

## Characterisation of gastrin receptors on a rat pancreatic acinar cell line (AR42J). A possible model for studying gastrin mediated cell growth and proliferation

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**SUMMARY** Trophic changes of the exocrine pancreas after *in vivo* gastrin (G)/CCK treatment are well documented but up to now the study of the mechanisms involved is restricted by the lack of a suitable *in vitro* model. Nevertheless the *in vivo* trophic effect induced by gastrin/CCK peptides has been associated with an increase of ornithine decarboxylase (ODC) activity. In the present work, using the AR42J cell line in which CCK receptors and stimulation of amylase release by CCK peptides has already been demonstrated, we investigated the presence of gastrin binding sites and the possible modulation of proliferation by an inhibitor of ODC activity. <sup>125</sup>I-BH-G17ns binding is saturable, reversible and specific. Potencies of the different analogues tested are G17ns > CCK<sub>8</sub> > CCK<sub>6</sub>ns ≥ G<sub>6</sub>s > G/CCK<sub>4</sub>. Furthermore dBt cGMP, a non-peptide antagonist for CCK receptors, does not compete for gastrin binding. This indicates the existence of a subclass of gastrin binding sites. Difluoromethyl ornithine (DFMO) (1 mM), an irreversible inhibitor of ODC, inhibits cell growth from day 3 up to day 7. This growth inhibition is dose dependent and closely related to an intracellular polyamine modulation. Putrescine and spermidine levels fell under detectable values while spermine levels increased. All these data suggest that this cell line could be a useful *in vitro* model to study the mechanisms of gastrin induced growth control.

Growth of the pancreatic tissue can be stimulated by gastrointestinal hormones such as cholecystokinin, gastrin, secretin, and analogues.<sup>2-4</sup> Morisset and coworkers<sup>1</sup> have recently demonstrated that caerulein induced pancreatic growth is associated with an increased accumulation of putrescine, spermidine and spermine, and that α-Difluoromethyl ornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), can specifically inhibit caerulein induced pancreatic hypertrophy. These data lend further support to the involvement of ODC and polyamines in induced pancreatic growth, but up to now the study of the mechanisms involved is restricted by the lack of a suitable *in vitro* model.

In the present study using the AR42J cell line in which CCK receptors and stimulation of amylase release by CCK peptides have already been demonstrated,<sup>5</sup> we investigated the presence of gastrin

binding sites and the possible modulation of cell proliferation by an inhibitor of ODC activity.

### Methods

#### CELL CULTURE

AR42J cells, originally developed by Jessop and Hay,<sup>6</sup> were obtained from Dr Logsdon (San Francisco, California, USA). These cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum. Cells were routinely plated at 2·10<sup>5</sup> cells/ml into 60 mm well dishes and the medium changed every two days. Cell growth was measured by cell counting on a coulter counter coultronics model ZM.

#### LIGAND BINDING STUDIES

Non-sulphated gastrin 2-17 was radioiodinated by conjugation of the peptide to <sup>125</sup>I-Bolton-Hunter reagent and purified by RP-HPLC as previously

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described.<sup>7</sup> Binding assays were carried out on cells harvested with 0.025% EDTA alone.  $2 \times 10^5$  cells were incubated with 60 pM  $^{125}\text{I}$ -BH-G<sub>17</sub>ns and various concentrations of analogues in a Krebs-Hepes buffer supplemented with 0.5% bovine serum albumin, 0.03% soybean trypsin inhibitor and 0.1% bacitracin, in a total volume of 0.5 ml at 37 °C for 30 min unless otherwise indicated in the Figures.

Dissociation kinetics were studied by incubating cells with the radioligand for the time required for equilibrium. Then, a saturable concentration of unlabelled peptide was added and residual binding was measured at various times. Specific binding was defined as the excess binding over that in blanks containing 1  $\mu\text{M}$  of unlabelled peptide.

