Peptide derived from insulin with regulatory activity of dopamine transporter

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Abstract

A nonapeptide derived from the C terminus of the insulin B chain, H2N-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-COOH, was found to strongly inhibit dopamine (DA) uptake by rat dopamine transporter (DAT) stably expressed in CHO cells (designated D8 cells). The kinetic experiments on D8 cells gave a curve typical of competitive inhibition with an \( IC_{50} = 6.9 \) \( \mu M \). This inhibitory effect was also confirmed by experiments on striatal synaptosomes. The rat administered with the nonapeptide unilaterally into substantia nigra showed dose-dependent velocity and duration of the round movement contralateral to the nonapeptide-injected side. In addition, the nonapeptide dose-dependently reduced the binding of the tritium-labeled cocaine analog (\( ^3H \)-WIN35,428) to DAT of D8 cells, which suggests that the nonapeptide may inhibit the transport activity of DAT in the way as cocaine does. Meanwhile, the peptide DOI (insulin with 8 amino acid residues deleted at the C terminus of the B chain) shows a significantly stimulating effect on DAT uptake activity in D8 cells. So insulin is proposed as a kind of neuropeptide precursor in the brain and insulin-derived peptides may be involved in the process of regulating the DA system, and these peptides may be developed into new medicines for disorders concerning the DA system such as Parkinson’s disease and cocaine addiction. © 2001 Elsevier Science Ltd. All rights reserved.

1. Introduction

Insulin is a peptide hormone involved in the regulation of glucose homeostasis. Its synthesis and function in the peripheral tissues have been extensively studied and are now well understood. However, the origin of insulin in brain is controversial. Brain insulin could be derived from blood through the brain–blood barrier (B.B.B.) (Frank et al., 1985). Recent studies suggest that insulin in the CNS could also be of local origin (Boyd et al., 1985; Devaskar et al., 1993). The clear physiological effect of insulin in the central nervous system (CNS) includes regulation of food intake, control of glucose uptake and trophic actions on neurons and glial cells. These actions of insulin are mediated by the insulin receptor coupled to tyrosine kinase signal transduction pathways, resembling closely those in peripheral tissues. Body weight and reproduction were affected in mice with a neuron-specific disruption of the insulin receptor gene (Bruning et al., 2000). Additionally, brain insulin has acute and chronic regulatory effects on members of the neurotransmitter transporter family, especially on the dopamine transporter (DAT) and the norepinephrine transporter (NET). Dysregulation of insulin signaling in the CNS has been linked to the pathogenesis of neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease (Takahashi et al., 1996; Frolich et al., 1998). Recent studies showed that dopamine and DAT were closely implicated in the latter disease (Takahashi et al., 1996; Frolich et al., 1998). Dopamine (DA) is an important neurotransmitter in the central nervous system, and is essential in such activities as movement, cognition and emotion. The synaptic signaling by dopamine is terminated by its reuptake into the presynaptic neuron. This reuptake is achieved via the function of DAT, which has been cloned from various species (Giros et al., 1991; Kilty et al., 1991; Shimada et al., 1991; Usdin et al., 1991; Figlewicz et al., 1994). The dopamine transport activity of
DAT can be inhibited by cocaine and that could induce potent cocaine addiction. DAT mRNA in the ventral tegmental area/substantia nigra pars compacta from rats that were treated with intraventricular insulin was significantly increased (Pristupa et al., 1994). Measurement in striatal slices from fasted rats revealed a significant decrease in dopamine uptake, and this effect could be reversed by pre-incubating the slices in vitro with a physiological concentration of insulin (Patterson et al., 1998). It would appear that insulin regulates DAT activity through multiple mechanisms. However, the mechanisms are still unclear. A question is naturally raised as to whether insulin can directly interact with (or bind to) DAT to regulate its function. In the current study, we studied the interaction between DAT and insulin as well as insulin-derived peptides, and demonstrated that peptides derived from insulin might interact with DAT and regulate its activity.

