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Slow reacting substances of anaphylaxis: Identification of leukotrienes C-1 and D from human and rat sources

(human lung/rat peritoneal leukocytes/peripheral airways/synthetic leukotrienes)

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ABSTRACT **Slow reacting substance(s) of anaphylaxis (SRS-A) was isolated from both human (lung) and rat sources and compared with three synthetic SRS-As of known structure—leukotrienes (LTs) C-1, C-2, and D. Reversed-phase liquid chromatography was used both as a final purification step and a means of comparison of biologically derived and synthetic substances. Two major peaks of SRS-A activity of both rat and human origin corresponded chromatographically with LTC-1 and LTD, respectively, and had equivalent specific activities on the guinea pig ileum. With guinea pig ileum, the specific activities (units/pmol) for synthetic leukotrienes and anaphylactic peaks were (mean ± SEM): synthetic LTC-1, 1.93 ± 0.13; SRS-A^{rat} peak I, 1.69 ± 0.43; synthetic LTD, 6.10 ± 1.15; SRS-A^{rat} peak II, 7.14 ± 0.51; and SRS-A^{hu} peak II, 1.90. Both synthetic LTC-1 and LTD and their SRS-A natural counterparts had a preferential contractile activity on guinea pig peripheral airway compared to central airways and were at least 200 times more active than histamine on peripheral airways on a molar basis. Leukotriene D is the major SRS-A of human lung and accounts for almost all of the biological activity. It likely is formed from leukotriene C-1 *in vivo* by an enzymic process of the well-known γ-glutamyltransferase type.**

Although "slow reacting substance of anaphylaxis" (SRS-A) has been recognized as a putative major mediator of immediate hypersensitivity reactions for 40 years (1), only in the past decade have both proof of potency and evidence of chemical structure emerged. Brocklehurst (2) distinguished SRS-A from histamine in an anaphylactic perfusate by its contractile action on an (H-1) antihistamine-blocked guinea pig ileum wth a slow progression to maximal effect. Subsequent investigations established it to be a polar lipid (3, 4) with strong ultraviolet absorbance and possibly containing sulfur (4, 5). Cysteine was subsequently observed to augment generation of SRS-A (6) and a calcium ionophore was shown to stimulate production of nonanaphylactic slow-reacting substance (SRS) (7, 8) with incorporation of radiolabeled arachidonic acid as the lipid precursor (9, 10). Recently, an ionophore-stimulated SRS from mouse mastocytoma cells designated as leukotriene C-1 (11) (LTC-1) was identified as 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*,11,14-*cis*-icosatetraenoic acid (**II** in Fig. 1) (12, 13) and was prepared in quantity by total synthesis.

This communication reports the use of synthetic leukotrienes C-1 (13) and D (IV) (LTD) to standardize retention times on high-performance liquid chromatography (HPLC) (14, 15) and to calculate the specific functional activities of SRS-A produced by an anaphylactic reaction in the rat peritoneal cavity or human lung. Additionally, naturally derived SRS-A components were compared with the synthetic leukotrienes in regard to differential and specific activities on the guinea pig tracheal and lung parenchymal muscle strips *in vitro* (14, 16), UV absorption, and enzymic oxidation.

MATERIALS AND METHODS

Materials. SRS-A was produced by IgG_a-dependent pathways in the rat peritoneal cavity (4) and by IgE-dependent activation of human lung fragments (17) and was isolated by elution from XAD-8, DE-52 cellulose, and silicic acid columns as described (14).

SRS-A fractions were dried by flash evaporation, resuspended in 1 ml of water, introduced onto a 4.6 × 250 mm C_{18} reversed-phase HPLC (C_{18} RP-HPLC) column with a 3.2 × 40 mm C_{18} precolumn (Altex Scientific, Berkeley, CA) at 1500 psi (10.35 megapascals), and eluted isocratically in 65% methanol/34.9% water/0.1% acetic acid, pH 5.6, at 1 ml/min; 1-ml samples were collected with on-line monitoring of absorbance at 280 nm for 45 min.

