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STIMULATION OF HUMAN FIBROBLAST COLLAGEN SYNTHESIS *IN VITRO* BY γ-AMINOBUTYRIC ACID

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Abstract—Human buccal mucosa fibroblasts were exposed in culture to γ-aminobutyric acid (GABA) and the areca alkaloid arecaidine. Both GABA and arecaidine stimulated collagen synthesis and proliferation in a concentration-dependent manner, with arecaidine consistently producing the greater stimulation. Prior exposure to GABA or arecaidine for 5 days caused the cells to become insensitive when challenged with either drug.

A variety of factors are known to alter the rate of collagen synthesis in fibroblasts. They include hormones, growth factors, drugs and serum [1, 2]. In addition, there is a large body of evidence indicating that collagen metabolism is markedly altered in a variety of connective tissue diseases [1, 3]. One such disease is oral submucous fibrosis (OSF), a chronic disabling disease, characterised by a chronic accumulation of submucosal collagen and causing stiffness of the oral mucosa and impairment of mouth opening. Although the aetiology of OSF is unknown, there is a strong association with the chewing of areca nuts [4–6], a common habit in South-East Asia.

Experimental evidence for an aetiological role of the areca nut in OSF has come from tissue culture studies where ethanolic extracts of the nut were found to stimulate collagen synthesis in human fibroblast cultures [7]. Also, arecoline, the major alkaloid of the areca nut, is hydrolysed by fibroblasts *in vitro* to the stable metabolite arecaidine, a potent stimulator of fibroblast activity [8]. Arecaidine, a minor constituent of areca nuts [9] has also been shown to be a potent competitive inhibitor of γ-aminobutyric acid (GABA) re-uptake in rat brain slices and it has been suggested that this may well be the cause of some of the psychological effects of betel nut consumption [10, 11]. As arecaidine shows pharmacological activity in GABAergic systems and shares certain structural similarities with GABA, we were prompted to investigate the effect of GABA on collagen synthesis and proliferation by human buccal mucosa fibroblasts.

MATERIALS AND METHODS

Tissue culture materials were obtained from Gibco-Europe (Uxbridge, U.K.). Fibroblasts were cultured from normal human buccal mucosa obtained during routine surgical removal of third molar teeth. They were grown in 25-cm^2 culture flasks containing Eagle's Minimal Essential Medium (MEM) supplemented with foetal calf serum (FCS; 20%), penicillin and streptomycin (100 U/ml each) and buffered with bicarbonate (3.5 g/l) in an atmosphere of 5% CO_2, 95% air. The cells were maintained in MEM + 10% FCS, subcultured with 0.25% trypsin solution at weekly intervals, then stored in liquid nitrogen until used for experiments when they were between the 4th and 10th passages.

Collagen synthesis was measured in 96-well microwell culture plates (Microtiter, Linbro, Gibco-Europe, Uxbridge, U.K.). Each well was inoculated with 15,000 cells in 200 μl of MEM + 10% FCS and incubated overnight before replacement with 200 μl of MEM containing the drugs and 2% FCS. The rate of collagen synthesis was estimated by the incorporation of [^{3}H]proline into pepsin-resistant native collagen [12] over 24 hr. Briefly, 0.2 μCi 5[^{3}H]proline (23 Ci/mmol, Amersham International, Amersham, U.K.) was added to each well with β-aminoproprionitrile fumarate and L-ascorbic acid (50 μg/ml each). On termination, the cell layer and culture medium together were extracted with pepsin (0.5 mg/ml in a final concentration of 0.5 M acetic acid) for 16 hr at 4°. Radioactively labelled collagen was purified by salt precipitation and scintillation spectrometry (Rackbeta, LKB, Sweden) with external standardisation, and expressed as disintegrations per minute (dpm). Each control and treatment group in the microwell plates comprised six replicate wells. Proliferation was also measured in 96-well culture plates, but the inoculum was 5000 cells/well. After 5 days incubation, the numbers of cells in the microwell cultures were estimated by a methylene blue staining method modified from Currie [13]. Culture medium was aspirated and the cell layers in each well fixed in methanol for 5 min, stained with 100 μl methylene blue (0.1% in 10 mM borate buffer, pH 8.5) for 30 min and rinsed 3 times with 400 μl borate buffer. The methylene blue was eluted from the cell layers with 100 μl 0.1 M HCl containing 20% ethanol, and the absorbance measured at 650 nm in an automated multichannel spectrophotometer (Titertek Multiskan, Flow Laboratories, Croydon, Surrey, U.K.), using the first column of wells which had contained medium alone as a reference blank. Each control and treatment group in the microwell plates comprised six replicate wells.