2. Materials and methods

2.1. Cloning of DAT cDNA by RT-PCR

Adult SD rat was decapitated and the substantia nigra was dissected out. Total RNA was extracted by Trizol solution (Gibco BRL Life Technologies) according to the manufacturer’s protocol. Two primers were synthesized according to the released sequence of rat DAT cDNA (gene bank accession number: M80570): 5’-AAA GAA TTC CCA TGA GTA AGA GCA AAT GC-3’ (10–29 nucleotides: 61–80); 5’-AAA CTC GAG CTT TAC AGC AAC AGC CAG TC-3’ (10–29 nucleotides: 1924–1906). First strain of cDNA was synthesized from 2 µg total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Gibco BRL Life Technologies). High fidelity Taq plus II polymerase (Sangon Ltd., Canada) was used for PCR with the procedure of: 94°C 1 min, 55°C 1 min, 72°C 2 min, 30 cycles on Perkin Elmer Cycles 9600. A desired 1.8 kb DNA fragment containing the full length DAT coding sequence was cloned into pBluescript II KS (+) between the restriction site of EcoR I and Xho I. The whole length of the sequence was checked to confirm that no mutation had occurred that would alter the amino acid sequence.

2.2. Stable expression of the rat dopamine transporter (rDAT), mouse amino-butyric acid transporter type 1 (mGAT1) and rat serotonin transporter (rSERT) in CHO cells

The EcoR I-Xho I fragment containing the complete coding sequence of the rat brain dopamine transporter (DAT), was cloned into the EcoR I-Xho I sites of the expression vector pCDNA3 under the control of the cytomegalovirus promoter. The resultant plasmid, designated DAT-pCDNA3, was used to transfect CHO cells using the Calcium Phosphate method (Schenborn and Goiffon, 2000). Selection of the transfected cells in 400 µg/ml active G418 began at 48 h after transfection and continued for 2 weeks. CHO cells stably expressing DAT were then subcloned by limiting dilution. Several subclones were selected and assayed for [3H]dopamine uptake. The clone with the highest uptake, designated D8, was chosen for further characterization. A clone of CHO cells stably expressing mGAT1 (designated G1 cells) and a clone of CHO cells stably expressing rSERT were obtained in a similar way as described above.

2.3. Dopamine, serotonin and amino-butyric acid transport assays and [3H]WIN35,428 binding assay

D8 cells, grown in RPMI1640 medium (Gibco BRL Life Technologies) containing 10% FBS (Gibco BRL Life Technologies) to near confluence in 48-well tissue culture plates (Costar) (approximately 60,000 cells per well in 48-well plates), were rinsed once with phosphate-buffered saline (PBS) and pre-incubated in 100 µl Hank’s balanced salt solution (HBSS) for 10 min at room temperature. [3H]dopamine (Amersham Pharmacia Biotech), ascorbic acid and pargyline were added to final concentrations of 151 nM, 100 µM and 100 µM, respectively. Unless otherwise noted, the cells were incubated for 20 min at room temperature. The reaction was terminated by aspiration of the HBSS and the cells were washed three times rapidly (10 s/wash) with cold PBS. The cells were then solubilized in 2N NaOH and an aliquot was measured by liquid scintillation counting (Beckman LS 5000 TA) to quantify the uptake of [3H]dopamine. IC50 curve and inhibition studies were performed by adding inhibitor at the beginning of the transport assay. For the study of the competitive inhibition curves, D8 cells were pre-incubated in HBSS for 10 min, and the buffer was then changed to HBSS containing increasing concentrations of dopamine while the nonapeptide was added to give final concentrations of 0, 3.68, 5.52 µM, respectively. For the study of the IC50 curve, D8 cells were pre-incubated in HBSS for 10 min, and the buffer was then changed to HBSS containing increasing concentrations of the nonapeptide. Control transport of D8 cells in HBSS alone was set at 100%. For the study of the stimulating effect of DOI, different concentrations of DOI were added at the beginning of the uptake assay. For the study of the partially reversible inhibiting effect of the nonapeptide on D8 cells, two D8 cell groups were pretreated for 10 min at room temperature with HBSS and 10 µg/ml (9.2 µM) nonapeptide in HBSS solution, respectively. The solution was aspirated and the cells were washed with PBS three times. The cells were pre-incubated in HBSS for 10 min. Assay was initiated with addition of [3H]dopamine, ascorbic acid
and pargyline to give final concentrations of 151 nM, 100 µM and 100 µM, respectively, and continued for 10 min. For serotonin and amino-butyric acid transport assays, the procedure was similar to that for dopamine transport assay in D8 cells except that 50 nM [3H]serotonin (Amersham Pharmacia Biotech) was used instead of [3H]dopamine for S6 cells and 50 nM [3H] amino-butyric acid (Amersham Pharmacia Biotech) was used instead of [3H] dopamine and ascorbic acid and pargyline in the system for G1 cells.

