行政院國家科學委員會專題研究計畫　成果報告

下下下下下下下下下下下下下酶酶下下下下下下下下下下下

厭食作用」在自由活動動下的效應

計畫類別：個別型計畫
計畫編號：NSC93-2320-B-040-016-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：中山醫學大學醫學系生理學科

計畫主持人：郭東益
共同主持人：謝易修
計畫參與人員：楊順下

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94 年 10 月 12 日

European Journal of Neuroscience, Vol. 22, pp. 715–723, 2005

Transcriptional involvement of protein kinase C-alpha isozyme in amphetamine-mediated appetite suppression

Yih-Shou Hsieh,[1] Shun-Fa Yang,[1] Hui-Ling Chiou[2] and Dong-Yih Kuo[3]
[1]Institute of Biochemistry, Chung Shan Medical University, Taichung, Taiwan, R.O.C.
[2]School of Medical Technology, Chung Shan Medical University, Taichung, Taiwan, R.O.C.
[3]Department of Physiology, Chung Shan Medical University, Taichung City 40201, Taiwan, R.O.C.

Keywords: dopamine agonist, feeding, neuropeptide Y, signal transduction

Abstract

Amphetamine (AMPH) is known as an anorectic agent. The anorectic action of AMPH has been attributed to its inhibitory action on hypothalamic neuropeptide Y (NPY), an appetite stimulant in the brain. The molecular mechanisms behind this anorectic action of AMPH are still unclear. This study investigated the possible role of protein kinase C (PKC) isotypes in this anorectic action. Results revealed that most PKC isotypes (α, βII, γ, δ, η, λ and ζ), except βI and ϵ isotypes, were stimulated during a repeated treatment of AMPH. Among these stimulated isotypes, three isotypes (α, δ, λ) were activated and expressed in a similar manner, while the other isotypes were expressed differently and specifically. To determine if PKCα was involved in the anorectic response of AMPH, the infusions of antisense oligonucleotide into the brain were performed 1 h before daily AMPH treatment in freely moving rats, and the results showed that PKCα knock down could block the anorectic response and restore NPY mRNA levels in AMPH-treated rats. These results suggest that PKC isotypes- (at least the α isotype), related modification of NPY gene expression in hypothalamus might play an essential role in the central regulation of AMPH-mediated feeding suppression.

Introduction

Amphetamine (AMPH) is known for its suppressive effect on appetite. Some previous studies (between 1937 and 1970) indicated that AMPH was widely prescribed for the treatment of obesity and had served as a prototype for the development of subsequent anorectic drugs, such as phentermine and phenylpropanolamine (Nichols, 1994). Humans treated with AMPH reported euphoria, increased attention, restlessness, difficulty sleeping, and headaches, while some might become anxious, feel confident, irritable, hostile, and aggressive (Biel & Bopp, 1978). Because of the effects of AMPH on human appetite, body weight, and psychosis, the mechanisms of AMPH-induced anorexia, weight loss or psychomotor effect have been investigated extensively.

The mechanism underlying the anorectic action of AMPH was implicated in the release of biogenic amines that might lead to an inhibitory action on hypothalamic neuropeptide Y (NPY) gene expression (Sulzer *et al*., 1995; Kuo, 2003). A daily treatment of AMPH lasting for several days could initially induce a marked decrease of food intake followed by a gradual recovery of food intake and eventually backed to the normal level (tolerant effect) (Leibowitz, 1975; Seiden *et al*., 1993; Kuo *et al*., 2001). Our previous study had shown that AMPH exerted an inhibitory action on hypothalamic NPY, an orexigenic neurotransmitter in the brain, to achieve an anorectic effect (Kuo, 2003). Moreover, a recent report also indicated that methamphetamine could decreased NPY mRNA expression in the area of hypothalamic arcuate nucleus (Crowley *et al*., 2005). Hypothalamic NPY played an important role in the regulation of feeding behaviour as an infusion of NPY into the brain of satiated rats might elicit a ravenous food intake and repeated infusions might lead to obesity (Billington *et al*., 1994; Woods *et al*., 1998).

Protein kinase C (PKC) is present abundantly in neuronal tissues and has been implicated in the neuronal signalling process of the brain (Huang & Huang, 1993; Tanaka & Nishizuka, 1994). PKC participated in a wide variety of physiological and pathophysiological processes in the brain, which might be explained, in part, by the heterogeneity of the PKC gene family, which encompasses at least ten functional isotypes (Nishizuka, 1995). These isotypes are subtly different in the aspects of enzymological properties, tissue expression, and intracellular localization (Tanaka & Nishizuka, 1994). Evidence has revealed that PKC could modulate the action of feeding behaviour (Kanoh *et al*., 2003) as well as AMPH-induced dopamine (DA) release (Giambalvo, 1992a,b; Giambalvo, 2004). Thus, it is possible that PKC is involved in the neuronal signalling of AMPH anorexia. Therefore, defining the specific isotype of PKC involved may provide more information for understanding the signalling mechanism of AMPH.

PKC isotypes have been divided by Nishizuka, (1988, 1995) into three subfamilies: conventional cPKCs (α, βI, βII, and γ), novel nPKCs (δ, ϵ, η, and θ), and atypical aPKCs (ζ and λ). The cPKCs require calcium, diacylglycerol (DAG) or exogenous phorbol esters, and the cofactor phosphatidyl-l-serine (PS) for full activation. Novel PKCs also require DAG (or TPA) and PS for activation; however, calcium does not enhance their activation. Atypical PKCs can not be activated by either DAG or TPA and are unresponsive to calcium (Toker, 1998). These different isoforms exhibit distinct tissue distributions, suggesting specific roles in neural functions (Huang *et al*., 1988; Tanaka & Nishizuka, 1994).

Until recently, PKC isotypes involved in the anorectic effect of AMPH have not been determined, nor their possible roles in the

Correspondence: Dr Dong-Yih Kuo, as above
E-mail: dykuo@csmu.edu.tw

Received 21 March 2005, revised 6 May 2005, accepted 23 May 2005

doi:10.1111/j.1460-9568.2005.04245.x

modulation of NPY gene expression. Therefore, this study was aimed at evaluating the involvement in the above-mentioned actions of nine PKC isotypes; θ isotype was ruled out due to the fact that it was expressed predominantly in muscle and haematopoietic cells (Altman & Villalba, 2002). Recently, approaches by modulating isotype-selective inhibitors or activators have been applied in the improvement of nervous diseases (Way *et al*., 2000; Battaini, 2001) therefore our results may be helpful for therapeutic research into AMPH-like anti-obesity drugs. In addition, our results may also be helpful in understanding the signalling mechanism of some nervous diseases induced by repeated administrations of AMPH.

In the current study, PKCα antisense oligodeoxynucleotides (ODN) was employed to disrupt α gene expression in order to examine its effect on AMPH-induced anorexia in freely moving rats. The method of antisense knock down had inherent difficulties. However, other investigators had used these antisense ODNs to specifically down-regulate PKCα gene expression (Dean & McKay, 1994; Hua *et al*., 2002). Moreover, antisense ODNs were preferentially taken up by neurons in the rodent brain after intracerebral administration (Yee *et al*., 1994) or intracerebroventricular (ICV) administration (Wahlestedt, 1994). In behavioural studies, ICV administration of antisense ODNs could be used to interrupt specific gene expression in the brain (Chiasson *et al*., 1992; Ghosh *et al*., 1993; Ogawa & Pfaff, 1998) or in the hypothalamus (Hulsey *et al*., 1996).

Materials and methods

Animal treatments

Male Wistar rats, with a weight of 200–300 g, were obtained from the National Laboratory Animal Center. They were housed individually in a cage, maintained at 22 ± 2 °C in a room with a 12-h light : 12-h dark cycle (light on at 06:00 h) and habituated to frequent handling. The administration of the drug and the checking of food intake were performed every day at the beginning of dark phase (18:00 h). All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. This study has been approved and reviewed by National Science Council, Taiwan, ROC.

To examine the effect of repeated treatments of AMPH (Sigma-Aldrich, MO, USA) on feeding behaviour, rats ($n = 6$–8 for each group) were injected intraperitoneally (i.p.) with the drug at doses of 0, 2 or 4 mg/kg daily for 4 days. The first time AMPH was injected was at the end of Day 0 (i.e. at the beginning of Day 1) and the intake data was calculated with respect to the food amount of the previous day.

