Three distinct anti-allergic drugs, amlexanox, cromolyn and tranilast, bind to S100A12 and S100A13 of the S100 protein family

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To investigate the roles of calcium-binding proteins in degranulation, we used three anti-allergic drugs, amlexanox, cromolyn and tranilast, which inhibit IgE-mediated degranulation of mast cells, as molecular probes in affinity chromatography. All of these drugs, which have different structures but similar function, scarcely bound to calmodulin in bovine lung extract, but bound to the same kinds of calcium-binding proteins, such as the 10-kDa proteins isolated in this study, calcyphosine and annexins I–V. The 10-kDa proteins obtained on three drug-coupled resins and on phenyl-Sepharose were analysed by reversed-phase HPLC. It was found that two characteristic 10-kDa proteins, one polar and one less polar, were bound with all three drugs, although S100A2 (S100L), of the S100 family, was bound with phenyl-Sepharose. The cDNA and deduced amino acid sequence proved our major polar protein to be identical with the calcium-binding protein in bovine amniotic fluid (CAAF1, S100A12). The cDNA and deduced amino acid sequence of the less-polar protein shared 95% homology with human and mouse S100A13. In addition, it was demonstrated that the native S100A12 and recombinant S100A12 and S100A13 bind to immobilized amlexanox. On the basis of these findings, we speculate that the three anti-allergic drugs might inhibit degranulation by binding with S100A12 and S100A13.

Key words: affinity chromatography, calcium-binding protein, cDNA cloning, degranulation, exocytosis.

INTRODUCTION

Release of chemical mediators by mast cells and basophils with degranulation has many important roles, especially in localized reactions. This release of mediators upon immunological stimulation is assumed to be the main pathological mechanism of immediate-type allergic reactions, for instance of bronchial asthma, urticaria and anaphylaxis. Despite improvements in the diagnosis and treatment of bronchial asthma, the number of patients suffering from the disease and the rates of morbidity and mortality have increased over the last two decades [1–3]. Therefore, elucidation of the mechanisms of degranulation and the intracellular signal transduction involved is important, so much research on the mechanisms of degranulation has been performed. In many papers it has been already indicated that calcium ions must initiate a number of different processes that lead to exocytosis, including the movement of secretory vesicles to the plasma membrane, docking of vesicles at the plasma membrane prior to fusion, and fusion of vesicles with the plasma membrane [4–6]. In addition, several proteins have been identified as candidates required in exocytosis, e.g. annexin VII (synexin) [7–9], annexin II [10,11], GTP-binding proteins [12,13], calcyclin [14,15] and others. Despite research on the progression of exocytosis, most of the details of this phenomenon still remain unknown.

To investigate the correlation between calcium-binding proteins and exocytosis, we previously attempted to use anti-allergic drugs, amlexanox and cromolyn, which inhibit the degranulation of mast cells, as molecular probes for affinity chromatography. We performed the drug-affinity chromatographic procedure with bovine lung extract, and reported that both of these drugs bind to the same kind of calcium-binding proteins, except calmodulin (CaM) [16]. In the present study, we have used three kinds of anti-allergic drug, amlexanox, cromolyn, and tranilast, as ligand in affinity chromatography, and examined binding of the drugs to the same kinds of protein, even though the structures of the drugs are different. Further, the specificity of these three drugs binding to 10-kDa proteins was compared with that of phenyl-Sepharose, as a negative model of anti-allergic drugs. As a result, it has been clarified that both of two kinds of 10-kDa protein bind characteristically to these drugs. These proteins are presumed to be major targets for these three anti-allergic drugs, and are thought to play a significant role in degranulation. Therefore, we elucidated the structure of these proteins by partial amino acid sequence analysis and cDNA cloning.

EXPERIMENTAL

Materials

Tranilast [rizaben, *N*-(3,4-dimethoxy-cinnamoyl)anthranilic acid] was a generous gift from Kissei Yakuhin Co. (Matsumoto, Japan). Phenyl-Sepharose 6 FF was bought from Pharmacia Biotech (Uppsala, Sweden). SDS/PAGE molecular mass standards (low range) and the prestained protein standard (broad range) were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). The pT7Blue T-Vector was obtained from Novagen

Abbreviations used: CaM, calmodulin; AF, AF-amino TOYOPEARL 650M; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride; TFA, trifluoroacetic acid; CAGC, calgranulin C.

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The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number AB001567.

(Madison, WI, U.S.A.). A bovine lung Uni-ZAP XR library was purchased from Stratagene (La Jolla, CA, U.S.A.). *Taq* polymerase and other modifying enzymes were obtained from Takara Shuzo Co. (Kyoto, Japan). Restriction endonucleases were bought from New England Biolabs (Beverly, MA, U.S.A.). Digoxigenin-11-dUTP was purchased from Boehringer Mannheim (Mannheim, Germany). All other chemicals were the same as in the report published previously [16].