The biological activities of the synthetic leukotrienes, rat SRS-A (SRS-A^{rat}), and human SRS-A (SRS-A^{hu}) were assessed in a dose–response fashion with the guinea pig ileum bioassay. Standardization was achieved by defining 1 unit of activity as that produced by a 5 ng/ml solution of histamine as described (2). The contractile effects of LTC-1, LTD, SRS-A^{rat}, and SRS-A^{hu} were additionally evaluated with guinea pig tracheal spirals and parenchymal strips (14, 16). Concentration–effect relationships for the constriction of each tissue by histamine were determined by exposing the tissue to histamine, subjecting it to overflow wash with Tyrode's solution, and allowing it to return to its baseline tension, usually in about 40–60 min. Small amounts of concentrated spasmogens were added serially to the organ bath to achieve final bath concentrations ranging between 4×10^{-13} and 4×10^{-6} M for the synthetic leukotrienes; a period of 120–180 sec was allowed between additions of the concentrated solutions to allow tissue constrictions to become fully developed.

Synthesis of LTC-1 (II) and LTD (IV). The synthesis of the SRS LTC-1 (**II**) from the methyl ester of LTA (**I**) by two different routes has previously been described (13). The LTC-1 used in this study was prepared by reaction of the methyl ester **I**, $[\alpha]_D^{25}$ −21.9° (in cyclohexane), with *N*-trifluoroacetylglutathione dimethyl ester. The *N*-trifluoroacetyl derivative of glutathione dimethyl ester (mp 160–162°C) was obtained by reaction of the tetramethyl ester of the disulfide form of glutathione (18) with trifluoroacetic anhydride and powdered

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Abbreviations: SRS-A, slow reacting substance of anaphylaxis; SRS, nonanaphylactic slow reacting substance; LTC-1, leukotriene C-1; LTD, leukotriene D; NTFA-LTD, *N*-trifluoroacetyl-LTD; HPLC, high-performance liquid chromatography; u, units; RP, reversed phase.

