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Analysis of the CCK_B receptor antagonism of virginiamycin in guinea-pig ileum longitudinal myenteric plexus

[1]M. Corsi, G. Pojani, G. Dal Forno, C. Pietra, G. Gaviraghi & D. Trist

Glaxo Research Laboratories, 37135 Verona, Italy

1 Virginiamycin, a macrolide reported to bind selectively to CCK_B/gastrin receptors has been studied in a functional test, namely cholecystokinin-induced contraction of guinea-pig ileum myenteric plexus (LMMP).

2 Virginiamycin (1–10 μM) antagonized the selective CCK_B agonist cholecystokinin tetrapeptide (CCK-4). The antagonism appeared not to be competitive as the highest concentration (10 μM) caused a reduction of its maximal effect. An apparent pA_2 of 6.64 ± 0.06 (s.e.) could be estimated if this depression was ignored. The selective CCK_B antagonist, L-365,260 (0.01–0.3 μM) antagonized competitively the CCK-4 induced contraction and a pK_B of 8.60 ± 0.16 (s.e.) was estimated.

3 The combined dose-ratio analysis for virginiamycin, tested at 3 and 10 μM in association with 0.03 and 0.1 μM L-365,260, respectively, resulted in observed log dose-ratios of 1.39 and 1.53. That was consistent with both antagonists acting on the same receptor in LMMP.

4 These data, represent the first evidence of the antagonism of virginiamycin in a functional assay and they support the hypothesis of homogeneity between CCK_B receptors in the CNS and in peripheral tissues.

Keywords: Guinea-pig ileum; cholecystokinin; L-365,260; virginiamycin; CCK_B receptor

Introduction

Cholecystokinin (CCK) causes marked peripheral and central effects by interacting with two different types of receptor, CCK_A and CCK_B (Bock, 1991). The former is largely distributed in the periphery and the latter is found in most abundance in the CNS (Bock *et al.*, 1989; Woodruff & Hughes, 1991). The two receptors have been distinguished by use of both selective agonists (e.g. CCK-4 for CCK_B receptors) and selective antagonists (e.g. L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3yl)-1H-indole-2-carboxamide) for CCK_A receptors and L-365,260 ((3R(+)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3yl)-N-(3-methylphenyl)-urea) for CCK_B receptors, Chang & Lotti, 1986; Lotti & Chang, 1989). Differences between CCK_B receptors and gastrin receptors, although suggested (Patel & Spraggs, 1992; Botella *et al.*, 1992), have, as yet, not been clearly demonstrated. Recently, the guinea-pig ileum longitudinal muscle myenteric plexus (LMMP) has been shown to contain neuronal receptors for both the CCK_A and CCK_B sub-types (Lucaites *et al.*, 1991; Dal Forno *et al.*, 1992). Thus, in the presence of a selective CCK_A receptor antagonist such as L-364,718, LMMP could be considered as a functional assay for CCK_B receptors.

Selective antagonists for the CCK_B receptor, so far described, tend to fall into a number of discrete chemical classes. These include the benzodiazepines, peptoids and the pyrazolidinones (Kervin, 1991). In addition, virginiamycin, a macrolide antibiotic produced by fermentation of a strain of *Streptomyces olivaceous*, has been reported to bind to CCK_B/gastrin receptors (Lam *et al.*, 1991). Thus, in radiolabelled binding studies virginiamycin has an IC_{50} (nM) of 571 and 710 for CCK_B (guinea-pig brain) and gastrin (guinea-pig gastric glands) receptors, respectively, with no apparent affinity for CCK_A (rat pancreas) receptors (> 100,000 nM). To date it has not been reported whether virginiamycin acts as an agonist or an antagonist at CCK_B receptors. Thus the objective of the study described below was to determine and to quantify the nature of the interaction of virginiamycin with CCK_B receptors in a functional assay, namely LMMP.

[1] Author for correspondence.