Coupling of ligand to matrix for drug-affinity chromatography

The coupling of anti-allergic drugs to AF-amino TOYOPEARL 650M (AF) resin was carried out as described previously [16]. Briefly, amlexanox (0.1 g) dissolved in 1 ml of *N*,*N*′-dimethylformamide was added to the AF resin (7 ml, $\approx$ 5 g wet mass), and *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC; 0.15 g) suspended in 10 ml of *N*,*N*′-dimethylformamide was added to the mixture. After adjusting the pH to 5.0, the mixture was incubated with gentle shaking for 48 h at 25 °C. The coupling of cromolyn (0.25 g) and tranilast (0.2 g) each to AF resin (5 g wet mass) by 0.5 g of EDC was carried out in a similar manner.

Drug-affinity chromatography of the bovine tissue extract

Drug-affinity column chromatography was carried out as described previously [16]. Briefly, bovine lung (200 g) was homogenized in 5 volumes of buffer A (20 mM Tris/HCl/0.1 mM EGTA, pH 7.5). The homogenate was centrifuged at 15000 ***g*** for 30 min, and the supernatant fluid was adjusted to a calcium concentration of 0.5 mM, followed by centrifugation at 15000 ***g*** for 30 min. The protein solution obtained above (150 ml) was applied to the tranilast-coupled AF column (1.5×3 cm) which was pre-equilibrated with buffer B (20 mM Tris/HCl/0.5 mM $CaCl_2$, pH 7.5). After these columns were washed with 500 ml of buffer C (20 mM Tris/HCl/0.2 mM $CaCl_2$, pH 7.5), proteins were eluted with 150 ml of buffer D (20 mM Tris/HCl/2.0 mM EGTA, pH 7.5). The same experiments were performed with cromolyn-coupled and amlexanox-coupled AF columns, and a control column (1.5×3 cm).

Phenyl-Sepharose column chromatography of the bovine lung extract

The protein solution obtained above (450 ml) was also applied to a phenyl-Sepharose column (2.5×5 cm) pre-equilibrated with buffer B. After the column was washed with buffer C (1000 ml), proteins were eluted with buffer D (250 ml).

Analytical procedures

Tricine/SDS/PAGE (12 %) was performed by the method of Schagger and von Jagow [17]. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. The protein concentrations of eluate from chromatography were measured by the method of Bradford [18]. For partial amino acid sequence, purified proteins were digested with lysylendopeptidase (EC 3.4.21.50) in 0.1 M Tris/HCl buffer (pH 9.0). After overnight incubation at 37 °C, the proteolytic fragments were separated by HPLC (model LC-10A, Shimadzu, Kyoto, Japan) with a C_{18} reversed-phase column (Tosoh TSKgel ODS-80, 4.6×25 cm) with a linear gradient from 0 to 80 % (v/v) acetonitrile in the presence of 0.1 % (v/v) trifluoroacetic acid (TFA) at flow rate of 1 ml/min. The amino acid sequence of each proteolytic fragment was determined with automated protein sequencers (model 476A, Applied Biosystems, Foster City, CA, U.S.A. or model PPSQ-21, Shimadzu).

Separation and purification of lower-molecular-mass proteins from the EGTA eluate

The EGTA eluates obtained from amlexanox–AF, cromolyn–AF and tranilast–AF columns and the phenyl-Sepharose column were concentrated 50-fold and separated preparatively by 12 % Tricine/SDS/PAGE using a pre-stained SDS/PAGE standard (broad range, Bio-Rad) as a molecular-mass marker. The lower molecular-mass area (7–13 kDa) of the electrophoresed gel was excised by a cutter and the proteins were recovered from the gel pieces using a electroelutor (Little Blue Tank, Isco, Inc., Lincoln, NE, U.S.A.). The protein solution containing 0.1 % TFA was subjected to reversed-phase HPLC on the C_{18} column equilibrated with 0.1 % (v/v) TFA, and eluted with a linear gradient from 25 to 64 % (v/v) acetonitrile containing 0.1 % (v/v) TFA at a flow rate of 1 ml/min, followed by a linear gradient from 64 to 80 % (v/v) acetonitrile. The eluates corresponding to peaks were collected and freeze-dried. The amino acid sequences of the purified proteins were analysed.

Synthesizing an authentic DNA probe for the 10-kDa anti-allergic-drug-binding protein 'a' and library screening

Bovine lung genomic DNA was isolated by a conventional method [19] and was used as a template for synthesizing the authentic DNA probe of the 10-kDa amlexanox-binding protein 'a'. Two PCR primers; a forward primer, a, 5′-ATHTTYCAYYTNGAYGCNGAYAA-3′, based on IFQDLDADK, and a reverse primer, b, 5′-YTTRTGDATRTCDATRTGNGCNGT-3′, based on TAHIDIHK, were designed according to the peptide sequence of the protein (see Figure 4 below). PCR amplification (35 cycles) was performed in 100 μl of reaction mixture containing 100 ng of bovine lung DNA, 100 pmol of the primers and the reagents described in Perkin-Elmer's amplification protocol. PCR cycles consisted of 1 min of denaturation at 94 °C, 1 min of annealing at 45 °C, and 1 min of polymerization at 72 °C in a thermocycler (Perkin-Elmer). The PCR product was cloned into the pT7Blue T-Vector and was used as the authentic DNA probe after confirming the probe by sequencing. About 5×10^4 plaques from a bovine lung Uni-ZAP XR cDNA library were screened with the authentic DNA probe labelled with digoxigenin-11-dUTP by PCR amplification. Hybridization was performed overnight with the probe in a solution of $5 \times$ SSC (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1 % sodium *N*-lauroyl sarcosinate/0.02 % SDS/1 % blocking reagent (Boehringer Mannheim) at 55 °C. Membranes were washed twice in $2 \times$ SSC/0.1 % SDS (5 min, 25 °C) and twice in $0.1 \times$ SSC/0.1 % SDS (15 min, 55 °C), blocked with buffer 1 (100 mM maleic acid/150 mM NaCl, pH 7.5) containing 1 % blocking reagent (30 min, 25 °C), incubated with primary antibody (1:5000 anti-digoxigenin-Fab fragments/buffer 1, 30 min, 25 °C), and then washed twice with buffer 1 (15 min, 25 °C). Three positive plaques were identified by chemiluminescence (Lumiphos 530 reagent, Wako Chemicals, Osaka, Japan). These inserts were used for sequencing.