#### CELL POLYAMINE CONTENT

Intracellular polyamines were extracted in 0.3 M HC10<sub>4</sub> and dansylated according to the procedure of Newton *et al.*<sup>8</sup> Separation of the dansylated polyamines was carried out on a  $\mu$ Bondapak C<sub>18</sub> column (Waters, Milford, USA) with a solvent composed of TEAP-CH<sub>3</sub>CN (pH 3.5) in the ratio (40:60) for solvent A and (20:80) for solvent B. Samples were eluted in the gradient mode using the concave gradient program number 9 (Waters, model 660). The gradient changed from 100% solvent A to 100% solvent B in 15 min at a flow rate of 21 ml/min. Fluorimetric detection used a model 420 (Waters) equipped with 280 and 338 nm filters for excitation and emission respectively. The area of the peaks was calculated on a Waters integrator Model 740 using a two point calibration curve.

$^{125}\text{I}$ -Bolton-Hunter reagent with a specific activity of 2000 Ci/mmol was purchased from Amersham, France. Acetonitrile was purchased from Fluka Lab.; Human gastrin-(2-17)ns from UCB Bioproducts, Brussels, Belgium; G/CCK-4 from Interchim, Montluçon, France, CCK-8 from CRB Laboratories, Cambridge, England; (Thr,Nle)-CCK<sub>9</sub> was a gift from Professor E Wünsch, Max Planck Institut für Biochemie, München, West Germany. Putrescine, spermidine, spermine, DNS-Cl were purchased from Sigma (St Louis, Mo, USA). DFMO was kindly provided by Dr J Wilkins, Merrel Dow Research Institute (Strasbourg, France).

## Results

#### BINDING STUDIES

As shown in Figure 1, specific binding of  $^{125}\text{I}$ -BH-(2-17)-G-17ns to AR42J cells reached a maximal level after a 20 min incubation period ( $94.12 \pm 18.6$  fmol/10<sup>6</sup> cells). Non-specific binding remained lower than 25% of total binding until 60 min of incubation. The addition of unlabelled G-17ns into the medium,

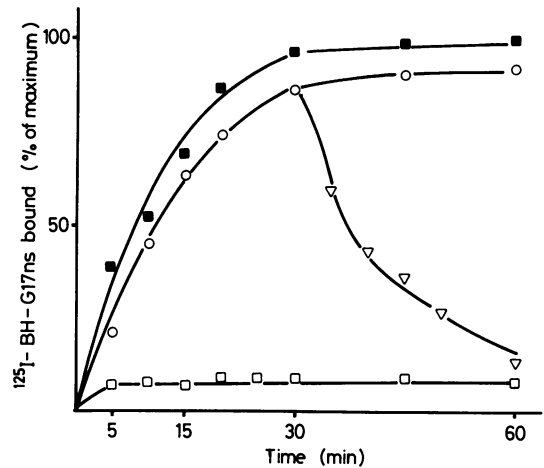


Fig. 1 Time course of association and dissociation of  $^{125}\text{I}$ -BH-G<sub>17</sub>ns to AR42J cells. Cells were incubated at 37 °C with the radioligand alone (■), or in the presence of 1  $\mu\text{M}$  G<sub>17</sub>ns (non specific binding) (□). At steady state 1  $\mu\text{M}$  ( $\nabla$ ) G<sub>17</sub>ns was added to the incubation medium for dissociation. Results are expressed on the percentage of total radioactivity bound in the absence of unlabelled gastrin. Each value was determined in triplicate and this experiment is representative of three others.

