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Dopamine regulates expression of the glandular-type kallikrein gene at the transcriptional level in the pituitary

(proopiomelanocortin/cDNA cloning/RNA blots/nuclear run-on assay)

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ABSTRACT **A glandular-like kallikrein enzyme, a member of a well-characterized family of specific arginyl endopeptidases that may be involved in prohormone processing, has previously been shown to be present in the anterior and neurointermediate lobes of the rat pituitary. We isolated glandular-like kallikrein cDNAs from cDNA libraries prepared from these two tissues. By nucleotide sequence, restriction endonuclease, solution hybridization/nuclease protection, and blot analyses, we showed that, of the 8–10 rat kallikrein-encoding genes, it is the true glandular kallikrein mRNA that is expressed in both pituitary lobes. RNA blot-hybridization analysis of anterior and neurointermediate lobe pituitary RNA revealed a kallikrein mRNA of ≈900 base pairs. As analyzed by blot-hybridization and solution hybridization/nuclease protection analyses, the true glandular kallikrein mRNA was present at low levels: ≈0.05% of total mRNA in both male and female neurointermediate lobes. Similar low levels of the glandular kallikrein mRNA were found in the male anterior lobe, whereas the levels were 10- to 15-fold higher in the female anterior lobe. *In vivo* administration of a dopamine agonist (bromocryptine) or antagonist (haloperidol) caused a decrease or increase, respectively, in the amount of true glandular kallikrein mRNA in the neurointermediate lobe of both sexes that closely paralleled changes in proopiomelanocortin mRNA levels. Bromocryptine decreased and haloperidol increased true glandular kallikrein mRNA levels in the female anterior lobe but had no effect in the male anterior lobe. Nuclear transcription run-on studies showed that the changes in mRNA were due, at least in part, to parallel effects of haloperidol on kallikrein gene transcription. Thus, these studies have demonstrated that the pituitary expresses the glandular-type member of the kallikrein gene family and that dopaminergic compounds elicit changes in kallikrein mRNA, at least in part, by modulating transcription. In the intermediate lobe, regulation of true glandular kallikrein gene expression is parallel to that of proopiomelanocortin gene expression, suggesting that the enzyme may play a physiological role in the production and/or secretion of the proopiomelanocortin peptides in this tissue.**

Glandular kallikrein is a member of a family of arginyl-specific endopeptidases, some of which have been shown to be involved in processing of specific prohormone precursors to their bioactive end products. This family of similar enzymes is encoded by a family of highly homologous genes consisting of 20 members in the mouse (1, 2) and 8–10 members in the rat genome (3, 4). These genes code for a set of very similar mRNAs, several of which have recently been sequenced, including the one that encodes true glandular kallikrein in the rat (also termed the PS mRNA) as well as tonin and other enzymes whose activity remains uncharacterized (3–5). Our interest in these proteases was stimulated by the possibility that they are involved in the processing of proopiomelanocortin (POMC) to its bioactive end-products (6) and by the finding of Powers and Nasjletti (7, 8) that glandular-like kallikrein enzymatic activity exists in two major sites of POMC gene expression: the intermediate and anterior lobes of the rat pituitary gland. In addition, recent studies have shown that the level of glandular kallikrein enzymatic activity in the intermediate lobe is regulated by dopaminergic compounds (9, 10) in parallel to the known regulation of POMC gene expression and secretion in this tissue (11). This phenomenon is similar to the coordinate regulation seen in the levels of POMC and of two of its known processing enzymes, amino-terminal acetyltransferase and carboxyl-terminal amidating enzyme (12, 13), and suggests that kallikrein plays a role in neurointermediate lobe-specific production and/or secretion of the POMC-derived peptide products. Thus, to address the question of which kallikrein gene(s) is expressed in the pituitary and to identify the mechanisms involved in the coordinate regulation of the rat pituitary kallikrein and POMC genes, we developed anterior- and neurointermediate-specific cDNA libraries, and isolated and sequenced or characterized kallikrein-related cDNAs from each. These isolated cDNAs were then used to characterize kallikrein mRNA levels and transcription rates in these tissues after dopaminergic manipulation and to compare them to those of POMC.

