Vol. 134, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1986 Pages 29-36

REVERSIBLE INHIBITION OF INTERCELLULAR JUNCTIONAL COMMUNICATION BY GLYCYRRHETINIC ACID

James S. Davidson, Ingrid M. Baumgarten and Eric H. Harley

Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, South Africa

Received November 14, 1985

Intercellular gap-junctional communication was measured using metabolic co-operation in co-cultures of argininosuccinate synthetase-deficient and argininosuccinate lyase-deficient human fibroblasts. 18-α-glycyrrhetinic acid (AGA) was found to inhibit communication by more than 95% at concentrations as low as 2 uM. Concentrations up to 100 uM were not cytotoxic over a period of 2 hours. Communication inhibition was of rapid onset and was readily reversible. Communication remained continuously yet reversibly blocked in cells cultured in the presence of AGA for 20 days. The related compounds 18-β-glycyrrhetinic acid and carbenoxolone also caused communication inhibition. The effect is probably not mediated via mineralocorticoid or glucocorticoid receptors since aldosterone and glucocorticoids had no effect on communication. AGA thus has properties of a useful inhibitor in the study of intercellular junctional communication.

INTRODUCTION: Gap junctions are thought to mediate the exchange of small molecules directly between cells in contact, by forming aqueous channels linking the cytoplasmic compartments of adjacent cells (1,2). A convenient system for measuring intercellular junctional communication (IJC) in cultured human fibroblasts uses two mutant cell lines derived from patients with inborn errors of the urea cycle (3). Neither argininosuccinate synthetase-deficient nor argininosuccinate lyase-deficient fibroblasts are able to incorporate ^{14}C-citrulline into protein, since both enzymes are required to convert citrulline into arginine. However co-cultures of the two cell types incorporate ^{14}C-citrulline efficiently by the transfer of argininosuccinate between cells via intercellular junctions (Fig. 1) and there is no detectable transfer via the medium (3). The rate of simultaneous incorporation of ^{3}H-phenylalanine serves as an index of toxicity for any added compound. Thus the ^{14}C-citrulline/^{3}H-phenylalanine ratio in co-cultures of the mutant cells is a measure of IJC. Because of the possiblity that compounds could inhibit one of the enzymatic

0006-291X/86 $1.50

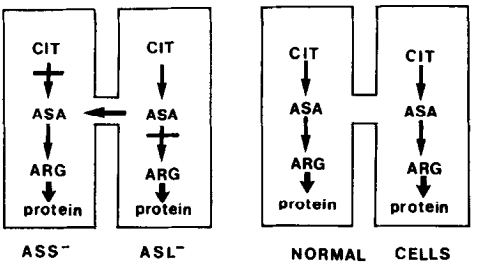

Fig. 1. Principle of assay of intercellular junctional communication. CIT = citrulline; ASA = argininosuccinate; ARG = arginine.

steps in citrulline metabolism, it is also necessary to monitor the effects of compounds on normal human fibroblasts (4).

Gap-junctional intercellular communication is known to be susceptible to pharmacological inhibition by several classes of compounds (5-9). The human fibroblast model used in the present study has previously been employed to investigate inhibition of IJC by organochlorine pesticides and related compounds (4,10), retinoids (11) and tumor-promoting phorbol esters (11). Here we report that 18-α-glycyrrhetinic acid (AGA) and the related compounds 18-β-glycyrrhetinic acid and carbenoxolone are novel inhibitors of IJC.

MATERIALS AND METHODS

Chemicals : The following were obtained from Sigma Chemical Co. : 18-α-glycyrrhetinic acid (AGA), 18-β-glycyrrhetinic acid, prednisolone, lupeol and betulin. Glycyrrhizic acid was obtained from Aldrich Chemical Co. Cholesterol and hydrocortisone were from BDH Chemicals Ltd. Carbenoxolone sodium was a kind gift from Berk Pharmaceuticals (Pty) Ltd. AGA, 18-β-glycyrrhetinic acid, lupeol and betulin were dissolved in dimethyl sulfoxide (DMSO). Glycyrrhizic acid and carbenoxolone were dissolved in water. The remaining chemicals were dissolved in ethanol. Final solvent concentration was 0.5% or less and appropriate solvent controls were included in each experiment. A fresh stock solution of AGA was prepared immediately before each experiment, as it was found that stored solutions had increased cytotoxicity. [Carbamoyl-^{14}C]citrulline and [2,3-^{3}H]phenylalanine were from the Radiochemical Centre, Amersham.