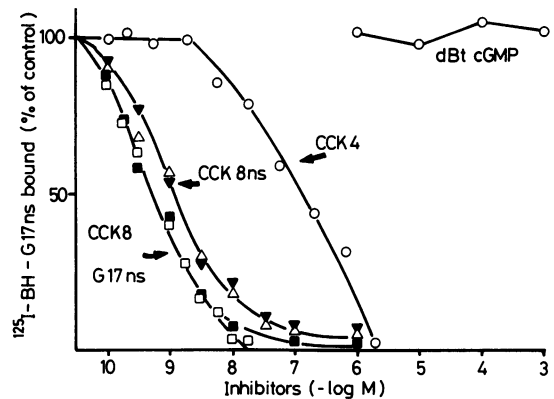


Fig. 2 Ability of various analogues to inhibit binding of  $^{125}\text{I}$ -BH-G<sub>17</sub>ns to AR42J cells. Cells were incubated for 30 min at 37 °C with the radioligand (60 pM) plus different concentration of G<sub>17</sub>ns (□), CCK<sub>8</sub> (■), CCK<sub>8</sub>ns (▼), G<sub>6</sub>s (△), G/CCK<sub>4</sub> (○) and dBT cGMP. Results are the mean of at least five separate experiments.

resulted in rapid dissociation of bound radioactivity with a half time of about 8.5 min. Analysis of the displacement curves of the labelled G-17ns by CCK and gastrin peptides showed that CCK<sub>8</sub> and G<sub>17</sub>ns inhibited the binding with the same potency (Fig. 2); IC<sub>50</sub> were respectively 4.2 and 5.6  $10^{-10}$  M and a total

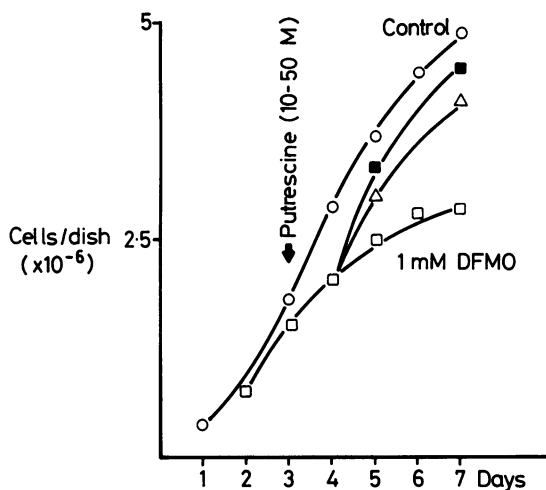


Fig. 3 Effects of DFMO on AR42J cell growth. AR42J cell number as a function of time, in the absence (○) or the presence of DFMO (1 mM) (□). At day 3, putrescine 10  $\mu$ M ( $\Delta$ ) or 50  $\mu$ M ( $\blacksquare$ ) was added to the culture medium of cells treated with DFMO 1 mM. Results are the mean of four separate experiments done in triplicate.

inhibition was reached with  $2 \cdot 10^{-8}$  M. CCK<sub>8</sub>ns, G<sub>6</sub>s and G/CCK<sub>4</sub> also inhibited <sup>125</sup>I-BH(2-17)-G<sub>17</sub>ns binding with C<sub>50</sub> of  $10^{-9}$  M,  $1 \cdot 6 \cdot 10^{-9}$  and  $10^{-7}$  M respectively. Dibutyl cyclic GMP, a non-peptidic antagonist specific for CCK receptors, did not affect the radioligand binding (Fig. 2).

<sup>125</sup>I-(Thr,Nle)-CCK<sub>9</sub> binding results were in good agreement with those obtained by Logsdon<sup>5</sup> using <sup>125</sup>I-CCK<sub>39</sub> a radioligand. Furthermore we showed that gastrin peptide inhibited <sup>125</sup>I-(Thr,Nle)-CCK<sub>9</sub> binding with a high affinity and that dibutyl cyclic GMP partially blocked (60%) CCK binding at the highest dose tested ( $10^{-3}$  M).

The ratio gastrin preferring receptors v CCK<sub>9</sub>-preferring receptors was found to be  $0 \cdot 107 \pm 0 \cdot 011$ .