MATERIALS AND METHODS

Isolation and Characterization of Kallkrein-Like cDNAs. RNA was isolated from anterior or neurointermediate pituitary lobes of young female rats using the procedure of Cathala *et al.* (14). Double-stranded cDNA was synthesized by published protocols (15). *Eco*RI linkers were ligated to *Eco*RI-methylated double-stranded cDNA, and, after digestion with *Eco*RI, high molecular weight cDNAs [>400 base pairs (bp)] were electrophoresed through and electroeluted from a 4% acrylamide gel. The cDNAs were then ligated to phage λ GT10 arms and packaged *in vitro* (16).

An aliquot containing 50,000 recombinant phage was plated prior to amplification. The remainder was amplified as described by Huynh *et al.* (16). The plaques were screened as described by Benton and Davis (17). A rat kallikrein gene fragment was nick-translated (18) and hybridized to filters (19). Positive plaques were isolated, and DNA was prepared by plate lysis (20). After restriction mapping, the cDNAs were sequenced by the Maxam–Gilbert method (21). In addition, plaques were screened with two oligomers specific for true glandular kallikrein (Fig. 1) (3, 4). The oligomers were

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Abbreviation: POMC, proopiomelanocortin.

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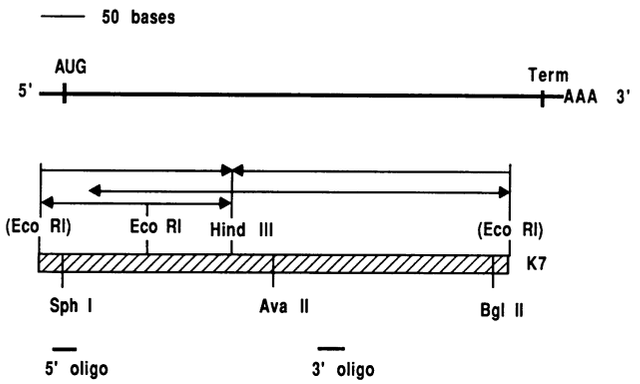

FIG. 1. Schematic of pituitary kallikrein cDNA cloning studies. The sequencing strategy and useful restriction endonuclease sites are shown above the cDNA (hatched bar). The synthetic oligonucleotides (5' probe: AACAGGCATGCAGGAGCA; 3' probe: TCAATCCGTCAGGTGTGATG) and restriction endonuclease sites used in identification of cDNAs as true glandular kallikrein are shown below the cDNA structure.

digested with kinase and hybridized to plaques, and the filters were washed in 3 M tetramethylammonium chloride at 59°C (22).

Hormonal Manipulation of Animals. Male or female Sprague–Dawley rats weighing 200 g (Camm Breeding Laboratories, Wayne, NJ) were housed in a room with a 12-hr light/12-hr dark cycle. The animals were killed by decapitation, and the pituitaries were removed immediately and separated into anterior and neurointermediate lobes under a dissecting microscope to minimize cross-contamination. RNA was then prepared from the separated lobes (14).

For the hormonal studies, rats were injected subcutaneously with 0.2, 2, or 10 mg/kg of body weight per day of haloperidol (Sigma) or 0.2, 2, or 10 mg/kg of body weight per day of 2-bromocryptine (Sigma). Both haloperidol and bromocryptine were dissolved in a solution of 15% ethanol in saline at concentrations of 0.2, 2, or 10 mg/ml. Control rats were injected with vehicle.