Synthesizing an authentic DNA probe for the 10-kDa anti-allergic-drug-binding protein 'e' and library screening

Single-stranded cDNA was prepared from the poly(A)$^+$ RNA, using reverse transcriptase with random 9-mers as primers, and used as a template for synthesizing the authentic DNA probe for

the 10-kDa anti-allergic-drug-binding protein 'e'. Three degenerate oligonucleotide primers; a forward primer, a, 5′-GGNWSNYTNWSNGTNAAYGARTTYAA-3′, based on GSLSVNEFK, and reverse primers, b-1, 5′-YTTNGCNARY-TCNCCDATNARNCKCC-3′, based on WRLIGELAK, and b-2, 5′-DATNARNCKCCARTAYTCNWSRA-3′, based on FSEYWRLI, were designed according to the peptide sequence of the protein (see Figure 5 below). With these primers, we performed a nested PCR to obtain a more specific product. The first round of PCR reactions was performed in a total volume of 100 μl containing 5 ng of single-stranded cDNA, 100 pmol of each primer (a and b-1), 1 × PCR buffer (Perkin-Elmer), 2 M of each dNTP, 3 mM $MgCl_2$ and 2.5 units of *Taq* polymerase. The 30 cycles were performed at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min in the thermocycler. A part of this reaction solution (3 μl of 100 μl) was used as a template for the second-round PCR reaction in a total volume of 100 μl containing 100 pmol of each primer (a and b-2). The other PCR cycles and conditions were all the same as above. The nested PCR product (150 bp) was ligated into the pT7Blue T-Vector. The *Bam*HI- and *Nde*I-cleaved fragments from the single clone were used as the authentic DNA probe after confirming the probe by sequencing. About 6×10^4 plaques from a bovine lung Uni-ZAP XR cDNA library were screened by the same method as described above. Three positive plaques were identified by chemiluminescence. These inserts were used for the sequencing analysis.

Affinity chromatography of native protein 'a' and recombinant proteins 'a' and 'e' on amlexanox–AF column

The purified native protein 'a' (0.2 mg) in buffer B (8 ml) was loaded into an amlexanox–AF column (1.5 × 3 cm) pre-equilibrated with buffer B. After washing the column with buffer C, the protein was eluted with buffer D containing EGTA followed by elution with 20 mM Tris/HCl buffer (pH 7.5) containing 6 M urea. Each 5 ml of eluate was collected. Each 0.5 ml of eluate was freeze-dried and used for SDS/PAGE. Affinity chromatography of the recombinant proteins 'a' (0.6 mg) and 'e' (0.6 mg) was performed with the amlexanox–AF column in a similar manner as the native protein 'a'.

RESULTS

Isolation of calcium-binding proteins from lung extract by anti-allergic drug-affinity chromatography

To examine the calcium-binding proteins that interact with anti-allergic drugs, we used three kinds of drug, amlexanox, cromolyn and tranilast, as ligands in affinity chromatography. The anti-allergic drug-coupled carriers were prepared by dehydrative coupling of these drugs to the AF resin, which is a hydrophilic polymer with amino groups. Extracts of bovine lung containing Ca^{2+} were applied to the drug-coupled columns. After the columns were washed, the retained proteins were eluted with an EGTA-containing buffer. Figure 1 shows 12% Tricine/SDS/PAGE patterns of the isolated proteins from the amlexanox–AF column (Figure 1, lane 3), cromolyn–AF column (Figure 1, lane 4), tranilast–AF column (Figure 1, lane 5) and a blank column for control (Figure 1, lane 2). The calcium-binding proteins obtained showed three major groups at about 10, 22 and 32–42 kDa (Figure 1, lanes 3–5). These SDS/PAGE patterns clearly indicate that the proteins isolated by these drug-coupled columns are essentially similar, and that these proteins bind to ligands, not to the matrix. The electrophoretic pattern indicates that low-molecular-mass proteins are quantitatively dominant in these binding proteins. For comparison, phenyl-Sepharose, which is often used in hydrophobic-interaction chromatography, was examined instead of the drug-coupled resins, and the Tricine/SDS/PAGE of the EGTA eluate from phenyl-Sepharose

