Biochemical Pharmacology, Vol. 30, No. 13, pp. 1781–1784, 1981.
Printed in Great Britain.

0006-2952/81/131781-04 $02.00/0

PRODUCTION OF SLOW REACTING SUBSTANCE IN RAT GRANULOMATOUS INFLAMMATION

YASUKO KOSHIHARA*, ICHIRO NAGASAKI and SEI-ITSU MUROTA

Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo-173, Japan

(*Received* 31 *October* 1980; *accepted* 15 *January* 1981)

Abstract—Production of slow reacting substance (SRS) in inflamed tissue was examined. Minced-pouch walls from carrageenin granulomas of 3, 7 and 14 days old in rats were incubated in the presence of cysteine and Ca^{2+}-Ionophore. SRS-like substance was found to be formed during the incubation. Production of the substance was diminished by the presence of BW 755C, but enhanced by indomethacin in the reaction mixture. The substance was partially purified by XAD-8 and silicic acid column chromatographies and was shown to be SRS by its typical contractile activity on guinea-pig ileum selectively antagonized by FPL 55712. Production of SRS by the granuloma was highest on day 3, and then decreased. No detectable SRS activity was present in exudates of granuloma at any stage.

Carrageenin granuloma is a good experimental model of both acute and chronic inflammation. The wet weight of the granulation pouch wall is maximal on day 5 and accumulation of pouch fluid occurs in the next few days; then both the weight of pouch wall and amount of fluid decrease gradually [1]. The granumola pouch wall mainly consists of fibroblasts, probably derived from monocytes and/or lymphocytes, macrophages, intercellular substances like collagen and acidic glycosaminoglycans. Inflammation oedema results from increased permeability of microvessels to macromolecules in the blood plasma. The increased permeability is probably partly due to the effect of released histamine on microvessels, but other endogenous substances, such as prostaglandins and kinins are also thought to contribute to the increased vascular permeability in most inflammatory conditions.

The effluent from challenger-sensitized guinea-pig and rat lung, which is associated with slow reacting substance of anaphylaxis (SRS-A), has also been reported to increase vascular permeability [2–4]. Recently, purified SRS-A from perfused lungs of sensitized guinea-pig was reported to increase vascular permability and also reduced blood flow in guinea-pig skin [5]. These findings suggest that SRS-A may be involved in some processes in inflammation. However, there is no direct evidence that inflammatory tissue can actually produce SRS (slow reacting substance) or SRS-A. The present paper describes studies on SRS production in inflammatory tissue.

MATERIALS AND METHODS

Formation of granulomas. Carrageenin granulomas were induced in male Sprague–Dawley strain rats (150 ± 10 g body weight) by the method of Fukuhara and Tsurufuji [1] except that Seakem 202 carrageenin (Marine Colloid Inc., Springfield, N.J., U.S.A.) was used instead of TS36 carrageenin. Four ml of 2% (w/v) carrageenin in saline was injected into an air sac (8 ml volume), previously formed on the back of the rats by injecting air subcutaneously.

* Author to whom correspondence should be addressed.

Formation of SRS from granulomas. Samples of 4 g of pouch wall from granulomas on days 3, 7, and 14 after carrageenin injection were washed twice with phosphate buffered saline and minced into pieces. The pieces were incubated at 37° in incubation buffer consisting of 150 mM NaCl, 3.7 mM KCl, 3.0 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 0.9 mM $CaCl_2$ and dextrose, adjusted to pH 7.0 with NaOH. Production of SRS was performed by the method of Murphy *et al.* [6] with slight modifications. Briefly, minced granuloma pouch wall (4 g) in 9 ml of incubation buffer supplemented with 0.01 M L-cysteine was incubated at 37° for 3 min. Then Ca^{2+}-Ionophore A23187 was added to a final concentration of 20 μg/ml. After gentle shaking for 20 min, the mixture was chilled and centrifuged and the supernatant was adjusted to 80% (v/v) ethanol concentration. The mixture was stood at 0° overnight, and then centrifuged at 2000 r.p.m. for 10 min, and the supernatant was evaporated. Granuloma pouch fluid was centrifuged to remove clusters of dead cells and adjusted to 80% concentration of ethanol.

Isolation of SRS. SRS was isolated by XAD-8 and silicic acid column chromatographies by the method of Murphy *et al.* [6] with slight modifications. Briefly the residue soluble in 80% ethanol was decomposed with 1 ml of 0.1 N NaOH for 30 min at 37°. The solution was neutralized and applied to an XAD-8 anion-exchange column (Rohm and Haas Co. U.S.A., 1.2 × 1.5 cm). The column was washed with 10 ml of H_2O, then materials were eluted with 10 ml of 80% ethanol. The ethanol eluate was evaporated, dissolved in 0.2 ml of 80% ethanol, and applied to a silicic acid column (100 mesh, Mallinkrodt Chemical Works, St. Louis, MO, U.S.A., 1.2 × 2.0 cm). Materials was eluted successively with 5 ml each of ether/hexane 3:7 (v/v), ethyl acetate, 5% (v/v) methanol, 10% methanol and 50% methanol in ethyl acetate, and methanol.