D8 cells were assayed for membrane binding of the tritium-labeled cocaine analog (−)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane (WIN35,428) as described (Kitayama et al., 1992). Briefly, [3H]WIN35,428 binding was determined by using intact cells incubated with 2 nM [3H]WIN35,428 (86 Ci/mmol; NEN) for 120 min at 4°C. 100 µM (−)-cocaíne was added to parallel incubations to provide estimates of nonspecific binding. Three washes with ice-cold buffer terminated the binding assay. Analysis of the effect of the nonapeptide on the WIN35,428 binding assay was performed in cells incubated with 2 nM [3H]WIN35,428 and increasing concentrations of the nonapeptide.

2.4. Uptake of dopamine by striatal synaptosomes

The procedure has been described elsewhere (Kokoshka et al., 1998) with some modifications.

Briefly, Male Sprague Dawley rats (200 g–250 g) were killed by decapitation and the striata were dissected out. Fresh striatal tissue was homogenized in ice-cold 0.32 M phosphate-buffered sucrose and centrifuged (800xg for 12 min at 4°C). The supernatant (S1) was then centrifuged (22,000xg for 10 min at 4°C), and the resulting pellets (P2) were resuspended in ice-cold 0.32 M phosphate-buffered sucrose. Aliquots (30 µl resuspended P2) were preincubated for 5 min at 37°C in KRH medium containing NaCl 120 mM, KCl 4.7 mM, CaCl2 2.2 mM, Heps 25 mM, MgSO4 1.2 mM, KH2PO4 1.2 mM, glucose 10 mM. The incubation was continued for 10 min in the same medium containing 100 µM pargyline, 100 µM ascorbic acid and 151 nM [3H]dopamine (300 µl final volume). The reaction was stopped by dilution with 1.2 ml of ice-cold Li+KRH medium (in which NaCl was substituted by LiCl) and centrifugation (12,000xg, 10 min, 4°C). Pellets were re-suspended with 1.2 ml of ice-cold Li+KRH medium and centrifuged again as above. The pellets were then decomposed by adding 50 µl 2N NaOH and an aliquot was used for determination of radioactivity. The specific uptake of dopamine was defined as the difference between the total uptake at 37°C in the Na+KRH medium and the non-specific accumulation determined at 37°C in the Li+KRH medium. For detecting the effect of different peptides on DA uptake by striatal synaptosomes, 10 µg/ml (1.7 µM) insulin, 10 µg/ml (2.1 µM) DOI and 1 µg/ml (0.92 µM) nonapeptide were added at the beginning of the uptake measurement.

