SLOW-REACTING SUBSTANCE OF ANAPHYLAXIS

Purification and characterisation

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Received 18 January 1978

1. Introduction

Slow-reacting substance of anaphylaxis (SRS-A) is a primary mediator of immediate-type hypersensitivity reactions such as asthma. It is released together with histamine, other primary mediators and prostaglandins, and its release was originally described over 30 years ago [1]. Since then the roles of histamine and prostaglandins in asthma and anaphylaxis have been extensively studied. It has been found, by the development of chemical antagonists and later by the synthesis of pure mediators, that histamine accounts for only part of the bronchoconstriction of anaphylaxis or asthma, and that while some prostaglandins are bronchoconstrictors, their main action seems to be as modulators of the other agonists [2,3].

Consequent on these findings the study of SRS-A (the remaining major bronchoconstrictor substance associated with the above immunological reactions) has taken on a new relevance and sense of urgency. To date, however, major difficulties with purification and structure elucidation of SRS-A have severely retarded an understanding of the biological significance of this important mediator.

At various times over some 20 years of study by several groups, SRS-A has been described in the literature as a hydroxy acid, a 'low molecular weight lipid', 'a carboxylic acid with hydroxyl groups and one or more double bonds' [4,5] and more recently as a compound containing 'a sulphate ester group' [6]. In fact, the chemical evidence for all of these generalisations is not entirely conclusive and reflects the difficulties encountered in the attempted determination of the structure of small quantities of impure biologically active substances.

We describe here an improved purification procedure which will yield highly purified SRS-A as judged by spectroscopic methods. This new procedure creates the possibility of structure determination, together with a re-examination of the pharmacological properties previously studied using only partially purified material.

Abbreviation: SRS-A, slow-reacting substance of anaphylaxis

2. Materials and methods

2.1. *Guinea pigs*

Guinea pigs (Dunkin Hartley strain, either sex) 250–300 g were sensitised using ovalbumin [7] (Sigma grade II). After 21 days the animals were killed and their lungs perfused for 15 min with Tyrode solution containing 1 μg/ml of indomethacin. The lungs were then challenged with 10 mg ovalbumin (Sigma grade III) and the effluent collected on ice over a period of 10 min [2]. Activated charcoal was added to the crude effluent (0.25 g/100 ml), which

was then shaken and centrifuged at 2000 × *g* for 20 min. The supernatant was discarded and the charcoal washed twice with 80% ethanol (80 mg/100 ml effluent). The washings, containing SRS-A activity, were then rotary evaporated at 35°C and freeze-dried.

2.2. *Gel filtration*

This was carried out in a column (180 × 1.0 cm) of G15 Sephadex developed in water/methanol/ammonia (0.880) (40/40/20, v/v/v) and monitored at 275 nm on a Cecil Spectrophotometer.

2.3. *Electrophoresis*

This was performed on DEAE and SG 81 paper (Whatman Ltd.) in pH 6.5 pyridine acetate buffers, at 3 kV for 1 h. Samples for bioassay were eluted with the solvent system used for gel filtration (see above).

2.4. *Acid extraction*

The crude ex-G15 SRS-A was rotary evaporated, freeze-dried and taken up in pH 2.8 acetic acid solution; it was extracted twice into ether and the ether phase was dried under nitrogen and finally on a vacuum pump.

2.5. *High pressure liquid chromatography*

This was carried out on a Waters instrument, using a Bondapak C_{18} column eluted with water followed by a gradient to 100% methanol.

2.6. *Ultraviolet spectra*

These were obtained for the purified SRS-A fraction and for standard compounds on a Unicam SP 800 spectrophotometer.

2.7. *Assay of SRS-A activity*

At all stages of purification, fractions were assayed for SRS-A activity on guinea-pig ileum [2]; slow-reacting substance of anaphylaxis (SRS-A) was quantitated in arbitrary units defined in terms of the activity of an initial batch of partially purified material. 0.068 ± 0.004 units SRS-A are equivalent to 2 units SRS-A measured in terms of histamine [8].

3. Results and discussion

The problems of purifying small quantities of biologically active molecules are three-fold:

(i) Losses by absorption and adsorption.
(ii) Destruction by acid, base, nucleophiles or electrophiles.
(iii) The sample can only be monitored by its biological activity and quantitated in units.

The following purification scheme reported here has been designed to minimise losses via (i) and (ii) above, but at the same time to maximise the physical data relevant to the structure of SRS-A.

The SRS-A is always observed to elute from a G15 column together with material absorbing strongly at 275 nm; this ultraviolet active material contains tyrosine and phenylalanine, thus giving an indication of the low molecular weight of SRS-A. The gel data would suggest mol. wt 200–400 (allowing for any aromaticity in the SRS-A structure).

