neurons were found in the pretectal area. In the transverse plane at the level of caudal hypothalamus, TH-positive neurons were localized in the dorsal part of the NID; yet, they were not found to protrude with their processes into the 3rd ventricle (fig. 2, a). After incubation of the consecutive section with 5-HT antiserum, 'liquor contacting' neurons with specific immunofluorescence appeared at the dorsal margin of the lateral infundibular recess (LIR) in the NID (fig. 2, b). In the more rostral sections of the hypothalamus, TH-positive neurons were found to be scattered in the area lateral to the PVO, the so called PVO-accompanying cells[6], but the liquor contacting neurons in the PVO lacked TH-positive immunofluorescence (fig. 3, a). The liquor contacting neurons of PVO were found to show 5-HT-positive immunofluorescence, but the PVO-accompanying cells were negative (fig. 3, b). 5-HT-positive processes of these cells penetrated into the 3rd ventricle and their intraventricular protrusions had strong immunofluorescence and formed palisades (fig. 3, c). In the preoptic region, TH-positive liquor contacting neurons were widely distributed between the lining of ependymal cells and in the subependymal layer (fig. 4, a). These cells were more abundant in the rostro-dorsal part than in the caudoventral part. Apart from these cells, TH-positive neurons were seen in the suprachiasmatic region and in the periventricular gray around the preoptic recess. However, 5-HT-immunofluorescent cells were not detected in these areas (fig. 4, b).

Discussion. The distribution of monoaminergic neuron systems, as elucidated in the present immunohistochemical study, generally agrees with previous findings obtained by histofluorescent techniques[2-9]. However, some differences and new findings were seen in the present study.

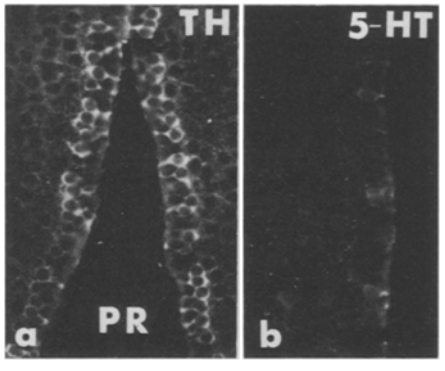

Figure 4. Immunofluorescent micrographs through the PRO in the transverse plane. *a* Numerous liquor contacting neurons with TH-positive immunofluorescence around the preoptic recess (PR) (× 190); *b* no 5-HT-positive immunofluorescence are present in the PRO (× 190).

1. In the caudal part of the 4th ventricle, there are TH-positive, CA-producing liquor contacting neurons but not 5-HT containing ones. 2. The liquor contacting neurons in the PVO and NID were found to be TH-negative but 5-HT-positive. 3. Small numbers of TH-positive neurons, but no 5-HT-positive neurons, were found in the pretectal area.

The liquor contacting neurons localized in the caudal brainstem, are similar in their morphology to the CA-producing, liquor contacting neurons in the PRO. Their localization suggests a phylogenetic relationship to the area postrema of mammals.

We also found besides 5-HT-containing liquor contacting neurons another type of neurons in the NID and around the PVO; especially in the NID, TH-positive neuronal perikaryas were noted to be situated near the 5-HT-positive liquor contacting neurons. Therefore, this region may be supplied with a specific innervation by both CA (dopamine) and 5-HT neurons.

From histofluorescent studies, it has been reported that 'liquor contacting' neurons of PVO contained both catecholamine and serotonin[2-9]. From our present immunofluorescent findings, it is now clear that 'liquor contacting' neurons of PVO contain only 5-HT but no TH-positive catecholaminergic neurons. This fact may suggest the possibility that monoamines (dopamine and 5-HT) were taken up from CSF into the liquor contacting neurons in PVO.

The functions of these 'liquor contacting' neurons are unknown. There may be 2 functions: 1. to discharge their neurosecretory material by way of an apocrine secretion from liquor contacting neurons into the ventricle; and 2. to receive information from the CSF that may influence the neurosecretory activity. Further studies are necessary to elucidate the physiological function of 'liquor contacting' neurons.

