Neurochemical Research, Vol. 18, No. 2, 1993, pp. 231–233

Specificity of Calcium-Activated Neutral Proteinase (CANP) Inhibitors for Human μCANP and mCANP

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(Accepted July 8, 1992)

We investigated the relative inhibition of purified human μCANP and mCANP by five cysteine proteinase inhibitors including N-acetyl-Leu-Leu-nor-leucinal (C-I) and N-acetyl-Leu-Leu-methioninal (C-II), calpeptin, E64, and leupeptin. Based on IC_{50} measurements, calpeptin and C-I were stronger inhibitors by one to two orders of magnitude than C-II, leupeptin or E64. None of the five inhibitors, however, exhibited greater specificity for human μCANP or mCANP. These results indicate that, although the inhibition of a given cellular event by these compounds may suggest CANP involvement, effects on μCANP cannot be discriminated from those on mCANP.

KEY WORDS: Calcium-activated neutral proteinases; calpains; calpeptin; leupeptin; E64; human brain; proteinase inhibitors.

INTRODUCTION

Two forms of calcium-activated neutral proteinases (CANPs, or calpains) exist in the brain which, as purified enzymes, require calcium levels either in the micromolar range (μCANP) or in the millimolar range (mCANP) for maximum activity in vitro (for review, 11, 20). CANP activation has been implicated as an important factor in the cascade of events that leads to the degeneration of neurons in various pathological states (2,8,10,17,19). Moreover, we have shown that abnormal degrees of CANP activation may be a widespread phenomenon in the brains of individuals with Alzheimer disease (Saito, K.-I., Elce, J., Hamos, J., and Nixon, R. A., unpublished data). Since μCANP and mCANP are products of two different genes and differ in cellular distribution (5), they are likely to have distinct physiological functions. Inhibitors specific for each enzyme type would be helpful in characterizing these roles. At present, several inhibitors isolated from microbial cultures have been most commonly used. Of these, leupeptin inhibits not only CANPs but also other cysteine proteinases and serine proteinases (9,14,22,24). A second inhibitor in this group, E64, irreversibly inhibits CANPs and other cysteine proteinases (12,15). Recently, additional CANP inhibitors have been synthesized, including N-acetyl-Leu-Leu-norleucinal (C-I) and N-acetyl-Leu-Leu-methioninal (C-II), which have been used as selective CANP inhibitors (1,3,4,13,18). A newer compound, calpeptin, is considered to be more cell permeable than other CANP inhibitors (22). The specificity and relative inhibitory potency of these newer compounds toward different CANP forms has not been studied in detail, particularly in relation to the older inhibitors. For this reason, we have compared the inhibitory activities of these five compounds toward human μCANP and mCANP.

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0364-3190/93/0200-0231$07.00/0

EXPERIMENTAL PROCEDURE

C-I and C-II were purchased from Boehringer-Mannheim, Indianapolis, IN. Leupeptin was obtained from Sigma Chemical, St. Louis, MO. Calpeptin and E64 were synthesized at the Research Center, Mitsubishi Kasei Co., Yokohama, Japan. Human erythrocyte μCANP was partially purified as follows: Human blood was collected and immediately heparinized. Erythrocytes were washed four times with 5 mM Tris HCl, pH 7.4, 0.1M KCl, 6 mM NaCl, and 0.01M sucrose. Washed packed cells were hemolyzed for 10 min in 10 mM Tris HCl, pH 7.4, 1 mM benzamidine, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 15,000 × g for 30 min at 4°C. The hemolysate was added to Sephadex A-50 (Pharmacia LKB Biotech, Piscataway, NJ) and stirred very slowly overnight at 4°C. The slurry of resin was transferred to the column and washed with 50 mM Tris HCl, pH 7.4, 2 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 2 mM EDTA (Buffer A). Proteins were eluted with 0.15 M KCl in Buffer A and concentrated using Centriprep-10 membrane filters (Amicon, Danvers, MA). The protein concentrate, adjusted to 0.4 M KCl with 4 M KCl, was applied to a 1.5 × 8 cm column of phenylsepharose CL-4B (Pharmacia LKB Biotech., Piscataway, NJ) equilibrated with 0.4 M KCl in 50 mM Tris HCl, pH 7.4, 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 1 mM benzamidine and 1 mM PMSF (Buffer B). The column was washed with 0.4 M KCl in Buffer B to elute endogenous CANP inhibitors and the μCANP fraction was eluted with Buffer B. Human brain mCANP was purified as described previously (23). The calcium concentrations required for half-maximal and maximal activities of μCANP were 15 and 100 μM, respectively (Fig. 1). In the case of mCANP, half-maximal and maximal activities were observed at calcium concentrations of 100 μM and 1 mM (Fig. 1), consistent with previous results (23).

Enzyme activities were assayed using [^{14}C]azocasein as substrate (21, 23). The test compounds were preincubated with 12.5 units of CANP at 0°C for 30 min. One unit of CANP activity was defined as the amount of enzyme that digests 1 μg of [^{14}C]azocasein in 30 min at 30°C. The enzyme was added to a reaction mixture containing 250 μg substrate (specific activity: 160 cpm/μg [^{14}C]azocasein), 50 mM Tris HCl, pH 7.4, and 1 mM DTT and the reaction was started by adding 5 μl of either 0.1 M or 2.3 mM $CaCl_2$ for the mCANP or μCANP assays, respectively. After a 30 min incubation at 30°C, the reaction was terminated by adding 300 μl of ice-cold 10% (w/v) trichloroacetic acid solution. Enzyme activity was measured as the radioactivity in the acid-soluble fraction after centrifugation. Background radioactivity was estimated from samples incubated in the absence of calcium and in the presence of 1 mM EGTA. To obtain the concentration of the compounds required to achieve a 50% inhibition of enzyme activity (IC_{50}), four or five concentrations of each compound were used. In each experiment, the IC_{50} value was measured from dose-response curve and the mean value was calculated after all the experiments were done.