To determine the effect of daily AMPH (0 or 2 mg/kg; i.p.) on hypothalamic NPY and PKC mRNA levels, rats were injected with the drug once a day for 1, 2, 3 or 4 days depending on the group of rats. Rats were divided into five groups ($n = 4$–6 for each group) according to the day to be killed. In other words, rats in Day 1 group were treated with AMPH daily for 1 day and then killed at the end of Day 1 (at 18:00 h), and rats in Day 2 group were treated with AMPH daily for 2 days and then killed at the end of Day 2 (at 18:00 h). Accordingly, rats of the other groups were treated with AMPH daily for 3–4 days until the day to be killed. Upon the day to be killed, rats received a treatment of 2 mg/kg AMPH at 40 min before killing to enhance the effect of the drug, and then rats were anaesthetized and decapitated. Following the decapitation, the hypothalamus was removed to determine the levels of NPY and PKC mRNA.

Studies to assess the effects of ICV injection of PKCα antisense on AMPH-induced anorexia and NPY mRNA as well as PKCα mRNA levels are described in the relevant sections.

RNA extraction

Hypothalamic NPY and PKC mRNA levels were measured in a block of mediobasal hypothalamic tissue as described previously (Morris, 1989). In brief, total RNA was isolated from tissues using a modified guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). Each hypothalamic block was homogenized in 1 mL of TRIZOL reagent (Life Technologies, Inc., Grand Island, USA) using an Ultrasonic Processor (Vibra Cell, Model CV17; Sonics & Materials Inc., Danbury, Connecticut, USA). After an incubation at 22 °C for 5 min, 0.2 mL of chloroform was added to each sample, the samples shaken vigorously for 15 s, incubated at 22 °C for 3 min, and then centrifuged at 12 000 × ***g*** for 15 min at 4 °C. After removal of the aqueous phase and precipitation with 0.5 mL isopropanol, samples were incubated at 22 °C for 10 min and centrifuged at 12 000 × g for 15 min at 4 °C. The gel-like RNA pellets were washed with 75% ethanol by vortexing and centrifugation at 7500 × ***g*** for 5 min at 4 °C. Thereafter, RNA pellets were dried briefly, dissolved in RNase-free water, and stored at −80 °C. The RNA was determined spectrophotometrically at 260 nm (Hitachi U-3210, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR)

Using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim GmbH, Germany), RNA was reversely transcribed into single-stranded cDNA. For each sample, 8 μL of sterile DEPC water containing 2 μg of RNA were added to oligo-p(dT)15 primer (0.8 μg/μL) followed by being denatured at 65 °C for 15 min, cooled at 25 °C for 10 min, then added to a reaction mixture consisting of 10× reaction buffer (100 mM Tris, 500 mM KCl; pH 8.3), deoxynucleotide mix (10 mM each), $MgCl_2$ (25 mM), RNase inhibitor (40 unit/μL), and AMV reverse transcriptase (25 unit/μL). Reaction mixtures were incubated at 42 °C for 2 h and then heated to 95 °C for 5 min to terminate the reaction followed by soaking at 16 °C. PCR was subsequently carried out by mixing 3 μL of cDNA product with mastermix solution consisting of DEPC water, 10× reaction buffer, $MgCl_2$ (25 mM), deoxynucleotide mix (10 mM each), P1 and P2 primers (1 μg/μL each), and Taq polymerase (5 unit/μL). Sequences of primers used in this experiment are shown in Table 1. With glyceraldehyde-3-phosphate dehydrogenase (GAPDH) being used as an internal standard calibrator, PCR reactions for NPY were carried out on a PCR thermocycler (GeneAmp PCR System 2700, CA, USA) with the following steps: 91 °C for 1 min (denaturing), 60 °C for 1 min (annealing), and 72 °C for 30 s (extension) for 28 cycles followed by a final elongation step at 72 °C for 7 min, and finally the PCR products were soaked at 16 °C. PCR reactions for other molecules analysed were carried out in steps similar to those described above except for changes to two steps (annealing and cycles) that were as follows: PKCα (52 °C, 28 cycles), PKCβI (61 °C, 28), PKCβII (60 °C, 28), PKCγ (56 °C, 28), PKCδ (55 °C, 25), PKCη(61°C, 25), PKCλ (60°C, 28), PKCϵ (55 °C, 25), PKCζ (60 °C, 28), GAPDH (52 °C, 25).

Gel electrophoresis

After RT-PCR, 8 μL of each PCR product was subsequently separated by flat-bed gel electrophoresis on a 3% agarose gel (Difco, Detroit, MC, USA). Gels stained by ethidium bromide (0.5 μg/mL, Sigma-Aldrich Co., MO, USA) were visualized under UV light, photographed, and then scanned densitometrically (Hoefer, San Francisco, CA, USA). Ratios of NPY and GAPDH mRNA for each treatment day were calculated to determine relative NPY mRNA levels. Contents of NPY mRNA in AMPH-treated group were indicated as the percentage

TABLE 1. The sequences of primers used in RT-PCR

	Primer	Sequence 5′ → 3′	Size of product (base pairs)
NPY	Sense	GGGCTGTGTGGACTGACC	264
	Antisense	GGAAGGGTCTTCAAGCCT	
PKC α	Sense	TGAACCTTCAGTGGAATGAGT	325
	Antisense	GGCTGCTTCCTGTCTTCTGAA	
PKC βI	Sense	TGTGATGGAGTATGTGAACGGGGG	639
	Antisense	TCGAAGTTGGAGGTGTCTCGCTTG	
PKCβII	Sense	GACCGGTTTTTCACCCGCCA	309
	Antisense	CCATCTCATAGAGATGCTCC	
PKC δ	Sense	CACCATCTTCCAGAAAGAACG	352
	Antisense	CTTGCCATAGGTCCCGTTGTTG	
PKC ϵ	Sense	CGAGGACGACTTGTTTGAATCC	389
	Antisense	CAGTTTCTCAGGGCATCAGGTC	
PKC η	Sense	CCCGCAACCACCCCTTCCTCA	561
	Antisense	AGCGCATGGTGGGGTTCTTGGTC	
PKC γ	Sense	CAGCATCGACCAAGCTGATTT	347
	Antisense	ATACCGCGCTCCCTGTCTGTGAAA	
PKC λ	Sense	ATACCGCGCTCCCTGTCTGTGAAA	408
	Antisense	ATGCTAACTGTGACCGGGCTAACG	
PKC ζ	Sense	CGATGGGGTGGATGGGATCAAAA	681
	Antisense	GTATTCATGTCAGGGTTGTCTG	
GAPDH	Sense	TCCCTCAAGATTGTCAGCAA	309
	Antisense	AGATCCACAACGGATACATT	

of control group. Similar steps were used to determine the contents of PKC mRNA of each isotype.

Lateral ventricular cannulation

Rat surgery was performed under anaesthesia with pentobarbital (30 mg/kg, i.p.) using stereotaxic apparatus (Kopf Model 900, Tujunga, CA, USA). The target of cannulation was close to the junction between the right lateral ventricle and the third ventricle (coordinates 0.8 mm posterior to Bregma, 1.5 mm from the midline, and 3.5–4.0 mm below the dura; Paxinos & Watson, 1986). A 23-g stainless steel guide cannula was implanted and secured to the skull using stainless-steel screws and dental cement. Correct placement was confirmed by observing a transient and rapid inflow of vehicle in the PE tube connected to a 28-g injector cannula. The cannula was then occluded with a 28-g stylet. For intracerebroventricular (ICV) infusion of antisense, the stylet was replaced with a 28-g injector cannula extending 0.5 mm below the tip of guide cannula. Behavioural testing began at 1 week after surgery. For all experiments verification of cannula placement was carried out by the administration of angiotensin II (100 ng/rat; Sigma-Aldrich Co., MO, USA). Angiotensin II reliably induced water drinking in non-deprived rats when administered into the ventricles (Ritter *et al*., 1981). Only data from rats drinking more than 10 mL within 30 min were included in this study.

