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CHLORDIAZEPOXIDE IS A COMPETITIVE THYROTROPIN-RELEASING HORMONE RECEPTOR ANTAGONIST IN GH_3 PITUITARY TUMOUR CELLS

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Addition of thyrotropin-releasing hormone (TRH) to [^{3}H]-inositol pre-labelled GH_3 pituitary tumour cells suspended in medium containing 10mM lithium chloride led to a rapid diminution in cellular [^{3}H]-inositol and increase in [^{3}H]-inositol 1-phosphate (InsIP), [^{3}H]-inositol bisphosphate ($InsP_2$) and [^{3}H]-inositol trisphosphate ($InsP_3$). In the presence of the benzodiazepine tranquillizer, chlordiazepoxide, the TRH concentration-response curves for these effects were shifted to the right in a parallel fashion. The K_i for chlordiazepoxide in inhibiting all four responses was 1.5×10^{-5} M. Chlordiazepoxide did not inhibit the small bombesin-induced rise in [^{3}H]-InsIP. Another benzodiazepine, diazepam, was less active. The TRH-induced rise in cytosolic free calcium monitored in Quin-2-loaded GH_3 cells was also blocked by chlordiazepoxide in a competitive manner, while that induced by high K^+-induced depolarisation was unaffected. It is suggested that chlordiazepoxide acts as a competitive antagonist at the level of the TRH receptor.

A variety of agonists, acting on different hormone and neurotransmitter receptors, stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdIns4,5P_2$) to yield 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (1,2). This appears to be a bifurcating signal mechanism which delivers two messages to the cell: the activation of protein kinase C, due to the elevation in 1,2-diacylglycerol content, and the mobilization of cell-associated calcium, as a result of the increase in $InsP_3$ levels (see refs. 2 and 3 for recent reviews). GH_3 pituitary tumour cells contain receptors for the hypothalamic releasing factor TRH which are coupled to $PtdIns4,5P_2$ hydrolysis, Ca^{2+}-mobilisation and prolactin release (4-9). There are, at present, no drugs which can be used to antagonise TRH at its receptor, a limitation which has retarded

Abbreviations:

TRH, thyrotropin-releasing hormone; InsIP, inositol 1-monophosphate; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; $PtdIns4,5P_2$, phosphatidylinositol 4,5-bisphosphate; PtdIns, phosphatidylinositol; $[Ca^{2+}]_i$, cytosolic free calcium concentration.

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our understanding both of the physiological effects of TRH and the detailed mechanisms that follow receptor activation. Recently, two preliminary reports have suggested that high concentrations of certain benzodiazepines, in particular, chlordiazepoxide, compete with TRH for binding to its receptor in the brain and pituitary gland (10,11). We now show that chlordiazepoxide interacts with the TRH receptor on GH_3 pituitary tumour cells, acting as a competitive antagonist.

MATERIALS AND METHODS

Materials. Culture media, antibiotics, sera and GH_3 cells were obtained from Flow laboratories, Irvine, Scotland. myo-[^{3}H]-inositol (16C_i/mmole) was from Amersham International, Amersham, England. Benzodiazepines were obtained from Roche Products, Welwyn Garden City, England. TRH was from Universal Biologicals, Cambridge, England. Bombesin and Dowex 1X8 (Cl^- form, 100-200 mesh) anion-exchange resin were from Sigma, Poole, England.

Cell culture. GH_3 cells were grown as previously described (6) in Ham's F10 medium supplemented with antibiotics, horse serum and foetal calf serum. Measurements of the cellular content of inositol metabolites were made on cells which had been labelled to isotopic equilibrium with [^{3}H]-inositol (5μC_i/dish) for 3-4 days. This was added to the growth medium on the day of the last medium change (usually 7-10 days after plating). For most experiments, cells were suspended in a balanced salt solution, as previously described (6). The experiments involving the measurement of inositol metabolites were conducted in balanced salt solution in which 10mM of the NaCl was replaced by 10mM LiCl.