mRNA Analysis. Total RNA or poly(A)$^+$ RNA, isolated by oligo(dT)-cellulose chromatography, was denatured with formaldehyde/formamide and electrophoresed through a 1% agarose gel containing 6% formaldehyde (23). RNA was transferred to nitrocellulose (24) after staining with ethidium bromide in water. The filter was baked for 3 hr at 80°C, prehybridized for 12 hr in Wahl prehybridization buffer (19), and then hybridized at 42°C for 24 hr with a ^{32}P-labeled nick-translated pituitary glandular-type kallikrein cDNA insert or ^{32}P-labeled single-stranded RNA probes. After hybridization, the filters were washed twice for 15 min in 0.3 NaCl/0.03 M sodium citrate, pH 7.0/0.1% $NaDodSO_4$ at room temperature. Filters were then washed twice in 0.015 M NaCl/0.0015 M sodium citrate/0.1% $NaDodSO_4$ at 45°C (85°C for the RNA probe) and exposed to x-ray film with Cronex intensifying screens at −70°C. To reprobe, filters were washed in boiling water for 20 min and then rehybridized. Solution hybridization of mRNA to single-stranded RNA probes and subsequent digestion with RNase A was done as described by Zinn *et al.* (25).

Nuclear Run-On Transcription Assay. Nuclei were isolated from pooled anterior or neurointermediate lobes of three rats injected as above with 2 mg of haloperidol per kg of body weight 1 or 16 hr before sacrifice. The nuclear isolation was performed as described (26) with a gentle centrifugation procedure to maintain the integrity of the nuclei. The nuclear transcription run-on assay was performed by a slight modification (26) of an established procedure (27). Kallikrein, prolactin, and POMC cDNA inserts were used to prepare the nitrocellulose filters for quantitation of transcripts.

RESULTS

Anterior and Intermediate Pituitary Kallikrein mRNA Is of the Glandular Type. cDNA libraries were prepared from poly(A)$^+$ RNA isolated from female rat anterior and neurointermediate lobes. With the phage λ GT10 system, several large cDNA libraries representing from 0.5 to 1 million individual clones were prepared. They were screened with a 300-bp genomic fragment derived from a rat kallikrein gene identified by cross-hybridization with the previously isolated mouse pMK1 kallikrein cDNA clone (generously provided by J. Shine of Australia National University). When the kallikrein gene fragment was used to probe a Southern blot of rat genomic DNA, ≈10 bands were observed, suggesting that the fragment would be useful as a probe for isolating any of the rat kallikrein-like cDNAs. With this probe, a single independent cDNA clone was detected in each of two complete screenings of two separate pituitary neurointermediate lobe libraries. Subsequent rescreening with the isolated pituitary kallikrein cDNA clones or the genomic fragment did not yield any different cDNA clones. The two independently isolated neurointermediate pituitary-specific cDNA clones (K7 and K719) were subjected to Maxam–Gilbert nucleotide sequence analysis. Both clone K719 and the larger K7, the structure of which is shown in Fig. 1, had exactly the same nucleotide sequence as that of the rat pancreatic kallikrein mRNA (true glandular kallikrein mRNA), as reported by Ashley and MacDonald (3). Using antisense single-stranded RNA for solution hybridization/RNase A protection studies with total neurointermediate lobe RNA from male or female pituitaries, we were able to show only a single protected band (Fig. 2), even at longer exposure times, which was indicative of true glandular kallikrein mRNA only.

Solution hybridization/nuclease protection studies similar to those described above for neurointermediate pituitary also showed true glandular kallikrein mRNA as the only identifiable kallikrein mRNA in male or female anterior pituitary (Fig. 2). Kallikrein-encoding cDNA clones were isolated from the female anterior lobe cDNA library, which contained an ≈10-fold higher level relative to the neurointermediate libraries of kallikrein clones. Of 25 individual positive plaques from the initial screening, all hybridized at high stringency to two synthetic DNA oligomers (Fig. 1) specific for true glandular kallikrein mRNA (Fig. 1) (3, 4). In addition, 2 different isolated cDNAs from this group showed restriction endonuclease sites (*Ava* II, *Bgl* II, *Sph* I) that are uniquely characteristic of the true glandular kallikrein mRNA.

Characterization and Pituitary Levels of Kallikrein mRNA. Kallikrein-like mRNA could be detected in both anterior and neurointermediate pituitary RNA as a single band of ≈900 bp on blots (Fig. 3) or as a single protected band of 498 bases in nuclease protection studies (Fig. 2). The amount of kallikrein-like mRNA was estimated to be ≈0.05% of the total mRNA in all tissues except the female anterior lobe, based on the length of exposure time required to give bands of equal intensity to POMC mRNA (Fig. 3). In female anterior lobe, kallikrein-like mRNA levels were about 10- to 15-fold higher.