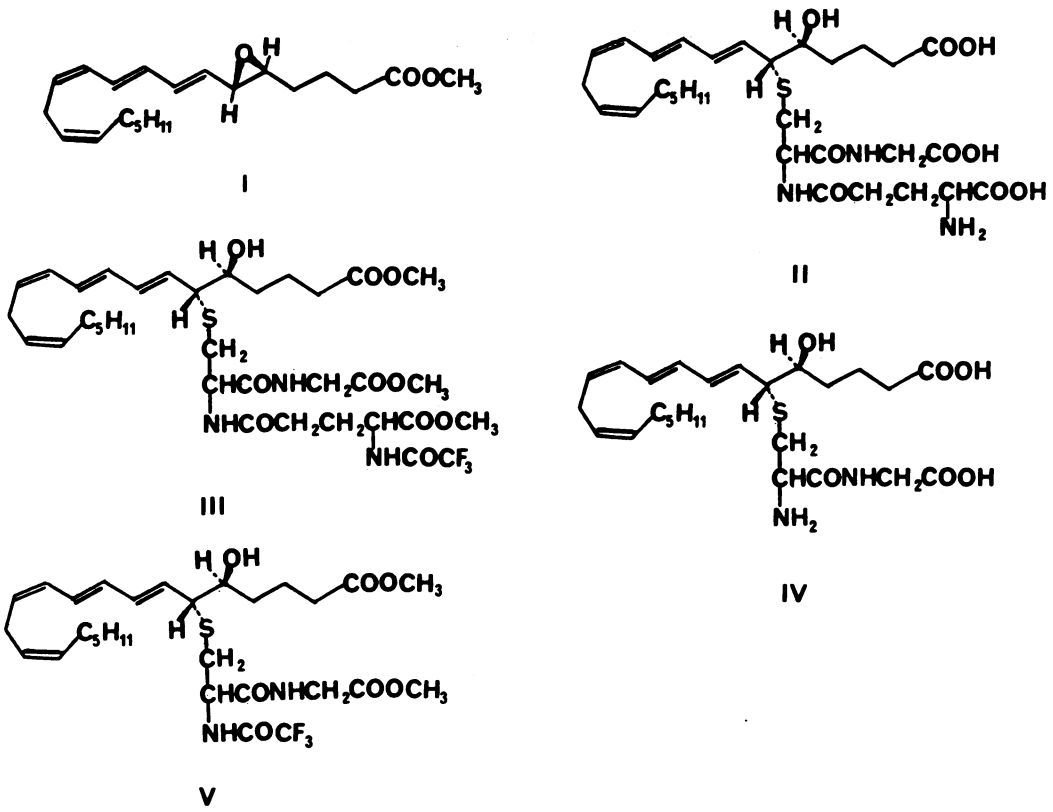

FIG. 1. Structures of leukotrienes and synthetic intermediates.

sodium carbonate in methylene chloride with stirring at 0°C for 30 min, followed by isolation and disulfide cleavage using triphenylphosphine in dimethoxyethane/water, 2:1 (vol/vol), at 23°C for 3 hr. Reaction of I with *N*-trifluoroacetylglutathione dimethyl ester (2 equiv) and triethylamine (3 equiv) in concentrated methanol solution under argon at 23°C for 4 hr gave the protected derivative III (80% yield) which was then hydrolyzed to II quantitatively with 0.05 M potassium carbonate in dimethoxyethane/water, 1:1 (vol/vol), at 23°C for 4 hr. For biological tests, synthetic II was purified by RP-HPLC using a Waters Associates μ-Porasil-C_{18} column with methanol/water, 65:35 (vol/vol), containing 0.1% acetic acid buffered to pH 5.6 with ammonium hydroxide for elution. Purified II could be stored for many days without appreciable decomposition in frozen pH 6.8 phosphate buffer solution (−20°C) under argon. Purified II was homogeneous by RP-HPLC analysis and showed characteristic absorption at 270, 280, and 290 nm (ϵ 32,000, 40,000, and 31,000) in methanol solution.

The Cys-Gly analog of LTC-1, (IV), now designated LTD, was synthesized from I in an analogous way by coupling with *N*-trifluoroacetylcysteinylglycine methyl ester. L-Cystine was converted to *N*-trifluoroacetylcystine (mp 166–167°C) in 75% yield by treatment either with trifluoroacetic anhydride and trifluoroacetic acid at 0°C for 0.5 hr (19) or with ethyl trifluoroacetate (25% excess) and sodium methoxide (2 equiv) in methanol at 23°C for 20 hr (20). The methyl ester of *N*-trifluoroacetyl L-cystinylglycine was prepared by conversion of the above acid to the acid chloride (phosphorus pentachloride in ether at 0°C for 0.5 hr), isolation, and subsequent reaction with glycine methyl ester in tetrahydrofuran at 0–23°C for 2 hr. Reduction of the crystalline cystine derivative so obtained (mp 188.5–189.5°C) with triphenylphosphine (1.1 equiv) in dimethoxyethane/water, 2:1 (vol/vol), at 23°C for 3 hr, extractive isolation, and recrystallization from ether gave *N*-trifluoroacetylcysteinylglycine methyl ester (mp 99–100°C).