Extraction and determination of inositol and inositol phosphates. 0.5ml aliquots of suspended cells (prelabelled with [^{3}H]-inositol) were incubated for 10 min at 37°C in the presence or absence of benzodiazepines and/or agonists (TRH or bombesin). At the end of this time, the reaction was terminated by the addition of an equal volume of 20% (w/v) trichloroacetic acid (ice-cold). The water-soluble metabolites of inositol were extracted and separated by anion-exchange chromatography as described by Berridge et al (12).

Measurement of cytosolic free calcium concentration in GH_3 cells. Cytosolic free calcium was estimated using Quin-2 (13). Cells (3-5 x 10^6/ml), loaded with 3x10^{-5}M Quin-2/AM, the tetraacetoxymethyl ester of Quin-2, for 15 min at 37°C, were washed 3 times in balanced salt solution and stored at room temperature. Immediately before use, cells were centrifuged (14000g; 5s) and resuspended in fresh balanced salt solution (pre-equilibrated at 37°C) at a cell concentration of 3-5 x 10^6/ml. Fluorescence measurements were made at 37°C in a stirred sample (2ml) of these cells using a Perkin-Elmer LS-3 spectrofluorimeter; excitation wavelength-339nm, emission wavelength-492nm. At the end of the analysis, 5x10^{-5}M digitonin was added to measure the maximum fluorescence (F_{max}) and then excess alkaline ethylene glycol bis(-aminoethylether) N,N,N',N'-tetraacetic acid was added to measure minimum fluorescence (F_{min}). The cytosolic free calcium was calculated from the equation: $[Ca^{2+}]_i = K_D \times (F-F_{min})/(F_{max}-F)$, taking a value of 115nM as the K_D of Quin-2 for Ca^{2+} (13). Chlordiazepoxide, at concentrations above 2x10^{-5}M quenches Quin-2-Ca^{2+} fluorescence. The highest concentration used for this part of the work was 5x10^{-5}M, which quenched the Ca^{2+}-signal by around 30%. The drug did not, however, affect resting $[Ca^{2+}]_i$ levels in cells, the degree of stimulation induced by high concentrations of TRH or alter the K_D of Quin-2 acid for calcium (data not shown, and see below). It seems likely, therefore, that the indicator still functions adequately in the presence of the benzodiazepine.

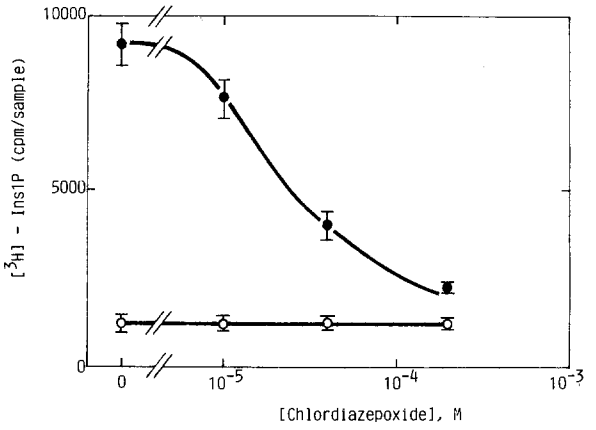

Fig.1: Effect of chlordiazepoxide on TRH-stimulated [^{3}H]-InsIP formation in GH_3 cells. 0.5ml aliquots of cell suspension were incubated in the presence (●) or absence (o) of TRH ($3x10^{-9}$M) for 10min at 37°C. Certain samples (as indicated in the figure) also contained either 10^{-5}M, $4x10^{-5}$M or $2x10^{-4}$M chlordiazepoxide. Reactions were terminated and [^{3}H]-InsIP measured as described in the Methods section. Values shown are the means (±S.E.M.) of triplicate samples from a representative experiment.