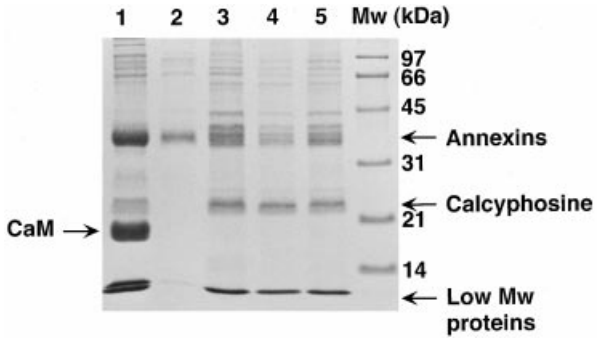

Figure 1 Tricine/SDS/PAGE (12%) of the proteins obtained from bovine lung tissues after drug-affinity chromatography

Details of these conditions have been described in the text. The gel was stained with Coomassie Brilliant Blue R-250. Molecular-mass (Mw) standards are shown in kDa. Lane 1, proteins obtained from phenyl-Sepharose by eluting with EGTA. Lanes 2–5, proteins obtained from the anti-allergic-drug-affinity columns by eluting with EGTA. Lane 2, acetylated amino column for control; lane 3, amlexanox–AF column; lane 4, cromolyn–AF column; lane 5, tranilast–AF column. Arrows on the right indicate positions of major binding proteins of these anti-allergic drugs.

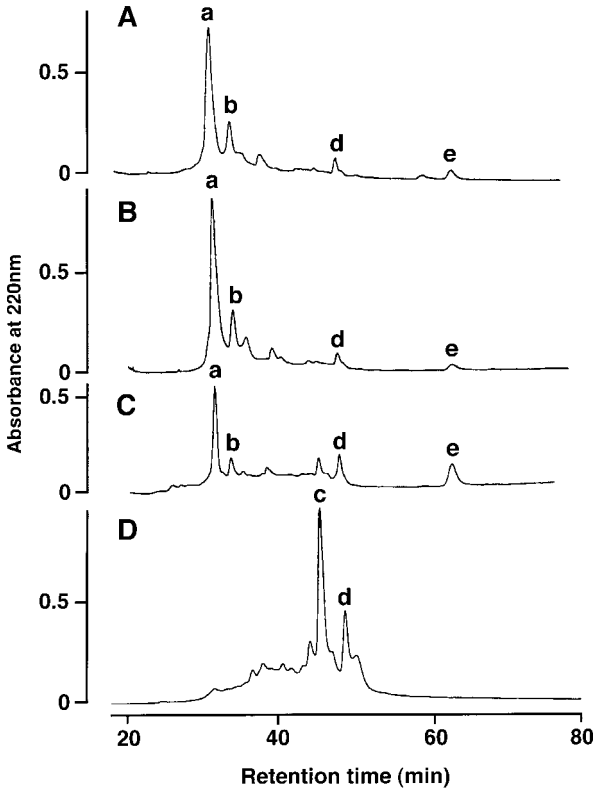

Figure 2 Elution profiles of C_{18} reversed-phase HPLC of the 10-kDa proteins in EGTA from drug-affinity chromatography of bovine lung extract

(**A**) Tranilast–AF column; (**B**) cromolyn–AF column; (**C**) amlexanox–AF column; (**D**) phenyl-Sepharose column. Details of the conditions are described in the text. Designated proteins 'a'–'e' were collected manually and digested with lysylendopeptidase for protein-sequence analysis.

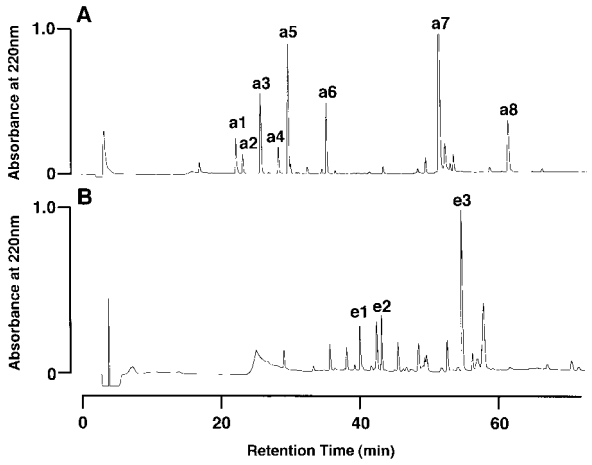

Figure 3 C_{18} reversed-phase HPLC elution profiles of lysylendopeptidase-digested peptides of the purified 10-kDa proteins 'a' and 'e'

The proteins 'a' and 'e' were digested and cleavage products were separated by HPLC on a C_{18} column. HPLC elution profiles of (**A**) protein 'a' and (**B**) protein 'e' are shown. Designated fragments (a1–a8) and (e1–e3) were then sequenced. Details of the condition are described in the Experimental section.

is shown in Figure 1 (lane 1). The three anti-allergic drugs scarcely bind CaM (Figure 1, lanes 3–5), although CaM was bound mainly by phenyl-Sepharose (Figure 1, lane 1).

