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European Journal of Pharmacology 511 (2005) 31–41

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Disconnection between the early onset anorectic effects by C75 and hypothalamic fatty acid synthase inhibition in rodents

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Received 25 October 2004; accepted 18 January 2005

Abstract

In order to explore the relationship between the anorectic effect of 3-carboxy-4-octyl-2-methylenebutyrolactone (C75) and its pharmacokinetic properties, studies of in vivo and in vitro pharmacological characterization of C75 were performed in Fischer rats. In a quantitative measurement of food intake, we determined that appetite suppression by C75 takes place within 4 h. The C_{max} for C75 of 2.6±1.5 μM was reached within 1–4 h after intraperitoneal administration at 30 mg/kg, a drug level that causes complete blockade of food intake. However, this concentration is substantially lower than the effective concentration used to inhibit rat fatty acid synthase enzyme activity in vitro (IC_{50}: ~200 μM) and hypothalamic enzyme activity was found not to be inhibited by intraperitoneal administration of C75 at 30 mg/kg. Instead, a dramatic induction of c-Fos expression was found in area postrema. Collectively, these data indicate that the anorectic effect of C75 is independent of its inhibition of fatty acid synthase in the hypothalamus.

Keywords: C75; Fatty acid synthase; Appetite suppression; Obesity

1. Introduction

In mammals, the endogenous synthesis of long-chain fatty acids is catalyzed solely by fatty acid synthase, a single, homodimeric, multifunctional enzyme (Wakil, 1989). It utilizes acetyl-CoA, malonyl-CoA, and NADPH in a stepwise and sequential manner to build long acyl chains. Long-chain fatty acids are important substrates for energy metabolism. Overaccumulation of fat, the storage form of long-chain fatty acids, results in obesity—an epidemic that is increasing at an alarming rate in the industrialized world (Friedman, 2000).

C75, a member of α-methylene-γ-butyrolactones, was first discovered as a fatty acid synthase inhibitor and anti-tumor agent (Kuhajda et al., 2000). When administered in rodents, it has a robust and sustained effect in reducing body weight (Loftus et al., 2000). This weight loss is purportedly realized through concerted actions in the central nervous system (CNS), resulting in appetite suppression (Kumar et al., 2002; Loftus et al., 2000), and in peripheral tissues by the activation of carnitine palmitoyltransferase-1 (CPT-1), a rate-controlling enzyme for mitochondrial β-oxidation, resulting in increased fatty acid β-oxidation (Thupari et al., 2002).

The detailed mechanism of action for the anorexia observed with C75 administration is a matter of controversy. At the transcriptional level, C75 blocks both the fasting-induced increase in mRNA for orexigenic neuropeptide mRNAs for neuropeptide Y and agouti-related protein and fasting-induced down-regulation of mRNA for anorexigenic neuropeptides pro-opiomelanocortin and cocaine-amphetamine-related transcripts (Loftus et al., 2000; Shimokawa et al., 2002). The altered expression of these appetite-controlling neuropeptides could explain the long-lasting C75

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doi:10.1016/j.ejphar.2005.01.034

appetite suppression. However, C75 has a rapid effect on food intake that may be explained by at least four hypotheses involving: 1) hypothalamic malonyl-CoA; 2) hypothalamic AMP-activated protein kinase (AMPK); 3) glucose; and 4) nonspecific neuronal activation.

The hypothalamic malonyl-CoA hypothesis postulates that fatty acid synthase inhibition by C75 increases the concentration of the fatty acid synthase's substrate, malony-CoA, in the hypothalamus. Malonyl-CoA in turn serves as the second messenger mediating the anorectic effect. This hypothesis is based on the observation that the simultaneous administration of 5-(tetradecyloxy)-2-furoic acid (TOFA), a known acetyl-CoA carboxylase inhibitor, by intracerebroventricular (i.c.v.) injection largely offsets the anorectic effect of C75 (Loftus et al., 2000). It is further supported by the observation that the concentration of malonyl-CoA increases in the hypothalamus after administration of C75 (Hu et al., 2003). The hypothalamic malonyl-CoA hypothesis is attractive in that it extends the "fuel-sensing mechanism" by malonyl-CoA in peripheral tissues (Ruderman et al., 1999) to the "satiety-sensing mechanism" in CNS.

The hypothalamic AMP-activated protein kinase (AMPK) hypothesis is based on the observation that the level of AMPK phosphorylation and activity is rapidly reduced upon C75 administration. Coadministration of 5-aminoimidazole-4-carboxamide 1-β-D-ribofluranoside (AICAR), an AMPK activator, reverses the C75 anorectic effect (Kim et al., 2004; Landree et al., 2004).

The glucose hypothesis proposes that the increase in glucose use, rather than decreased use of fatty acid per se, is important for the anorexic effects (Wortman et al., 2003). The last hypothesis, nonspecific neuronal activation hypothesis, has support from the observation that C75 elicits widespread neuronal activation for appetite-controlling neurons regardless if they are orexigenic (neuropeptide Y neurons) or anorexigenic (pro-opiomelanocortin neurons), and also caused activation of non-relevant neurons such as Purkinje neurons (Takahashi et al., 2004).

Despite a number of reports that have appeared in recent years characterizing the metabolic consequences of C75 administration, surprisingly, there are no data available regarding its pharmacokinetic properties. Also, the correlation between the in vitro potency of C75 for fatty acid synthase inhibition and its in vivo anorectic effect has not been explored. This report directly addresses these issues and resolves questions regarding the mechanistic role of fatty acid synthase inhibition in the anorectic effect of C75.

2. Materials and methods

2.1. Materials

We obtained [2-^{14}C]malonyl-CoA (55 mCi/mmol, 98% purity) (Amersham Pharmacia Biotech UK Limited), acetyl-CoA, malonyl-CoA, NADPH, *S*(+)-fenfluramine, and phentermine (all from Sigma), sibutrimine (Bristol-Myers Squibb Medicinal Chemistry Synthesis), Bio-Spin 30 column (Bio-Rad), mouse anti-fatty acid synthase monoclonal IgG (BD Transduction Laboratories), rabbit anti-c-Fos polyclonal antibody (Santa Cruz Biotechnology), and NorthernMax Kit (Ambion). C75 was either synthesized by our colleague Ian Mitchell or obtained from Calbiochem.