RESULTS

The effect of chlordiazepoxide on TRH-stimulated [^{3}H]-inositol metabolites.

Addition of TRH to lithium-treated GH_3 cells leads to a reduction in the cellular PtdIns content and an elevation of [^{3}H]-InsIP (7,14). The data presented in fig.1 indicate that the effects of a rather low concentration of TRH ($3x10^{-9}$M) on [^{3}H]-InsIP accumulation are inhibited in a concentration-dependent manner by chlordiazepoxide. The benzodiazepine, by itself, does not affect the cellular content of [^{3}H]-InsIP (fig.1). Since the concentration of chlordiazepoxide necessary to block the TRH-induced response is rather high by CNS standards, the nature of the antagonism was further investigated. As shown in fig.2, TRH altered the cellular content of all four water-soluble inositol metabolites in lithium-treated GH_3 cells. Lithium is known to block the conversion of InsIP to inositol (12,15), and, as would be expected, [^{3}H]-inositol levels declined in the presence of the peptide, while levels of the phosphorylated derivatives of inositol, in particular [^{3}H]-InsIP, increased (fig.2). Interestingly, the decline in cellular [^{3}H]-inositol content occurred at lower TRH concentrations than those which affected the other metabolites such as [^{3}H]-$InsP_2$ and [^{3}H]-$InsP_3$. The concentration of TRH

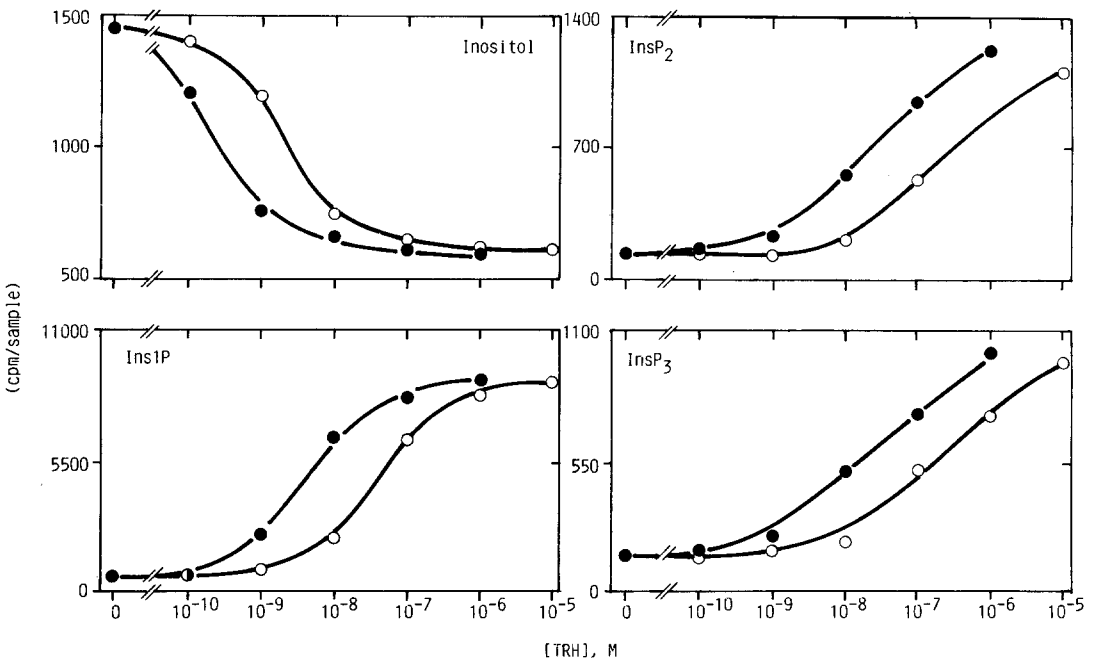

Fig.2: Concentration-response relationships for TRH-stimulated [^{3}H]-inositol metabolism in GH_3 cells: the effect of chlordiazepoxide. 0.5ml aliquots of cell suspension were incubated with various concentrations of TRH in the presence (o) or absence (•) of chlordiazepoxide ($2x10^{-4}$M) for 10 min at 37°C. The measurement of the different [^{3}H]-inositol-containing metabolites used the method of Berridge et al (12). Values are the means of duplicate determinations in an experiment which was repeated three times with similar results.

