Use of Receptor Antagonists in Elucidating the Mechanism of Action of TRH in GH_3 Cells[a]

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The role of thyrotropin-releasing hormone (TRH) in the physiological control of prolactin release from the anterior pituitary gland is well established.[1] Only in the past decade, however, have substantial advances been made in understanding the cellular mechanisms by which the releasing hormone achieves these effects. Much credit for our present enlightenment is due to the pioneering work of Armen Tashjian and his colleagues who, over 20 years ago, developed a number of rat pituitary tumor cell lines, collectively termed GH cells.[2] Certain of these clonal cell lines, in particular GH_3 and GH_4C_1 cells, contain many receptors for TRH on their cell surface[3] and respond to their occupancy by enhancing both prolactin release and synthesis.[1] Although it is naive to imagine that these cells are entirely homogeneous in terms of their sensitivity to the tripeptide, there is little doubt that analysis of biochemical data obtained from these cells is much less open to misinterpretation than is corresponding data derived from hemipituitaries or cultured anterior pituitary cell preparations. This report will outline recent advances in TRH signal transduction mechanisms and will emphasize that TRH receptor antagonists, albeit of limited selectivity, are available and can contribute to these studies.

TRH-STIMULATED INOSITOL LIPID METABOLISM

In recent years, the ubiquitous association of stimulated inositol lipid breakdown and cell activation in response to a wide variety of receptor stimulants has become evident.[4] The characteristic intracellular signals—an increase in cytosolic free calcium and activation of protein kinase C—are known to derive from the formation within the cell of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and 1,2-diacylglycerol (DAG). These result from hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) by a phosphodiesterase enzyme that Downes and Michell[4] have termed phosphoinositidase C. The production of two intracellular messengers as an immediate response to occupation of one receptor is a novel

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finding in the field of cell signaling and presumably allows the cell great flexibility in the control of its response. Although knowledge of this signaling system is now substantial, there is a reluctance by investigators in the field to claim that we comprehend the full extent of its physiological role. Major questions remain to be answered; these will be illustrated by reference to the status of the inositol lipid cycle in TRH-stimulated GH cells.

Little is known regarding the nature of the TRH receptor, although it has been evident for many years that its cellular complement on GH cells is subject to marked variation in response to both homologous and heterologous factors.[2] Ramsdell and Tashjian[5] recently used an ingenious selection procedure that exploits the ability of the tripeptide to alter cell adhesiveness to its growth substratum as a means to clone GH_4C_1 cell variants exhibiting marked differences in their TRH receptor number. These clones are already proving to be of use in studies of TRH action and may also facilitate attempts to isolate cDNA clones encoding the receptor itself. Recent work on the sequence deduction of the β-adrenergic, muscarinic, and substance K receptors indicates that the TRH receptor, like those just cited, may contain seven membrane–spanning α-helices in its structure and that this may be a common fingerprint in all receptors that couple to GTP-binding proteins.[6] Evidence that the latter is the case for the pituitary TRH receptor is now considerable: (1) binding of TRH to its receptor can be modulated by GTP,[7] (2) TRH stimulates a membrane GTPase in GH cells,[8] (3) GDP is replaced on the G-protein by GTP in a TRH-dependent manner,[9] and (4) the addition of GTP to isolated GH cell membrane synergizes with TRH in activating inositol lipid hydrolysis.[10,11] The nature of G_p, the putative G-protein involved in this process, remains unknown; it is not sensitive to either cholera or pertussis toxin[10–12] and thus differs both from those involved in the regulation of adenylate cyclase (G_s and G_i) and from that which couples to inositol lipid changes in certain cell types, such as blood cells.[13] Equally obscure is the nature of the effector enzyme that activates inositol lipid hydrolysis, the phosphoinositidase C, although there is evidence that the mechanism by which it is activated is related to a change in its affinity for calcium ions that occurs on interaction with the activated (GTP-liganded) G-protein.[10] The result of this interaction is that the enzyme can now function well at the level of cytosolic free calcium that prevails in the unstimulated cell, that is, an increase in calcium is unnecessary and also insufficient for phosphoinositidase C activation in GH cells.