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A specific endogenous inhibitor of two forms of Ca^{++} activated neutral proteases in platelets

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Summary. An endogenous, specific inhibitor of high molecular weight has been isolated from bovine blood platelets, which inhibits the activity of the 2 forms of platelet Ca^{++} activated neutral proteases reported previously by us. The inhibition is not due to chelation of Ca^{++} but results from a stoichiometric complex formation.

The presence of a Ca^{++} activated neutral protease (CANP) has been reported in various tissues and it has been termed as KAF[1], CaAF[2], CANP[3], RTF[4]. Their physiological roles may consist in myofibrillar protein degradation[2,5] or activation of kinases[1,6], but these reported CANPs require a relatively higher concentration of Ca^{++}

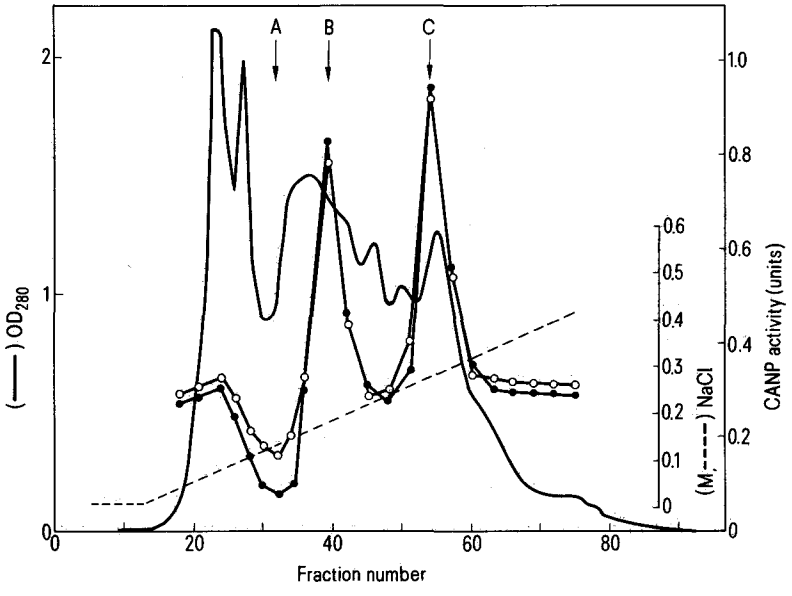

Figure 1. Resolution of 2 forms of CANPs and the inhibitor in bovine platelets on DEAE-Sepharose CL-6B column chromatography. The dialyzed supernatant of platelet lysate (20 ml, 67.8 mg of protein) was applied to a DEAE-Sepharose CL-6B column (1.5 × 17 cm) previously equilibrated with 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT (buffer A). The column was washed with 150 ml of the same buffer. Elution was performed with a 150 ml linear concentration gradient of NaCl (0–0.6 M) in buffer A. Each fraction contained 1.5 ml. A 30 µl aliquot of each fraction was added to the assay mixture containing 0.3 units of µ-CANP or m-CANP and then CANP activity was assayed in the presence of 4 mM $CaCl_2$ which activated both enzymes. CANP activity in the presence of 0.3 units of either µ-CANP (○——○) or m-CANP (●——●) indicates that fractions under arrow A possess the lowest CANP activities suggesting the presence of inhibitor against both enzymes.

for their activity than that physiologically attained in the cell. Recently, the presence of another type of CANP has been reported[7–9], which is fully activated in the presence of a micromolar level of Ca^{++}.