2.2. Recording of overnight food consumption

All animal experiments were approved by Bristol-Myers Squibb Company Animal Care and Use Committee and followed National Research Council Guidelines. Male Fischer rats weighing 150–180 g upon arrival (Charles River Breeding Laboratories, Kingston, NY) were housed in pairs in polystyrene cages on Alpha-Dri bedding (Shepherd Specialty Papers, Kalamazoo, MI) at 22 °C and 50% humidity. They were maintained on a 12/12 light/dark schedule with lights on at 0400 h. Rats received ad libitum water and food (rodent diet; PMI Nutrition International, Brentwood, MO). At the time of the study, rats weighed ~300 g and were ~4 months old.

Experiments were conducted in 16 Habitest modular test cages (30×30 cm) (Coulbourn Instruments LLC, Allentown, PA). Each cage was equipped with a removable grid floor and drop pan, a house light, an optical lickometer, a lever with a triple cue light, a pellet feeder, and a pellet trough module with a magazine light. The test cages were each housed within a ventilated isolation chamber. Food consumption was recorded using a Compaq DeskPro 4000 computer running Graphic State software (Coulbourn Instrument LLC), and connected to the test cages with Lablinc (Coulbourn Instrument LLC) interface modules.

In preparation for drug treatment, a group of 16 rats was trained to lever press for food on a Fixed Ratio 3 schedule of reward (45 mg of food pellet; Bio-Serv, Frenchtown, NJ). A feeding session ran for 15 h, from 1600 h until 0700 h the next day. This included the normal 12 h of the dark cycle and 3 h of the next day's light cycle. Each animal was trained until its response characteristics were stable.

For intraperitoneal (i.p.) and per os (p.o.) drug treatment sessions, the rats were randomized into four groups. For i.p. drug sessions, each group was treated with vehicle RPMI 1640 medium (Invitrogen Corporation), C75 1.0, 3.0, 10, or 30 mg/kg, i.p., 1 h before being placed in the test cage at 1600 h. For p.o. drug sessions, each group was treated with saline or the appropriate dose of fenfluramine, phentermine, or sibutramine. All compounds were administered at a rate of 0.1 ml/100 g body weight 1 h prior to the onset of the dark part of the day/night cycle when food became available.

Experiments employing i.c.v. administration were conducted in the same manner using a different group of 16 rats that were surgically implanted with lateral ventricular cannulae approximately 1 week prior to drug testing. These rats were first trained to lever press for food for approx-

imately 10 days before cannula implantation in the lateral ventricle. For implantation, the rats were anesthesized with xylazine (9 mg/kg) and ketamine (55 mg/kg), i.m., in a volume of 1 ml/kg body weight. After full induction of anesthesia, the rats were placed in a stereotaxic apparatus (Kopf Instrument Co.) and 26-gauge guide cannulae (Plastic Products Co., Roanoke, VA) were stereotaxically positioned at 1.0 mm caudal and 1.4 mm lateral to bregma, and 2.4 mm below the dura mater. The guide cannulae were fixed in position with stainless steel screws and dental cement. At the end of the experiment, the location of each cannula was verified by injection of 5 μl of methylene blue through the cannula, the rat euthanized with CO_2, the brain removed and sectioned, and the location of the cannula verified visually.

Food consumption was recorded as lever presses in 1-h intervals for the full 15-h feeding session. For statistical purposes, the data from two experiments were combined with the session total and each 3-h period of the 15-h experiment analyzed using analysis of variance (ANOVA) and Fisher PLSD statistics.

2.3. Testing effects of anorexigenic compounds using 22-h food-deprived rats

Fischer rats weighing 200–300 g were food-deprived for 22 h prior to the beginning of the experiment. Compounds were injected either through p.o. or i.p. administration at a rate of 0.1 ml/100 g body weight, 1 h prior to food being made available. Regular rat chow in the normal feeding hopper was placed on the cage and the amount of food consumed was measured at 1 h.

2.4. Fatty acid synthase enzymatic assays

Purified rat fatty acid synthase was used to characterize the inhibition of fatty acid synthase activity by C75 in vitro. The purification procedure followed the five-step protocol described by Linn (1981). Before sacrifice, male rats weighing 200–250 g were fasted for 48 h and then fed a high-carbohydrate diet (20% vitamin-free casein, 70% sucrose, 5.15% fiber, and 1% vitamin mix) for another 48 h. The livers from diet-treated rats were removed for purification. After the final step, the purity of fatty acid synthase was assessed by Coomassie blue staining as a single band on SDS-PAGE gel. The purified fatty acid synthase enzyme activity was assayed in Buffer A (100 mM KH_2PO_4, pH 7.0, 200 μM NADPH, 20 μM acetyl-CoA, and 60 μM malonyl-CoA) by the rate of oxidation of NADPH through monitoring OD_{340} in 96-well plates at room temperature. To assay fatty acid synthase activity in crude extract from total brain or hypothalamic homogenates of Fischer rats, a radioactive assay format was adopted from Arslanian and Wakil (1975). For each assay, 0.05 μCi of malonyl-CoA was used. In a typical assay, 300 μg of cytosol extract (100,000×*g* centrifugation supernatant) was assayed in 0.5 ml of Buffer A. The assays were initiated by the addition of malonyl-CoA, and incubated at 37 °C for 10 min. To terminate the reaction, aliquots of 0.1 ml of 0.5 N NaOH were added. After the addition of another 0.2 ml of ethanol, the samples were boiled for 15 min to saponify. Aliquots of 0.1 ml of 1 N HCl were then added to acidify the samples. The samples were extracted three times with 2 ml of pentane. Combined extracts were washed with 2 ml of 0.1% acetic acid, blown dry by N_2, dissolved in 0.5 ml of pentane, and subjected to scintillation counting.

2.5. Irreversible inhibition of fatty acid synthase by C75 studied by Bio-Spin 30 gel filtration columns

An aliquot of 15 μg of rat fatty acid synthase enzyme was incubated with C75 (typically 1 mM C75 delivered by DMSO, final vehicle concentration 2%) in 60 μl of Buffer B (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, and 2% glycerol). After an incubation at room temperature for 1 h, the enzyme/inhibitor mixture was loaded onto Bio-Spin 30 column pre-equilibrated with Buffer B. Gel filtration was performed by centrifugation for 4 min at 1000×*g* at room temperature according to manufacturer's instructions. Aliquots (20 μl) of flowthrough fractions were subjected either to fatty acid synthase assay with OD_{340} decay method or to an analysis with Coomassie blue stain to assess the recovery of fatty acid synthase protein.