Comparisons of the composition of lower-molecular-mass proteins by reversed-phase HPLC

The EGTA-eluates obtained from amlexanox–AF, cromolyn–AF and tranilast–AF columns, and by phenyl-Sepharose column, were separated preparatively by 12% Tricine/SDS/PAGE using pre-stained molecular-mass markers as an index. The lower-molecular-mass proteins ($\approx$ 7–13 kDa) were recovered from the electrophoresed gel using a electroelutor. The protein solutions obtained were subjected to C_{18} reversed-phase HPLC and the eluates corresponding to peaks were collected. Figure 2 shows the chromatographic profile of the lower-molecular-mass proteins obtained from the tranilast–AF (Figure 2A), cromolyn–AF (Figure 2B) and amlexanox–AF columns (Figure 2C), and the phenyl-Sepharose column (Figure 2D). The chromatographic patterns of the lower-molecular-mass proteins from the three anti-allergic drug-coupled columns looked like, whereas that of the phenyl-Sepharose column differed from the drug-coupled AF columns. As shown in Figure 2, all of the anti-allergic drugs showed three characteristic protein peaks; 'a', 'b' and 'e', which were eluted at different retention times. The major and more polar protein 'a' was eluted after approximately 32 min, and the minor and less polar one, 'e', was eluted after 64 min. A protein which was eluted at $\approx$ 34 min was named 'b'. The major two peaks from phenyl-Sepharose were named 'c' and 'd'. Composition (%) of each protein determined by HPLC was as follows ('a'–'e' respectively): tranilast, 41.6, 12.7, 0.1, 2.8 and 2.9%; cromolyn, 44.4, 13.0, 0.1, 2.7 and 1.8%; and amlexanox, 32.1, 7.5, 5.6, 9.4 and 15.9%.

Amino acid sequences of the lower-molecular-mass proteins

Amino acid sequences of the purified proteins 'a'–'e' obtained from the drug-coupled AF columns and the phenyl-Sepharose column were analysed partially after lysylendopeptidase digestion followed by HPLC separation. Peptide mappings on HPLC of the proteins 'c' and 'd' from phenyl-Sepharose were similar. Five peptides from the protein 'c', YSGQEGDK, ELLHK, ELPSFVGEK, VDEEGLK and LMGDLDENSDQQVDFQEY, could be sequenced. A computer homology search revealed the protein 'c' to be S100A2 (S100L) of S100 protein family [20]. The sequence of protein 'd' was the same as that of protein 'c'. Thus both proteins 'c' and 'd' are considered to be S100A2 variants. In the same way, it was confirmed that the protein 'd' from the drug-coupled columns was a S100A2 variant.

The reversed-phase HPLC of the digested peptides of proteins 'a' and 'e' are shown in Figure 3. Designated fragments were sequenced. The sequencing of protein 'a' determined the 13 N-terminal amino acids to be TKLEDHLEGIINI. Furthermore,

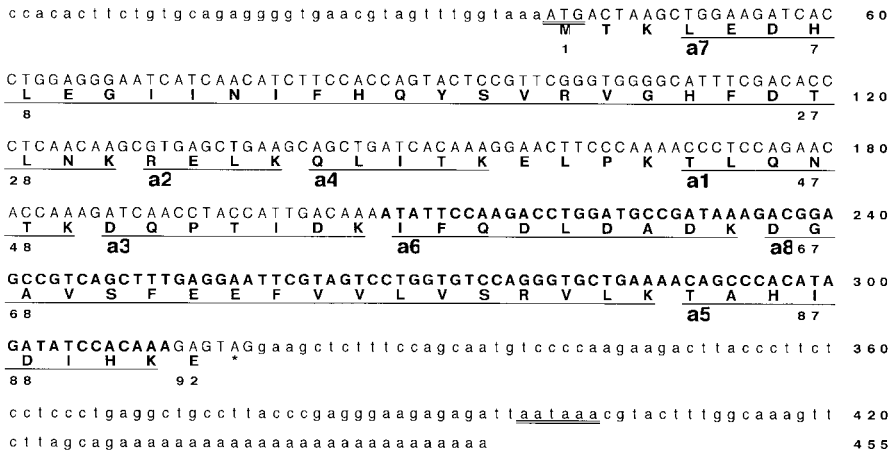

Figure 4 The nucleotide sequence of the cDNA coding the bovine 10-kDa anti-allergic-drug-binding protein 'a' and the deduced amino acid sequence

The predicted protein sequence is shown below the nucleotide sequence in one-letter code. The nucleotide sequence is numbered from the initiation site of this clone (see numbering on the right). The translated region is shown in capital letters and the 5′- and 3′-non-coding regions are shown in lower-case letters. The sequences obtained by micro-sequencing of peptides a1–a8 are underlined. Fragment designations correspond to those shown in Figure 3(**A**). The 105-bp DNA fragment used as a probe is shown in bold. The initial methionine residue and the polyadenylation signal are double underlined. An asterisk indicates the termination codon.