Methods

Male Dunkin-Hartley guinea-pigs weighing approximately 300–400 g were allowed free access to food and water until just before use. The animals were killed by cervical dislocation and 10 cm of terminal ileum was rapidly removed and placed in a modified Krebs solution (composition, mM: NaCl 118.5, $CaCl_2$ 1.2, KCl 4.7, $MgSO_4$ 0.6, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11.1). A 4 cm length of ileum was placed over a glass-pipette and the longitudinal muscle with adherent myenteric plexus was removed with a cotton tip. The preparations were set-up in 2 ml sylanized (5% dimethylchloroxylane 95% toluene) baths at 37°C containing the Krebs solution gassed with a 5% CO_2:95% O_2 mixture. The muscle was attached to an isotonic transducer (Ugo Basile 7006) coupled to a polygraph (Ugo Basile Gemini 7070 and Unirecord 7050). The tissues were washed and adjusted to a tension of 0.5 g. After a resting phase of 60 min the tissues were challenged several times with 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) 30 μM at a cycle time of 10 min until a constant response was achieved.

Experimental protocol

All the experiments described here were performed with Krebs solution containing L-364,718 at 10 nM. Each tissue received only one treatment. Serial concentration-response curves (CRC) for the agonist CCK-4 were run at intervals of 10 min. When evaluating the effect of the antagonist virginiamycin, L-365,260 or the mixture of both, a 60 min contact time was allowed before the first administration of the agonist. The antagonist or the mixture of antagonists was left in contact with the tissue throughout the duration of the experiment. The contractions measured were expressed as a percentage of the effect obtained with 30 μM DMPP.

Analysis of data

Curve fitting The CRC data were routinely fitted by a four parameter logistic equation (De Lean *et al.*, 1978):

$$\text{response} = a + \frac{R_{max}\,[A]^n}{[A]^n + [A_{50}]^n} \quad (1)$$

where a and R_{max} are the minimum and maximal asymptotes respectively, [A] the agonist concentration, n the slope factor and $[A_{50}]$ the concentration of the agonist that induces 50% of the maximal effect (ED_{50}). Antagonism were tested for significant differences in parallelism and of maximal responses in comparison with the control. If no significant difference was found, the analysis of the antagonist effect was performed.

Analysis of individual antagonist effects Curve displacements by antagonists applied singly were analysed fitting CRC data to the equation:

$$\text{response} = \frac{R_{max}\,[A]^n}{(([A_{50}]^n)(1 + ([B]^m/K_B))^n + [A]^n)} \quad (2)$$

where R_{max}, [A], n and $[A_{50}]$ are as above defined, B is the antagonist concentration, m is the equivalent to the Schild slope parameter and K_B is the antagonist dissociation constant when m is not significantly different from one.

Combined dose-ratio analysis This analysis was performed according to the procedure developed by Shankley *et al.* (1988). The theory predicts that when two antagonists (A and C) act through an interaction at the same receptor to produce their effect (additive model), then the measured dose-ratio should be:

$$DR_{A+C} = DR_A + DR_C - 1 \quad (3)$$

where DR_A and DR_C are the dose ratio obtained independently in the presence of the antagonist A and C. The model was tested by comparing the discrepancy between observed and expected statistics for the models with estimates of their standard errors. Thus, for the additive model:

$$S_A = \log ED_{50AC} - \log(ED_{50A} + ED_{50C} - ED_{50}) \quad (4)$$

where ED_{50AC}, ED_{50A}, ED_{50C} and ED_{50} are the concentrations of agonist that induce 50% of response in the presence of the mixture of the antagonists AC, only A or only C and in the absence of both (control) respectively. The term S_A is the test statistic of the model which will have a value of zero when the data comply with the model.