2.6. Determination of pharmacokinetic properties of C75 in Fischer rats

The pharmacokinetic properties of C75 were evaluated in male Fischer rats. Rats were administered with 10 and 30 mg/kg, i.p., C75 (n=3 for each dose group). A single blood sample at each time point was drawn via retro-orbital eye bleed at a volume of 250 μl at 20 min, 1 h, 2 h, 4 h, and 24 h after C75 administration. Plasma samples were immediately frozen and stored in −20 °C freezer. For analysis, plasma samples were thawed, treated with 3 vol of acetonitrile, and centrifuged to eliminate precipitates; 60 μl of supernatant was mixed with 150 μl of water and 10-μl aliquots were subjected to analysis.

Plasma levels of C75 were determined by liquid chromatography tandem mass spectrometry (LC/MS/MS). Briefly, the LC/MS/MS system included binary Shimadzu LC-10AD pumps (Shimadzu Corp., Columbia, MD), a CTC PAL autosampler (Leap Technologies, Switzerland), and a Sciex API 4000 mass spectrometer (Applied Biosystems, Foster City, CA). A Phenomenex Luna C18 column (2.0 mm×50 mm, 3 μm; Phenomenex, Torrance, CA) was used for separation at a flow rate of 0.3 ml/min. Mobile phases consisted of 10 mM ammonium formate and 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The initial mobile phase composition was 95% A:5% B. After 1 min of equilibrium at 5% B, it was ramped up to 95% B over 6 min and then held at 95% B for 1 min. ESI mass spectra were acquired in the negative ion mode with

multiple reaction monitoring (MRM) at ion pair of 253.1:208.9. The plasma levels of C75 were calculated by comparison of peak areas of the acquired mass chromatograms with an external calibration curve in the same matrix. Pharmacokinetic parameters including area under curve$_{0-\infty}$, C_{max}, T_{max}, and elimination half-life ($T_{1/2}$) were determined using a noncompartmental analysis package from Kinetica 4.2 (Innaphase, Philadelphia, PA).

2.7. Northern and immunoblot analysis

Northern blot analysis was performed as previously described in Meegalla et al. (2002). The specific activities of [^{32}P]-labeled cDNA probes for blot hybridization were ~10^6 cpm/ml for fatty acid synthase and ~10^5 cpm/ml for cyclophilin, respectively. Immunoblot analysis was performed as previously described (Cheng et al., 2001) using mouse anti-fatty acid synthase monoclonal at a concentration of 1 μg/ml. Bound antibodies were revealed with HPR-conjugated secondary antibody using SuperSignal CL horseradish peroxidase substrate (Pierce).

2.8. Immunohistochemistry staining

Immunohistochemistry was performed as previously described (Fritschy et al., 1991) with minimal modifications. Three hours after drug administration, Fischer rats were anesthesized with ketamine (90 mg/kg, i.p.) and xylazine (3 mg/kg, i.p.) and perfused transcardially with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde/0.1% glutaraldehyde in PBS. Brains were removed immediately and postfixed in 4% paraformaldehyde overnight at 4 °C. After treatment with 10% dimethylsulfoxide for 2 h for cryoprotection, 40-μm sections were cut from frozen brain on a sliding microtome and collected in PBS. The sections were then pre-incubated in 1.5% normal goat serum for 30 min, which was followed by incubation overnight at 4 °C in 0.2% Triton X-100 containing anti-c-Fos rabbit polyclonal antibody (1:10,000). Brain sections were then processed according to the ABC method (Vectastain Elite kit, Vector Laboratories), using diaminobenzidine hydrochloride as substrate. After immunostaining, sections were mounted on gelatin-coated slide, air-dried, dehydrated, and coverslipped.

3. Results

3.1. Quantitative characterization of appetite suppression by C75

A comparison was made between the effects of C75 with standard anorectic agents fenfluramine, phentermine, and sibutramine in a food-deprived model. In order to assess the acute effect, rats were conditioned to consume their entire daily ration of food in a 2-h interval (1300–1500 h). Once food consumption during this interval was stable, rats were deprived of food for 22 h. Drugs were injected at 1200 h, and food consumption was monitored for 1 h beginning at 1300 h (Table 1). C75 produced a dose-dependent decrease in food consumption. Fenfluramine, phentermine, and sibutramine appeared to be more potent and effective than C75 in this model.

In order to establish the quantitative relationship between C75 administration and its effect on food consumption, we established a computer-assisted model that records the food consumption in a time-dependent manner over a duration of 15 h. A group of 16 rats was trained to lever press for food on a Fixed Ratio 3 schedule of reward (45 mg of food pellet). A feeding session ran from 1600 h until 0700 h the next day, including the regular 12 h of the dark cycle and 3 h of the light cycle. Importantly, for all the drugs tested, changes in food consumption were faithfully reflected by changes in lever pressing in the FR3 schedule (Table 1).

C75 administered at 1, 3, 10, and 30 mg/kg, i.p., reduced total food consumption in a dose-related manner by 9%, 35%, 67%, and 97%, respectively. The reduction in

Table 1
The effects of C75, *S*(+)-fenfluramine, phentermine, and sibutramine on food consumption in food-deprived rats and on lever presses by FR3 schedule of food reward

	% Decrease of food consumption in 22-h food-deprived rats (n=10)				% Decrease of lever presses by FR3 schedule of food reward (n=7–8)							
Drugs[a]	C75	Fen	Phe	Sib	C75		Fen		Phe		Sib	
Duration measured	1 h	1 h	1 h	1 h	3 h	15 h	3 h	15 h	3 h	15 h	3 h	15 h
Dose (mg/kg)												
0.1		10										
0.3		47*					32*	14				
1		73*		0	0	9	69*	72*			16	
3	0		19	12	49*	35*	73*	86*	31*	0	38*	13
10	10		56*	49*	68*	67*	80*	72*	80*	25*	85*	23
30	55*		98*	89*	95*	97*,[b]			99*	63*		59*

[a] Drug administration: C75, i.p.; Fen (fenfluramine), p.o.; Phe (phentermine), p.o.; Sib (sibutrimine), p.o.
[b] The 30 mg/kg dose of C75 was tested in a separate experiment.
* P<0.05.

food consumption was statistically significant ($P<0.05$) at the 3, 10, and 30 mg/kg doses of C75 (Table 1 and Fig. 1A). The effect of C75 on food consumption in the FR3 paradigm parallels the effects reported by Clegg et al. (2002) using direct measurement of food uptake for a period of 24 h in rats.