There continues to be considerable debate as to which of the three inositol-containing phospholipids can act as a substrate for the phosphodiesterase that yields DAG. *In vitro* studies suggest that both PtdIns(4,5)P_2 and phosphatidylinositol 4-phosphate (PtdIns4P) can act as substrates for the enzyme, but they do not support the contention that the selectivity of the enzyme extends to phosphatidylinositol (PtdIns) itself.[10,11] Credence of the *in vitro* experiments in broken cells depends on the accurate maintenance of proportions of the three lipids that are found in the plasma membrane of the intact cell. Data are not available to assess whether this condition is fully met by the experimental conditions that have been used *in vitro*, but there is good reason to suggest that the most highly phosphorylated inositol lipid (PtdIns(4,5)P_2) will be the most likely, and PtdIns the least likely, to be affected by the inevitable use of less than perfect conditions *in vitro*.

Thus, it is unlikely that a potential substrate role of PtdIns will have been overlooked and likely that the role of PtdIns(4,5)P_2 will have been underestimated in this work. Whether such considerations explain the large amount of PtdIns4P hydrolysis observed during stimulation with TRH *in vitro*[10,12] remains to be clari-

fied. The data from intact cell studies that bear on this question are inconclusive, although once again they support the view, initially at least, that the polyphosphoinositides, and in particular PtdIns(4,5)P_2, are the preferred substrates for phosphoinositidase C. (See reference 14 for a recent discussion on this topic.) However, the TRH-stimulated hydrolytic response apparently is not desensitized markedly over the first 30 minutes of stimulation,[15] and a far more difficult problem is to determine whether the polyphosphoinositides remain the primary substrates for the phosphoinositidase C over this entire period. That this may not be the case is suggested by the work of Imai and Gershengorm[16] who, on the basis of experiments that monitor the specific activity of ^{32}P-labeled inositol lipids during stimulation by TRH, have concluded that PtdIns(4,5)P_2 hydrolysis is only associated with the early stages of receptor activation and that PtdIns subsequently becomes the preferred substrate. The distinction is important because if this is true, only one second messenger, namely DAG, will be produced in the later stages of receptor stimulation. This area of stimulated inositol lipid metabolism in GH cells is considered also in a later section of this review.

A further area of this topic, which is in the process of rapid reevaluation, is the metabolic fate of Ins(1,4,5)P_3. Work from the laboratories of Irvine, Downes, and their colleagues has revealed an unsuspected level of complexity in the metabolism of this substance which, because of its apparent wastefulness, presumably indicates that a further physiological mediator or element of control is present.[17] In GH cells, HPLC analysis of acid extracts from [^3H]inositol-labeled cells indicates that at least 9–10 distinct inositol phosphate isomers are present and that the cellular content of each is increased by treatment with TRH.[18,19] Irvine and Moor[20] recently suggested that inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) may have a second messenger role in regulating calcium influx across the plasma membrane. In GH_3 cells, this substance is produced after a lag of 10–15 seconds after agonist addition, appreciably slower than Ins(1,4,5)P_3 accumulation.[19] There is good evidence from a variety of sources that regulated calcium influx in these cells appears to follow calcium mobilization by about 10 seconds: the addition of TRH to EGTA-treated GH cells mimics faithfully the calcium response in full calcium medium for about 10 seconds and then falls rapidly back into the unstimulated range.[14] In the presence of extracellular calcium, a second, long-lived "plateau" phase of cytosolic free calcium rise is present.[1] Although the kinetics of this calcium influx undoubtedly fit well with those of Ins(1,3,4,5)P_4 formation in GH_3 cells, much of the calcium influx is due to the opening of voltage-sensitive calcium channels that are dihydropyridine sensitive and apparently indirectly regulated by protein kinase C.[21] There have been some indications, however, that a portion of the influx is insensitive to blockers of voltage-sensitive calcium channels,[22] and further work will no doubt ascertain whether this represents a process that is in any way modulated by Ins(1,3,4,5)P_4.