Metabolic co-operation assay : The origins of the human mutant fibroblast cell lines were as described (3). They were maintained on Dulbecco's Minimal Essential Medium (DMEM, Gibco) with 10% fetal calf serum (Gibco) in 4% CO_2 at 37°C. Preparation of co-cultures and the assay of IJC were as described (4,10). Briefly, trypsinised suspensions of the two cell types were mixed in equal proportions, plated at high density (confluent or subconfluent) and incubated for 18-22 hours. The co-cultures were then incubated in labelling medium containing radioisotopes and test chemical(s). Where the labelling medium was hepes-buffered saline (medium B in ref. 4) incubation time was 2 hours, ^{14}C-citrulline was used at 0.25 uCi/ml and ^{3}H-phenylalanine at 0.6 uCi/ml. Where the cells were labelled in growth medium (DMEM with 10% fetal calf serum) incubation time was 5 hours with ^{14}C-citrulline at 1 uCi/ml and ^{3}H-phenylalanine

at 2 uCi/ml. After labelling, radioactivity in acid-insoluble cellular material was determined as described (10). All experiments were performed in duplicate and repeated at least once with similar results. Each point shown is the mean of duplicate wells. Bars on the figures are of a length equal to the difference between duplicates and are omitted where this difference was less than 5%.

RESULTS: Exposure to AGA caused a dose-dependent inhibition of ^{14}C-citrulline incorporation in co-cultures of mutant cells, but not in normal cells (Fig. 2a), indicating that AGA inhibits IJC in this human fibroblast system. In serum-free medium, IJC as measured by citrulline/phenylalanine incorporation ratio, was inhibited by 97% at 2 uM AGA. ^{3}H-phenylalanine incorporation, reflecting overall protein synthesis, was essentially unaffected by concentrations as high as 100 uM over a 2-hour period. No morphological changes were apparent by phase contrast microscopy during a 2-hour exposure to up to 100 uM AGA.

In the presence of 10% serum, higher doses of AGA were required to elicit communication inhibition (Fig. 2b). A similar effect was seen in the presence of 0.5% bovine serum albumin (not shown), suggesting that AGA binds to albumin, and that such bound AGA is not effective in inhibiting IJC.

Longer exposure (20 h) of cultures to AGA before labelling produced very similar dose-response curves to those shown in Fig. 2 (data not shown). However rounding-up of the cells and decreased phenylalanine incorporation, indicating

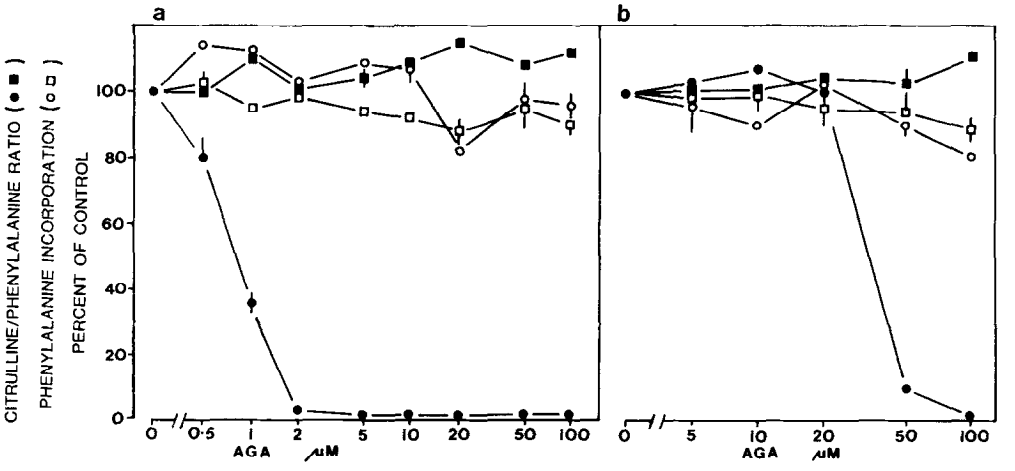

Fig. 2. Inhibition of communication by AGA. Co-cultures (●,○) and normal cells (■,□) were labelled for 2 hours in medium B containing AGA at the indicated concentrations without serum (a), or with 10% dialysed fetal calf serum (b).