One of the strengths of FR3 schedule is a detailed dissection of food consumption pattern throughout the recording. As shown in Fig. 1B, food consumption is highest in the early part of the dark cycle and declines throughout the night. The light cycle feeding falls to very low levels. For rats dosed with 3 mg/kg C75, significant decreases in food consumption were observed during the first and second 3 h periods. For rats dosed with 10 mg/kg, the significant decrease in food consumption was more pronounced and persisted throughout the dark part of the day/night cycle. Thus, C75-mediated decrease in food consumption was an early onset event (occurs within 4 h) and, at higher doses, can persist for more than 12 h.

In order to test whether the effect of i.p. administered C75 on food consumption was due to a CNS effect, presumably through the hypothalamus, overnight food recording was assessed after administration C75 by i.c.v. injection into the lateral ventricle (Fig. 1C and D). Indeed when rats were dosed with 3 μg and 10 μg i.c.v., there was a decrease in total food consumption. However, this was not statistically significant. Importantly, when food consumption data were assessed in 3-h time blocks, no change was noted for the first 3 h. This was in contrast to peripheral administration of C75. Instead, during the 4–6 h period, statistically significant, dose-independent decreases in food consumption were observed (compare Fig. 1B and D).

In summary, C75 administered peripherally showed a more robust and a more rapid-onset (within 4 h) suppression of food consumption as compared to direct central administration into the lateral ventricle of the CNS.

3.2. Determination of C75 potency in inhibiting fatty acid synthase enzyme activity in vitro

As reported earlier, inhibition of fatty acid synthase by C75 is time-dependent (Kuhajda et al., 2000). This characteristic of C75 inhibition is reproduced in Fig. 2A. Without pre-incubation, C75 only decreased fatty acid synthase activity by ~20% at a concentration of 1 mM. However, after being pre-incubated for 60 min at room temperature, the potency of C75 increased dramatically. The half-maximal inhibitory concentration (IC_{50}) in this con-

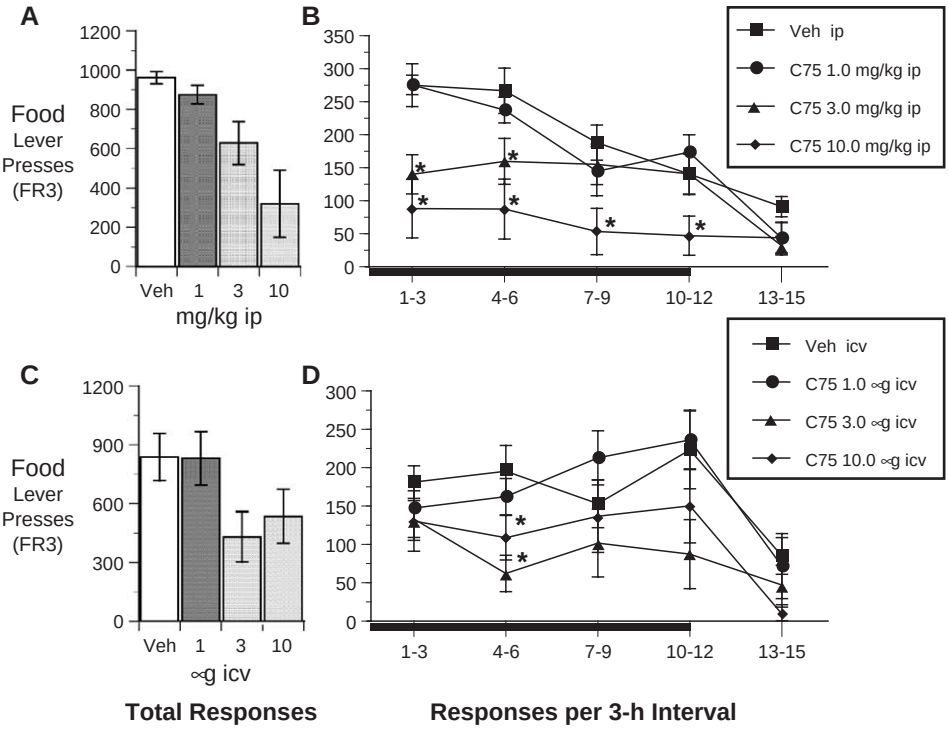

Fig. 1. (A and B) Effect of C75 i.p. administration on overnight feeding. Fischer rats trained to lever press for food were randomized into four groups. Each group was treated with vehicle (RPMI), C75 1.0, 3.0, or 10 mg/kg, i.p., 1 h before being placed in the test cage at 1600 h. A feeding session ran for 15 h, from 1600 h until 0700 h the next day. This included the normal 12 h of the dark cycle and 3 h of the next day's light cycle. Food consumption was recorded as lever presses. (A) Lever presses for the full 15 h feeding session. (B) Lever presses placed into 3 h blocks. Data for two experiments were combined and were analyzed using ANOVA and Fisher PLSD statistics. (C and D) Effect of C75 i.c.v. administration on overnight feeding. Experiments employing i.c.v. administration were conducted using Fischer rats trained to lever press for food, which were surgically implanted with lateral ventricular cannulae approximately 1 week prior to drug testing. The cannulated rats were randomized into four groups and i.c.v. administration with either 3 μl of RPMI vehicle or with 1, 3, or 10 μg of C75 in the same volume of vehicle. Food consumption was recorded as described (A and B).