There is little published data on the routes by which Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 are metabolized in GH cells, although, since at least four bisphosphate isomers have been reported to exist,[18,19] a number of pathways must be involved. Although Ins(1,4)P_2, Ins(1,3)P_2, and Ins(3,4)P_2 have been described in other tissues and cells, the presence of a bisphosphate isomer that co-elutes with Ins(4,5)P_2[18,19] and its production from Ins(1,4,5)P_3 in GH_3 cell sonicates (Ruiz-Larrea and Drummond, unpublished data) makes it likely that a third route of Ins(1,4,5)P_3 metabolism, by 1-phosphatase, exists in addition to the well-characterized 5-phosphatase and 3-kinase. Further work is necessary to gauge the generality of this route in other tissues. However, a bisphosphate isomer with similar characteristics is a major metabolite in mouse B lymphocytes (unpublished data).

This metabolic sequence may also explain the presence in CNS and GH_3 cells of inositol 5-phosphate, in addition to the 1- and 4-phosphate isomers (reference 23 and Ackermann, Sherman, Hughes, and Drummond, unpublished work). The cellular levels of all three monophosphates are increased after TRH simulation.

BENZODIAZEPINES AND THE TRH RECEPTOR

The clinical actions of the benzodiazepines are adequately explained by their ability to enhance GABAergic transmission in the CNS.[24] This notwithstanding, there is a growing appreciation that the basic benzodiazepine nucleus can be used as a template for the production of drugs targeted against a number of neuropeptide receptors in the CNS and periphery. Thus, tifluodom, which is a benzodiazepine, can bind with reasonably high affinity to kappa opiate receptors[25] and, in a piece of inspired medicinal chemistry, a series of these derivatives, such as L-364718, have been developed as antagonists at cholecystokinin receptors and, hence, as novel probes of cholecystokinin function.[26] The reason for the widespread use of the benzodiazepines in this area is unclear, but from a pharmaceutical point of view the attractions are obvious: this is a well-characterized chemical nucleus from a pharmacokinetic and toxicological perspective and, in addition, one whose most likely complication, that is, the interaction with the $GABA_A$ receptor-ionophore complex, is extremely well understood in molecular terms.[24]

Work with L-364718 and its antagonism of cholecystokinin responses demonstrates the importance of having a specific receptor antagonist available. In its absence, the pharmacological and physiological effects of any receptor stimulant cannot be fully catalogued. The action of TRH remains in this somewhat unsatisfactory state: much is known regarding its physiological role in the body from studies with, for example, TRH antisera, but there is a lingering doubt that its full spectrum of activity and importance will only be clear when it is possible to reverse its action using a small molecule antagonist.

In 1983, the first progress was made along what is still an unfulfilled path when Sharif *et al.*[27] reported that a number of benzodiazepines were able to compete with TRH for binding to its receptor. This report was rapidly confirmed and extended.[28,29] Until this point, two general types of benzodiazepine binding sites had been reported: (1) the clinically relevant site that is located on the $GABA_A$ receptor-Cl^- ionophore complex,[24] and (2) a low-affinity site whose properties and pharmacology differ markedly from those of the former.[30] The clinical relevance and nature of this second site still remain to be established. Interestingly, the pharmacology of neither of these sites parallels that found when benzodiazepine affinity for the TRH receptor is examined, thus dispelling the view that the anxiolytic role that has been ascribed to TRH[31] is in some way connected with a common site at which the benzodiazepines also bind. A wide range of benzodiazepines have subsequently been investigated for their ability to compete with [^{3}H]TRH for binding to its CNS receptor. These data, obtained from work conducted in a number of laboratories, and which generally agree well, have been collated, and averaged values are presented in TABLE 1.