2.5. Substantia nigra administration and locomotor activity

The procedures for substantia nigra administration have been described elsewhere (Martin et al., 1996). Here some steps were modified. Male Sprague Dawley rats weighing 200–250 g were housed in cages in a temperature-controlled room with a 12 h light–12 h dark cycle and were given access to food and water ad libitum. Rats were anaesthetized with 2% tribromoethanol (8.0 ml/kg) and mounted in a small animal stereotaxic apparatus (Kopf) with the incisor bar set at –2.5 mm. The skull was exposed and a 0.5 mm diameter burr hole was made using a dental drill. The nonapeptide was prepared as 1 µl 0 mM, 2.3 mM, 4.6 mM, 9.2 mM, 18.4 mM in a base of Heps buffer solution (10 mM Heps, 100 mM NaCl, pH 7.0) and administered into the right substantia nigra (SN) when the rat nearly recovered using coordinates of AP 5.2 mm, R 2.2 mm, V –7.8 mm by a 26 gauge Hamilton Syringe (0.5 µl/min). The Hamilton Syringe was left in place for an additional 10 min before removal. The skin was sutured with wound clips and the behavior of the animals was observed. 1 µl of 450 mM cocaine in the same medium was administered as a positive control. Locomotor activity was...
assessed by observing the direction of rotation and by counting the maximum rotation velocity and the duration of rotation. Rotation was counted by visual observation. One rotation was defined by the animal’s completing a 360° circle without turning back to the opposite direction. All animal procedures were in accordance with the guidelines for care and use of laboratory animals and were approved by the institutional animal care and use committee.

2.6. Data analysis

The data were analyzed using Origin software (Northampton, MA, USA). Values are the mean ±SEM of three samples. Difference between groups was assessed by two-way analysis of variance (ANOVA) and Student’s t-test.

3. Results

3.1. Cloning of DAT cDNA and stable expression of DAT, SERT and GAT1 in CHO cells

The full length DAT cDNA from adult SD rat substantia nigra was obtained by RT-PCR. The PCR product was sequenced to confirm that no mutations had been introduced during the PCR process and then cloned into pCDNA3 vector (designated DAT-pCDNA3). A cell line stably expressing DAT was constructed by transfecting DAT-pCDNA3 into CHO cells. [3 H]DA uptake was measured to evaluate the activity of DAT expressed in CHO cells. The cell clone with the highest uptake, designated D8, was chosen for further experiments. In the same way, a clone highly expressed GAT1 (designated G1 cells) and a clone highly expressed SERT (designated S6 cells) were obtained.

3.2. Effect of different peptides on dopamine uptake by D8 cells, serotonin uptake by S6 cells and GABA uptake by G1 cells and effect of the nonapeptide on WIN35,428 binding to DAT of D8 cells

Figure 1 shows that 10 µg/ml (1.7 µM) insulin does not affect DAT activity. However, concentration of 10 µg/ml, a nonapeptide derived from the C terminus of the insulin B chain, (H2N-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala-COOH), strongly inhibited the DA uptake activity of DAT expressed in D8 cells (Student’s t-test, P<0.05). A 7 amino acid peptide (the nonapeptide with N-terminal two amino acid residues (Arg and Gly) deleted) also slightly inhibited the DA uptake activity (Student’s t-test, P<0.05). Meanwhile, the peptide DOI (insulin with 8 amino acid residues deleted at the C terminus of the B chain) shows significant stimulation of DA uptake on D8 cells (Student’s t-test, P<0.05). Insu-
lin C-terminal deleted 4 peptide (DTI), insulin C-terminal deleted 5 peptide (DPI) and insulin C-terminal deleted 6 peptide (DHPI) had no effect on DA uptake. 100 µM cocaine, as a positive control, strongly inhibited DA uptake.

The kinetic character of the nonapeptide’s effect on DAT was also analyzed. Figure 2 shows a curve typical of competitive inhibition with an IC$_{50}$=6.9 µM (7.5 µg/ml). The inhibition effect of the nonapeptide was only partially reversible. After washing the D8 cells treated with the nonapeptide, about 64% of activity of DA uptake was recovered (Fig. 3). The effect of the nonapeptide on WIN35,428 binding to the DAT of D8 cells was also analyzed. At concentrations of 1, 2, 4, 6, 8 and 10 µM, the nonapeptide increasingly reduced [³H]WIN35,428 binding to the DAT of D8 cells (Fig. 4). The dose-dependent effect of DOI on DAT uptake was also tested on D8 cells. At a concentration of 0.021 µM, DOI significantly improved the uptake activity of the DAT (Student’s t-test, P<0.05). The stimulating effect of DOI on DAT uptake is dose-dependent and saturates above 0.042 µM (Fig. 5). A mixture of DOI and the nonapeptide with a stoichiometry of 1:1 (at the concentration of 10 µM) shows an inhibiting effect on DAT in D8 cell just as 10 µM nonapeptide used alone does (Fig. 6), while DOI used alone at the same concentration shows a stimulating effect.