Arecaidine was prepared by acid hydrolysis of arecoline hydrobromide and purified by TLC as previously described [8]. GABA was obtained from

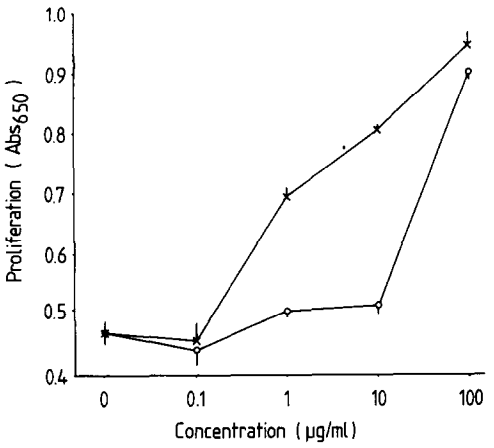

Fig. 1. The effect of GABA (○) and arecaidine (×) on fibroblast proliferation. Fibroblasts (5000 per well) were exposed to GABA or arecaidine (0, 0.1, 1, 10 or 100 μg/ml) for a period of 5 days and the cells stained with methylene blue. Results are expressed as absorbance units ± SEM (N = 6 per group).

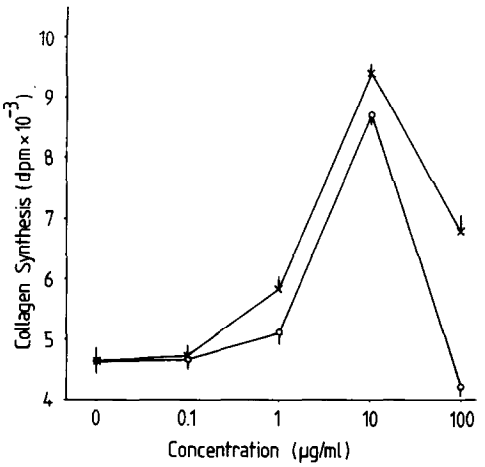

Fig. 2. The effect of GABA (○) and arecaidine (×) on collagen synthesis by fibroblasts. Results are expressed as dpm ± SEM (N = 6 per group).

Sigma Chemical Co. (Poole, Dorset, U.K.) and used without further purification.

In order to assess the effect of arecaidine and GABA on fibroblast activity, three types of experiment were performed: (1) fibroblasts were exposed to culture medium containing arecaidine or GABA (0, 0.1, 1, 10 and 100 μg/ml) for 24 hr and assayed for collagen synthesis simultaneously; (2) fibroblasts were exposed to arecaidine or GABA (0, 0.1, 1, 10 and 100 μg/ml) for a period of 5 days and cell proliferation assayed by methylene blue staining; (3c) fibroblasts were incubated with culture medium containing arecaidine or GABA (0, 1 and 10 μg/ml) for a period of 5 days. The medium was then aspirated and the cells re-challenged with either control medium or medium containing GABA or arecaidine (10 μg/ml) for a period of 24 hr and collagen synthesis measured over this period.

RESULTS

Proliferation

Both arecaidine and GABA stimulated the proliferation of fibroblasts over a 5-day period in a concentration-dependent manner (Fig. 1). Arecaidine caused more stimulation than GABA at each concentration.