which produced 50% of the maximum response was $3x10^{-10}$M (versus the decline in cellular [^{3}H]-inositol), $4x10^{-9}$M (versus [^{3}H]-InsIP accumulation) and $\geq 2x10^{-8}$M (versus [^{3}H]-$InsP_2$ and $InsP_3$ formation). In the presence of chlordiazepoxide ($2x10^{-4}$M), the TRH concentration-response relationship for stimulation of all four responses was shifted to the right in a parallel fashion, indicative of competitive antagonism (fig.2). In each case, a K_D of $1.4x10^{-5}$M could be calculated for the chlordiazepoxide-TRH receptor interaction.

The specificity of the benzodiazepine-TRH receptor interaction.

GH_3 cells contain receptors for the tetradecapeptide bombesin which are coupled to changes in inositol lipid metabolism and prolactin release (16,17). Bombesin, in our hands, is a much weaker agonist than TRH with respect to [^{3}H]-InsIP formation (Table 1). Even 10^{-7}M of the peptide, a supramaximal

Table 1. The effect of benzodiazepines on peptide-induced [^{3}H]-InsIP formation in GH_3 cells

Benzodiazepine	control	TRH(10^{-9}M)	Bombesin(10^{-7}M)
	InsIP (cpm/sample)		
control	395±4	771±40***	480±19*
chlordiazepoxide (10^{-4}M)	376±17	461±14$^{*,++}$	489±9**
diazepam (10^{-4}M)	385±16	574±28$^{**,+}$	474±14*

$^{***}p<0.005$, $^{**}p<0.01$, $^{*}p<0.05$ versus appropriate untreated or benzodiazepine-treated control.

$^{++}p<0.01$, $^{+}p<0.05$ versus TRH-treated control.

Cells, prelabelled with [^{3}H]-inositol (5μC_i/dish for 48hrs) were stimulated for 10min at 37°C with the indicated concentrations of benzodiazepine and/or peptide. [^{3}H]-InsIP was extracted and measured as described in the Methods section. Values shown are the means (±S.E.M.) for triplicate samples from a representative experiment.

concentration (16, and data not shown) elicits only a 20% increase in the cellular content of [^{3}H]-InsIP. Nevertheless, this small increase was insensitive to concentrations of chlordiazepoxide which markedly reduced the elevation in [^{3}H]-InsIP induced by TRH (Table 1). The data in table 1 also indicate that diazepam, a related benzodiazepine, is a weaker TRH antagonist than chlordiazepoxide. Like the latter drug, diazepam is also ineffective against bombesin-induced [^{3}H]-InsIP formation (Table 1).

The effect of chlordiazepoxide on GH_3 cell calcium changes.

A number of groups have recently reported that TRH and high K^+ induce an elevation of cytosolic free calcium ($[Ca^{2+}]_i$) in Quin-2 or aequorin-loaded GH_3 cells (8,18-20). The elevation in $[Ca^{2+}]_i$ induced by 10^{-9}M TRH was almost totally abolished by $5x10^{-5}$M chlordiazepoxide (fig.3, left-hand panels). The block, however, could be overcome by increasing the concentration of TRH (fig.3, centre panels). A similar-sized response to high K^+-induced depolarisation was unaffected by chlordiazepoxide (fig.3, right-hand panels).