ICV injection of antisense ODN

To determine the effect of PKCα antisense on the anorectic response of AMPH, rats (n = 6–8 for each group) were daily injected with missense or antisense (20 μg in a 10-μL vehicle; ICV) 1 h before daily treatment of AMPH (4 mg/kg; i.p.) for 4 days. Before AMPH treatment, rats should be ICV injected with a similar dose of missense or antisense daily for 2–3 days until the response of feeding behaviour was slightly reduced in antisense group. This is due to the fact that either continuous or repeated ICV injections of antisense may be necessary to maximize the behavioural effect and especially to block the synthesis of constitutively active gene products (Zhang & Creese, 1993; Ogawa & Pfaff, 1998). 20-mer oligodeoxynucleotides targeting rat PKCα mRNA were screened as described (Dean & McKay, 1994). The sequences of the PKCα antisense and missense ODN were 5′-GAC ATCCCTTTCCCCCTCGG-3′ (ISIS22703, beginning at position 1973 in the rat PKCα mRNA), and 5′-CGTCCTCAGTCGTCCCT CAC-3′ (ISIS22702), respectively. We used ODNs that were phosphorothioate-modified (S-ODN) only on the three terminal bases of both the 5′ and 3′ ends (Proligo Pty Ltd, Singapore), because these S-ODNs had been shown to produce sequence-specific effects without detectable toxicity in brain region and was regarded as a well-established agent in several vertebrate systems (Widnell *et al*., 1996a; Ogawa & Pfaff, 1998). Both antisense and missense S-ODN were dissolved in artificial cerebrospinal fluid containing 140 mM NaCl, 3.35 mM KCl, 1.15 mM $MgCl_2$, 1.26 mM $CaCl_2$, 1.2 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, pH 7.4.

Another experiment was designed to determine the effect of pretreatment of PKCα antisense or missense on NPY mRNA levels in AMPH-treated rats. Rats (n = 6–8 for each group) were daily injected with antisense or missense (20 μg in a 10-μL vehicle; ICV) at 1 h before daily AMPH (4 mg/kg; i.p.) for 2 days (Day 0 and Day 1). Before AMPH treatment, rats should be ICV injected with a similar dose of antisense or missense daily for 2–3 days until the response of feeding behaviour was slightly reduced in antisense group. At 40 min after AMPH treatment, the hypothalamus was removed from the brain and its NPY mRNA content was determined by RT-PCR.

To determine the effect of pretreatment of PKCα antisense or missense on PKCα mRNA level in saline-treated rats, rats (n = 4–6 for each group) were daily injected with antisense or missense (20 μg in a 10-μL vehicle; ICV) for 2–3 days until the response of feeding behaviour was slightly reduced in the antisense group. At 40 min after the last treatment of antisense or missense, the hypothalamus was removed from the brain and its PKCα mRNA content was determined by RT-PCR.

Western blotting

Protein samples extracted from hypothalamus tissue were separated in a 12.5% polyacrylamide gel, transferred onto a nitrocellulose membrane and then incubated separately with specific PKCα antibodies (Transduction Laboratories, Lexington, KY, USA) and α-tubulin antibodies (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA). After incubation with horseradish peroxidase goat anti-rabbit IgG, the colour signal was developed by 4-chloro-1-napthol/3,3′-diaminobenzidine, 0.9% (w/v) NaCl in Tris-HCl (Sigma Chemical Co., St. Louis, MO, USA). Relative photographic density was quantified by scanning the photographic negative film on a Gel Documentation and Analysis System (AlphaImager, 2000, Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis

Data were presented as mean ± SEM. Two-way or one-way ANOVA followed by Dunnett's test were used to detect significances among groups. $P < 0.05$ was considered to be statistically significant.

Results

The effect of AMPH treatment on feeding behaviours

The changes of food intake in rats receiving AMPH are shown in upper panel of Fig. 1. Using two-way ANOVA to repeatedly measure

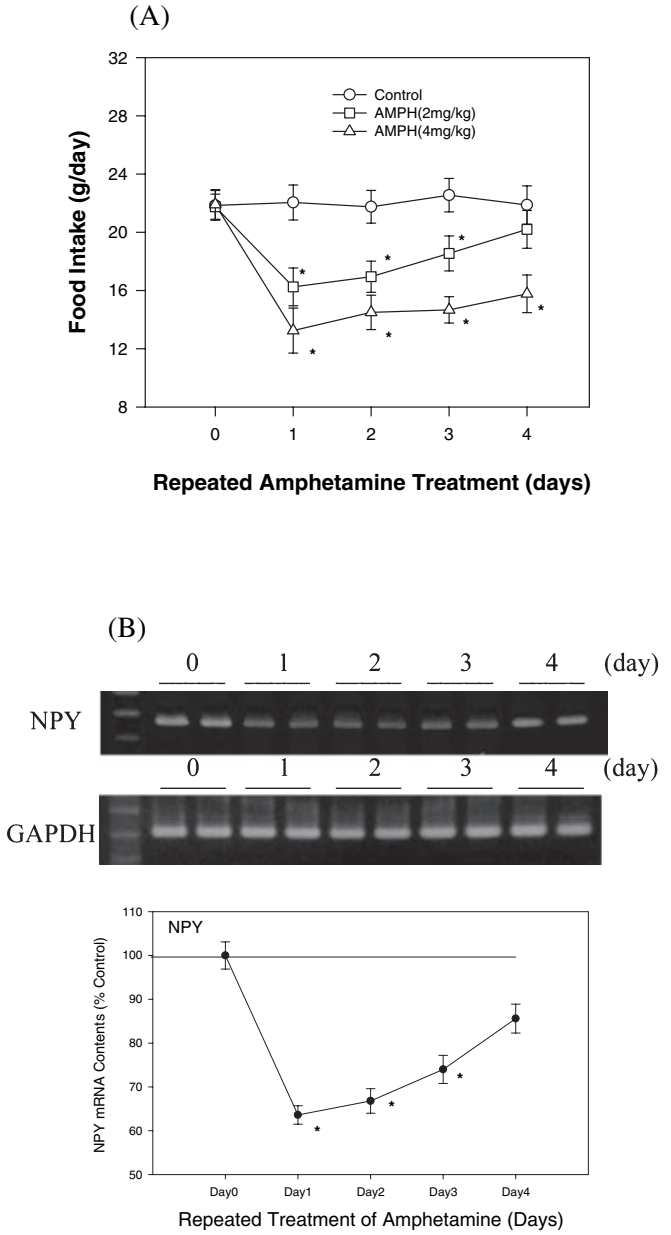

FIG. 1. (A) Effects of repeated treatments of amphetamine (AMPH) on daily food intake over a four-day period. The first injection of AMPH was performed at the end of Day 0 (i.e. at 18:00 h). Each point represents the mean ± SEM of 6–8 rats. *$P < 0.05$ vs. control group of each treatment day. (B) Effects of repeated amphetamine (AMPH) treatments on hypothalamic NPY mRNA levels over a four-day period. Upper panel, the results of RT-PCR analysing hypothalamic mRNA for NPY and GAPDH in stained ethidium bromide gels. Lower panel, relative densitometric values for RT-PCR products of hypothalamic NPY mRNA of AMPH- and saline-treated groups. The content of NPY mRNA in AMPH-treated group was indicated as the percentage of control. Bars were mean ± SEM. $n = 4$–6 each group. *$P < 0.05$ vs. control.

the effect of AMPH from Day 1 to Day 4, a significant dose effect ($F_{2,20} = 10.8$, $P < 0.01$) and time effect ($F_{4,35} = 3.6$, $P < 0.01$) was revealed, but it was not inclusive of the interaction effect. Dunnett's test ($P < 0.05$) revealed that a treatment with 2 mg/kg AMPH reduced the food intake from Day 1 to Day 3, and that treatment with 4 mg/kg AMPH reduced food intake from Day 1 to Day 4 when compared to controls. This result revealed that repeated treatments of 2 mg/kg AMPH could produce a marked anorectic response on Day 1 and induced a gradual tolerant effect on the following days. However, with a dose of 4 mg/kg AMPH, it could produce a continuous anorectic response during a four-day period.

The effect of AMPH treatment on hypothalamic NPY mRNA levels

Results shown in lower panel of Fig. 1 revealed that a daily treatment of AMPH (2 mg/kg) for 4 days resulted in a significant decrease in NPY mRNA levels. Analysis with one-way ANOVA ($F_{4,25} = 5.7$, $P < 0.01$) followed by Dunnett's test ($P < 0.05$) indicated a significant decrease of NPY mRNA contents on Day 1, Day 2 and Day 3 as compared with the control group. Moreover, the changes of NPY mRNA levels were consistent with the changes of feeding behaviour during four-day repeated treatments of AMPH, revealing the involvement of NPY gene in AMPH anorexia. In addition, this result also revealed that the development of AMPH tolerance was relevant to the restoration of NPY gene expression.