Similarly, when the two antagonists act independently (multiplicative model) the measured dose-ratio should be:

$$DR_{A\cdot C} = DR_A * DR_C \quad (5)$$

For the multiplicative model the test statistic of the model is:

$$S_M = \log ED_{50AC} + \log ED_{50} - \log ED_{50A} - \log ED_{50C} \quad (6)$$

so that S_M, the test statistic will be zero when the data comply with the model.

From the combined dose ratio analysis the dissociation constant (K_Bv) for virginiamycin was estimated using the equation (Black *et al.*, 1986; Leff & Morse, 1987; Trist *et al.*, 1987):

$$DR_{AC} = 1 + \frac{[A]}{K_B I(1 + [C]/K_B v)} \quad (7)$$

where DR_{AC} in this case is the dose-ratio obtained with both antagonists (A and C) together, measured from the curve produced in the presence of C alone, [A] is the concentration of the standard (L-365,260) and K_BI is its dissociation constant.

Values are given ± s.e.mean.

Drugs used

The following drugs were used: cholecystokinin tetrapeptide (CCK-4) and DMPP, both from Sigma. L-364,718, L-365,260 and virginiamycin were synthesized by the chemistry department of Glaxo Italy. DMPP was dissolved in water, whereas CCK-4 (at 30 mM) and L-364,718, L-365,260 and virginiamycin (all three at 100 μM) were dissolved in dimethylsulphoxide. Further dilutions were made in distilled water. Vehicles were found not to produce significant effects on the CCK-4-induced contraction.

Results

CCK-4 (0.01–300 μM), as has already been reported (Dal Forno *et al.*, 1992), contracted the LMMP showing a biphasic CRC. In the same study it was demonstrated that this effect was due to the activation of both types of CCK receptors, with a higher potency of CCK-4 for the CCK_B type. In the presence of L-364,718 at 10 nM, the CCK-4 CRC changes into a mono-phasic curve with a suppression of the upper part, the CCK_A-activated region (Figure 1). To avoid possible interaction with CCK_A receptors, all further experiments described in the present study were carried out in the presence of 10 nM L-364,718.

Analysis of individual antagonistic effects

L-365,260 (0.01–0.3 μM) antagonized CCK-4-induced contractions in a competitive manner (Figure 2). The Schild slope parameter was 1.25 ± 0.15 (95% CL 0.93–1.56) which was not significantly different from one ($P = 0.10$). The analysis of the antagonism resulted in a pK_B of 8.60 ± 0.16 (Figure 2). Virginiamycin (1–10 μM) antagonized in a dose-related manner the CCK-4 CRC. However, at the highest concentration, virginiamycin caused a significant ($P = 0.001$) reduction of the maximal response to the agonist (Figure 3). An apparent dissociation constant (pA_2) was, however, estimated without any assumption of the mechanism of action. It was 6.64 ± 0.06. Comparing the two antagonists, virginiamycin is about 100 fold less potent than L-365,260. Virginiamycin over the range 0.3 to 10 μM did not contract the LMMP (data not shown).

Combined dose-ratio analysis

The effect of the two antagonists acting alone and in combination are shown in Figures 4 and 5. The concentrations were chosen to give a similar individual displacement. Figure 4 demonstrates the effect of L-365,260 0.03 μM ($n = 4$) and virginiamycin 3 μM ($n = 4$) when given alone or in combination ($n = 4$). Thus, if addition of the dose-ratio occurred then