Reaction of the methyl ester I with 2 equiv of *N*-trifluoroacetylcysteinylglycine methyl ester and 4 equiv of triethylamine in a minimal volume of methanol under argon at 23°C for 4 hr and extractive isolation afforded a single major product which was purified by thin-layer chromatography to yield the dimethyl ester of *N*-trifluoroacetyl LTD (V) (58%) as homogeneous material (by HPLC analysis on μ-Porasil) with characteristic UV absorption at 270, 280, and 290 nm (ϵ 31,000, 40,000, and 31,000). Reaction of V under argon with 0.13 M potassium carbonate in water/methanol, 3:1 (vol/vol), at 23°C for 18 hr followed by isolation and purification as described above for LTC-1 afforded pure LTD (IV) in *ca.* 90% yield. Purification of IV by RP-HPLC served to separate IV (retention volume, 9.3) from a minor impurity (<5%, UV max at 278 nm, probably the $\Delta^{11,12}$-*trans* isomer of IV) which showed a relative retention volume of 10.0. Use of the milder conditions, which were adequate for the quantitative conversion of III to LTC-1 (II), for the hydrolysis of V resulted in incomplete deprotection to give a mixture of IV and its *N*-trifluoroacetyl derivative. The latter was synthesized unequivocally from I by coupling with *N*-trifluoroacetylcysteinylglycine followed by selective cleavage of the resulting monomethyl ester using 0.05 M potassium carbonate in aqueous dimethoxyethane at 23°C for 4 hr. Samples prepared by both methods were identical and could be converted to LTD (IV) by hydrolysis with 0.13 M potassium carbonate in water/methanol, 3:1.

RESULTS

Chromatography of LTC-1, LTC-2, LTD, and *N*-trifluoroacetyl-LTD (NTFA-LTD) on C_{18} RP-HPLC with on-line spectrophotometric monitoring at 280 nm yielded absorbance peaks with retention times (mean ± SEM) of 16.5 ± 0.7, 18.5 ± 0.6, 25.5 ± 0.3, and 32.0 ± 1.0 min; the biologic activities of the eluates corresponded directly to the absorbance peaks. Biological activities of the synthetic compounds assessed on the