Table 1

Preincubation 0.5h	Wash	Labelling 2h	^{3}H-phenylalanine cpm	^{14}C-citrulline cpm	$^{14}C/^{3}H$ ratio x 100
DMSO 0.5%	not washed	DMSO 0.5%	21045 20996	4050 4309	19.2 20.5
AGA 30 uM	not washed	AGA 30 uM	19922 20292	59 59	0.3 0.3
AGA 30 uM	washed with serum	DMSO 0.5%	20661 20497	3532 3845	17.1 18.8
AGA 30 uM	washed without serum	DMSO 0.5%	19465 19344	148 112	0.8 0.6

Reversibility of communication inhibition by AGA. 20 hours after plating, co-cultures were preincubated in medium B (hepes-buffered saline) with or without AGA as indicated. After 30 min the cells were washed 3 times with medium B with or without 10% dialysed serum, or not washed, as indicated. Labelling with radioisotopes was for 2h in serum-free medium B with or without AGA. Results for duplicate wells are shown.

cellular toxicity, occurred after 20 h exposure to concentrations of AGA above 30 uM in serum-free medium and above 150 uM in medium with 10% serum.

Communication inhibition by AGA was reversible by washing the monolayers with medium containing serum (Table 1). The rate of reversal was rapid, since cultures washed immediately before labelling showed nearly as great a degree of IJC as control cultures not exposed to AGA. Such washed cultures showed no signs of cellular damage by phase-contrast microscopy, and continued to proliferate at the same rate as control cultures. Washing with serum-free medium failed to reverse communication inhibition, suggesting that AGA partitions strongly into the lipid phase of cell membranes.

Communication inhibition was maintained for up to 20 days during continuous exposure to AGA and was still reversible after 17 days (Fig. 3). For this type of long-term experiment, the dose of AGA was found to be critical. For example, in the experiment shown in Fig. 3, where the medium contained 10% serum, 75 uM AGA was insufficient to induce complete communication inhibition whereas 150 uM was toxic. At the concentration used, 100 uM, the rate of increase of cell number was slowed by 24% compared to control cells (data not shown).

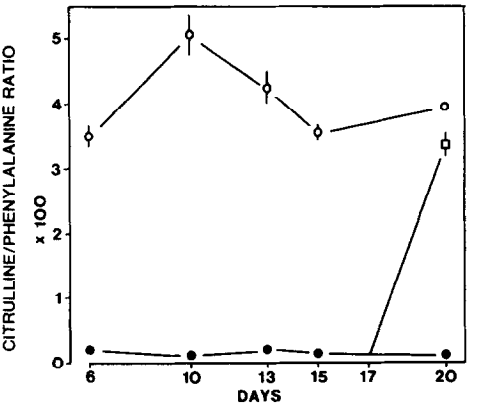

Fig. 3. Effect of continuous long-term exposure to AGA. Co-cultures were plated on day 1 and cultured in growth medium (DMEM plus 10% fetal calf serum) with 100 uM AGA (●) or vehicle only (0.3% DMSO, ○) from the time of plating. The medium was renewed every 3 days. On day 17 some wells (□) which had been grown on AGA-medium were washed twice with growth medium and replaced with medium without AGA. Labelling was for 5h in growth medium.