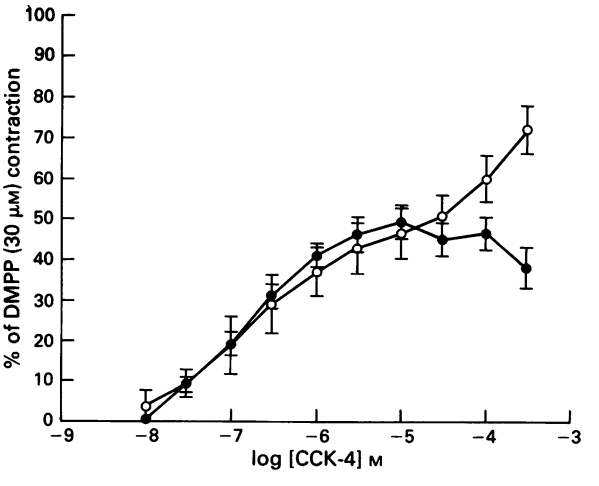

Figure 1 Concentration-response curve for cholecystokinin tetrapeptide (CCK-4) alone (○) and in the presence of 10 nM L-364,718 (●). Each point represents the mean ± s.e. of 7 replications.

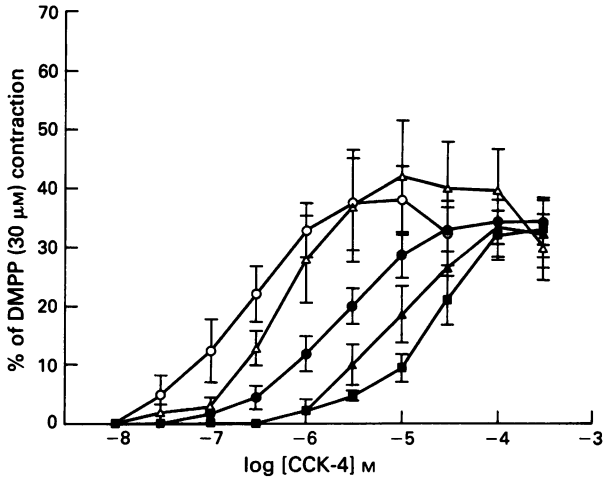

Figure 2 Concentration-response curve for cholecystokinin tetrapeptide (CCK-4) alone (○) and in the presence of L365,260: 0.01 μM (△), 0.03 μM (●), 0.1 μM (▲) and 0.3 μM (■). All the experiments were carried out in the presence of 10 nM L-364,718 and each point is the mean ± s.e. of 5–10 replications.

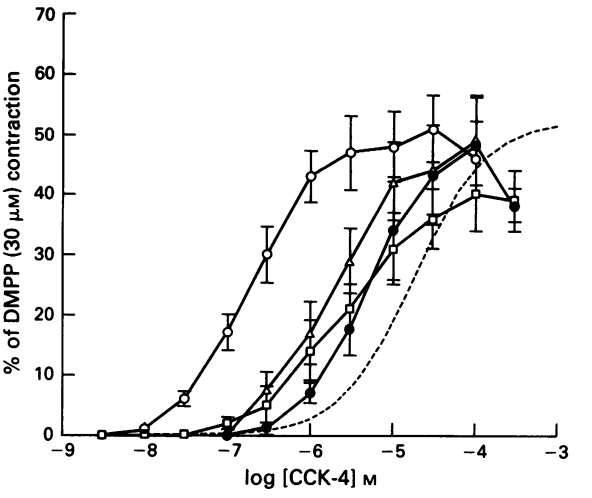

Figure 4 Concentration-response curve for cholecystokinin tetrapeptide (CCK-4) alone (○) and in the presence of 3 μM virginiamycin (△), 0.03 μM L-365,260 (□) and a combination of 3 μM virginiamycin and 0.03 μM L-365,260 (●). All the experiments were carried out in the presence of 10 nM L-364,718 and each point is the mean ± s.e. of 4 replications. The dashed line shows the location of the CCK-4 concentration-response curve which was predicted by assuming that the antagonists acted independently.