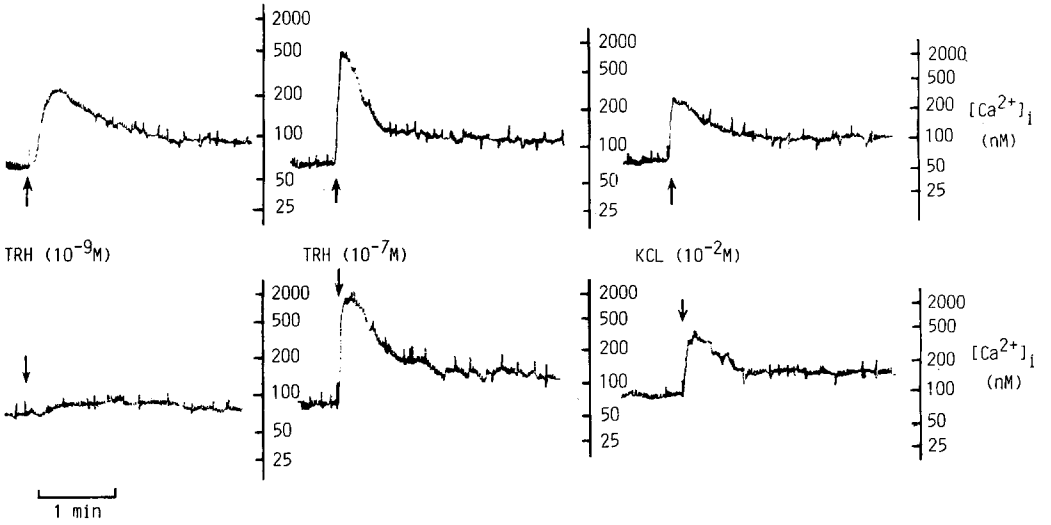

Fig.3: The effect of chlordiazepoxide on the increase in cytosolic free calcium induced by TRH or K^+-depolarisation in Quin 2-labelled GH_3 cells. 2ml aliquots of cell suspension (preloaded with Quin 2) were stirred with TRH (10^{-9}M; left-hand panels), TRH (10^{-7}M; centre panels) or KCl (10^{-2}M; right-hand panels). In all cases, the lower traces were from samples which also contained chlordiazepoxide ($5x10^{-5}$M). The method used to calibrate the signal is indicated in the Methods section. These are representative traces which were obtained on at least three different cell suspensions.

DISCUSSION

In the past few years, a number of peptide or non-peptide antagonists have been found for peptide hormones and releasing factors (21-23). To my knowledge, however, there are no reports of a selective TRH receptor antagonist-a limitation which has significantly slowed our evaluation of the physiological role of the tripeptide. The data presented in this paper agree qualitatively with the recent TRH receptor binding studies which show that certain benzodiazeines compete with TRH for binding to its receptor (10,11). They show, in addition, that the interaction is antagonistic in nature i.e. benzodiazepines such as chlordiazepoxide are competitive TRH receptor antagonists. The antagonism is specific since neither bombesin- nor high K^+ depolarisation-induced responses are sensitive to the drug. It is unlikely that these benzodiazepine effects are mediated by an interaction with the GABA receptor-Cl^--ionophore, as appears to underlie many of the central actions of this class of minor tranquillizers (24):- a) the concentrations necessary to block the actions of TRH are, in the case of diazepam, in particular, around 1000-fold higher than

for GABA-related effects (25), b) Chlordiazepoxide is more active at the TRH receptor than is diazepam, a reversal of their potencies in the CNS (25) and c) GABA, itself, does not affect TRH-induced responses in GH_3 cells (data not shown). Moreover, the fact that diazepam is less potent than chlordiazepoxide in binding to the TRH receptor is also inconsistent with the notion that the "peripheral-type" benzodiazepine binding sites, observed in a variety of tissues, represent TRH receptors - diazepam is also much more potent than chlordiazepoxide at these sites (26).