A number of compounds with structures related to AGA were tested for their ability to inhibit IJC. 18-β-glycyrrhetinic acid (not shown) and the water-soluble derivative, carbenoxolone (Fig. 4a) were both able to inhibit IJC with dose-response curves very similar to AGA. However, both of these compounds were more toxic than AGA at the higher doses. Glycyrrhizic acid, a glycoside of

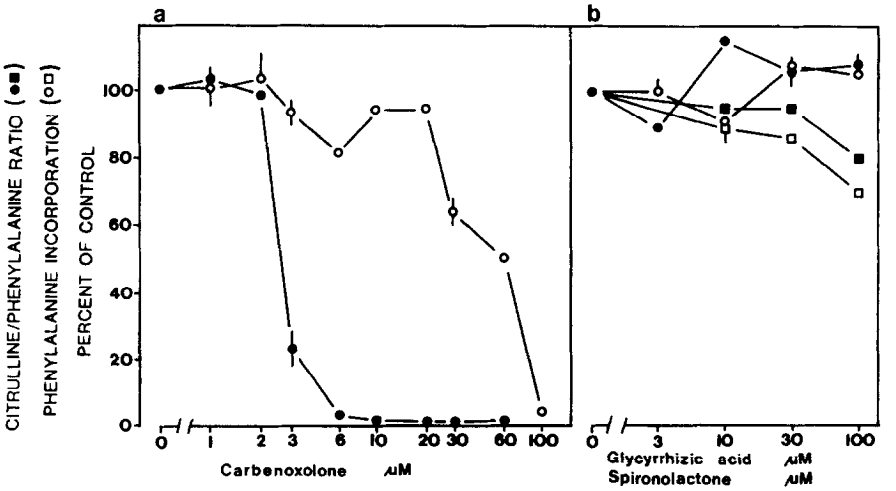

Fig. 4. Effect on co-cultures of carbenoxolone (a), glyccyrrhizic acid (b; ●,○) and spironolactone (b; ■,□). Experimental conditions as for Fig. 2a.

Table 2

Pre-treatment (20h)	Labelling medium (2h)	^{14}C-citrulline/^{3}H-phenylalanine ratio x 100
Normal medium	vehicle only	28.2 ± 0.7
"	TPA	11.0 ± 0.4
"	AGA	0.6 ± 0.3
"	TPA + AGA	0.3 ± 0.1
TPA 1000 ng/ml	vehicle only	30.2 ± 0.8
"	TPA	30.1 ± 1.4
"	AGA	1.2 ± 0.4
"	TPA + AGA	1.0 ± 0.1

Inhibition of junctional communication by AGA in co-cultures refractory to phorbol ester-induced inhibition. Cultures were made refractory to phorbol esters by exposure to 1000 ng/ml TPA for 20h prior to labelling. They were washed twice with medium with 10% serum and once with serum-free medium, and labelled for 2h in serum-free medium B in the presence of TPA (66 ng/ml) and/or AGA (10 uM). Results are mean ± difference of duplicate wells.

18-β-glycyrrhetinic acid had no effect on LJC at concentrations up to 100 uM (Fig. 4b).

Aldosterone (50 uM), spironolactone (100 uM), prednisolone (100 uM), hydrocortisone (100 uM), 7-deoxycholate (60 uM), cholesterol (100 uM), lupeol (30 uM) and betulin (30 uM) all showed no tendency to inhibit LJC at concentrations up to those shown in brackets, which were the highest tested. For example, the dose-response curve for spironolactone is shown in Fig. 4b. In addition, the inhibitory effect of AGA (10 uM) was not diminished by the simultaneous presence of a tenfold excess of the mineralocorticoid antagonist spironolactone (100 uM) (data not shown).

Tumor-promoting phorbol esters such as TPA (12-0-tetradecanoylphorbol-13-acetate) have been previously shown to induce a partial inhibition of LJC in this co-culture system (11). However, prolonged exposure to high concentrations of TPA results in a refractory state in which the cells are no longer responsive to phorbol ester (11). As shown in Table 2, co-cultures which had been made refractory to TPA retained their sensitivity to AGA-induced communication inhibition.

DISCUSSION: The present results demonstrate inhibition of LJC by AGA and the related compounds 18-β-glycyrrhetinic acid and carbenoxolone, in a human

fibroblast system. Of these, AGA was the least cytotoxic and its properties were investigated in detail.