In order to detect the specificity of the nonapeptide and DOI on DAT, the effect of these two peptides was examined on the serotonin transporter and the GABA transporter. The results show that neither nonapeptide nor DOI had any significant effect on the serotonin trans-

Fig. 4. Effect of different concentrations of the nonapeptide on WIN35,428 binding to DAT of D8 cells. The value of [³H]WIN35,428 binding of D8 cells was set at 100%. [³H]WIN35,428 binding of CHO cells and that of D8 cells dealt with 100 µM cocaine were taken as negative controls. Values are the mean±SEM of three samples. *, compared to D8 cells group, the significant difference, p<0.05.

Fig. 5. Effect of different concentrations of DOI on the dopamine transporter expressed in D8 cells. Values are the mean±SEM of three samples. *, compared to D8 cells group, the significant difference, p<0.05.

port in S6 cells or on the amino-butyric acid transport in G1 cells (Fig. 7).

3.3. Effect of different peptides on dopamine uptake by striatal synaptosomes

In order to confirm the effect of the nonapeptide and DOI on DAT in brain, the uptake of [³H]DA by striatal synaptosomes was also assayed. 1 µg/ml (0.92 µM) nonapeptide significantly inhibited the uptake of [³H]DA by striatal synaptosomes (Student’s t-test, P<0.05) similar...
Fig. 7. Effect of nonapeptide and DOI on serotonin transport in S6 cells and amino-butyric acid transport in G1 cells. A, effect of nonapeptide and DOI on the serotonin transport in S6 cells. B, effect of nonapeptide and DOI on the amino-butyric transport in G1 cells. Nonapeptide, 9 µM; DOI, 2 µM. Values are the mean±SEM of three samples.

to its effect on D8 cells. 10 µg/ml (2.1 µM) DOI showed a slight inhibition which contradicts the result obtained from D8 cells (Fig. 8).

3.4. Microinjection into substantia nigra of male adult rat and locomotor activity

The nonapeptide was also administered unilaterally into the rat substantia nigra to determine its effect on locomotor activity. 1 µl of the nonapeptide in different concentrations was administered unilaterally into the substantia nigra. The rat showed an asymmetric concaving posture with a posture bending toward the contralateral nonapeptide-injected side. The rat immediately began to rotate continuously after recovery, contralateral to the nonapeptide-injected side. The nonapeptide induced a dose-dependent increase in the maximum velocity and the duration of the round movement (Fig. 9). The rat injected with 0 µM nonapeptide (just Hepes buffer solution), as a negative control, did not display meaningful contralateral or ipsilateral rotation. The maximum velocity of the rats injected with 1 µl 4.6 mM nonapeptide was significantly different from that of the negative control (Student’s t-test, P<0.01). The duration of round movement of the rats injected with 1 µl 2.3 mM nonapeptide was significantly different from that of the negative control (Student’s t-test, P<0.01). The rat injected with 1 µl 450 mM cocaine, as positive control, showed contralateral rotation.

4. Discussion

It is very interesting that peptides (for example, the nonapeptide and DOI) derived from insulin could alter the transport activity of the dopamine transporter. The function of the nonapeptide and DOI is sequence-dependent. The Arg and the Gly in the N terminus of the nonapeptide were important for its inhibiting effect on the DA uptake activity of DAT, because the inhibiting effect was dramatically reduced (Fig. 1) if these two amino acid residues were deleted from the nonapeptide. Further work is needed to elucidate the details of the role that each amino acid residue plays in the activity of the nonapeptide, and a peptide with higher affinity for DAT may be obtained finally by changing some residues. We also tested a peptide of (Arg)₅ without effect on D8 cells to show that the conformation, rather than the positive charge, plays a key role in the interaction of the nonapeptide and DAT (data not shown). Extension of the C terminus of the B chain of DOI to that of insulin gradually reduced and finally even totally abolished its stimulating effect on DA uptake in D8 cells. The effect of DOI
and the nonapeptide on DAT are specific because neither of them had any effect on GABA transporter subtype I (GAT1) and serotonin transporter (SERT), both of which belong to the same transporter family as DAT.