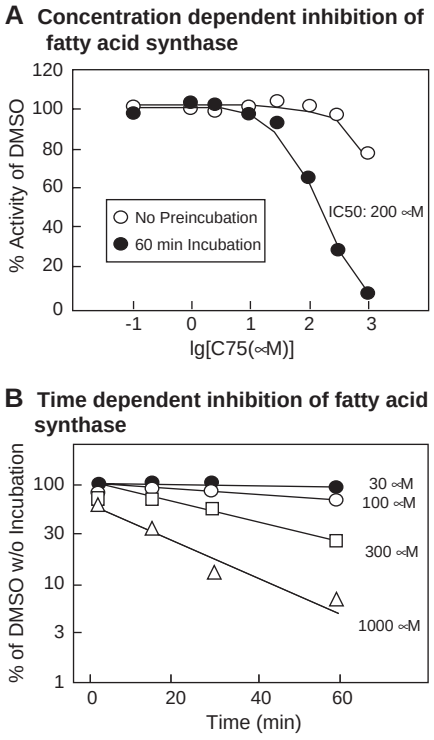

Fig. 2. (A) Concentration-dependent C75 inhibition of fatty acid synthase in vitro. Aliquots of rat fatty acid synthase (2 μg in each tube) were either pre-incubated with the indicated concentration of C75 (delivered by DMSO, final concentration 2% vol/vol) for 60 min at room temperature or mixed with C75 briefly in Buffer A without fatty acid synthase substrates in 100 μl. Equal volumes of Buffer A with fatty acid synthase substrates were added to the mixtures to initiate fatty acid synthase assay to monitor the decay of OD_{340}. The fatty acid synthase activity with 2% DMSO incubation (no difference from that without DMSO) was expressed as 100%. Fatty acid synthase activities with C75 incubation at various concentrations were expressed as a percentage of that value. IC_{50} was calculated with PRISM software. (B) Time-dependent inhibition of fatty acid synthase by C75. Large aliquots of rat fatty acid synthase were incubated with C75 at indicated concentrations. After the indicated time of incubation, small aliquots (~2 μg of fatty acid synthase) were removed from the mixture and assayed for fatty acid synthase activities.

dition was determined to be 200 μM with a Hill slope of 2 consistent with the notion that a covalent interaction between C75 and fatty acid synthase might be required for optimal inhibition.

A time course study was conducted to determine the rate of inactivation of rat fatty acid synthase by C75 (Fig. 2B). C75 concentrations at or below 30 μM demonstrated no detectable fatty acid synthase inactivation throughout the incubation. In contrast, at C75 concentrations of 100, 300, and 1000 μM, fatty acid synthase was inhibited in a dose- and time-dependent manner. The exponential decay of fatty acid synthase activity can be fitted into the equation $v/v_0=\exp(-k_{obs}t)$ (v=remaining fatty acid synthase catalytic velocity at given incubation time, v_0=fatty acid synthase catalytic velocity without incubation). The parameter k_{obs} was determined to be 0.0077, 0.024, and 0.044 min^{-1} at concentrations of 100, 300, an 1000 μM, respectively.

3.3. Pharmacokinetic properties of C75 in vivo

For the pharmacokinetic evaluation of C75 in Fischer rats, we administered C75 by i.p. injection at 10 mg/kg (which caused a significant reduction of food consumption) or 30 mg/kg (which caused a complete suppression of food consumption). The mean plasma concentration-versus-time profile for C75 at 10 and 30 mg/kg i.p. is shown in Fig. 3. After i.p. administration, C75 was rapidly absorbed into the systematic circulation. At 10 mg/kg, a C_{max} level of 0.95±0.37 μM was reached at the first sample collection time (20 min). At 30 mg/kg, a C_{max} level of 2.6±1.5 μM was reached at 1–4 h (mean T_{max} is 2.3 h). $T_{1/2}$ of C75 was relatively long in both dose groups, with mean values of 7.0±0.8 and 8.2±2.1 h for 10 and 30 mg/kg, respectively. Area under $curve_{0-\infty}$ was 5.4±0.8 and 21.6±4.6 μM h for 10 and 30 mg/kg doses, respectively, indicating a good dose proportionality of C75 exposure.

3.4. Lack of hypothalamic fatty acid synthase enzyme inhibition by i.p. C75

The substantial difference between the maximal plasma concentration of anorectic doses of C75 as compared with the concentration required to inhibit fatty acid synthase in vitro suggests that in vivo administration of C75 would not demonstrate inhibition of fatty acid synthase enzyme activity.

To measure fatty acid synthase inhibition by C75 in vivo, we took advantage of the fact that C75 is an irreversible inhibitor (Kuhajda et al., 2000). Because the nature of inhibition of fatty acid synthase by C75 is through covalent interaction, we reasoned that if C75 administered in vivo inactivated fatty acid synthase in a given tissue, the status of inactivated fatty acid synthase would be maintained during tissue homogenization and would be reflected by an in vitro fatty acid synthase activity measurement. To test this rationale directly, we examined a scenario in which fatty acid synthase was first incubated with C75, and then either C75 was diluted

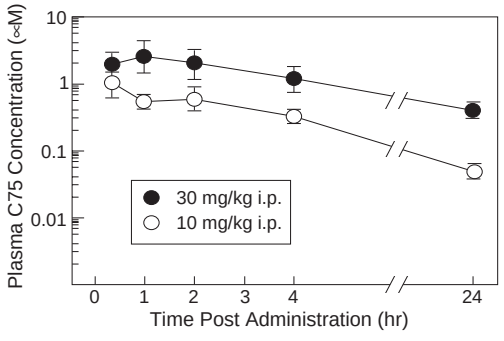

Fig. 3. Plasma concentration versus time profile after i.p. administration of C75 at 10 and 30 mg/kg. Plasma concentrations were shown as the mean values with standard deviations from three rats from each dose group.

directly to a concentration below the IC_{50} (as determined in Fig. 2A) or C75 was purified away from the fatty acid synthase/C75 complex. As shown in Fig. 4A, in conditions (b) and (c), aliquots of purified fatty acid synthase were incubated with C75 at 1 mM for 1 h, a condition that abolishes the majority of the fatty acid synthase activity (Fig. 2A). In (b), we directly diluted the mixture of fatty acid synthase and C75 by 10-fold in the assay condition so that the C75 concentration was ~100 μM, which is half of the IC_{50}. In (c), we purified the potential fatty acid synthase/C75 complex (molecular mass: ~520 kDa) from free C75 (molecular mass: 0.254 kDa) through a gel filtration column. In both of these conditions, the enzymatic activity of fatty acid synthase remained undetectable. These results indicate that once fatty acid synthase is inactivated through interaction with C75, even in a condition where free C75 is substantially diluted or separated away or from the fatty acid synthase/C75 complex, the fatty acid synthase remains inactive and its inactive state can be reflected by the in vitro enzymatic measurement.