The potency of AGA as an inhibitor of IJC was of the same order as retinoic acid (11) and greater than that of tetraphenylboron (10) which are the most potent representative compounds of two other classes of inhibitors of IJC. However AGA was less cytotoxic than these compounds, as assessed by its effect on protein synthesis rate. Communication inhibition by AGA was of rapid onset indicating that AGA acts on existing gap junctions, not only on new junction formation. Inhibition was readily reversible by washing the cells with albumin-containing medium. The observed rate of reversal is too rapid to be due to new junction formation alone, and therefore must reflect the re-opening of existing junctions. Communication inhibition could be maintained continuously for 20 days with only a minor slowing of the rate of cell proliferation, provided the concentration of AGA in the culture medium was carefully chosen.

AGA, 18-β-glycyrrhetinic acid and glycyrrhizic acid are derived from the liquorice root, Glycyrrhizia glabra, and carbenoxolone (18-β-glycyrrhetinic acid sodium hemisuccinate) is a derivative used in the treatment of peptic ulcer. These compounds have steroid-like structure and affinity for mineralocorticoid receptors and intrinsic mineralocorticoid activity (12,13). Their anti-inflammatory properties may be mediated via glucocorticoid receptors, for which glycyrrhetinic and glycyrrhizic acids also have low but demonstrable affinity (13,14,15). None of the steroids tested had any inhibitory effect on junctional communication. In addition, communication inhibition by AGA was not diminished by simultaneous exposure to a mineralocorticoid antagonist. Thus the ability of AGA to inhibit IJC appears not to be mediated either by mineralocorticoid or glucocorticoid receptors.

Another group of substances capable of inhibiting IJC are the tumor-promoting phorbol esters such as TPA (5-7,11), which are thought to activate protein kinase C (16). Communication inhibition by phorbol esters may therefore represent pharmacological activation of a physiological junction-regulatory mechanism. However, co-cultures which had been made

refractory to TPA remained sensitive to the effect of AGA, indicating that AGA probably does not act via the protein kinase C pathway.

We conclude that AGA is a novel inhibitor of IJC, an action which is not mediated via steroid receptors or protein kinase C. The reversibility of action and lack of toxicity of AGA should make it a useful investigative tool in the study of junctional communication.

REFERENCES

1. Hertzberg, E.L., Lawrence, T.S., and Gilula, N.B. (1981) Ann. Rev. Physiol. 43, 479-491.
2. Loewenstein, W.R. (1981) Physiol. Rev. 61, 829-913.
3. Davidson, J.S., Baumgarten, I.M., and Harley, E.H. (1984) Exp. Cell. Res. 150, 367-378.
4. Davidson, J.S., Baumgarten, I.M., and Harley, E.H. (1985) Cancer Res. 45, 515-519.
5. Yotti, L.P., Chang, C.C., and Trosko, J.E. (1979) Science 206, 1089-1091.
6. Fitzgerald, D.J., and Murray, A.W. (1980) Cancer Res. 40, 2935-2937.
7. Enomoto, T., Sasaki, Y., Shiba, Y., Kanno, Y., and Yamasaki, H. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 5628-5632.
8. Williams, G.M., Telang, S., and Tong, C. (1981) Cancer Lett. 11, 339-344.
9. Tsushimoto, G., Trosko, J.E., Chang, C.C., and Aust, S.D. (1982) Carcinogenesis 3, 181-185.
10. Davidson, J.S., Baumgarten, I.M., and Harley, E.H. Biochim. Biophys. Acta, in press.
11. Davidson, J.S., Baumgarten, I.M., and Harley, E.H. (1985) Carcinogenesis 6, 645-650.
12. Armanini, D., Karbowiak, I., Krozowski, Z., Funder, J.W., and Adam, W.R. (1982) Endocrinology 111, 1683-1686.
13. Armanini, D., Karbowiak, I., and Funder, J.W. (1983) Clin. Endocrinol. 19, 609-612.
14. Molhuysan, J.A., Gerbrandy, J., DeVeries, L.A., Dejong, J.C., Lenstra, J.B., Turner, K.P., and Borst, J.G.G. (1950) Lancet 259, 381-386.
15. Amagaya, S., Sugishita, E., Ogihara, Y., Ogawa, S., Okada, K., and Aizawa, T. (1984) J. Pharm. Dyn. 7, 923-928.
16. Nishizuka, Y. (1984) Nature 308, 693-698.