The nonapeptide significantly inhibits the uptake of $[^3H]$DA by striatal synaptosomes in agreement with its action on D8 cells, but it is worth noting that the inhibiting effect on DA uptake is much stronger on synaptosomes than on D8 cells. The IC$_{50}$ of the nonapeptide tested on D8 cells is a little higher than that reported for cocaine (Giros et al., 1993). This may be due to the different method and cells used. Considering the results from the synaptosomes and those from in vivo experiments, the nonapeptide seems more potent than cocaine if at the same concentration.

DOI shows a slightly inhibiting effect on synaptosomes which contradicts the result from D8 cells. The result of DOI on synaptosomes was repeated and the reason for the contradiction remains unknown. We explain this by the different characters of CHO cells and synaptosomes, and it is more likely that DOI will bind to targets other than DAT at the level of synaptosomes. The saturation property of the stimulating effect of DOI on D8 cells, the inhibiting effect of DOI on striatal synaptosomes and the apparent effect of the nonapeptide indicate that the pattern of the interaction of DOI and DAT is different from that of the nonapeptide and DAT, and the former pattern may be more complicated.

Rats administered unilaterally with the nonapeptide into the substantia nigra show dose-dependent maximum velocity and duration of contralateral round movement. The results from in vivo experiments indicate that a bilateral imbalance of DA concentration in the substantia nigra has occurred, perhaps because the nonapeptide inhibits the activity of DAT and increases the concentration of DA on the injected side. We also injected 2.2 $\mu$l 40 $\mu$g/$\mu$l nonapeptide intracerebroventricularly in rats. After injection the rats showed significantly increased frequency of vacuous chewing. This effect could also be demonstrated by intraperitoneal administration of 400 $\mu$l 0.5 $\mu$g/$\mu$l nonapeptide. This means that the nonapeptide in peripheral tissues can pass the blood brain barrier and affect the function of the CNS. In this experiment, the effect of the nonapeptide was different from that of cocaine. After 5 $\mu$l 300 mM cocaine was injected into the brain ventricle, the rat was induced to shake its head (data not shown).

Our previous unpublished work showed that the nonapeptide and DOI did not bind to the insulin receptor and had no effect on glucose regulation, therefore their effect on DAT is not mediated by insulin receptor signaling. It has been reported that brain insulin could regulate mRNA synthesis of members of the neurotransmitter transporter family, especially of the dopamine transporter (DAT) and the norepinephrine transporter (NET), which may be mediated by insulin receptor signaling or by DAT signaling. According to our results, insulin itself has no direct interaction with DAT on D8 cells. Our data suggest that insulin regulates the DA system through binding to the insulin receptor, or through binding different degraded fragments directly to DAT (the nonapeptide, for example), or by an unknown mechanism (DOI, for example). Whether these pathways exist naturally and what are the underlying biological mechanisms needs further investigation. It be that, in addition to its role as a whole molecule acting via the insulin receptor, insulin may play a new role in the CNS as a neuropeptide precursor, with its fragments interacting with new target molecules, to regulate brain function.

Because the nonapeptide could inhibit the binding of WIN35,428 to DAT, it is interesting to hypothesize that the nonapeptide may mimic the conformation of cocaine and occupy the binding positions of cocaine to disrupt dopamine’s binding to DAT. The exact binding model...
is still unknown and needs to be established. It is valuable in theoretical research that two parts of one molecule with certain functions, the big part with stable conformation and the small part with a flexible one that could be induced by contacting the target, play opposite roles. This research may add to our knowledge of structure–function relationship. These insulin-derived peptides could, after optimization, have important therapeutic implications for disorders concerning the DA system such as Parkinson’s disease and cocaine addiction.

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References