The effect of AMPH treatment on hypothalamic PKC mRNA levels

Results shown in Fig. 2 revealed that a daily treatment of AMPH (2 mg/kg) for 4 days resulted in a significant increase in mRNA levels of α, δ and λ isotypes. Analysis with one-way ANOVA followed by Dunnett's test ($P < 0.05$) indicated a significant increase of α ($F_{4,25} = 4.1$, $P < 0.05$), δ ($F_{4,25} = 2.9$, $P < 0.05$) and λ ($F_{4,25} = 3.2$, $P < 0.05$) isotypes on Day 1 and Day 2 as compared with the control group. The increased values of mRNA content were approximately 210 ± 42% in α isotype, approximately 132 ± 35% in δ isotype, and approximately 140 ± 22% in λ isotype as compared to the control group. This result revealed that the α, δ and λ genes were activated in a similar manner during a repeated treatment of AMPH.

Results shown in Fig. 3 revealed that a daily treatment of AMPH (2 mg/kg) for 4 days resulted in a significant effect on mRNA levels of γ, βII, η and ζ isotypes. Analysis with one-way ANOVA followed by Dunnett's test ($P < 0.05$) indicated a significant effect on the increases of βII isotype on Day 1 ($F_{4,25} = 3.9$, $P < 0.05$), and η isotype from Day 2 to Day 4 ($F_{4,25} = 4.2$, $P < 0.05$) as compared with the control group. However, it also revealed an inhibitory effect on γ isotype on Day 3 and Day 4 ($F_{4,25} = 4.9$, $P < 0.01$), and ζ isotype on Day 4 ($F_{4,25} = 2.8$, $P < 0.05$) as compared with the control group. This result suggests that γ, βII, η and ζ genes are expressed in a manner different from that of α, δ and λ isotypes during a repeated treatment of AMPH. Moreover, these four isotypes are expressed in a specific manner when compared with each other.

Finally, there were no significant changes in mRNA levels of βI and ϵ isotypes during a four-day treatment of AMPH (data not shown).

Effects of ICV injection of antisense S-ODN on AMPH anorexia

As shown in the upper panel of Fig. 4, a pretreatment of PKCα antisense S-ODN in AMPH-treated rats partially blocked the anorectic response of AMPH, indicating the involvement of the α gene in AMPH anorexia. Using two-way ANOVA to analyse the effect of PKCα antisense pretreatment on AMPH anorexia from Day 1 to Day 4, significant dose-dependent ($F_{3,27} = 4.1$, $P < 0.01$) and time-dependent effects ($F_{4,34} = 3.6$, $P < 0.01$) were revealed; however, it was not inclusive of the interaction effect. Comparing the food intake between antisense/AMPH-treated and AMPH alone-treated rats every day, significant effect was seen on Day 1, Day 2 and Day 3. Furthermore, a significant effect was also seen from Day 1 to Day 4

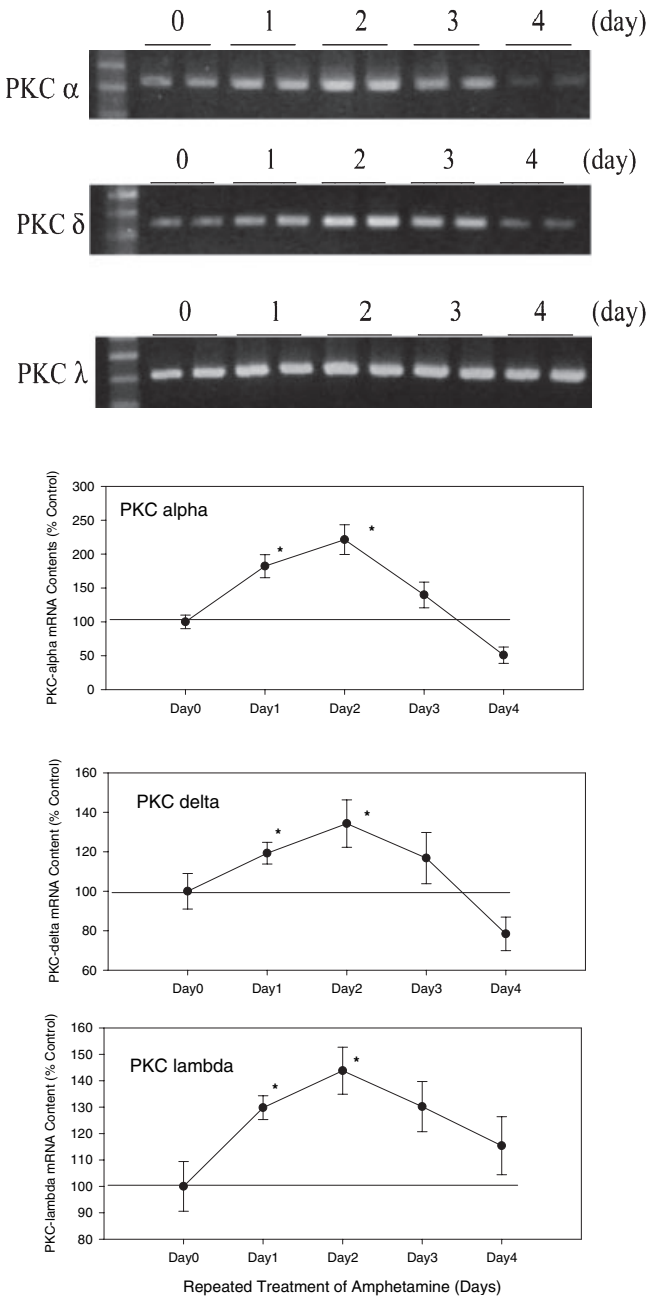

FIG. 2. Effects of repeated amphetamine (AMPH) treatments on hypothalamic PKC mRNA levels over a four-day period. Upper panel, the results of RT-PCR analysing mRNA levels of PKC isotype (α, δ and λ) in stained ethidium bromide gels. Lower panel, relative densitometric values for RT-PCR products of PKC mRNA in AMPH- and saline-treated groups. The content of PKC mRNA in AMPH-treated group (Day 1 to Day 4) was indicated as the percentage of control (Day 0). Bars were mean ± SEM. $n = 4$–6 each group. *$P < 0.05$ vs. control.