Bioassay. Smooth muscle contraction induced by SRS was assayed on isolated guinea-pig ileum in a 8-ml cuvette in Tyrode's solution containing atropine (6×10^{-6} M) and pyrilamine maleate (10^{-7} M) by a slight modification of the procedure of Orange and

Table 1. Column chromatography of SRS from granuloma pouch wall

Fraction	SRS (units)	Yield (%)
0.1 N NaOH (37°C for 30 min)	1289.4	100
XAD column chromatography		
water	0	0
80% ethanol	1182.7	91.7
Silicic acid column chromatography		
ether/hexane (3:7)	0	0
ethyl acetate	—	—
5% methanol/ethyl acetate	0	0
10% methanol/ethyl acetate	0	0
50% methanol/ethyl acetate	142.5	11.1
methanol	0	0

Austen [3]. One unit of SRS activity was defined as the amount giving the same response as 5 ng of histamine hydrochloride.

The criterion of SRS activity used was that on maximal contraction of the ileum by addition of 0.1 μg of FPL 55712 (kindly supplied from Fison Co., Leicestershire, England) it caused rapid relaxation of the ileum of more than 50 per cent.

Histamine concentration was measured by the same method except that pyrilamine maleate was omitted from the Tyrode's solution.

RESULTS

Isolation of SRS from carrageenin-induced granuloma

The pouch wall of 3 day granulomas was minced and incubated with cysteine and Ca^{2+}-Ionophore as described in the Materials and Methods. The SRS released into the incubation medium was isolated by two kinds of column chromatography. The amount of SRS produced was assayed by smooth muscle contracting activity in the presence of an antagonist of histamine.

Table 1 shows the elution profile of SRS. SRS from granuloma pouch wall was eluted with 50% methanol in ethyl acetate (50% MeOH fraction) from the silicic acid column. The considerable activity for contraction of smooth muscle eluted with ethyl acetate was due to prostaglandins not SRS, because it was insensitive to a low concentration of FPL 55712. Prostaglandin $F_{2\alpha}$ and some prostaglandin B_1 produced by the granuloma [7], were also eluted in this fraction (data not shown).

Since SRS, leukotriene D and C are eluted from the silicic acid column with 50% methanol in ethyl acetate and methanol only, respectively [6, 8], the 50% MeOH fraction obtained in this experiment must also contain leukotriene C or D. The yield of SRS in the 50% MeOH fraction was 11.1 per cent of the starting amount after decomposition with alkali.

Identification of the substance produced by granuloma as SRS

To identify the material produced by the granuloma as SRS, inhibitors of SRS biosynthesis were added to the incubation mixture. Indomethacin is known to inhibit prostaglandin synthesis, but not SRS production [9]; it has a stimulatory effect on SRS production by inhibiting 5-HPETE peroxidase [10]. On the other hand, BW 755C (kindly supplied by Ono Pharmaceutical Co., Japan) inhibits SRS and prostaglandin biosyntheses through inactivation of lipoxygenase and cyclooxygenase [11]. The requirements of cysteine and Ca^{2+}-Ionophore for SRS production by granuloma were also investigated.

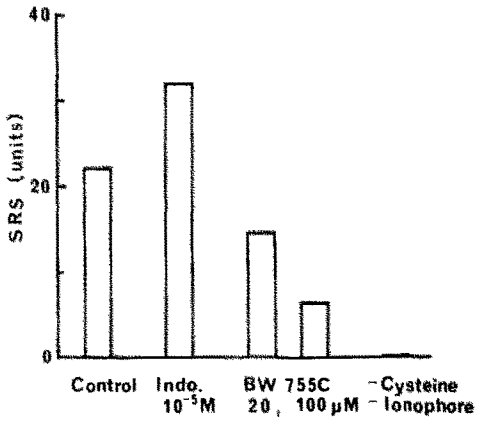

Fig. 1. Effects of indomethacine and BW 755C on SRS production by granuloma pouch wall. Minced granuloma pouch wall was incubated with 10^{-5} M indomethacin (Indo.), or 20 μM or 100 μM BW 755C at 37° for 5 min. Then the mixture was incubated with cysteine and Ca^{2+}-Ionophore at 37° for 20 min, and SRS was isolated as described in Materials and Methods. The amount of SRS was determined by bio-assay.