A second issue that impacts accurately measuring fatty acid synthase inhibition in vivo is the fluctuation of fatty acid synthase protein levels in various metabolic conditions. It is well documented that fatty acid synthase expression levels in liver and adipose tissue are dramatically induced in the fed condition and decrease after starvation (Meegalla et al., 2002). We examined whether hypothalamic and brain fatty acid synthase mRNA are altered after C75 administration at anorectic doses. As shown in Fig. 4B, three groups of Fischer rats were treated with vehicle (fed ad libitum) or C75 (i.p. at 30 mg/kg, fed ad libitum), or fasted for 24 h. At this dose, C75 almost completely blocked food consumption and had an effect very similar to fasting on fatty acid synthase expression in the liver (compare lanes 5 and 6 to lane 4). However, in contrast to the effect in liver, fatty acid synthase expression in the hypothalamus and the rest of brain remained constant, indicating that the transcriptional mechanisms controlling the fatty acid synthase expression in the liver in response to various metabolic conditions are not the same in the CNS. Furthermore, there was no compensatory effect at the level of fatty acid synthase expression after C75 administration. Likewise, fatty acid synthase protein level was also maintained constant after C75 dosing in the hypothalamus and brain (data not shown). Hence, fatty

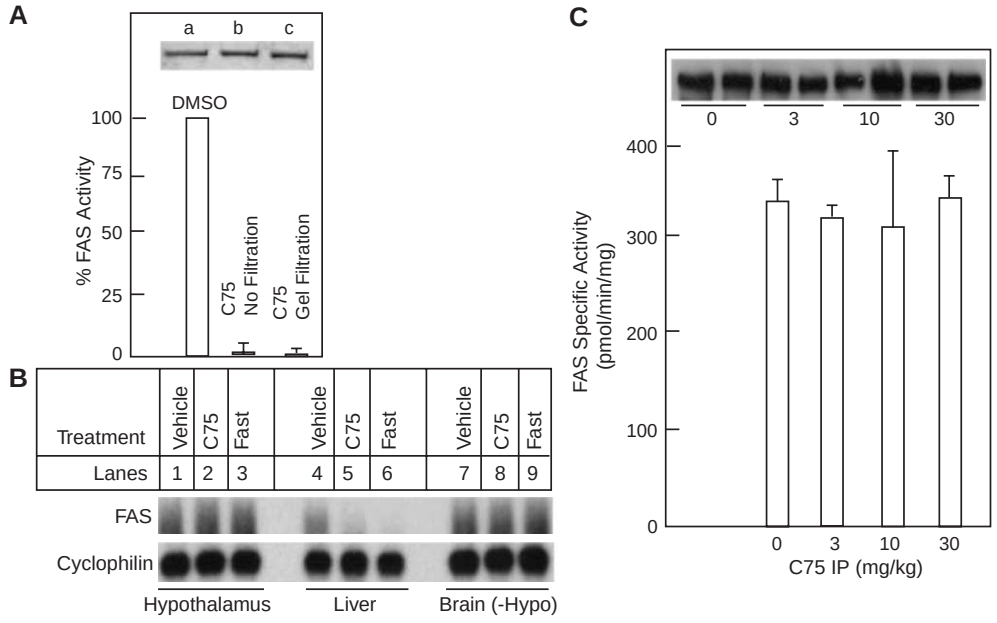

Fig. 4. (A) Irreversible inhibition of rat fatty acid synthase by C75. Three aliquots of 15 μg of rat fatty acid synthase enzyme were incubated with: (a) 2% DMSO or (b and c) with 1 mM C75 for 1 h at room temperature. For (a) and (c), the mixtures were loaded onto Bio-Spin 30 column and chromatography was performed. The flowthrough fractions were assayed or stained with Coomassie blue (inset). Proportional amount of fatty acid synthase/C75 mixture in (b) was used in parallel without going through the chromatography procedure. (B) RNA blot analysis for various tissues after C75 treatment. Male Fischer rats were administered, via i.p., either with vehicle RPMI (lanes 1, 4, and 7), or with 30 mg/kg body weight C75 in RPMI (lanes 2, 5, and 8), or food-deprived (lanes 3, 6, and 9). After 22 h, food consumption and body weight were measured. From the sacrificed animals, tissues of the hypothalamus, the rest of the brain without hypothalamus, and the livers were taken and total RNA was extracted. For samples of the hypothalamus (lanes 1–3) and brain (lanes 7–9), aliquots of 20 μg of RNA were loaded. For samples of the liver (lanes 4–6), aliquots of 10 μg of RNA were loaded. The RNA blot hybridization was performed as described in Materials and methods. (C) Measurement of hypothalamic fatty acid synthase activity after acute C75 i.p. administration. Male Fischer rats were administered with C75 i.p. delivered by RPMI at various concentrations as indicated. Three hours after dosing, the rats were sacrificed and the hypothalamus, brain (without hypothalamus), and liver were taken. Aliquots of 300 μg of crude cytosol extracts were subjected to radioactive fatty acid synthase enzymatic assays. Two animals were used for each dosing. For each animal sample, duplicated enzyme assays were performed. The data represent the average of the four data points and the bars represent standard deviation from the mean. FAS=fatty acid synthase.

acid synthase activities measured in vitro from the hypothalamus or other parts of brain would only reflect the level of enzyme activity, not fatty acid synthase protein quantity.

To assess the in vivo inhibition of hypothalamic fatty acid synthase after C75 administration, four groups of Fischer rats were dosed with vehicle, or C75 at 3, 10, and 30 mg/kg, i.p. After 3 h, we sacrificed the vehicle or C75-treated rats, prepared cytosol from the hypothalamus, and measured fatty acid synthase activity using a radioactive assay protocol. Consistent with Northern blot data in Fig. 4B, the fatty acid synthase protein content was constant among the animals throughout the dosing groups as assessed by an immunoblot analysis (Fig. 4C, inset). However, throughout the treatment, fatty acid synthase enzymatic activity was not diminished. These data provide direct evidence that hypothalamic fatty acid synthase was not inhibited at an anorectic dose of C75.