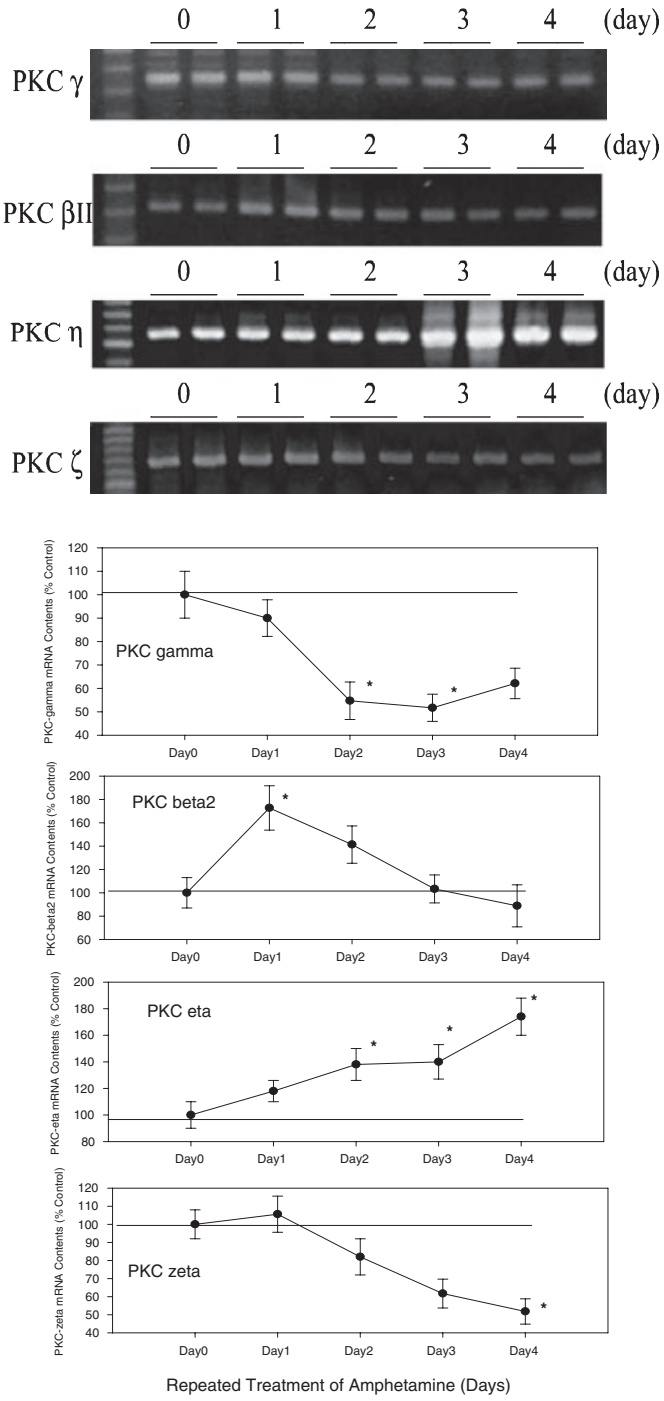

FIG. 3. Effects of repeated amphetamine (AMPH) treatment on hypothalamic PKC mRNA levels over a four-day period. Upper panel, the results of RT-PCR analysing mRNA levels of PKC isotype (γ, βII, η and ζ) in stained ethidium bromide gels. Lower panel, relative densitometric values for RT-PCR products of PKC mRNA in AMPH-treated and control groups. Content of PKC mRNA in AMPH-treated group (Day 1 to Day 4) was indicated as the percentage of control (Day 0). Bars were mean ± SEM. $n = 4$–6 each group. *$P < 0.05$ vs. control.

by comparing antisense/AMPH-treated and missense-treated (control) rats. The feeding response in missense alone-treated rats was similar to that in saline-treated rats (shown in Fig. 1) during a four-day period of treatment. Moreover, the anorectic response in missense/AMPH-treated rats was not significantly changed when compared to that in AMPH alone-treated rats during a four-day period of treatment. These results reveal the non-interference of missense treatment in this study. This result indicates that PKCα knock down modify the feeding responses of repeated AMPH treatments.

Effects of ICV injection of antisense S-ODN on NPY mRNA levels

Results shown in the lower panel of Fig. 4 revealed that pretreatment of PKCα antisense in AMPH-treated rats resulted in a partial restoration of hypothalamic NPY mRNA content to the normal level.

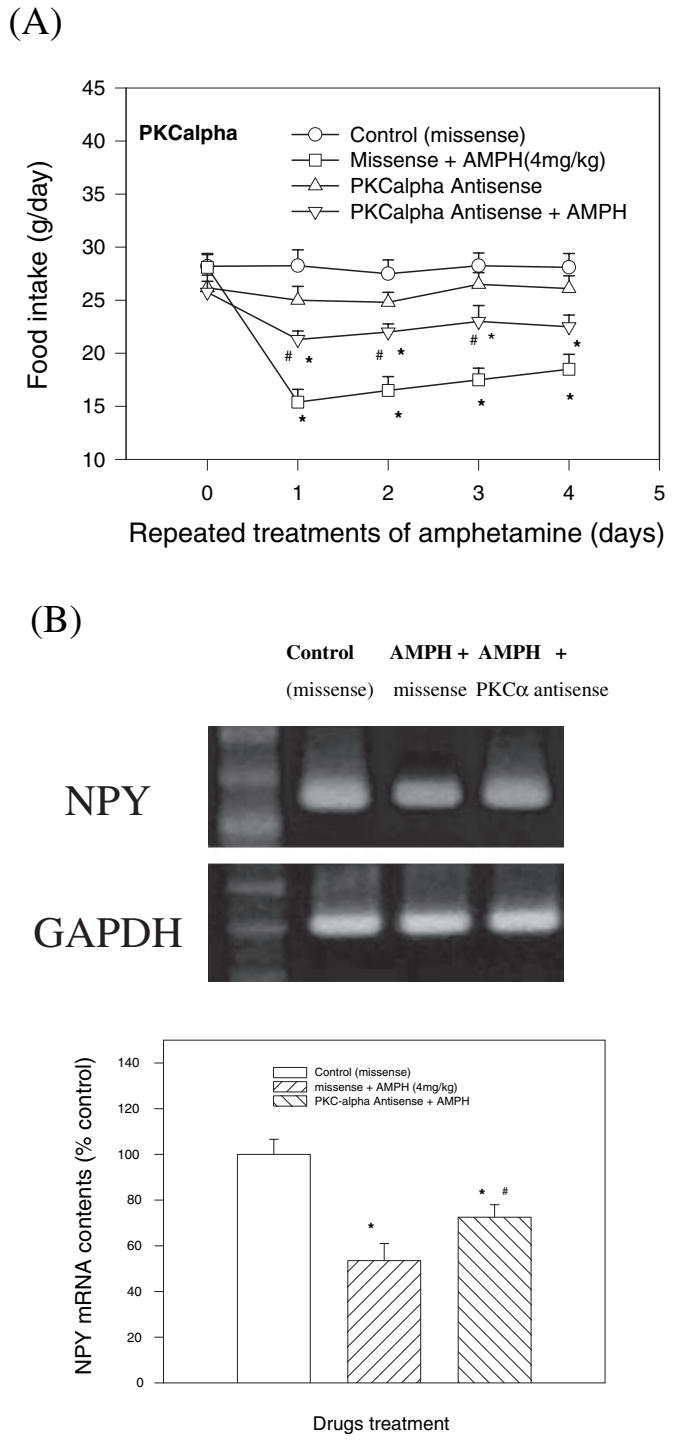

FIG. 4. Upper panel, the effects of PKCα antisense or missense pretreatment on AMPH-induced (4 mg/kg, i.p.) feeding behaviour over a four-day period. Daily missense or antisense (20 μg/10 μL/day, i.c.v.) was administered one hour before daily AMPH treatment. *$P < 0.05$ vs. the missense groups of each treatment day. #$P < 0.05$ vs. the AMPH-treated groups of each treatment day. Bars are mean ± SEM. $n = 6$–8 per group. Lower panel, the effects of PKCα antisense or missense pretreatment on NPY mRNA level on Day 1 in AMPH-treated (4 mg/kg, i.p.) rats. NPY mRNA levels were measured by RT-PCR. Content of NPY mRNA in drugs-treated rats was indicated as the percentage of control. Bars are mean ± SEM. $n = 4$–6 per group. *$P < 0.05$ vs. control, #$P < 0.05$ vs. AMPH-treated group.

Using GAPDH as the internal standard, the ratio of NPY mRNA over GAPDH mRNA in each group was calculated and compared. By one-way ANOVA ($F_{2,18} = 3.5$, $P < 0.05$) followed by Dunnett's test ($P < 0.05$), it revealed that the NPY mRNA content was decreased in both AMPH-treated and antisense/AMPH-treated rats as compared with the control (missense-treated) group. Moreover, significant decrease was also observed in antisense/AMPH-treated rats as compared with the AMPH-treated group. These results reveal that PKCα signalling is involved in the regulation of NPY gene expression in AMPH-treated rats.

Effects of ICV injection of antisense S-ODN on PKCα mRNA and protein levels

Results shown in the left panel of Fig. 5 revealed that pretreatment of PKCα antisense in saline-treated rats resulted in a significant decrease of PKCα mRNA levels. Using GAPDH as the internal standard, the ratio of PKCα mRNA over GAPDH mRNA in each group was calculated and compared. Statistical analysis using the *t*-test ($P < 0.05$) revealed that the PKCα mRNA content was decreased in antisense-treated rats as compared with the control (missense-treated) group. This result reveals that ICV injection of PKCα antisense is effective in reducing the hypothalamic PKCα mRNA levels in rats.

Results shown in the right panel of Fig. 5 revealed that pretreatment of PKCα antisense in saline-treated rats resulted in a significant decrease of PKCα protein levels. Using α-tubulin as the internal standard, the ratio of PKCα over α-tubulin in each group was calculated and compared. Statistical analysis using the *t*-test ($P < 0.05$) revealed that the PKCα content was decreased in antisense-treated rats as compared with the control (missense-treated) group. This result reveals that ICV injection of PKCα antisense is effective in reducing the hypothalamic PKCα protein level in rats.