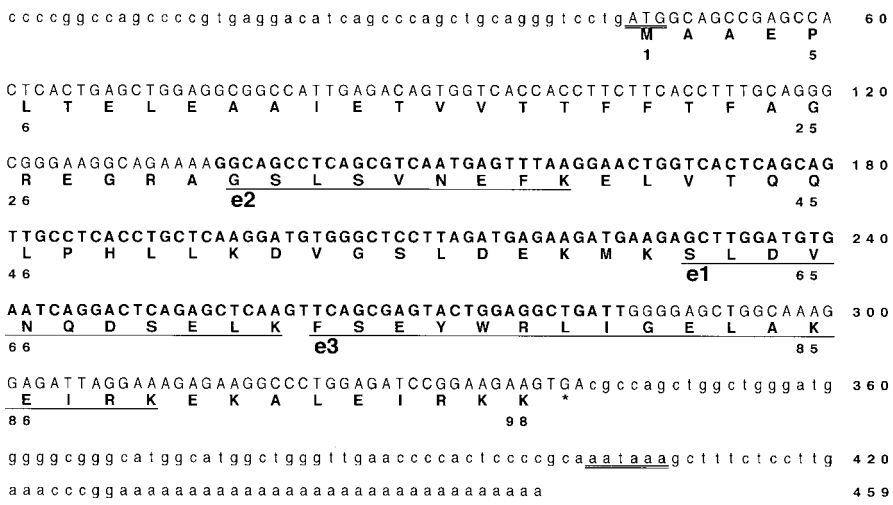

Figure 5 The nucleotide sequence of the cDNA encoding the bovine 10-kDa anti-allergic-drug-binding protein 'e' and the deduced amino acid sequence

Nucleotide sequences and predicted protein sequences of protein 'e' (accession number AB001567) are shown. The nucleotide sequence is numbered starting at the initiation site of this clone (see numbering on the right). The translated region is shown in capital letters and the 5′- and 3′-non-coding regions are shown in lower-case letters. The predicted protein sequence is shown below the nucleotide sequence in one-letter code. The sequences obtained by micro-sequencing of peptides e1–e3 are underlined. Fragment designations correspond to those shown in Figure 3(**B**). The 150-bp DNA fragment used as a probe is shown in bold. The initial methionine residue and the polyadenylation signal are double underlined. An asterisk indicates the termination codon.

eight fragments from the HPLC separation: a1, TLQNTK; a2, RELK; a3, DQPTIDK; a4, QLITK; a5, TAHIDIHK; a6, IFQDLDADK; a7, LEDHLEGIINIFHQYSVRVGHFDTL-NK; and a8, DGAVSFEEFVVLVSRVLK, could be sequenced (Figure 3A and Figure 4). A computer homology search revealed that protein 'a' was 81% similar to calgranulin C (CAGC), previously identified in pig granulocytes [21]. The protein 'a' was apparently a bovine homologue of CAGC. Partial amino acid sequence analysis of protein 'e' determined three fragments from the HPLC separation: e1, SLDVNQDSELK; e2, GSLSVNEFK; and e3, FSEYWRLIGELAK (Figure 3B and Figure 5). The sequencing of protein 'e' revealed that one of the fragments (e2) had 77.8% similarity with the sequence from residues 36 to 44 (GSLSVDEFM) of calcineurin B-like protein [22], but other fragments (e1 and e3) did not show great similarity with any other proteins. Therefore, we cloned and sequenced a cDNA encoding proteins 'a' and 'e' from a bovine lung cDNA library. Peptide mapping results and the amino acid sequence of proteins 'a' and 'e' obtained from cromolyn–AF and tranilast–AF columns were the same as those of proteins 'a' and 'e' from the amlexanox–AF column. Furthermore, the peptide mapping results and amino acid sequence of the protein 'b' from the three drug-coupled columns were extremely similar to those of the protein 'a'. Thus the protein 'b' is considered to be a variant of the protein 'a'.

cDNA cloning and sequencing of the 10-kDa anti-allergic-drug-binding protein 'a'

Isolation of a cDNA clone coding for protein 'a' (CAGC homologue) was carried out to determine the characteristics of the primary structure of the protein. At first, the authentic DNA probe of the protein was obtained by PCR using two primers synthesized according to the peptide sequence of the protein. A 105-bp band of expected size was isolated and cloned into the pT7Blue T-Vector. The 105-bp fragment, identified by sequencing, was used as the authentic probe (Figure 4, indicated in bold type). In screening about 5×10^4 plaques from a bovine lung Uni-ZAP XR cDNA library performed with the authentic probe, we obtained a positive clone of 455 bp in length and analysed it. Figure 4 delineates the nucleotide and the deduced amino acid sequences of the entire cDNA insert from the recombinant plasmid clone of the bovine CAGC homologue. The plasmid clone was 455 bp in length and contained 39 bp of a 5′-leader region, a 279-bp coding region including a termination codon, TAG, and 137 bp of a 3′-non-translated region. The polyadenylation signal sequence (AATAAA) is shown at nucleotide position 398–403. The cDNA and deduced amino acid sequence of the CAGC homologue was initially not found to have significant similarity with any protein except pig CAGC in the GenBank database. During preparation of this manuscript, the cDNA sequence of the calcium-binding protein in amniotic fluid-1 (CAAF1) was published [23], and the deduced amino acid sequence of the protein was identical with that of our protein 'a'.