AR42J cells, when grown in DMEM medium presented a logarithmic growth over a five day period with a population doubling time of 25–30 hours. Continuous treatment of AR42J cells with DFMO (1 mM) produced growth inhibition from day 3. The per cent inhibition was about 40% between days 5 and 7 in which growth seemed to be progressively arrested (Fig. 3). Viability of treated or control cells was tested by their ability to exclude trypan blue and was found to be higher than 95%. Furthermore this growth inhibition was dose dependent and totally reversed when exogenous putrescine was added to the culture medium.

Intracellular polyamine contents were determined under the same experimental conditions (Table). In control cells, polyamines were raised in the first days of culture and reached basal values at day 3. DFMO (0.5 mM) completely prevented the accumulation of putrescine and decreased spermidine content under 10% of control values. Spermidine level became undetectable for upper DFMO concentrations. This depletion was well correlated with growth inhibition. Spermine levels showed a two-fold increase during 1–5 mM DFMO treatment.

## Discussion

The present study investigates the interaction of <sup>125</sup>I-BH-(2-17)-G<sub>17</sub>ns with a rat pancreatic acinar cell line (AR42J) and demonstrates the ability of DFMO to modulate cell growth and intracellular polyamine content.

<sup>125</sup>I-BH-(2-17)-G<sub>17</sub>ns binding data indicated that gastrin receptor sites are present in AR42J cells. The ability of the different molecules tested to inhibit gastrin binding is similar to that found in dog pancreatic acini.<sup>7</sup> In AR42J cells radioiodinated gastrin binds with a high affinity to specific sites, in contrast to that observed in rat acini.<sup>9</sup> CCK<sub>9</sub> is about as potent as G<sub>17</sub>ns, whereas CCK<sub>8</sub>ns, G<sub>6</sub>s and CCK<sub>4</sub>

Table Effect of DFMO treatment on the polyamine levels of cultured AR42J cells

Treatment	Concentration (nmol/10 <sup>6</sup> cells)		
	Putrescine	Spermidine	Spermine
None	$0 \cdot 144 \pm 0 \cdot 015$	$1 \cdot 212 \pm 0 \cdot 065$	$0 \cdot 473 \pm 0 \cdot 004$
DFMO 0.5 mM	< 0.030	$0 \cdot 157 \pm 0 \cdot 05$	$0 \cdot 763 \pm 0 \cdot 113$
DFMO 1 mM	< 0.030	< 0.030	$1 \cdot 114 \pm 0 \cdot 174$
DFMO 5 mM	< 0.030	< 0.030	$0 \cdot 992 \pm 0 \cdot 143$
DFMO 10 mM	< 0.030	< 0.030	$1 \cdot 150 \pm 0 \cdot 259$
DFMO 1 mM + putrescine 10 $\mu$ M	< 0.030	$1 \cdot 316 \pm 0 \cdot 461$	$1 \cdot 202 \pm 0 \cdot 070$

AR42J cells ( $2 \cdot 10^6$  cells/ml) were plated and treated as described in materials and methods. At day 4, 10  $\mu$ M putrescine were added in culture medium of cells treated with DFMO 1 mM and, 24 hours later, cells were scraped out, counted and used for polyamine content determination. Results are the means  $\pm$  SEM of four separate experiments in duplicate.

are less potent to inhibit the binding of labelled gastrin. Furthermore, dBtcGMP, a non-peptidic antagonist specific for CCK receptors, did not affect the binding of gastrin, whereas it inhibits CCK<sub>1</sub> binding. All together these data suggest the existence of a subclass of CCK/gastrin binding sites displaying a high affinity for gastrin. These results are in agreement with those found by Logsdon<sup>5</sup> who noted that CCK receptors in AR42J cells seemed to be different from those in normal rat pancreatic acini. Results obtained with DFMO indicate that AR42J cell growth depends on adequate intracellular polyamine concentration, and that AR42J cell line which possesses two classes of different binding sites for CCK and gastrin may represent a useful *in vitro* model for studying the mechanisms of CCK/gastrin-induced growth control mediated by ODC activation.

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