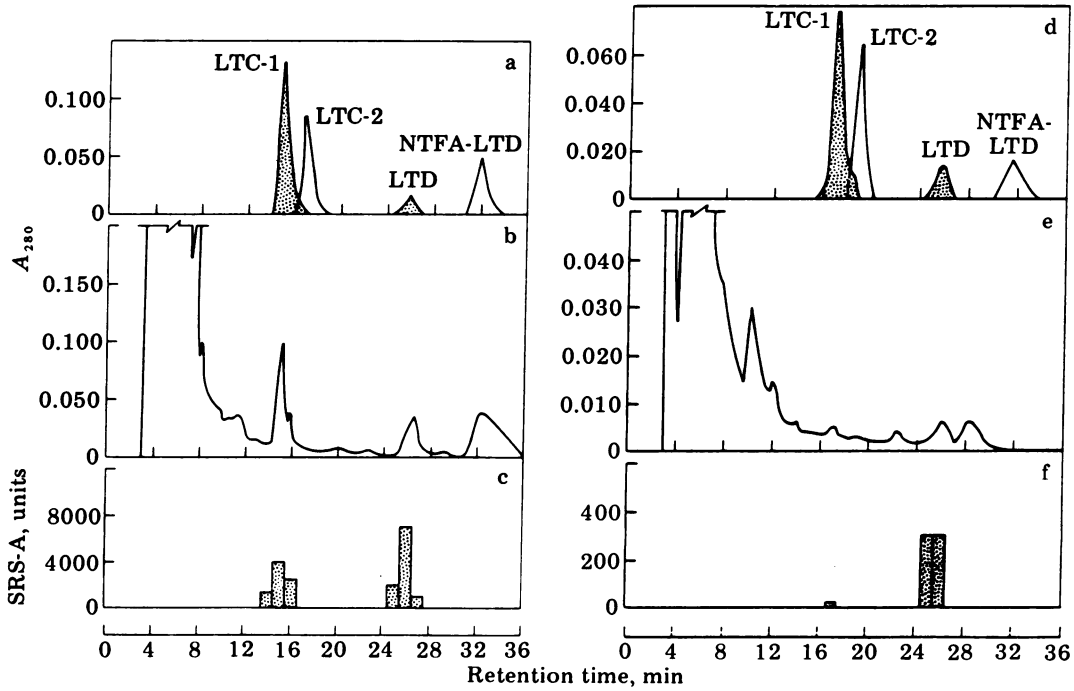

FIG. 2. (*a–c*) C_{18} RP-HPLC chromatogram of SRS-A^{rat}. (*a*) Retention times (280 nm detection) of three leukotrienes (LTC-1, LTC-2, and LTD) and a synthetic intermediate (NTFA-LTD). LTC-1 and LTD were applied to the column together (stippled peaks; LTC-2 and NTFA-LTD were applied separately. (*b*) Elution pattern (280 nm detection) for a single preparation of SRS-A^{rat}. (*c*) Retention times for biological activity of SRS-A^{rat}. (*d–f*) C_{18} RP-HPLC chromatogram of SRS-A^{hu}. As in *a–c* but SRS-A^{hu} instead of SRS-A^{rat}.

guinea pig ileum bioassay both before and after C_{18} RP-HPLC were unchanged. In eight experiments, mean (±SEM) activities of LTC-1 and LTD were 1.93 ± 0.13 and 6.10 ± 1.15 units (u)/pmol, respectively; in two experiments, each, LTC-2 and NTFA-LTD had mean specific activities of 0.91 and 1.00 u/pmol, respectively.

SRS-A produced by an anaphylactic reaction in the rat peritoneal cavity was purified in five separate experiments. A representative C_{18} RP-HPLC profile (Fig. 2*b*) displays a large quantity of 280-nm-absorbing material in the front with residual salt from the final DE-52 elution that was carried through the silicic acid step. Of the major subsequent peaks, the first was sharp with a descending shoulder and the other two were more broad. Biological activity was assayed on the guinea pig ileum for sequential 1-ml eluates, each of which corresponded to 1 min of retention time up to 45 min and included the subsequent gradient to 100% methanol. Biologic activity corresponded exactly to and was limited to the first and second retained A_{280} peaks. In the five experiments, 11–42% of the biological activity eluted at a retention time of 15–18 min and 55–89% at 25–27 min (Fig. 2*c*; Table 1). Minor variations from the theoretical percentages of HPLC solvent components occurred due to evaporation of methanol and caused alterations of up to 2 min in SRS-A retention times assessed by absorbance and biological activity. It thus was necessary to chromatograph samples of LTC-1 and LTD before or after chromatography of each SRS-A sample to calibrate the retention time of the synthetic compounds in each experiment (Fig. 2*a*). The analytic resolving capacity of this HPLC system was demonstrated by a reproducible retention time for LTC-2 that was 2 min longer than that of LTC-1 (Fig. 2*a*) (LTC-2 is the 11,12-*trans* isomer of LTC-1). In two of six experiments with rat SRS-A, a third area

Table 1. Recoveries and specific activities of SRS-A^{rat} and SRS-A^{hu} during purification

Column	SRS-A^{rat} 1	2	3	4*	5†	SRS-A^{hu} 1	2
XAD-8	ND	75,000	100,000	125,000	ND	10,000	ND
DE-52	ND	ND	70,000	75,000	ND	5,000	ND
Silicic acid	ND	15,000	25,000	40,000	13,200	3,000	1,500
C_{18}RP-HPLC:							
Peak I							
Total activity	1,500	6,500	4,500	12,500	700	20	150
Specific activity‡	2.27	2.17	1.09	1.59	1.96	§	§
Peak II							
Total activity	3,700	9,000	15,600	19,500	5,600	600	965
Specific activity‡	6.67	5.55	7.14	7.14	9.09	1.89	1.92

SRS-A of rat and human origins were purified in five and two experiments, respectively, in four sequential steps. Recoveries after each purification step, where determined, were assessed on the guinea pig ileum bioassay in units; 1 unit of SRS-A equals that amount causing a contraction equal to that produced by histamine at 5 ng/ml.

* Of the biological activity, 6% eluted in a third peak at a retention time of 33 min.

† Of the biological activity, 9% eluted in a third peak at a retention time of 32 min.

‡ Specific activities were calculated in units/pmol for the biologically active peaks I and II eluted from the RP-HPLC. ND, not determined.

§ A_{280} absorbance peak areas were too small for accurate determination.