cDNA cloning and sequencing of the bovine 10-kDa anti-allergic-drug-binding protein 'e'

We isolated a cDNA clone coding for protein 'e' to determine the characteristics of the primary structure of the protein. First, the authentic DNA probe of the protein was obtained by nested PCR using three degenerate oligonucleotide primers synthesized according to the peptide sequence of the protein. A 150-bp band of the expected size was isolated and cloned into the pT7Blue T-Vector. The 150-bp fragment identified by sequencing was used as the authentic probe (Figure 5, indicated in bold type). By screening about 6×10^4 plaques from a bovine lung Uni-ZAP XR cDNA library with the authentic probe, we obtained a positive clone of 459 bp in length and analysed it. Figure 5 shows the nucleotide and the deduced amino acid sequences of the entire cDNA insert from the recombinant plasmid clone of the bovine 10-kDa protein. The protein was 459 bp in length and contained 45 bp of a 5′-leader region, a 297-bp coding region including a termination codon, TAG, and 117 bp of a 3′-non-translated

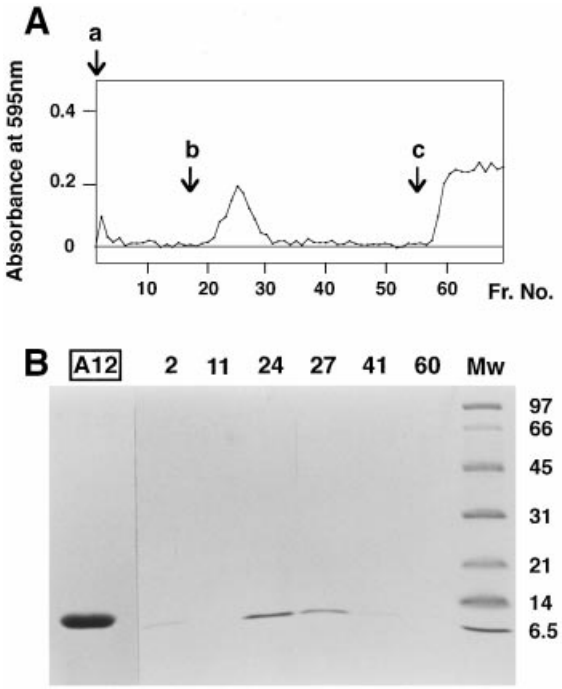

Figure 6 Affinity chromatography of native S100A12 (protein 'a')

(**A**) Elution curve of the native protein from the amlexanox–AF column. a, Sample load followed by washing; b, addition of buffer containing EGTA; c, addition of buffer containing urea. (**B**) Tricine/SDS/PAGE (12%) analysis of native S100A12 and the fractions (2, 11, 24, 27, 41 and 60) from amlexanox–AF column chromatography (see **A**). Size standard (Mw) is shown in kDa.

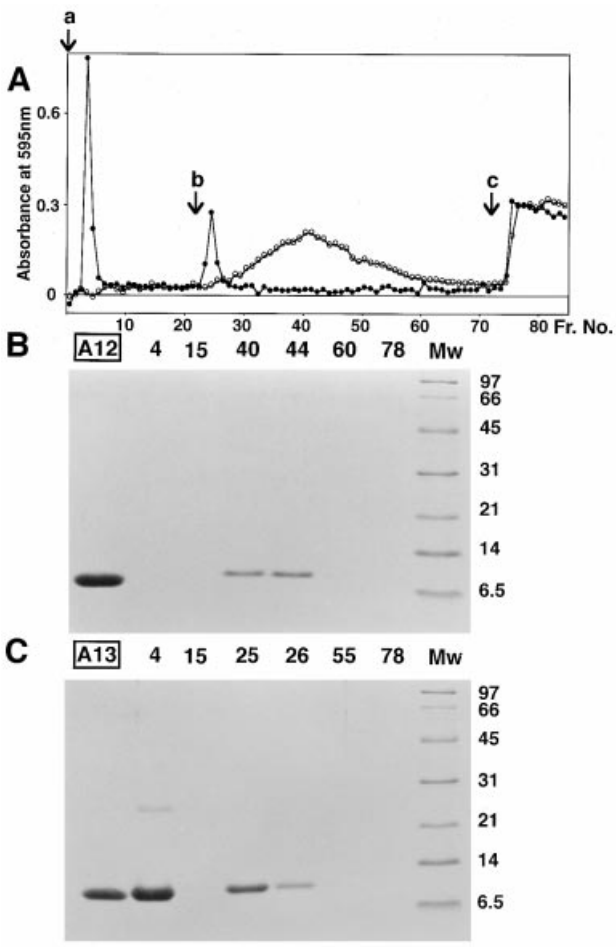

Figure 7 Affinity chromatography of the recombinant S100A12 (protein 'a') and S100A13 (protein 'e') and Tricine/SDS/PAGE analysis of selected fractions