Following the gel step we have developed two alternative pathways for further purification involving either electrophoresis or organic solvent extraction prior to high pressure liquid chromatography. Electrophoresis was chosen following the description of SRS-A as an acidic substance [4]. It was found that activity could not be recovered from normal Whatman No. 1 or 3MM papers, but could be eluted from DEAE-treated paper, and from SG 81 (silica-treated) paper in reasonable (>60%) yields. SRS-A runs towards the anode with a mobility relative to aspartic acid of 0.27. This mobility would suggest a mass of 500–1000 for SRS-A, but the behaviour on paper may be anomalous for other reasons, e.g., binding to the paper, and thus the gel data is believed to be a more reliable estimate. Organic extraction of SRS-A into acidified ether has been reported [8]. In our hands we find that extraction into ether from pH 2.8 acetic acid, following the gel filtration, gives overall yields in the ether layer of greater than 95%, with no activity left in the aqueous layer. Extraction at earlier stages in the purification meets with lesser success, and indeed the attempted extraction of lung effluent failed, the activity remaining in the aqueous phase. We interpret our high recovery to mean that the ex-G 15 SRS-A preparation is already more pure than reported [6]. High pressure liquid chromatography: As the final purification step leading to (as far as we can determine) absolutely pure SRS-A we have developed an important procedure using high pressure liquid chromatography (HPLC).

In this system two regions of activity are recovered

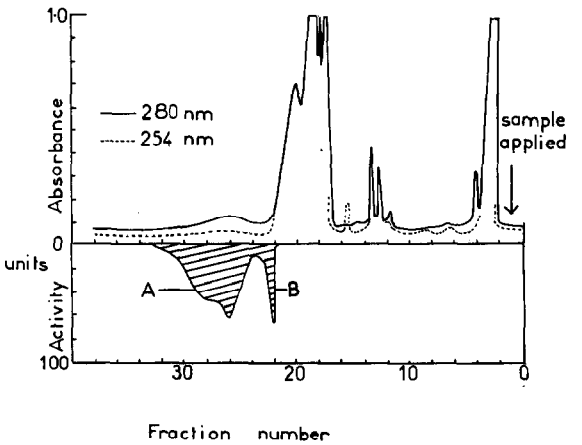

Fig.1. High pressure liquid chromatography profile of ether-extracted guinea-pig SRS-A. A Bondapak C_{18} column, developed in water:methanol was used and the effluent monitored at 254 nm and 280 nm. All fractions were assayed for SRS-A activity.

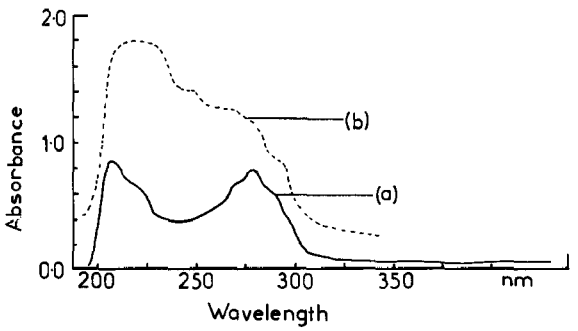

Fig.2. (a) Ultraviolet absorption spectrum of highly purified SRS-A (fraction A, fig.1) in methanol; 300 units/ml, 10 mm pathlength. (b) Ultraviolet absorption spectrum of SRS-A taken from [4].

from the column (A and B in fig.1) and indeed multiple activities for chromatographed SRS-A have been reported [10]. We interpret this as being due to the presence of a protonated and an unprotonated form of the same material. We have supported this conclusion by rechromatographing the active material eluted first from the column (B) which we assigned to unprotonated SRS-A. After acidification we now observe that this material chromatographs to give the major region of activity eluting much later from the column at a position corresponding with A in fig.1, i.e., assigned to protonated SRS-A. This ability to move the elution position of SRS-A on protonation can be used in a double purification step running unprotonated and then protonated SRS-A.

From this behaviour it appears likely that previous assignments of multiply active forms of SRS-A are due to the different physico-chemical properties of protonated/unprotonated SRS-A.

It should be noted that the SRS-A activity as found by bioassay coincides exactly with the small ultraviolet absorption at 280 nm and 254 nm indicated in fig.1, and that this material is the last to be eluted from the column. The relative purity of SRS-A is self-evident from the accompanying ultraviolet trace (fig.1) (i.e., a symmetrical ultraviolet trace at 280 nm and 254 nm). The purity is further emphasised by comparing the complete ultraviolet spectrum (fig.2a) with [4] (fig.2b).

Our spectrum suggests that the majority of ultraviolet activity previously assigned to SRS-A derives from an impurity. We find that SRS-A in methanol as a λ max at 279 nm (consistent with certain aromatics and conjugated chromophores). It has been suggested that SRS-A may be derived from arachidonic acid [11] and therefore may be related to the prostaglandins and thromboxanes [12]; however, examination of the ultraviolet spectra of some products of both the cyclo-oxygenase and lipoxygenase pathways indicates no close relationship with SRS-A.

4. Conclusion

An advanced purification technique has been described for SRS-A and evidence of high purity demonstrated. Suspected multiple forms of SRS-A are seen to be protonated or unprotonated versions of the same material, protonation probably taking place at a carboxyl group. The full ultraviolet spectrum of SRS-A is given, clearly discounting some classes of molecule for the SRS-A structure. Structural work on larger quantities of SRS-A is now progressing using mass spectrometric and nuclear magnetic resonance techniques.

Acknowledgements

We wish to thank Fisons Ltd for financial support and Mr John Green for valuable technical assistance.

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