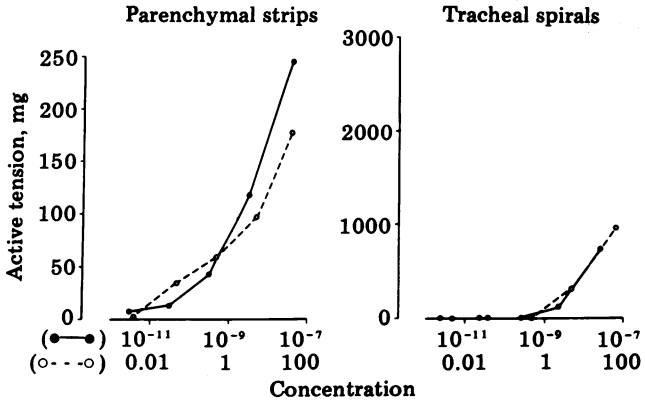

FIG. 3. Contractile effects of LTC-1 and SRS-A peak I on parenchymal strips (*Left*) and tracheal spirals (*Right*). ○, Mean of data from two separate tracheal spirals and parenchymal strips; ●, mean contractile effects of LTC-1 assayed on the same tissue strips. The maximal achievable active tension, as determined by contraction in response to 100 μM histamine, was 249 mg for the parenchymal strips and 2950 mg for the tracheal spirals. Concentration is shown on abscissa as molarity (upper line) and units/ml (lower line).

of activity eluted at more than 32 min and constituted 6% and 9%, respectively, of the total recovered biologic activities.

The specific activities of the biologically active peaks of rat SRS-A were calculated by utilizing the integrated areas from the HPLC chromatograms (detection at 280 nm), biological activities measured on the guinea pig ileum, and an assumed molar extinction coefficient at 280 nm (ϵ) of 40,000, as calculated for each of the leukotrienes (13). Molar concentrations (C) of SRS-A were calculated for each activity peak by using the formula $A = \epsilon BC$, in which A is the integrated absorbance at 280 nm and B is the path length of the spectrophotometer cell (1 cm) (Table 1). The mean (± SEM) specific activities for SRS-A^{rat} peaks I and II, respectively, were 1.69 ± 0.43 and 7.14 ± 0.51 u/pmol and compared favorably to 1.93 ± 0.13 and 6.10 ± 1.15 u/pmol for LTC-1 and LTD, respectively. In the two human SRS-A preparations tested, 87% and 97% of the biological activity eluted in peak II and corresponded to LTD by retention time (Table 1). The specific activities of 1.92 and 1.89 u/pmol in the two experiments were lower for SRS-A^{hu} peak II compared to SRS-A^{rat} peak II and LTD. Specific activities for SRS-A^{hu} peak I could not be calculated because of the small areas on the A_{280} absorbance peaks.

A 50% maximal contractile response of the parenchymal strip was achieved with bath concentrations of 3 nM LTC-1 or 7 units of SRS-A^{rat} peak I per ml, thus establishing 1 unit of SRS-A^{rat} peak I as equivalent to 0.43 pmol or 0.27 ng of LTC-1 (Fig. 3). On a molar basis, LTC-1 was 200-fold more active than histamine on parenchymal strips. The concentrations of LTC-1 and SRS-A peak I that gave half-maximal response on the parenchymal strips gave less than 5% of maximal activity on the tracheal spirals. A 50% maximal contractile response of parenchymal strip was achieved with a bathing solution containing freshly purified LTD at 20 fM or SRS-A peak II (rat or human) at 22 u/ml (Fig. 4). One unit of SRS-A peak II was equivalent to 9.1×10^{-19} mol (0.45 fg) of pure LTD. The concentrations of LTD and SRS-A peak II that gave a half-maximal response on the parenchymal strips had less than 1% and 9%, respectively, of maximal activity on the tracheal spirals.