The presence of a Ca^{++} dependent protease in blood platelets was first suggested in 1977 by Phillips and Jakábová[10] and a platelet CANP requiring a millimolar Ca^{++} concentration was purified by Truglia and his colleagues in 1981[11]. Subsequently, we identified 2 forms of CANPs in the soluble fraction of bovine platelets[12]. One requires a millimolar concentration of Ca^{++} as seen in the report by Truglia et al.[11], while the other is fully activated in the presence of a micromolar level of Ca^{++}. Therefore in the present report, the former enzyme is termed m-CANP and the latter µ-CANP. During our purification of CANPs, an increase in total enzyme activity was encountered with both CANPs, in accordance with a similar increase in activity observed by Truglia et al.[11].

These observations suggest the presence of an endogenous inhibitor of CANPs in platelets. In other tissues, a specific, endogenous inhibitor against m-CANP has been identified by several investigators[14–16]. However, the inhibitor of CANP in platelets has never been identified. This prompted us to search for inhibitor(s) for the 2 forms of CANPs which might be present in the soluble fraction isolated from bovine platelets.

Methods and results. The activity of µ-CANP was assayed with heat-denatured casein as a substrate in 50 mM imidazole-HCl buffer (pH 7.0) containing 50 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM ethyleneglycol bis (β-aminoethylether)-N,N,N′, N′-tetraacetic acid (EGTA), 0.4 mM $CaCl_2$ according to the method described before[12]. The activity of m-CANP was measured by the same procedure as in µ-CANP except that 4 mM $CaCl_2$ was added. In the presence of 0.4 mM $CaCl_2$, µ-CANP is fully activated but the activity of m-CANP cannot be detected (results not shown). 1 unit of CANP activity was defined as reported previously[12]. Assay for the inhibitor was performed by the same method as CANP assay except that 0.3 units of µ-CANP or m-CANP was added to the reaction mixture containing the test sample and followed by the substrate.

Washed platelets (1.4×10^{11}) were obtained from fresh bovine blood (4.2 l) according to the method described before[12] and suspended in 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5 mM EDTA, 5 mM EGTA, 1 mM DTT. The platelets were disrupted by sonification (15 sec × 8 times) with a sonifier (Branson Sonic Power Company, Model W-185E) on a setting of 7. The platelet lysate was then centrifuged at 105,000 × g for 1 h and the resultant supernatant was dialyzed overnight against 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT. The dialyzed supernatant was fractionated on DEAE-Sepharose CL-6B column chromatography as shown in figure 1. 2 peaks of CANP activity (µ-CANP in peak B and m-CANP in peak C) were obtained. Both peak fractions were further purified by Sephadex G-150 column chromatography and used as µ-CANP or m-CANP in subsequent experiments. As seen in figure 1 material eluted with 0.1 M NaCl and indicated by arrow A contained apparent inhibitory activity against µ-CANP and m-CANP when tested subsequently with purified preparations of both enzymes. The fractions with inhibitory activity (arrow A) were pooled, concentrated by precipitation with $(NH_4)_2SO_4$ added to reach 70% saturation and chromatographed on Sepharose CL-6B column. In each of the elution profiles, the inhibitory activity against µ-CANP and m-CANP was eluted exactly in the same fraction (results not shown). The inhibitory fractions obtained from the 2nd

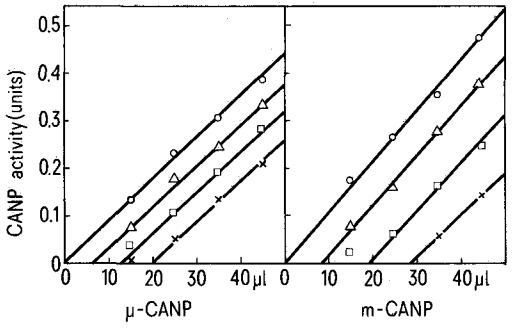

Figure 2. Interaction between 2 forms of CANPs and the inhibitor. Increasing amounts of µ- or m-CANP were added to tubes containing 0 µl (○——○), 8 µl (△——△), 16 µl (□——□), 24 µl (x–x) of the inhibitor. And µ- or m-CANP activity was assayed in the presence of 0.4 mM or 4.0 mM $CaCl_2$ using heat-denatured casein as a substrate.