(**A**) Elution curves of the recombinant proteins from the amlexanox–AF column (○, S100A12; ●, S100A13). a, Sample loading followed by washing; b, addition of the buffer containing 2 mM EGTA; c, addition of the buffer containing 6 M urea. (**B**) Tricine/SDS/PAGE (12%) analysis of the recombinant S100A12 and the fractions (4, 15, 40, 44, 60 and 78) from amlexanox–AF column chromatography (see **A**). (**C**) 12% Tricine/SDS/PAGE analysis of the recombinant S100A13 and the fractions (4, 15, 25, 26, 55 and 78) from amlexanox–AF column chromatography. Size standards (Mw) are shown in kDa.

region. The polyadenylation signal sequence (AATAAA) is shown at nucleotide positions 403–408. The cDNA and deduced amino acid sequences of the 10-kDa protein 'e' were initially not found to have significant similarity with any protein in the GenBank database. During the preparation of this manuscript, the human and mouse cDNA sequences of S100A13 were published [24], and the deduced amino acid sequences of the S100A13 protein was found to have 95% homology (88.8% identity) with that of our 10-kDa protein 'e'.

Affinity of native S100A12 and recombinant S100A12 and S100A13 to amlexanox

The affinity of the isolated proteins 'a' (S100A12) and 'e' (S100A13 homologue) to the anti-allergic drug, amlexanox, was examined. Because only a small amount of the native protein 'e' had been obtained, the recombinant S100A13 homologue was used. The recombinant proteins were expressed from the bovine lung cDNA [25]. The native protein 'a' was subjected to the amlexanox–AF column in the presence of calcium ions. After washing the column, the protein was eluted with EGTA. As shown in Figure 6, the elution pattern and 12% Tricine/SDS/PAGE of the native protein 'a' indicated that it bound to amlexanox in the presence of calcium ions, and dissociated from the drug on removing calcium from the protein. The recombinant S100A12 bound also with the drug-coupled column in a calcium-dependent manner (Figures 7A and 7B). The recombinant S100A13 homologue was examined in a similar manner and it was found that this protein also bound to the drug in a calcium-dependent manner, although part of protein 'e' passed through the column (Figures 7A and 7C).

DISCUSSION

In this study, we isolated two characteristic proteins binding to three different anti-allergic drugs, amlexanox, cromolyn and tranilast, by drug-affinity chromatography and identified the proteins to be S100A12 and S100A13. Previously, we reported that 22 and 32–42 kDa proteins binding to amlexanox and cromolyn were calcyphosine and annexins I–V, respectively [16]. The low-molecular-mass proteins of near 10 kDa (Figure 1) were quantitatively dominant and thus might be targets of these drugs. Reversed-phase HPLC analysis of the 10-kDa proteins from the three drug-coupled columns and from a phenyl-Sepharose column revealed that the elution profiles of the proteins from the three drug-coupled columns were similar, although they were different from that eluted from the phenyl-Sepharose column (Figure 2). Proteins 'a' and 'e' scarcely bound to phenyl-Sepharose, but bound specifically to the anti-allergic drugs.

Although amlexanox, cromolyn and tranilast have different structures, they have similar efficacy in inhibition of IgE-mediated degranulation of mast cells and basophils. These findings imply that these three compounds might interact with these proteins when working as anti-allergic drugs.

We determined the nucleotide sequence of the cDNA coding protein 'a' and deduced amino acid sequence as shown in Figure 4. The sequence of the protein 'a' was identical with that of the calcium-binding protein in amniotic fluid-1 (CAAF1) [23]. Thus, protein 'a' was identified to be S100A12. Figure 5 shows the nucleotide sequence of the cDNA and deduced amino acid sequence of the 10-kDa protein 'e'. Protein 'e' had 95% similarity with the proteins derived from the human and mouse cDNAs coding for S100A13 [24]. Thus, the protein 'e' was found to be a bovine homologue of S100A13.

The binding of the native and recombinant S100A12 proteins and the recombinant S100A13 to the immobilized amlexanox was examined and confirmed that these proteins interacted alone with the drug in a calcium-dependent manner and that the affinity of S100A12 to amlexanox was higher than that of S100A13. The latter observation implies that the spatial arrangement of the amino acid residues that interact with the drug differs in S100A12 and S100A13.

In a preliminary experiment on the structure–affinity correlation between the drugs and proteins, affinity chromatography by immobilized chromene-2-carboxylic acid and *N*-acetyl anthranilic acid, which have, respectively, the partial structures of cromolyn and tranilast, showed that these compounds bind proteins of 32–42 kDa but do not bind proteins of near 10 kDa (results not shown). This suggests that the whole structures of the anti-allergic drugs are necessary for binding of the drugs to the proteins.

Among the S100-protein family members, calcyclin [14,15] and S100A10 [26] have been identified as candidates required for exocytosis. Our results indicate that S100A12 protein is chiefly bound to the drugs in a calcium-dependent manner. The same result was obtained when bovine spleen extract was applied to an amlexanox-coupled affinity column (results not shown). Among the same group of anti-allergic drugs, a basophilic membrane protein [27] and nucleoside diphosphate kinase [28] have already been reported as cromolyn/cromoglycate binding proteins; however, few target proteins of amlexanox and tranilast have been identified. Our finding that three anti-allergic drugs bind to S100A12 and S100A13 proteins invites speculation that these drugs might inhibit degranulation by binding to these proteins.

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