Discussion

It has been reported that NPY is involved in the anorectic action of AMPH. However, the contribution of the PKC signal pathway in this action is still unknown. In this study, we found that several isotypes of PKC, including cPKC isotypes (α, βII and γ, but not βI), nPKC isotypes (δ and η, but not ϵ) and aPKC isotypes (ζ and λ) were stimulated during a four-day period of repeated AMPH treatment.

From the response of feeding behaviours, it appears that daily AMPH induces a marked anorectic response on Day 1 followed by a gradual tolerant response on the subsequent days. This alteration of feeding responses is consistent with changes of NPY gene expression. This result confirms that NPY is involved in regulating the AMPH anorexia, and it is therefore rational to speculate that the induction of AMPH tolerance is relevant to the restoration of NPY gene expression.

The α gene was activated during repeated AMPH treatments, and the mRNA levels of α after AMPH treatment were increased during Day 1 and Day 2 and returned gradually to normal level on the following days. Moreover, the mRNA level in the α isotype increased approximately two-fold, which was the most significant when compared with the other activated isotypes in AMPH-treated rats. It has been reported that the α isotype is distributed in all tissues, unlike other isoforms whose expression is restricted in the particular tissues (Dempsey *et al.*, 2000). This ubiquitously expressed isotype is activated in response to many kinds of stimuli and is therefore implicated in a variety of cellular functions (Nakashima, 2002). Thus, it is rational to predict that α isotype is activated during AMPH

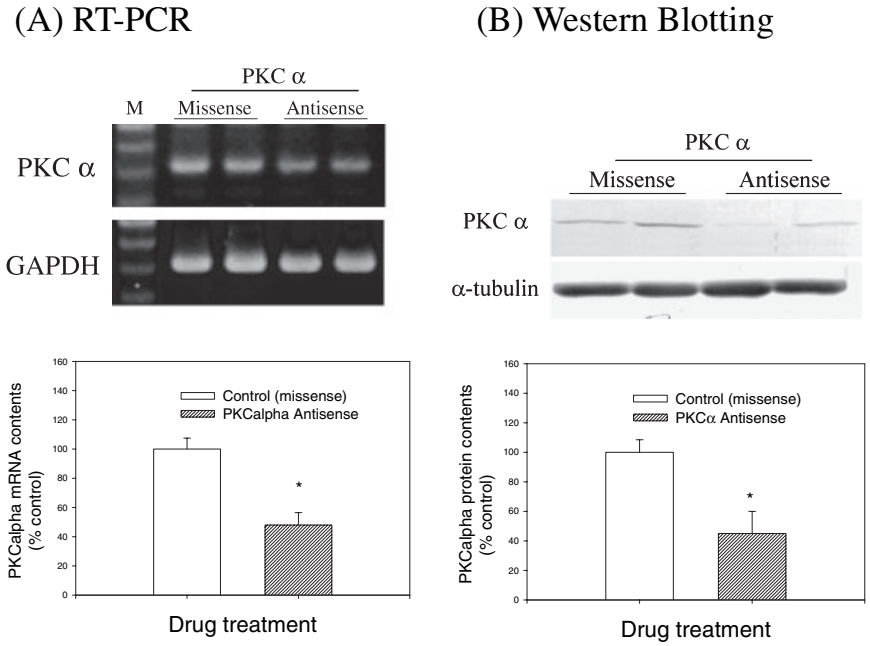

FIG. 5. Effects of PKCα antisense or missense treatment on hypothalamic (A) PKCα mRNA and (B) PKCα protein levels. (A) Upper panel, the results of RT-PCR analysing mRNA levels of PKCα isotype in stained ethidium bromide gels. Lower panel, relative densitometric values for RT-PCR products of PKCα mRNA in missense- and antisense-treated groups. Content of PKCα mRNA in antisense-treated group was indicated as the percentage of control (missense-treated). (B) Upper panel, the results of Western blotting analysing the contents of PKCα protein. Lower panel, relative densitometric values for Western blotting of PKCα protein in missense- and antisense-treated groups. Content of PKCα protein in antisense-treated group was indicated as the percentage of control (missense-treated). Bars were mean ± SEM. *nn* = 4–6 each group. $*P < 0.05$ vs. control.

treatment. Furthermore, the alteration of α mRNA levels is almost reciprocal to changes of NPY mRNA levels during AMPH treatment. This result implies that α signalling might play an inhibitory role in reducing NPY gene expression during AMPH treatment. To test this possibility, we examined the effects of ICV injection of PKCα antisense on AMPH-induced anorexia and NPY mRNA level. The results reveal that PKCα knock down blocks the feeding behaviour and restores the NPY mRNA levels in AMPH-treated rats. Thus, it is suggested that α signalling is involved in the regulation of AMPH anorexia by a negative modulation of NPY gene expression.

Based on this finding, it is possible that AMPH might activate the PKCα signalling in a distinct population of hypothalamic neurons and in turn inhibit NPY-producing neurons. For example, AMPH might at first activate the PKCα signalling in CART (cocaine- and amphetamine-regulated transcript)-producing neurons and then inhibit NPY-producing neurons to produce appetite suppression. CART is a potent appetite-suppressing peptide, which can be induced by an acute dosing of cocaine or AMPH and is highly expressed in the hypothalamus (Koylu *et al*., 1997). It has been reported that CART is closely associated with the actions of NPY as immunohistochemical studies reveal that NPY-positive varicosities are observed around CART peptide-positive cell bodies in the hypothalamus (Lambert *et al*., 1998). Similar to the role of CART-producing neurons, the involvement of other appetite-suppressive systems, such as proopiomelanocortin (POMC), should also be considered.

An ICV injection of PKCα antisense S-ODN in this study was sufficient to block the expression of both PKCα mRNA and protein *in vivo* in hypothalamus. Moreover, this antisense could modulate hypothalamic NPY gene expression that in turn could modify the AMPH-induced feeding behaviour. Compared to unmodified ODNs, S-ODNs are known to exert greater effects at much lower concentrations due to their higher intracellular stability (Ghosh *et al*., 1993). For *in vivo* application, S-ODNs were also more stable than ODNs after both ICV and intracerebral administration (Whitesell *et al*., 1993). These findings suggest that the antisense S-ODN method can be used as a tool to study causal relationships between molecular processes in the brain and behaviour. As the treatment of methamphetamine decreased NPY mRNA expression in the hypothalamic arcuate nucleus (Crowley *et al*., 2005), which was the original site of the NPYergic pathway, located next to the third ventricle in the brain, it is possible that PKCα antisense S-ODN, which was administered into the lateral ventricle of the brain in this study, might circulate via the cerebrospinal fluid into the third ventricle and then penetrate into the arcuate nucleus to interfere with the NPY gene expression.

The PKCα isozyme might play its role at the site of postsynaptic neurons rather than presynaptic nerve terminals. This hypothesis can be supported by two recent researches. Firstly, it has been reported that the PKCα isozyme is not relevant to the regulation of dopamine transporter (DAT), which is a presynaptic protein and the substrate of AMPH, because the coimmunoprecipitation study and the study with overexpression of PKCα strongly suggested that this isozyme neither bound to DAT nor affected DAT-mediated dopamine efflux in rat brain (Johnson *et al*., 2005). Secondly, another report indicated that an induction of long-term depression (LTD) required a postsynaptic cascade involving the activation of PKCα. LTD was absent in Purkinje cells in which the PKCα had been reduced by targeted RNA interference or in cells derived from PKCα null mice. However, in both cases, LTD could be rescued by expression of PKCα but not other PKC isoforms (Leitges *et al*., 2004). Thus, it is possible that PKCα functioned at the site of postsynaptic neurons, which might be NPY, CART or POMC neurons or other appetite-related neurons, but not at the site of presynaptic terminals interfering with AMPH-induced anorexia.