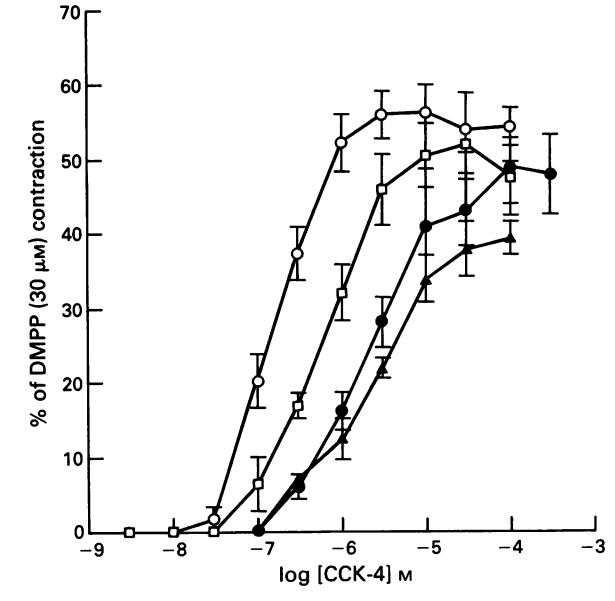

Figure 3 Concentration-response curve for cholecystokinin tetrapeptide (CCK-4) alone (○) and in the presence of virginiamycin: 1 μM (□), 3 μM (●) and 10 μM (▲). All the experiments were carried out in the presence of 10 nM L-364,718 and each point is the mean ± s.e. of 4 replications.

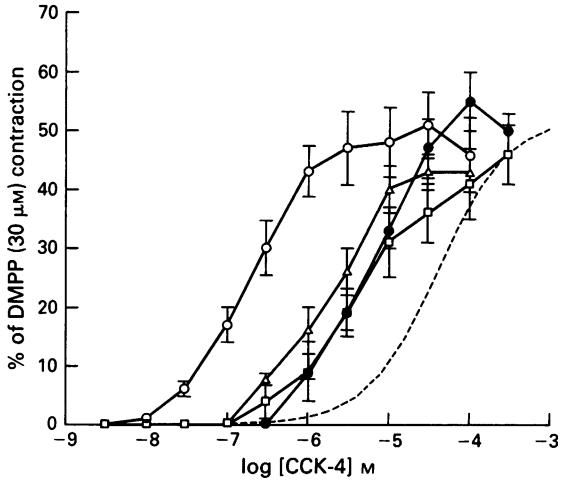

Figure 5 Concentration-response curve for cholecystokinin tetrapeptide (CCK-4) alone (○) and in the presence of 10 μM virginiamycin (△), 0.1 μM L-365,260 (□) and a combination of 10 μM virginiamycin and 0.1 μM L-365,260 (●). All the experiments were carried out in the presence of 10 nM L-364,718 and each point is the mean ± s.e. of 4 replications. The dashed line shows the location of the CCK-4 concentration-response curve which was predicted by assuming that the antagonists acted independently.

the predicted combined log dose-ratio would have been 1.32. On the other hand if the dose-ratios were multipled, then the log dose-ratio would have been 2.06. Figure 5 shows the effect of higher concentrations of the two antagonists, L-365,260 0.1 μM ($n = 4$) and virginiamycin 10 μM ($n = 4$) when given alone or in combination ($n = 4$). With these concentrations, if addition of the dose-ratios occurred then the predicted log dose-ratio would have been 1.49. For the multiplicative model the predicted log dose-ratio would have been 2.37. The data obtained in both experiments were consistent with the additive model and rejected the multiplicative model. In fact the observed log dose-ratios were 1.39 and 1.53 (Table 1). A simulation of the multiplicative model for both experiments have been included in both Figures 4 and 5. From the combined dose-ratio analysis the pK_B for virginiamycin estimated from equation (7) was 6.20 and 6.14 when virginiamycin was tested at 3 and 10 μM respectively.