The results emphasize that chlordiazepoxide and midazolam have the highest affinity for the receptor, but they illustrate also that certain benzodiazepine derivatives that are potent anxiolytics, such as flunitrazepam and flurazepam, are weak in binding to the TRH receptor. A second aspect of these data that emerges from a number of publications is that the ability of the drugs, such as midazolam, to

depress TRH receptor binding shows considerable regional specificity in its action.[29,32] Rinehart *et al.*[32] demonstrated that although midazolam and chlordiazepoxide compete effectively for binding in membranes prepared from rat amygdala, they are about two orders of magnitude less effective in the striatum. Moreover, the displacement curves deviate significantly from the sigmoid relationship expected when the data are presented in log transformation. The most likely explanation for these results is that CNS TRH receptors are heterogeneous with respect to their ability to interact with benzodiazepine ligands. This is an interesting finding that deserves further investigation. A further conclusion made by Rinehart *et al.*,[32] namely, that because midazolam does not affect a number of TRH-induced responses, these receptors are not physiologically relevant, may, with the benefit of hindsight, be less tenable. It seems likely, as will be described, that the affinity of the benzodiazepines for the TRH receptor is overemphasized

TABLE 1. Pharmacology of the TRH Receptor-Benzodiazepine Interaction[a]

Drug	CNS Binding (K_i, μM)	GH_3 Cell Response (K_i, μM)
Midazolam	0.07	3.2
Chlordiazepoxide	0.94	15.0
Diazepam	7.1	30.0
(±) 4-Methylmidazolam	—	25.0
(±) 4-Hydroxymidazolam	—	30.0
Flurazepam	55.0	—
Lorazepam	135.0	—
Demoxepam	—	>100.0
Clonazepam	>200.0	—
Kenazepine	>200.0	—
Temazepam	>200.0	—
Oxazepam	>400.0	—
Chlorazepate	>400.0	—
Adinazolam	—	>400.0
Flunitrazepam	>400.0	—

[a] Data from CNS binding assays at 4°C and contained in references 27, 28, 29, and 32 have been averaged and a mean K_i value is presented. Results from GH_3 cell studies are from references 33 and 36 and relate to K_i values for the inhibition of TRH-stimulated [^{3}H]$InsP_1$ formation measured at 37°C.

by the low temperature binding assay used and that the plasma level of the drugs achieved was simply too low to inhibit TRH binding to its receptor.

In response to published binding data, my laboratory became interested in determining the generality of the TRH receptor-benzodiazepine interaction by extending the studies to GH_3 and other clonal pituitary cell lines (see above). Although previous workers had discussed the competitive interaction from the point of chlordiazepoxide being a mimic of TRH action,[29] it seemed equally likely that the drug might act as a competitive antagonist, a possibility that was attractive to us in that it might facilitate our studies of TRH receptor mechanisms.

Accordingly, we tested chlordiazepoxide and diazepam as inhibitors and activators of the TRH-induced inositol lipid response in GH_3 cells.[33] These data showed clearly that the drugs were active, although at slightly higher concentrations than had been reported in the CNS binding assays. Moreover, both benzodiazepines were competitive antagonists at the TRH receptor and almost entirely

lacked any intrinsic receptor-stimulating property. The effect was specific in that neither a bombesin-induced inositol lipid response nor the ability of high concentrations of K^+ to elevate cytosolic free calcium levels in GH_3 cells were sensitive to the presence of chlordiazepoxide. The data were swiftly confirmed by other laboratories working with GH_3 cells,[12,34] and essentially similar data were provided for the antagonism of TRH responses in the isolated guinea pig antrum and duodenum.[35]