The δ and λ isotypes were activated in a manner similar to the α isotype and thus might be involved in the modulation of

AMPH-induced anorexia. Recently, δ and λ isotypes were emerging as an oxidative stress-sensitive kinase (Kanthasamy *et al*., 2003; Kitazawa *et al*., 2003; Fujita *et al*., 2004). AMPH has been regarded as a neurotoxin as it can increase the oxidative state of animals and produce oxidative damage to dopaminergic neurons (Yamamoto & Zhu, 1998; Cubells *et al*., 1994). Unpublished data in our laboratory (D.Y. Kuo & Y.S. Hsieh, unpublished observations) showed that an antioxidative enzyme superoxide dismutase (SOD) was also activated during a four-day period of repeated AMPH treatment, and the alterations of SOD mRNA levels during this period were parallel with those of PKCα, δ and λ. Thus, it is possible that α, δ and λ signalling in dopaminergic neurons is activated due to the increased oxidative stress induced by AMPH treatment, which in turn would attenuate NPY gene expression. However, this possibility needs to be investigated further.

The changes of δ mRNA levels were consistent with the changes of α mRNA levels, reflecting that there could be a certain cross-regulation between α and δ isotypes (Romanova *et al*., 1998; Dempsey *et al*., 2000; Mandil *et al*., 2001). Several reports indicated that both isoforms could promote a number of biological effects and could mutually regulate the expression and activity of other isoforms (Romanova *et al*., 1998; Murakami *et al*., 2002). Thus, α and δ isotypes might play their functional role in a manner of mutual cooperation to modulate AMPH anorexia.

The βII gene was activated on Day 1 during AMPH treatment. The β isotype has been reported to be involved in the regulation of stress-activated protein kinase activation in myeloid leukaemia cells (Kaneki *et al*., 1999) and the regulation of NPY gene expression in neuroblastoma cells (Troller *et al*., 2001). Thus, it is possible that the activation of βII signalling is involved in the inhibition of NPY gene expression. The η gene is also activated during AMPH treatment. However, unlike other isotypes, the level of PKCη mRNA increased gradually during AMPH treatment, revealing a different and specific mechanism of η signalling in AMPH-treated rats.

Instead of being activated, γ and ζ genes were inhibited during repeated AMPH treatments, implying that γ and ζ signalling are expressed in a different pathway during AMPH treatment. It is possible that the decreased expression of γ and ζ genes induced by AMPH treatment might disinhibit some neuromodulatory systems that play an inhibitory role in NPY gene expression (Barnea & Cho, 1993; Corbit *et al*., 2000; Fujita *et al*., 2004).

The present data provide a molecular basis for the anorectic effect of AMPH and imply that manipulations at the level of PKC might allow the development of therapeutic agents to improve the undesirable properties of AMPH. As most studies examining PKC and drugs of abuse did not differentiate between isoforms, it may be possible that targeting one particular isoform could avoid many undesirable side-effects. However, only the signalling of the PKCα isozyme is appropriately demonstrated in this study. It is still uncertain whether other PKC isoforms stimulated in the present findings might be involved in the modulation of NPY gene expression or the anorectic effect of AMPH. The administration of the corresponding PKC antisense S-ODNs into the brain will be carried out in further studies.

In summary, the present results show that at least seven PKC isotypes in the hypothalamus are stimulated during a repeated treatment of AMPH and that the α isotype is involved in the modulation of NPY gene expression, which participates in the anorectic response of AMPH.

Acknowledgements

This study was supported in part by a grant from the National Science Council (NSC-93–2320-B-040–016).

Abbreviations

AMPH, amphetamine; CART, cocaine- and amphetamine-regulated transcript; DAT, dopamine transporter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICV, intracerebroventricle; LTD, long-term depression; NPY, neuropeptide Y; ODN, oligodeoxynucleotide; PKC, protein kinase C; POMC, proopiomelanocortin; RT-PCR, reverse transcription-polymerase chain reaction; SOD, superoxide dismutase; S-ODN, phosphorothioate-modified ODN.

References

Altman, A. & Villalba, M. (2002) Protein kinase C-theta (PKC theta): a key enzyme in T cell life and death. *J. Biochem.*, **132**, 841–846.

Barnea, A. & Cho, G. (1993) Basic fibroblast growth factor selectively amplifies the functional state of neurons producing neuropeptide Y but not somatostatin in cultures of fetal brain cells: evidence for a cooperative interaction with insulin-like growth factor I. *Endocrinology*, **133**, 1895–1898.

Battaini, F. (2001) Protein kinase C isoforms as therapeutic targets in nervous system disease states. *Pharmacol. Res.*, **44**, 353–361.

Biel, J.H. & Bopp, B.A. (1978) Amphetamine: Structure-activity relationship. In Iversen, L.L., Iversen, S.D. & Snyder, S.H., (Eds), *Handbook of Psychopharmacology*. Plenum Press, New York, pp. 1–39.

Billington, C.J., Briggs, J.E., Harker, S., Grace, M. & Levine, A.S. (1994) Neuropeptide Y in hypothalamic paraventricular nucleus: a center coordinating energy metabolism. *Am. J. Physiol.*, **266**, R1765–R1770.

Chiasson, B.J., Hooper, M.L., Murphy, P.R. & Robertson, H.A. (1992) Antisense oligonucleotide eliminates *in vivo* expression of c-fos in mammalian brain. *Eur. J. Pharmacol.*, **227**, 451–453.

Chomczynski, P. & Sacchi, N. (1987) Single-step method of RNA isolation by acid quanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.

Corbit, K.C., Soh, J.W., Yoshida, K., Eves, E.M., Weinstein, I.B. & Rosner, M.R. (2000) Different protein kinase C isoforms determine growth factor specificity in neuronal cells. *Mol. Cell Biol.*, **20**, 5392–5403.

Crowley, W.R., Ramoz, G., Keefe, K.A., Torto, R., Kalra, S.P. & Hanson, G.R. (2005) Differential effects of methamphetamine on expression of neuropeptide Y mRNA in hypothalamus and on serum leptin and ghrelin concentrations in *ad libitum*-fed and schedule-fed rats. *Neuroscience*, **132**, 167–173.

Cubells, J.F., Rayport, S., Rajendran, G. & Sulzer, D. (1994) Methamphetamine neurotoxicity involves vacuolation of endocytic organelles and dopamine-dependent intracellular oxidative stress. *J. Neurosci.*, **14**, 2260–2271.

Dean, N.M. & McKay, R. (1994) Inhibition of protein kinase C-alpha expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides. *Proc. Natl Acad. Sci. USA*, **91**, 11762–11766.

Dempsey, E.C., Newton, A.C., Mochly-Rosen, D., Fields, A.P., Reyland, M.E., Insel, P.A. & Messing, R.O. (2000) Protein kinase C isozymes and the regulation of diverse cell responses. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **279**, L429–L438.

Fujita, T., Ikuta, J., Hamada, J., Okajima, T., Tatematsu, K., Tanizawa, K. & Kuroda, S. (2004) Identification of a tissue-non-specific homologue of axonal fasciculation and elongation protein zeta-1. *Biochem. Biophys. Res. Commun.*, **313**, 738–744.

Ghosh, M.K., Ghosh, K., Dahl, O. & Cohen, J.S. (1993) Evaluation of some properties of a phosphorodithioate oligodeoxyribonucleotide for antisense application. *Nucl. Acids Res.*, **21**, 5761–5766.

Giambalvo, C.T. (1992a) Protein kinase C and dopamine transport – 1. Effects of amphetamine *in vivo*. *Neuropharmacology*, **31**, 1201–1210.

Giambalvo, C.T. (1992b) Protein kinase C and dopamine transport – 2. Effects of amphetamine *in vitro*. *Neuropharmacology*, **31**, 1211–1222.

Giambalvo, C.T. (2004) Mechanisms underlying the effects of amphetamine on particulate PKC activity. *Synapse*, **51**, 128–139.

Hua, X.Y., Moore, A., Malkmus, S., Murray, S.F., Dean, N., Yaksh, T.L. & Butler, M. (2002) Inhibition of spinal protein kinase C alpha expression by an antisense oligonucleotide attenuates morphine infusion-induced tolerance. *Neuroscience*, **113**, 99–107.

Huang, K.P. & Huang, F.L. (1993) How is protein kinase C activated in CNS. *Neurochem. Int.*, **22**, 417–433.

Huang, F.L., Yoshida, Y., Nakabayashi, H., Young, W.S. & Huang, K.P. (1988) Immunocytochemical localization of protein kinase C isozymes in rat brain. *J. Neurosci.*, **8**, 4734–4744.

Hulsey, M.G., Pless, C.M., White, B.D. & Martin, R.J. (1996) ICV administration of anti-NPY antisense oligonucleotide: effects on feeding behavior, body weight, peptide content and peptide release. *Regul. Peptides*, **59**, 207–214.

