassay technique was purchased from the Radiochemical Centre (Amersham, U.K.). This antiserum was raised in rabbits against SS-(14) conjugated to bovine serum albumin and is specific for SS, but since SS-(14) constitutes the C-terminal portions of both SS-(25) and SS-(28), the antiserum does not distinguish between these 3 forms. Cross-reactivity with other peptides is less than 0.5%. Cross-reaction with several somatostatin analogues demonstrated that neither the *N*-terminal glycine nor the *C*-terminal cysteine residue is required for antibody binding, suggesting that the antigen site is directed towards the central part of the molecule containing the tryptophan residue.

RESULTS

The mean plasma glucose level was 4.79 ± 0.33 mmol/liter in the normoglycemic control group, and 0.41 ± 0.12 mmol/liter in the hypoglycemic group.

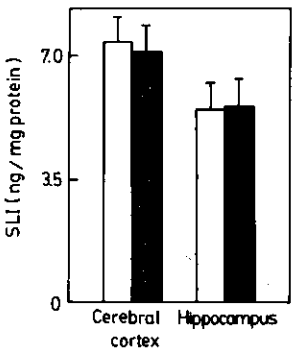

Fig. 1. SLI levels in the cerebral cortex and hippocampus in control (open bars) and insulin-injected rats (dotted bars). Values are expressed as the mean ± S.E.M. of 5 separate experiments. In each of the experiments, determinations were made in duplicate. No statistically significant differences are obtained when compared with the control animals.

Glucose treatment produced return of righting ability in 88% of the rats within 30–60 s, and 98% recovered within 5 min. Once righting was regained, there was a slow steady increase in motor activity.

Insulin-induced hypoglycemia produced no changes in SLI level in either the cerebral cortex or the hippocampus as compared with the control group (Fig. 1).

Cerebral cortex and hippocampal membrane preparations from control and insulin-treated rats bound $[^{125}I]Tyr^{11}$-SS in a time-dependent process. An apparent equilibrium was reached between 50 and 180 min of the 30 °C-incubation period. A given 60-min period was chosen during this time for the stoichiometric study of the binding process. In order to rule out the possibility of different SS-degrading activities in the membrane preparations that could have affected interpretation of the results, the peptide degradation rate was determined[4]. The peptide degrading capacity in the two membrane preparations was about 10% in both control and insulin-treated rats.

Increasing concentrations of unlabeled SS competitively inhibited the specific binding of $[^{125}I]Tyr^{11}$-SS to brain membrane preparations from both control and insulin-injected rats (Figs. 2 and 3, left panel). The specific binding of the tracer to the membranes prepared from the hippocampus was significantly lower in the insulin-injected rats than in the control animals, in both the absence and presence of unlabeled SS throughout the whole range of

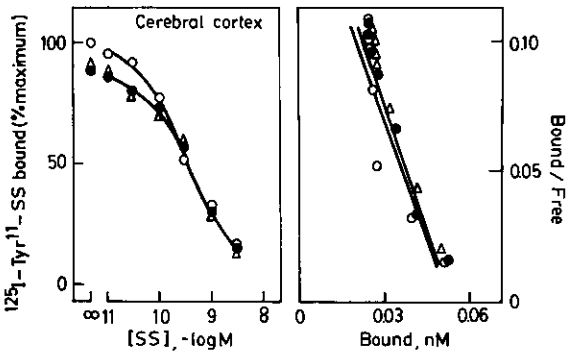

Fig. 2. Left panel: competitive inhibition of specific [^{125}I]Tyr^{11}-somatostatin ([^{125}I]Tyr^{11}-SS, 250 pM) binding to membranes of the cerebral cortex by unlabeled SS. Membranes (1.5 mg protein/ml) were incubated for 60 min at 30 °C in the presence of 250 pM [^{125}I]Tyr^{11}-SS and increasing concentrations of native peptide. Points correspond to control animals (●), insulin-treated (○) and insulin- and glucose-treated (△); each point is the mean of 5 replicate experiments. For the sake of clarity S.E.M.s are not represented but were always below 10% of the mean values. Right panel: Scatchard analysis of the same data.

TABLE I

Effect of insulin-induced hypoglycemia and subsequent glucose treatment on specific SS receptors in the cerebral cortex and hippocampus

Binding parameters were obtained by Scatchard[27] analysis of data from Figs. 2 and 3, right panel. K_d is the dissociation constant, nM; B_{max} is the binding capacity, fmol SS/mg protein. Each value is the mean ± S.E.M. of the 5 experiments.

Groups	*Cerebral cortex*	*Hippocampus*
Control ($n = 5$)		
K_d	0.33 ± 0.06	0.41 ± 0.05
B_{max}	373 ± 49	399 ± 28
Insulin-treated ($n = 5$)		
K_d	0.35 ± 0.04	0.36 ± 0.10
B_{max}	364 ± 22	263 ± 63*
Insulin- and glucose-treated ($n = 5$)		
K_d	0.34 ± 0.07	0.39 ± 0.12
B_{max}	369 ± 52	389 ± 35

* Significance of difference with respect to corresponding control values: $P < 0.05$.

concentrations studied. To determine whether this decrease in specific binding exhibited by insulin-induced hypoglycemic rats was due to a change in either receptor affinity or number, the stoichiometric data were interpreted by the method of Scatchard[27] (Figs. 2 and 3, right panel). These studies revealed that there was a decrease in the number of specific SS receptors in the hippocampus, but not in the cerebral cortex (Table I). However, no difference could be established with respect to the affinity values. The K_d values were in the nanomolar range, and Scatchard analysis indicated a single receptor class.