All of these data confirm that chlordiazepoxide might be a lead compound in developing a *specific* TRH receptor antagonist, but they emphasize the lack of utility of the substances at the whole body level because of their more powerful GABA-enhancing properties. With this disadvantage in mind, we set out to explore further the pharmacology of the TRH receptor-benzodiazepine interaction. This work is still underway and owes much to the report by Rinehart *et al.*[32] that midazolam is a more effective competitor than chloridazepoxide at the TRH receptor. However, it confirms this later report and once again shows that benzodiazepine is a competitive inhibitor of TRH action.[36] Studies with a number of available derivatives—imidazo- and triazolo-benzodiazepines—have shown that midazolam remains the most effective antagonist available (TABLE 1).[36] Interestingly, when the enantiomers of 4-methylmidazolam were examined separately as antagonists, both were active.[36] This finding contrasts with the data at the GABAergic site, in which the (+)-isomers are greater than 100-fold more effective than (-)-isomers.[24] Thus, one way forward may be to focus exclusively on the (-)-isomers as potential TRH antagonists that might have low or absent GABA-enhancing ability. One other interesting piece of data that emerged from this series of experiments was the finding that when TRH-induced inositol phosphate metabolism was examined in dose-ratio experiments conducted at 25°C in addition to 37°C, the antagonists were all significantly more potent at the lower temperature, four- to five-fold in the case of midazolam.[36] This raises the possibility that the higher affinity receptor binding observed in CNS assays at 4°C may not be relevant to physiological temperatures.

Thus, the disappointing conclusion at present must be that the benzodiazepines, while useful for those of us who investigate TRH receptor mechanisms, are of limited values to those who seek to study more fully the physiological role of TRH in the body.

The TRH antagonists can be used in a variety of ways to elucidate the mechanism of cell activation initiated by the hypothalamic peptide. The most obvious approach is to evaluate the degree of flux that occurs through the inositol phosphate metabolic pathways during receptor activation. Addition of midazolam or chlordiazepoxide at some point subsequent to TRH application can abrogate the peptide response completely, especially if a submaximal stimulant concentration is used (the affinity of midazolam for the TRH receptor is still about 100-fold less than that for TRH itself). In consequence, it is possible to examine the rate of metabolism, in the intact cell, of the different inositol phosphate isomers and to compare these with data obtained from broken cell studies. Another example of this approach is in testing the hypothesis of Imai and Gershengorn[16] that TRH-stimulated PtdIns(4,5)P_2 hydrolysis, unlike that of PtdIns, is transient. A number of laboratories have reported that there are sustained TRH-induced increases in cellular InsP_3 levels when the application of the peptide is continued,[15,37] and this is also found when the HPLC-separated InsP_3 isomers are monitored.[19] Nevertheless, it remains possible that this sustained increase does not represent a dynamic equilibrium, that is, that both production *and* metabolism of InsP_3 have ceased. That this is not the case is shown by experiments in which chlordiazepoxide is

added to TRH-stimulated GH_3 cells 30 or 60 minutes following initiation of the reaction.[36] It is evident that TRH receptor blockade leads to an immediate decrease in cellular $InsP_3$ levels regardless of the time of addition of the antagonist; thus, the sustained increase in $InsP_3$ content depends on continued synthesis and metabolism. It seems unlikely, therefore, that the thesis of Imai and Gershengorn[16] that PtdIns(4,5)P_2 hydrolysis is transient after TRH receptor stimulation is correct. The proof or disproof of the idea that direct PtdIns hydrolysis occurs is much more difficult and will require a different experimental approach.

In summary, competitive TRH antagonists are available for studies of TRH action but fall some way short of the specificity required for investigation of TRH action in the body. It seems likely that further advances in this area will only be possible by a more rigorous approach based in medicinal chemistry and conducted either by or with considerable collaboration from a pharmaceutical company. It is our opinion that only if such an approach is successful will the full physiological role of TRH be known.

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