Johnson, L.A., Guptaroy, B., Lund, D., Shamban, S. & Gnegy, M.E. (2005) Regulation of amphetamine-stimulated dopamine efflux by protein kinase C beta. *J. Biol. Chem.*, **280**, 10914–10919.

Kaneki, M., Kharbanda, S., Pandey, P., Yoshida, K., Takekawa, M., Liou, J.R., Stone, R. & Kufe, D. (1999) Functional role for protein kinase C beta as a regulator of stress-activated protein kinase activation and monocytic differentiation of myeloid leukemia cells. *Mol. Cell Biol.*, **19**, 461–470.

Kanoh, Y., Sajan, M.P., Bandyopadhyay, G., Miura, A., Standaert, M.L. & Farese, R.V. (2003) Defective activation of atypical protein kinase C zeta and lambda by insulin and phosphatidylinositol-3,4,5-(PO4)(3) in skeletal muscle of rats following high-fat feeding and streptozotocin-induced diabetes. *Endocrinology*, **144**, 947–954.

Kanthasamy, A.G., Kitazawa, M., Kanthasamy, A. & Anantharam, V. (2003) Role of proteolytic activation of protein kinase C delta in oxidative stress-induced apoptosis. *Antioxid. Redox. Signal.*, **5**, 609–620.

Kitazawa, M., Anantharam, V. & Kanthasamy, A.G. (2003) Dieldrin induces apoptosis by promoting caspase-3-dependent proteolytic cleavage of protein kinase C delta in dopaminergic cells: relevance to oxidative stress and dopaminergic degeneration. *Neuroscience*, **119**, 945–964.

Koylu, E.O., Couceyro, P.R., Lambert, P.D., Ling, N.C., DeSouza, E.B. & Kuhar, M.J. (1997) Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. *J. Neuroendocrinol.*, **9**, 823–833.

Kuo, D.Y. (2003) Further evidence for the mediation of both subtypes of dopamine D1/D2 receptors and cerebral neuropeptide Y (NPY) in amphetamine-induced appetite suppression. *Behav. Brain Res.*, **147**, 149–155.

Kuo, D.Y., Hsu, C.T. & Cheng, J.T. (2001) Role of hypothalamic neuropeptide Y (NPY) in the change of feeding behavior induced by repeated treatment of amphetamine. *Life Sci.*, **70**, 243–251.

Lambert, P.D., Couceyro, P.R., McGirr, K.M., Dall Vechia, S.E., Smith, Y. & Kuhar, M.J. (1998) CART peptides in the central control of feeding and interactions with neuropeptide Y. *Synapse*, **29**, 293–298.

Leibowitz, S.F. (1975) Catecholaminergic mechanisms of the lateral hypothalamus: their role in the mediation of amphetamine anorexia. *Brain Res.*, **98**, 529–545.

Leitges, M., Kovac, J., Plomann, M. & Linden, D.J. (2004) A unique PDZ ligand in PKC alpha confers induction of cerebellar long-term synaptic depression. *Neuron*, **44**, 585–594.

Mandil, R., Ashkenazi, E., Blass, M., Kronfeld, I., Kazimirsky, G., Rosenthal, G., Umansky, F., Lorenzo, P.S., Blumberg, P.M. & Brodie, C. (2001) Protein kinase C alpha and protein kinase C delta play opposite roles in the proliferation and apoptosis of glioma cells. *Cancer Res.*, **61**, 4612–4619.

Morris, B.J. (1989) Neuronal localization of neuropeptide Y gene expression in rat brain. *J. Comp. Neurol.*, **290**, 358–368.

Murakami, M., Horowitz, A., Tang, S., Ware, J.A. & Simons, M. (2002) Protein kinase C (PKC) delta regulates PKC alpha activity in a Syndecan-4-dependent manner. *J. Biol. Chem.*, **277**, 20367–20371.

Nakashima, S. (2002) Protein kinase C alpha (PKC alpha): regulation and biological function. *J. Biochem. (Tokyo)*, **132**, 669–675.

Nichols, D.E. (1994) Medicinal chemistry and structure-activity relationships. In Cho, A.K. & Segal, D.S., (Eds), *Amphetamine and its Analogs: Psychopharmacology, Toxicology, and Abuse*. Academic Press, San Diego, pp. 3–41.

Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implication for cellular recognition. *Nature*, **334**, 661–665.

Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.*, **9**, 484–496.

Ogawa, S. & Pfaff, D.W. (1998) Current status of antisense DNA methods in behavioral studies. *Chem. Senses*, **23**, 249–255.

Paxinos, G. & Watson, C. (1986) *The Rat Brain in Stereotaxic Coordinate*, 2nd Edn. Academic Press, Sydney, Australia.

Ritter, R.C., Slusser, P.G. & Stone, S. (1981) Glucoreceptors controlling feeding and blood glucose: location in the hindbrain. *Science*, **213**, 451–452.

Romanova, L.Y., Alexandrov, I.A., Nordan, R.P., Blagosklonny, M.V. & Mushinski, J.F. (1998) Cross-talk between protein kinase C-alpha (PKC-alpha) and – delta (PKC-delta): PKC-alpha elevates the PKC-delta protein level, altering its mRNA transcription and degradation. *Biochemistry*, **37**, 5558–5565.

Seiden, L.S., Sabol, K.E. & Ricaurte, G.A. (1993) Amphetamine: effects on catecholamine systems and behavior. *Annu. Rev. Pharmacol. Toxicol.*, **23**, 639–677.

Sulzer, D., Chen, T.K., Lau, Y.Y., Kristensen, H., Rayport, S. & Ewing, A. (1995) Amphetamine redistributes dopamine from synaptic vesicles to the cytosol and promotes reverse transport. *J. Neurosci.*, **15**, 4102–4108.

Tanaka, C. & Nishizuka, Y. (1994) The protein kinase C family for neuronal signaling. *Annu. Rev. Neurosci.*, **17**, 551–567.

Toker, A. (1998) Signaling through protein kinase C. *Front Biosci.*, **3**, D1134–D1147.

Troller, U., Zeidman, R., Svensson, K. & Larsson, C. (2001) A PKC beta isoform mediates phorbol ester-induced activation of Erk1/2 and expression of neuronal differentiation genes in neuroblastoma cells. *FEBS Lett.*, **508**, 126–130.

Wahlestedt, C. (1994) Antisense oligonucleotide strategies in neuropharmacology. *TIPS*, **15**, 42–46.

Way, K.J., Chou, E. & King, G.L. (2000) Identification of PKC-isoform-specific biological actions using pharmacological approaches. *TIPS*, **21**, 181–187.

Whitesell, L., Geselowitz, D., Chavany, C., Fahmy, B., Walbridge, S., Alger, J.R. & Neckers, L.M. (1993) Stability, clearance, and disposition of intraventricularly administered oligodeoxynucleotides: implications for therapeutic application within the central nervous system. *Proc. Natl Acad. Sci. USA*, **90**, 4665–4669.

Widnell, K.L., Self, D.W., Lane, S.B., Russell, D.S., Vaidya, V., Miserendino, M.J.D., Rubin, C.S., Duman, R.S. & Nestler, E.J. (1996a) Regulation of CREB expression: *in vivo* evidence for a functional role in morphine action in the nucleus accumbens. *J. Pharmacol. Exp. Ther.*, **276**, 306–315.

Woods, S.C., Figlewicz, D.P., Madden, L., Porte, D. Jr, Sipols, A.J. & Seeley, R.J. (1998) NPY and food intake: discrepancies in the model. *Regul. Peptides*, **75**, 403–408.

Yamamoto, B.K. & Zhu, W. (1998) The effects of methamphetamine on the production of free radicals and oxidative stress. *J. Pharmacol. Exp. Ther.*, **287**, 107–114.

Yee, F., Ericson, H., Reis, D.J. & Wahlestedt, C. (1994) Cellular uptake of intracerebroventricularly administered biotin- or digoxigenin-labeled antisense oligodeoxynucleotides in the rat. *Cell Mol. Neurobiol.*, **14**, 475–486.

Zhang, M. & Creese, I. (1993) Antisense oligodeoxynucleotide reduces brain dopamine D2 receptors: behavioral correlates. *Neurosci. Lett.*, **161**, 223–226.