Both synthetic and natural origin LTDs were considerably less stable to handling in air and subsequent storage than was LTC-1. This deterioration was clearly evidenced by loss of activity in the parenchymal strip assay; it was not as apparent by assay with either tracheal spirals or ileum. As indicated in Fig. 4 *Upper*, repeated cycles of thawing, aeration, sampling, argon flushing, and freezing over a 5-day period produced substantial inactivation.

DISCUSSION

The interest for many years in purifying SRS-A and identifying its chemical structure reflects a belief that it is a likely mediator of human reversible airway disease. SRS-A is generated by IgE-dependent pathways in human lung fragments and dispersed lung cells (17), and human small airway muscle is exquisitely reactive to SRS-A^{hu} (3). The prominence of peripheral airway constriction in bronchial asthma (21) may relate to the selective action of SRS-A on the peripheral airways, as shown *in vivo* in both the guinea pig (22) and the rhesus monkey (23) and *in vitro* in guinea pig tissues (14).

In addition to the evidence of a multicellular origin for SRS-A (17, 24–26), there is also evidence that the mediator represents a family of related activities. Two biological activities derived from ionophore treatment of rat mononuclear cells in the presence of cysteine were separated by straight-phase HPLC (27) and two activities generated by anaphylactic challenge of guinea pig lung were resolved on RP-HPLC (15). Furthermore, multiple SRSs were generated from rat mononuclear cells with A23187 in the presence of different thiols (28). Although the analytic studies demonstrated that LTC-1 was the major SRS produced by ionophore-stimulated mouse mastocytoma cells

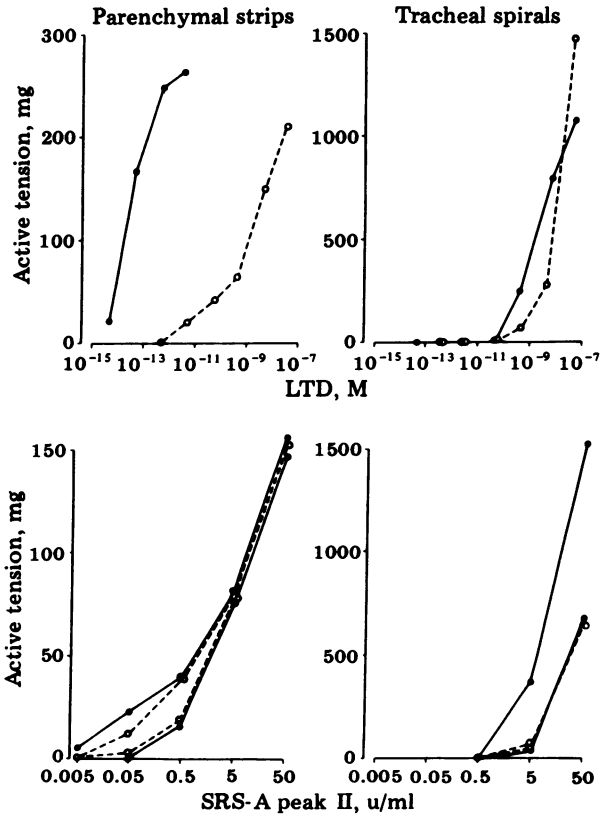

FIG. 4. Effects of LTD (*Upper*) and SRS-A peak II from various sources (*Lower*) on contractile activity of parenchymal strips (*Left*) and tracheal spirals (*Right*). (*Upper*) ●—●, LTD, assayed on two parenchymal strips and two tracheal spirals, after one cycle of sampling and storage with thawing twice. ●- -●, LTD assayed 5 days later after repeated cycles of sampling and storage. Results are the mean responses of two tissues of each type. (*Lower*) Contractile activities of rat (●—●) and human (●- -●) SRS-A peak II. Each line represents the concentration–effect relationship for SRS-A peak II from a separate preparation. Each preparation was assayed on at least two parenchymal strips and two tracheal spirals. The maximal achievable active tension (induced by 100 μM histamine) was 291 mg for the parenchymal strips and 3801 mg for the tracheal spirals.