Discussion

To date, little has been reported on CCK_B antagonists in *in vitro* functional assays. Of those antagonists so far described, L-365,260, the first selective CCK_B antagonist discovered, has become somewhat of a standard, particularly in biochemical and *in vivo* assay (Bock *et al.*, 1989). Also, L-365,260 has

Table 1 Combined dose-ratio analysis of virginiamycin with L-365,260 used as a standard CCK_B antagonist

	A	L-365,260 (0.03 μM)	L-365,260 (0.1 μM)
	C	VGM (3 μM)	VGM (10 μM)
Observed	$\log R_A$	1.11	1.33
	$\log R_C$	0.94	1.06
Multiplicative expected	$\log R_{A\cdot C}$	2.06	2.37
Additive expected	$\log R_{A+C}$	1.32	1.49
observed	$\log R_{AC}$	1.39	1.53
$S_A \pm$ s.e.		0.075 ± 0.167	0.027 ± 0.155
$S_M \pm$ s.e.		0.660 ± 0.292*	0.810 ± 0.076*

*Significantly different from zero ($P<0.05$).
VGM = virginiamycin
S_A and S_M were derived as described in the methods.

been analysed functionally *in vitro* in the guinea-pig ileum (Lucaites *et al.*, 1991; Dal Forno *et al.*, 1992).

In these studies its estimated affinity (pK_B) was similar to that reported in binding studies using brain homogenates (Bock *et al.*, 1989). Thus, in this study L-365,260 has been taken as the standard antagonist against which to test virginiamycin.

The structure of virginiamycin is considerably different from other known CCK_B receptor ligands. This raises the questions as to the nature of its interaction with the receptor (agonist or antagonist) and whether virginiamycin might discriminate between central and peripheral CCK_B receptors. These questions do not appear to have been addressed previously, as until now, only binding data have been reported.

In the LMMP, virginiamycin showed antagonistic activity for CCK-4, in conditions where CCK_A receptor activation had been blocked. No agonism was observed. However, the antagonism was not of a simple competitive nature as at 10 μM a significant reduction of the maximal response was seen. To evaluate further whether this depression is related to another mechanism of action of virginiamycin, combined dose ratio analysis was applied. This was justified since L-365,260 was available as a reliable standard antagonist. Combined dose-ratio analysis has become a powerful method for disecting out complex antagonism. In this case the results are consistent with a model of L-365,260 and virginiamycin both acting on the same receptor. In fact, in experiments where L-365,260 and virginiamycin were applied in combination, a protection of the depressant effect was observed. Thus, the reduction of the maximal response seems to be related to an interaction of virginiamycin with the CCK_B receptor. This might indicate that virginiamycin at high concentrations has a relatively slow off rate, or that a mechanism similar to that described by Liu *et al.* (1992) is involved, where antagonists might change the efficacy of an agonist. Further experiments are needed to elucidate those possibilities. The pK_B estimated for virginiamycin from the combined dose-ratio analysis was 6.2 which seemed to reflect its true affinity for virginiamycin on the CCK_B receptor in LMMP as this value was not significantly different from the affinity estimated in binding assays.

This was calculated from the IC_{50} values reported by Lam *et al.* (1991) as 6.24 using a concentration of radioligand equal to or less than its K_A value. The pA_2 calculated for virginiamycin using Schild analysis, where the depressant property has been ignored, gives a larger value of 6.64. The difference between this and the estimated pK_B is significant ($P<0.05$) using the standard error on the pA_2 estimation. It is not possible to define the standard error from the combined dose-ratio analysis as all of the data was pooled to perform the analysis. The difference probably reflects the influence of the depressant effect.

A comparison between the pK_B estimated in our study with the pK_i obtained from binding data, as decribed above (Lam *et al.*, 1991), suggests that virginiamycin does not discriminate between peripheral and central CCK_B receptors. The close convergence between the two estimates (6.2 and 6.24, respectively), however, does rely on the assumption that the pK_i can be obtained from the IC_{50} using the Cheng & Prusoff equation (1973). This itself requires that the antagonist–receptor interaction follows the law of mass-action.

In conclusion, these results have shown, for the first time, that virginiamycin acts as CCK_B receptor antagonist in a functional assay.

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