3.5. C75 activation of area postrema

Fenfluramine is a classic agent that is known to suppress food consumption. Acutely, it releases serotonin and blocks its reuptake. Although the actions of serotonin in feeding are thought to include sites in the gut, medulla, pons, amygdala, and hypothalamus, recent experiments indicate that the primary action might be mediated through the direct effect in the arcuate nucleus of hypothalamus (Heisler et al., 2002). To compare the patterns of CNS activation by C75 and fenfluramine, we examined the pattern of c-Fos induction by these two agents. In the hypothalamus, both fenfluramine and C75 caused a similar pattern of c-Fos induction, both in the arcuate nucleus and paraventricular nucleus (PVN) (Fig. 5A–D). A major difference was found in the area postrema where the induction of c-Fos was much more pronounced for C75 than fenfluramine (Fig. 6A and C). These results suggest

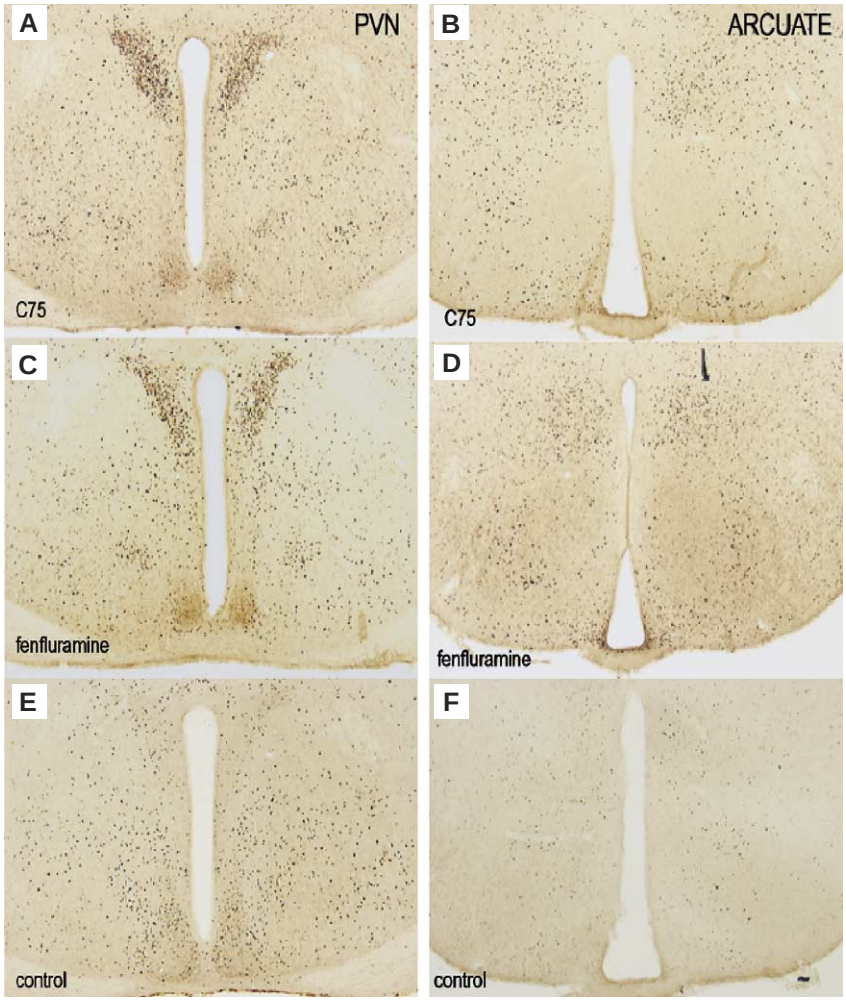

Fig. 5. Immunohistochemistry staining of anti-c-Fos for brain sections through the hypothalamus. Male Fischer rats were dosed with C75 at 10 mg/kg in vehicle RPMI (A and B) or with fenfluramine at 1 mg/kg in saline (C and D) through i.p. administration. *Control* is Fischer rats treated with both vehicles of RPMI and saline (E and F). Two hours after the drug administration or vehicle treatment, immunohistochemical staining was conducted according to the procedure as described in Materials and methods. For all the cohorts, four rats were treated and processed for immunohistochemistry analyses. Representative images are shown.

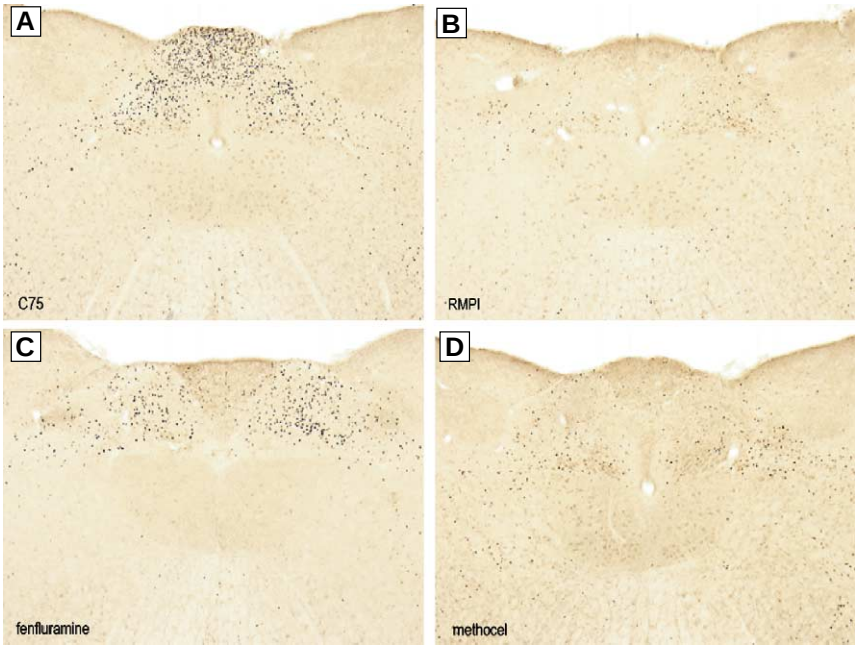

Fig. 6. Immunohistochemistry staining of anti-c-Fos for brain sections through the brainstem area postrema. Pharmacological treatment and immunohistochemistry staining were conducted as described in Fig. 5.

that the CNS effect of C75 may not solely be attributed to the hypothalamus. The activation of c-Fos in the area postrema, which may reflect satiety signals from periphery, or dysphoria or nausea, must be considered as a contributor to the C75 anorectic effect.