(11), complete synthesis of LTC-1 was necessary to define both the peptide side chain (12) and the stereochemistry of the ethylenic bonds (13). Synthetic LTD differs from LTC-1 in the absence of a glutamyl in the peptide side chain (Fig. 1) and may be derived naturally from LTC-1 by the action of a γ-glutamyltransferase (29).

In five separate experiments after four purification steps, rat peritoneal SRS-A chromatographed on C_{18} RP-HPLC with two major peaks of biological activity that had retention times of 16.5 ± 0.8 and 25.5 ± 0.4 min (mean ± SEM); these times could be superimposed on the retention times of LTC-1 and LTD, respectively. Additionally, both the peak contours for absorbance at 280 nm and the calculated specific activities (based on $\epsilon = 40{,}000$) were coincident and similar for peak I (1.60 ± 0.43 u/pmol) and LTC-1 (1.93 ± 0.13 u/pmol) and for peak II (7.14 ± 0.51 u/pmol) and LTD (6.10 ± 1.15 u/pmol), with the guinea pig ileum assay used to determine biological potency. In each experiment, SRS-A peak II contained the major proportion of biologically active material, a mean (±SEM) of 69% ± 10 of the assayable activities eluted from HPLC. Relative to the ethanol eluate from the XAD-8 column, total recovery after HPLC was 21% to 28%, indicating that the active peaks described represent a significant proportion of SRS-A (Table 1).

The UV absorption of SRS-A^{rat} peaks I and II and of LTC-1 and LTD were identical (λ_{max} 280 nm, shoulders at 270 and 290 nm). Treatment with soybean lipoxygenase in each case changed the UV absorption to λ_{max} 308 nm, shoulders at 295 and 323 nm.

Compared to SRS-A^{rat}, SRS-A^{hu} was more predominantly peak II (i.e., 87% and 97%) which corresponded to the retention time of LTD. The calculated recovery of SRS-A^{hu} was lower than that of SRS-A^{rat} and, in addition, the specific activity of SRS-A^{hu} peak II was 26% and 27% of the mean specific activity of SRS-A^{rat}. This divergence is probably due to instability in the presence of air and to differences in extraction with regard to contaminants and procedure.

LTC and SRS-A^{rat} peak I have selective activity on parenchymal strips in that a half-maximal response of this tissue was achieved, with 3 nM LTC-1 or 7 u of SRS-A^{rat} peak I per ml, whereas these concentrations had less than 5% of maximal activity on tracheal spirals.

It is clear that both synthetic LTD and SRS-A peak II are selective and potent peripheral airway agonists. The contractile profile of SRS-A peak II resembles that of partially inactivated LTD (Fig. 4). The extraction process involved in the isolation of the former from the anaphylactic diffusate may be accompanied by a chemical modification similar to that which occurs with synthetic LTD. It is noteworthy that the specific activity of SRS-A^{rat} peak I, based on the activity of LTC-1, is comparable on both the guinea pig ileum (0.32 ng/unit) and the parenchymal strips (0.45 ng/unit), whereas that for SRS-A^{rat} peak II compared to LTD handled in the same fashion is substantially lower on the guinea pig ileum (0.081 ng/unit) than on the parenchymal strip (0.42 fg/unit).

Present evidence indicates that SRS-A of rat and human origin is a mixture of LTC-1 and LTD, possibly with further catabolites. Although nonenzymatic conversion of LTC-1 to LTD in principle could occur during purification, studies on the chemical stability of LTC-1 in aqueous methanol solution under argon at 23°C for several days preclude this possibility. It is likely that LTC-1 is the SRS-A initially synthesized and that a proportion is converted to LTD *in vivo*, most probably by the ubiquitous enzyme γ-glutamyltransferase (29). The formation of LTD from LTC-1 in rat basophilic leukemia cells by enzymatic γ-glutamyl cleavage has been proposed by Örning *et al.* and demonstrated *in vitro* with γ-glutamyltransferase from porcine kidney (30). Clearly, LTD is the major SRS-A of both the rat and human and accounts for nearly all of the biologic activity of the latter.

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