Sepharose CL-6B column were pooled and used as a source of purified CANP inhibitor.

The molecular weight of this inhibitor was estimated to be $3.5\text{–}4.0 \times 10^5$ by gel filtration on Sepharose CL-6B according to a previously described procedure[12]. The inhibitor for CANP was heat stable since after heating at 98 °C for 20 min, more than 80% of its inhibitory activity remained. The inhibitory effect is apparently not due to chelation of Ca^{++} since the inhibition was not reversed by a high concentration of Ca^{++} (10 mM). Furthermore, the activity of trypsin, α-chymotrypsin, plasmin, thrombin, papain or ficin was not suppressed by the inhibitor, indicating its specificity to platelet CANPs.

As shown in figure 2, activities of μ-CANP and m-CANP were decreased in proportion to the amount of CANP inhibitor, which indicated that the inhibitor interacted stoichiometrically with CANP in a manner similar to a formation of the α_2-macroglobulin-protease complex[17]. In order to see whether inhibitor and CANPs form a high molecular weight complex in the absence of Ca^{++}, platelet extract was applied to the Sephadex G-200 column equilibrated with buffer containing EGTA. Inhibitor was eluted separately from CANPs, thus indicating a requirement for Ca^{++} in inhibitor-CANP complex formation (results not shown). When inhibitor was first preincubated with CANPs in the absence of Ca^{++} and then tested in the standard assay system containing 0.4 mM $CaCl_2$ or 4.0 mM $CaCl_2$, a similar decrease in activities of μ-CANP and m-CANP was observed. Thus, preincubation in the absence of Ca^{++} did not affect inhibitory activity.

Discussion. In the present study, a high molecular weight endogenous inhibitor of platelet CANPs was identified and characterized. This inhibitor was found to be specific for platelet CANPs in that it did not interfere with other enzymes, such as trypsin, etc. The interaction of the inhibitor with CANPs from other sources has not been studied. The newly described platelet CANP inhibitor is different from thiol protease inhibitors identified in the skin and leukocytes, since molecular weights of these inhibitors are low and they suppress the activity of papain or ficin[18,19].

Although CANP and its inhibitor were dissociated in the absence of Ca^{++} during gel chromatography on Sephadex G-200, the inhibitory effect was exerted by forming a stoichiometric complex of CANP and the inhibitor in the presence of Ca^{++}, indicating that Ca^{++} is involved in the binding of inhibitor to CANPs.

A similar inhibitor of m-CANP has been identified in cardiac muscle, brain and liver[14–16]. In these tissues, inhibition of μ-CANP by its endogenous inhibitor has not been studied but we have demonstrated in this study that the inhibitor can also block the activity of μ-CANP which is considered to be physiologically more significant than m-CANP because of its higher affinity for Ca^{++}.

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Synthesis and RES-stimulating activity of bacterial cell-wall peptidoglycan peptides related to FK-156

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Summary. The bacterial cell-wall peptidoglycan peptides and related compounds coupled with some fatty acids were synthesized, and their immunostimulating property was examined by carbon clearance assay. All the new compounds (**2–6**) proved to possess significant potencies superior to that of FK-156 (**1**).

A unique immunostimulating activity displayed by bacterial cell-wall peptidoglycans has attracted considerable attention in recent years[3]. In the preceding papers[4], we reported the synthesis of FK-156 (**1**), an immunostimulating microbial metabolite with a structurally close resemblance to the bacterial cell-wall peptidoglycan peptides. In connection with a major ongoing program on this immunostimulant, we were interested in preparing some analogues bearing D-analine instead of glycine in **1**, because such a peptide sequence comprises the fundamental unit of the peptidoglycan peptides in most gram-negative bacterial cell-walls[5]. Herein we report the syntheses of compounds (**2–6**) of this D-alanine series and their biological activity. The immunostimulating property of these compounds proved to be significantly superior to that of **1**.

The compounds of interest were synthesized starting from