Collagen synthesis

When fibroblasts were exposed to arecaidine or GABA for 24 hr, collagen synthesis was stimulated in a concentration-dependent manner (Fig. 2), with arecaidine producing more stimulation than GABA at each concentration. However, preincubation with arecaidine at 10 μg/ml caused the cells to become less sensitive to stimulation by GABA and arecaidine. Re-challenge with both drugs after preincubation with arecaidine produced a lower rate of collagen synthesis when compared with the group re-challenged with control media (Fig. 3a). Similarly,

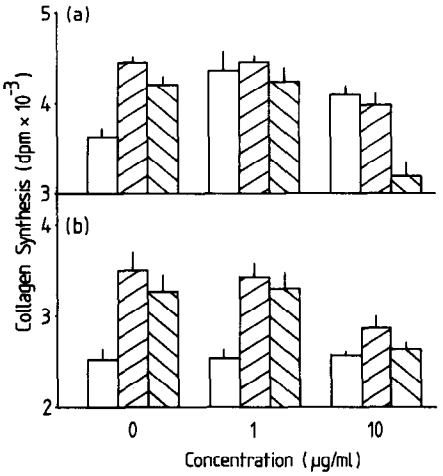

Fig. 3. The effect of long-term exposure to GABA or arecaidine on collagen synthesis by fibroblasts. Fibroblasts were exposed to (a) arecaidine or (b) GABA (0, 1 or 10 μg/ml) for a period of 5 days. They were then re-challenged with control media □, arecaidine (10 μg/ml) ▨ or GABA (10 μg/ml) ▧ for a period of 24 hr and collagen synthesis measured over this period. Results are expressed as dpm ± SD (N = 6 per group).

when the cells were exposed to GABA at 10 μg/ml for 5 days and then re-challenged with GABA or arecaidine the response was markedly reduced compared with cells preincubated with control medium (Fig. 3b). Preincubation with GABA or arecaidine at 1 μg/ml had little effect on subsequent stimulation with GABA or arecaidine (Fig. 3a and b).

DISCUSSION

In this report we have demonstrated for the first time that GABA stimulates collagen synthesis and proliferation in cultures of human fibroblasts. That fibroblasts respond to a drug better known as an inhibitory transmitter in the CNS is not necessarily surprising since fibroblasts have already been shown

to respond to adrenaline and isoprenaline [14, 15]. In addition, it has been known for some time that fibroblasts in culture can both synthesise and sequester GABA [16, 17] and it has been previously suggested that some neurotransmitters such as GABA may well act on non-neuronal cells as metabolic regulators [18]. We have previously shown that the response of fibroblasts to areca alkaloids is time dependent [8], with the stimulation of collagen synthesis falling progressively after a peak at 24 hr. This also appears to be the case for GABA. This suggests that desensitisation or "down-regulation" is occurring, and this was supported by the results of the rechallenge experiments (Fig. 3). The desensitisation to GABA was accompanied by a cross-desensitisation to arecaidine and vice versa, indicating that GABA and arecaidine share a common mechanism of fibroblast stimulation. Cross-desensitisation to hormones has been reported in several cell lines [15] and tends to be associated with the adenylate cyclase system. In this case, however, the addition of arecaidine, GABA or crude extract of areca nut had no effect on cyclic AMP levels in fibroblasts (A. Scutt and W. Harvey, unpublished results). On the other hand, the existence of GABA receptors on other cell types [19] and the fact that GABA is actively sequestered by fibroblasts, make it likely that GABA and arecaidine act via receptor mediated mechanisms. As both drugs show cross-desensitisation, it is possible that they may share a common receptor.

In a previous communication we have suggested that the areca alkaloids may stimulate fibroblast collagen synthesis *in vivo* and so contribute towards the fibrotic reaction in OSF. These results suggest that the stimulation may well be receptor mediated and that the areca alkaloids may be interfering with a GABA-mediated mechanism for controlling proliferation and collagen synthesis. The physiological role of GABA in fibroblasts is unclear. In the CNS, it is thought to be important as a neurotransmitter and in the production of energy. GABA can be metabolised by fibroblasts and so it is feasible that it is involved in the energy metabolism of these cells as well. However, the stimulation of fibroblast collagen synthesis and proliferation by GABA and the fact that fibroblasts can both synthesize and sequester it suggests that it may well have a more specialised role controlling fibroblast activity.

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