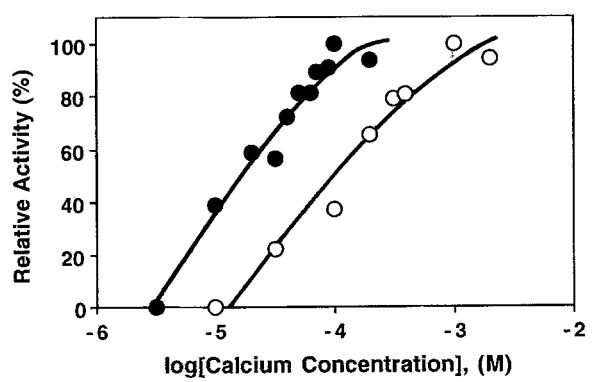

Fig. 1. Calcium requirement of μCANP and mCANP. μCANP and mCANP were prepared from human erythrocytes and human brain, respectively, as described in the text. The activities of 12.5 units of μCANP or mCANP against [^{14}C]azocasein were measured at varying calcium concentrations of Ca/EGTA buffer prepared by adding varying amounts of $CaCl_2$ to mixtures of 0.1 mM or 20 μM EGTA, respectively. The highest activity in the presence of calcium was taken as 100%. O: μCANP; O: mCANP.

RESULTS AND DISCUSSION

The inhibitory activities of these compounds toward μCANP and mCANP are indicated in Table I. None of the compounds showed a significant difference in their relative inhibitory potencies toward μCANP or mCANP, based on IC_{50}. Calpeptin, the most potent of the inhibitors, displayed IC_{50} values of 10 and 14 nM for μCANP and mCANP, respectively. The IC_{50} values for C-I toward μCANP and mCANP were 23 and 22 nM respectively, and C-II had 5-10 fold less potency than that of C-I. These results are consistent with other observations that C-I is a stronger inhibitor of human μCANP than is C-II (7), which could explain differences in their relative inhibitory effects on long-term potentiation in the hippocampus (3). In another report, however, C-I and C-II were equally potent toward μCANP or mCANP (14,18). A possible reason for this discrepancy may be the dif-

Table I. Inhibition of Human μCANP and mCANP by Proteinase Inhibitors

Compounds	IC_{50} (μM)	
	μCANP	mCANP
Calpeptin	0.010 ± 0.005 (4)	0.014 ± 0.002 (4)
C-I	0.023 ± 0.008 (6)	0.022 ± 0.004 (6)
C-II	0.129 ± 0.019 (6)	0.228 ± 0.002 (6)
Leupeptin	0.270 ± 0.006 (6)	0.375 ± 0.006 (6)
E64	1.53 ± 0.17 (4)	1.09 ± 0.10 (4)

μCANP and mCANP were prepared from human erythrocyte and human brain, respectively, as described in the text. Each compound was preincubated with 12.5 units of μCANP or mCANP at 0°C for 30 min. After adding the reaction mixture (substrate (specific activity: 160 cpm/μg [^{14}C]azocasein), 50 mM Tris HCl, pH 7.4, and 1 mM DTT), the reaction was started by adding $CaCl_2$. The enzyme activity was measured as radioactivity in the acid-soluble fraction after centrifugation. These values are means ± SEM for the number of determinations shown in parentheses.

ferent source of enzyme in the latter study (porcine [14,16]) vs. human [7, this study]).

Leupeptin is a less potent inhibitor of μCANP and mCANP. Since it also has inhibitory activity toward other cysteine proteinases and serine proteinases (18,26,36,38), its pharmacological effects in vivo cannot easily be ascribed to effects on CANPs. Although measurement of IC_{50} values is usually not appropriate for irreversible inhibitors, we also measured the IC_{50} value for E64, because Parkes et al. (12) demonstrated a close correspondence between the IC_{50} value for E64 and its rate constant for calpain activation. This epoxide inhibitor was the least inhibitory among the compounds we examined.

These results demonstrate that recently synthesized CANP inhibitors, especially calpeptin and C-I, have more potent inhibitory activity than leupeptin or E64. They do not show, however, greater specificity for μCANP or mCANP, even though certain of the compounds may be more specific for CANPs than for other cysteine proteinases (24). The results call for appropriate caution in interpreting the effects of these synthetic inhibitors in terms of the inhibition of specific protease systems.

ACKNOWLEDGMENTS

The authors wish to thank William Griffin for purifying human brain mCANP and Johanne Khan for help in preparing the manuscript for publication. These studies were supported by grants from the National Institute on Aging (AG08278 and AG05134) and by the Mitsubishi Kasei Corporation. Human brain tissue used for the isolation of mCANP was obtained from the McLean Hospital Brain Tissue Resource Center, which is supported by PHS Grant RO1-MH/NS31862 and the Seidel Research Fund.

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