Glucose administration to insulin-treated rats completely reversed the hypoglycemia-induced decrease of SS receptors in the hippocampus (Figs. 2 and 3, Table I).

To assess whether insulin exerts a direct action on somatostatin receptors, it was included in the incubation medium at the time of the binding assay. The addition of 1 μM of insulin to the incubation medium did not change either the binding capacity or the affinity of the somatostatin receptors (data not shown).

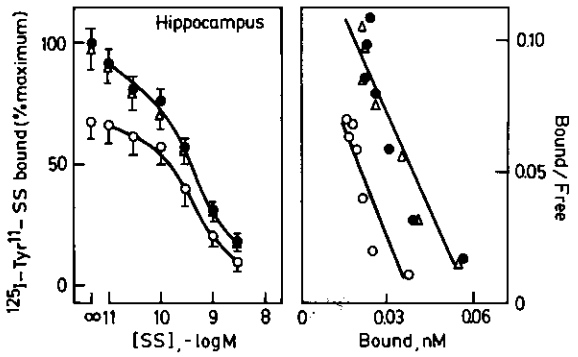

Fig. 3. Left panel: competitive inhibition of specific [^{125}I]Tyr^{11}-somatostatin ([^{125}I]Tyr^{11}-SS, 250 pM) binding to membranes of the hippocampus by unlabeled SS. Membranes (1.5 mg protein/ml) were incubated for 60 min at 30 °C in the presence of 250 pM [^{125}I]Tyr^{11}-SS and increasing concentrations of native peptide. Points correspond to control animals (●), insulin-treated (○) and insulin- and glucose-treated (△); values are expressed as the mean ± S.E.M. of 5 replicate experiments. Right panel: Scatchard analysis of the same data.

DISCUSSION

These experiments induced hypoglycemic coma through insulin administration. Because [^{125}I]Tyr^{11}-SS acts biologically like the native peptide[28], it was chosen as the tracer. During the course of this study, it was determined that freezing the membrane suspension at −70 °C resulted in about a 7% loss in specific binding of [^{125}I]Tyr^{11}-SS after 1 month.

Although some investigators use freshly prepared membrane suspensions[9,10], we like others[26,30], use frozen membrane suspensions, that are not more than 2 weeks old.

In comparison to the control group, there was no change in the SLI levels of the hippocampus and cerebral cortex and the number of specific SS receptors in the cerebral cortex of hypoglycemic rats at the onset of coma. However, the number of specific SS receptors in the hippocampus of hypoglycemic rats decreased during the first 5–10 min of hypoglycemic coma.

The blood glucose concentrations under basal conditions and 5–10 min after the onset of coma were similar to those previously reported in other studies[2,3,8]. In addition, the levels of SLI and the binding parameters of specific SS receptors in cerebral cortex and hippocampus of control rats were similar to those previously reported by other authors[22,25,29]. It should be mentioned that the Scatchard analysis demonstrated the existence of only one type of SS receptor. This feature agrees with some studies[9,10,29], but differs from others[24,25]. It is conceivable that differences in [^{125}I]Tyr^{11}-SS specific activities and the use of different SS-labeled analogues could be related to this disagreement.

This unchanged SLI level in the cerebral cortex and hippocampus of hypoglycemic rats is consistent with the studies of Berelowitz et al.[5] which did not find a change in SLI release after decreasing the extracellular glucose in large cerebral cortex explants from adult rats. Recently, however, Agnati et al.[3], have shown a reduction in SLI in the frontoparietal cortex and dorsal hippocampal formation in rats with insulin-induced hypoglycemia. This discrepancy may be explained by the fact that as well as using the whole cerebral cortex and hippocampus, our studies were performed at different time periods.

On the other hand, it might be that even though the overall SS content in the hippocampus was unchanged, the rate of SS synthesis and release may have changed. If this were the case, increased SS release or turnover from the hippocampal formation could lead to down-regulation of SS receptors in the hippocampus but we did not detect any change in SS content with radioimmunoassay. Furthermore, hypoglycemic coma is associated with a breakdown of energy and ion metabolism[14,18] and any changes observed in receptor characteristics would be secondary to such alterations. The finding that insulin-induced hypoglycemia causes a decrease in SS receptor binding in the hippocampus, and that this effect can be reversed by subsequent glucose treatment agrees with the findings that intravenous administration of glucose to stuporous animals in hypoglycemic coma produced recovery of near-normal neurological function[14].

We found no change in the number of specific SS receptors in the cerebral cortex after insulin injection, but, as we mentioned earlier, the fact that we studied the whole cerebral cortex may possibly have masked changes in the number of SS receptors in specific cortical brain areas.

These results suggest that glucose modulates SS receptors in the rat hippocampus. The physiological significance of these findings remains to be clarified.

An alternative approach might be receptor autoradiography studies (control vs hypoglycemic rats) which should provide us with an image of the hippocampal SS receptors.

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