Granuloma pouch wall at various stages was pre-incubated for 5 min with each inhibitor, and then incubated with cysteine and Ca^{2+}-Ionophore. The mixture was then subjected to column chromatography as described above, and the SRS activity of the 50% MeOH obtained was assayed. Control preparations were incubated without inhibitor. As shown in Fig. 1, 10^{-5} M indomethacin increased SRS production to 46 per cent over the control, whereas 100 μM BW 755C inhibited it 71 per cent. Since 10^{-5} M indomethacine is a sufficient concentration to inhibit prostaglandin synthesis completely, this experiment showed that the 50% MeOH fraction did not contain a cyclooxygenase metabolite. When cysteine and Ca^{2+}-Ionophore were omitted from the incubation mixture, no SRS production was detected, as reported previousiy [6, 12]. The above results suggest that the 50% MeOH fraction must contain leukotriene C and/or D.

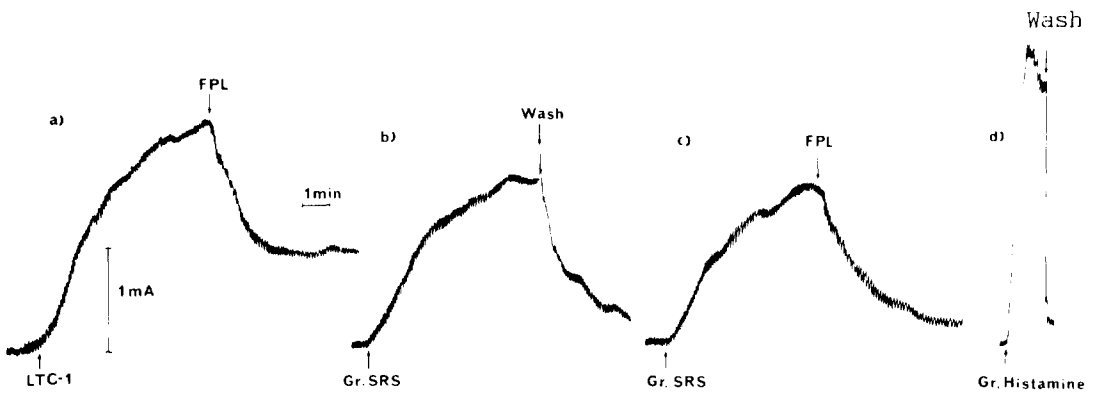

Fig. 2. Recordings of contractions of guinea-pig ileum after addition of leukotriene C-1 (LTC-1), SRS and histamine from granuloma pouch wall (Gr.). Amounts of 3 ng of synthetic LTC-1, Gr. SRS produced by 0.1 g of granuloma pouch wall and Gr. histamine produced by 0.02 g of granuloma pouch wall were used. FPL 55712 (FPL) was added at a final concentration of 0.01 μg/ml.

Figure 2 shows the contractile response of guinea-pig ileum to granuloma SRS. The time required to reach the maximum contraction was about 5 min. The contraction induced by the granuloma sample showed a typical slow reaction which was very similar to that induced by synthetic SRS (kindly supplied from Ono Pharmaceutical Co., Japan), and both contractions were reduced by addition of FPL 55712 at as low a concentration as 0.01 μg/ml.

Change of SRS producing activity with the granulation stage

SRS production by granulomas of various stages was examined by incubation of the granuloma pouch wall under standard conditions and bio-assay of the resulting 50% MeOH fraction from the silicic acid column. Figure 3 shows that SRS production was highest in the early granulation stage 3 days after carrageenin injection, and then gradually decreased. The wet weight of the granuloma pouch wall reached a maximum on day 7 after carrageenin injection, and then gradually decreased, and the volume of the pouch fluid increased with the granulation stage.

During chromatography of SRS, histamine was eluted with H_2O from the XAD-8 column [13] and its amount was determined by assay of ileum contraction without pyrilamine maleate, using authentic histamine as a standard. Histamine production by granuloma wall was almost the same between days 3 and 7, and then decreased to 25 per cent of this amount on day 14. Thus, the courses of production of SRS and histamine during granulation were different.

In the granuloma pouch fluid, no SRS activity was detectable and the histamine concentration was as low as 20–89 ng (data not shown).