The concentrations of chlordiazepoxide found necessary to interact with TRH receptors in the present study are higher than those which are found in plasma after drug administration (around micromolar with the highest doses of the benzodiazepine, ref.27). It is unlikely, therefore, that these findings will be immediately of relevence to therapeutics. Nevertheless, the demonstration that chlordiazepoxide is a TRH antagonist is significant for two reasons. Firstly, it seems likely that it will be possible, using the drug as a prototype, to develop more potent TRH antagonists which will, hopefully, be devoid of the classical effects on GABA-associated mechanisms. This will allow a more thorough examination of the physiological role of TRH than is currently feasible. Secondly, the availability of a TRH antagonist will be of immense benefit to those studying the mechanisms by which TRH receptor occupancy elicits a cellular response and, in particular, those of us who use GH_3 pituitary tumour cells as a model system for studying the role of inositol lipids in cell function.

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REFERENCES

1. Downes, C.P., and Michell, R.H. (1982) Cell Calcium 3, 467-502.
2. Berridge, M.J. (1984) Biochem. J. 220, 345-360.
3. Nishizuka, Y. (1984) Nature (London) 308, 693-698.
4. Rebecchi, M.J., and Gershengorn, M.C. (1983) Biochem. J. 216, 287-294.
5. Martin, T.F.J. (1983) J. Biol. Chem. 258, 14816-14822.
6. Macphee, C.H., and Drummond, A.H. (1984) Mol. Pharmacol. 25, 193-200.
7. Drummond, A.H., Bushfield, M., and Macphee, C.H. (1984) Mol. Pharmacol. 25, 201-208.

8. Gershengorn, M.C., and Thaw, C. (1983) Endocrinology 113, 1522-1526.
9. Tashjian, A.H. (1979) Methods Enzymol. 58, 527-535.
10. Sharif, N.A., Zuhowski, E.G., and Burt, D.R. (1983) Neurosci. Letts. 41, 301-306.
11. Simasko, S.M., and Horita, A. (1984) Eur. J. Pharmacol. 98, 419-423.
12. Berridge, M.J., Downes, C.P., and Hanley, M.R. (1982) Biochem. J. 206, 587- 595.
13, Tsien, R.Y, Pozzan, T., and Rink, T.J. (1982) J. Cell. Biol. 94, 325-334.
14. Drummond, A.H., and Raeburn, C.A. (1984) Biochem. J. 224, 129-136.
15. Hallcher, L.M., and Sherman, W.R. (1980) J. Biol. Chem. 255, 10896-10901.
16. Sutton, C.A., and Martin, T.F.J. (1982) Endocrinology 110, 1273-1280.
17. Westerdorf, J.M., and Schonbrunn, A. (1982) Endocrinology 110, 352-358.
18. Albert, P.R., and Tashjian, A.H. (1984) J. Biol. Chem. 259, 5827-5832.
19. Snowdowne, K.W., and Borle, A.B. (1984) Am. J. Physiol. 246(E9), E198-201.
20. Schlegel, W., and Wollheim, C.B. (1984) J. Cell. Biol. 99, 83-87.
21. Peikin, S.R., Costenbader, C.L., and Gardner, J.D. (1979) J. Biol. Chem. 254, 5321-5327.
22. Folkers, K., Horig, J., Rosell, S., and Bjorkroth, U. (1981) Acta Physiol. Scand. 111, 505-506.
23. Fries, J.L., Murphy, W.A., Sueiras-Diaz, J., and Coy, D.H. (1982) Peptides 3, 811-814.
24. Braestrup, C., Honore, T., Nielsen, M., Petersen, E.N., and Jensen, L.H. (1984) Biochem. Pharmacol. 33, 859-862.
25. Squires, R.F., and Braestrup, C. (1977) Nature (London) 226, 732-734.
26. Bowling, A.C., and DeLorenzo, P.J. (1982) Science 216, 1247-1251.
27. Greenblatt, D.J., and Shader, R.I. (1974) Benzodiazepines in Clinical Practice. Raven Press, New York.