4. Discussion

In the current study, we designed a series of in vivo and in vitro pharmacological experiments to assess the relationship between the inhibition of fatty acid synthase and the anorectic effect of C75. The results of these studies do not support the view that inhibition of fatty acid synthase in the hypothalamus is the mechanism of action for appetite suppression by C75.

This conclusion is based on the following lines of evidence: 1) the maximal plasma concentration of C75 when dosed at 30 mg/kg, which caused total blockade of food intake, was determined to be 2.6±1.5 μM; 2) with in vitro studies, no inhibitory activity of C75 at or below 30 μM of C75 (a level >10-fold of C_{max}) could be detected against purified rat fatty acid synthase enzyme; 3) when C75 was dosed at 30 mg/kg (exhibiting total suppression of food consumption), no inhibition of hypothalamic fatty acid synthase could be demonstrated.

We used an acute animal feeding model as the pharmacodynamic assessment for C75. In a computer-assisted food consumption assay, we established that C75 decreased food consumption within the first 4 h after the animals were dosed i.p. In the same model, we also established a linear dosing range in which the appetite suppression was evident. The advantage of using an acute animal model is to avoid complications such as adaptation or other secondary responses that animals might develop with chronic C75 administration.

How do we reconcile the absence of hypothalamic fatty acid synthase inhibition by C75 at anorectic exposures? Evidence has recently emerged that inactivation of hypothalamic AMPK is involved in the leptin-mediated appetite suppression signaling pathway (Andersson et al., 2004; Minokoshi et al., 2004). Interestingly, C75, in ex vivo neuronal culture, caused fluctuation of AMP/ATP level (Landree et al., 2004). When administered to animals, C75 caused rapid reduction of AMPK phorphorylation of the α-subunit, whereby the AMPK activity is reduced (Kim et al., 2004). Inasmuch as acetyl-CoA carboxylase (ACC) is a substrate of AMPK, whose phosphorylation diminishes its enzyme activity (Hardie and Carling, 1997), the net effect of decreased AMPK level by C75 translate into the decreased level of phosphorylation in acetyl CoA carboxylase (Landree et al., 2004), which would increase acetyl-CoA carboxylase enzyme activity and its product malonyl-CoA. By this pathway (Fig. 7), C75 administration could increase levels of malonyl-CoA. Indeed hypothalamic malonyl-CoA levels were shown to be

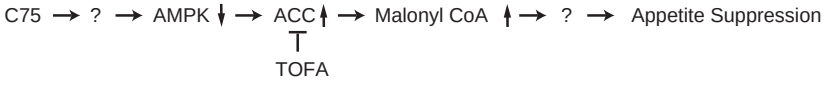

Fig. 7. Schematic drawing for the mechanisms of C75-elicited effect on malonyl-CoA concentration in the hypothalamus.

increased when C75 was delivered i.c.v. (Hu et al., 2003). When TOFA, an acetyl-CoA carboxylase inhibitor, was delivered, C75-induced appetite suppression was reduced, presumably through the blockade of C75-induced malony-CoA increases via acetyl-CoA carboxylase (Loftus et al., 2000). The increase in acetyl-CoA carboxylase activity by C75 reduction in AMPK activity is sufficient to explain the apparent increase of malonyl-CoA levels in the hypothalamus (Hu et al., 2003). At the transcription level, modulation of AMPK activity in the hypothalamus is sufficient to alter the expression levels of peptides involved in the appetite regulation. The infection of dominant-negative AMPK adenovirus in the hypothalamus reduced the mRNA expression of neuropeptide Y and agouti-related protein (Minokoshi et al., 2004).

From the literature and our current study, the identity of the direct molecular target of C75 in CNS remains elusive. Recently, Takahashi et al. demonstrated that C75 activates a wide spectrum of neurons, including pro-opiomelanocortin neurons, neuropeptide Y neurons, and Purkinje neurons. This observation led the authors to conclude that C75 is a nonspecific neuronal activator (Takahashi et al., 2004). The nonspecific neuronal activation could well be the reason for the fluctuation of AMP/ATP levels, which in turn modulate AMPK activity, leading to the anorectic effect.

Of possible importance in understanding the mechanism of anorectic action of C75 is to determine the biodistribution of C75 in CNS. It is possible that C75 elicits its action primarily through the area postrema, an area of the brainstem that is part of the dorsal vagal complex. This view is supported by the strong c-Fos staining of area postrema as demonstrated in the current study (Fig. 6) and by others in the field (Gao and Lane, 2003; Miller et al., 2004). Neuronal activation in a brainstem region that transmits satiety signals to the hypothalamus could lead to appetite suppression (Miller et al., 2004). The rather paradoxical observation that i.c.v. administration of C75 causes less anorexia than i.p. is consistent with this view (Fig. 1). Interestingly, c-Fos staining of area postrema is much stronger with C75 than fenfluramine, a classic anorectic agent whose site of action is generally considered to be at the level of the hypothalamus. The contribution of neuronal activation in area postrema by C75 may possibly reflect satiety signals from the periphery, or dysphoria or nausea (Clegg et al., 2002), and should be considered as a potential contributor to the anorectic effect of C75.

Our data do not rule out fatty acid synthase as a therapeutic target to treat metabolic syndrome via a peripheral effect. As fatty acid synthase is necessary for the synthesis of long-chain fatty acids, inhibiting fatty acid synthase could lead to the reduction of de novo synthesis of free fatty acid and triglyceride, which contributes greatly to fat deposition both in normal and obese human patients (Lammert et al., 2000; Strawford et al., 2004) as well as in experimental animals (Swierczynski et al., 2000). As the current study suggests that the C75 anorectic mechanism of action is not fatty acid synthase inhibition, obtaining a pharmacological proof-of-principle for fatty acid synthase as a therapeutic target awaits novel and potent fatty acid synthase inhibitors whose structures are sufficiently dissimilar to C75.

Acknowledgments

We thank our colleagues Reinhard Grzanna, Rupalie Meegalla, Cathy Kieras, Ian Mitchell, Jon Hangeland, James Devenny, Mary Ann Crissey, Susan Glick, and Rudolph Krause for contributing to the FAS project.

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