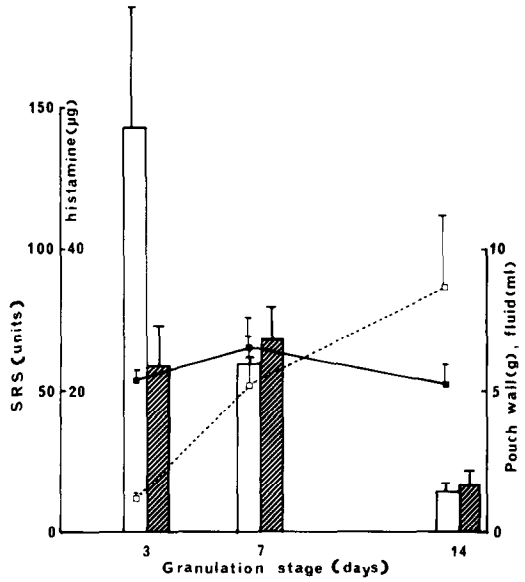

Fig. 3. SRS and histamine production by granuloma pouch wall at various granulation stages. Amounts of SRS and histamine were determined as described in the Materials and Methods. ▭ Units of SRS, ▨ amount of histamine, ■——■ wet weight of granuloma pouch wall, and □---□ granuloma pouch fluid. Five rats were used for 3 day granulomas, and 3 rats for 7- and 14-day granulomas.

DISCUSSION

The present study showed that inflammatory tissue produces most SRS activity in the early stage of inflammation. This may be partly due to change in the proportion of various cell types in the tissue. The pouch wall consists mainly of macrophage and fibroblasts, probably derived from monocytes and/or lymphocytes, and also contains mast cells, endothelial cells of blood vessels, etc. It was reported that three of these types of cells, monocyte, macrophages and mast cells, produce leukotriene C and/or D [14, 15]. Since SRS-A was found to increase vascular permeability and reduce blood flow in the skin, potentiating the activity of prostaglandin E_1, it may have widespread implications in inflammation [5]. So, it is very interesting that granulation tissue, which is a model of inflammatory tissue, produces SRS.

Under our assay conditions, endogenous arachidonic acid was used for SRS production by granuloma. Therefore, changes in the amount of endogenous arachidonic acid or phospholipase A_2 activity and the lipoxygenase activity may also contribute to change in SRS production at different stages.

The SRS produced by granuloma pouch wall was confirmed to have all the following properties of

leukotriene C and/or D: (1) it was eluted with 50% methanol in ethyl acetate from a silicic acid column [6]; (2) it was not produced in the absence of Ca^{2+}-Ionophore as a stimulant of phospholipase A_2 and cysteine as a substrate; (3) its production was increased by indomethacin and inhibited by BW 755C; and (4) it caused typical slow contraction of the ileum, which was antagonized by 0.01 ng/ml FPL 55712 (Fig. 2). In addition, the retention time of SRS produced by granuloma coincided with those of authentic leukotriene C and D on reversed phase polygosil high performance liquid chromatography (unpublished data), and triene in the SRS fraction from the chromatogram was detected by nuclear magnetic resonance analysis (data not shown).

Acknowledgements—We thank Ono Pharmaceutical Co. Ltd., Osaka, Japan for generous gifts of BW 755C and synthetic leukotriene C-1 and Fison Co., Leicestershire, England for a gift of FPL 55712.

REFERENCES

1. M. Fukuhara and S. Tsurufuji, *Biochem. Pharmac.* **18**, 475 (1969).
2. W. E. Brocklehurst, The Third International Symposium on Vaso-active Polypeptides: *Bradykinin and Related Polypeptides.*, p. 189. Pergamon Press, Oxford (1967).
3. R. P. Orange and K. F. Austen, *Adv. Immun.* **10**, 105 (1969).
4. R. Patterson, R. Orange and K. E. Harris, *J. Allergy Clin, Immun.* **62**, 371 (1978).
5. T. J. Williams and P. J. Piper, *Prostaglandins* **19**, 779 (1980).
6. R. C. Murphy, S. Hammarström and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **76**, 4275 (1979).
7. K. Ohuchi, L. Levine, H. Sato and S. Tsurufuji, *Prostagl. Med.* **2**, 293 (1979).
8. L. Örning, S. Hammarström and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **77**, 2014 (1980).
9. D. M. Engineer, U. Neiderhauser, P. J. Piper and P. Sirois, *Br. J. Pharmac.* **62**, 61 (1978).
10. M. I. Siegel, R. T. McConnell, N. A. Porter and P. Cuatrecasas, *Proc. natn. Acad. Sci. U.S.A.* **77**, 308 (1980).
11. O. Rådmark, C. Malmsten and B. Samuelsson, *FEBS Lett.* **110**, 213 (1980).
12. J. F. Burka and R. J. Flower, *Br. J. Pharmac.* **65**, 35 (1979).
13. R. P. Orange and P-L. Chang, *J. Immun.* **115**, 1072 (1975).
14. M. K. Bach, J. R. Brashler, S. Hammarström and B. Samuelsson, *Biochem. biophys. Res. Commun.* **93**, 1121 (1980).
15. C. A. Rouzer, W. A. Scott, Z. A. Cohn, P. Backburn and J. M. Manning, *Proc. natn. Acad. Sci. U.S.A.* **77**, 4928 (1980).