It is possible that this sexually dimorphic expression in the female anterior pituitary lobe was due to sex-specific expression of a different kallikrein gene. Because of extensive homology among the kallikrein gene family mRNAs, blot-hybridization analysis would not distinguish between different kallikrein mRNAs. Thus, we utilized a "gene-specific" technique of solution hybridization/RNase digestion (25) of pituitary glandular kallikrein cDNA in a pSP65 vector system. Because there are several regions within our single-stranded RNA probe of nonhomology with the nonglandular kallikrein mRNAs (3), this approach should measure only true glandular kallikrein mRNA with the full-length protected band. As shown in Fig. 2, a band of 498 nucleotides was

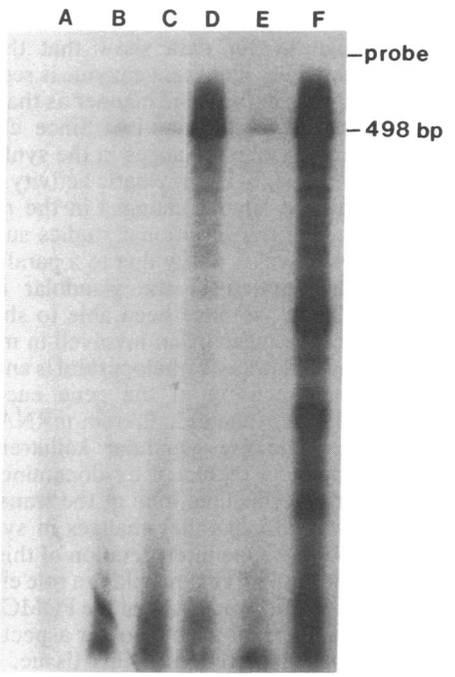

FIG. 2. Solution hybridization/nuclease protection analysis of pituitary RNAs. Rat total RNA (1 μg) from each tissue and yeast RNA (10 μg) were hybridized to a ^{32}P-labeled antisense kallikrein RNA probe and digested with RNase. The samples were then electrophoresed on a 4% acrylamide gel. Lanes: A, no rat RNA; B, male anterior pituitary; C, male neurointermediate pituitary; D, female anterior pituitary; E, female neurointermediate pituitary; F, female rat kidney. The position of the undigested 550-base probe is shown as "probe."

protected in male and female anterior and neurointermediate pituitary tissues, as expected for glandular kallikrein mRNA. Rat kidney RNA protected a band of the same size and several smaller bands as expected, since kidney expresses other kallikrein mRNAs in addition to the glandular type (R. McDonald, University of Texas at Dallas, personal communication). The relative abundance in the different lobes and sexes was essentially the same as that seen by the blot-hybridization analysis described above.

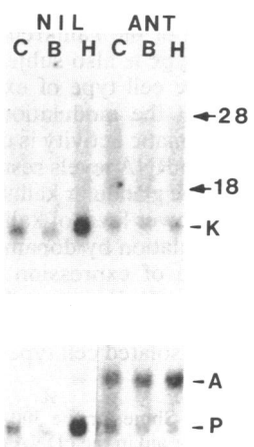

FIG. 3. Blot-hybridization analysis of anterior and neurointermediate RNA isolated from male rats. Blots were run with 5 μg of total RNA from neurointermediate lobe (NIL) or 7 μg of total RNA from anterior lobe (ANT) from each of the treatments. Injections of drug or vehicle were given daily for 3 days. Lanes: C, control; B, bromocryptine at 2 mg/kg per day; H, haloperidol at 2 mg/kg per day. (*Upper*) Probe of the blot with the K7 probe and exposure for 1 week. The K denotes the position of kallikrein mRNA and 28 and 18 denote the positions of the 28S and 18S ribosomal RNAs. (*Lower*) Reprobe of the same blot (after stripping off the kallikrein probe) with both POMC (P) and actin (A) probes. Only the relevant part of the blot is shown.

Regulation by Dopaminergic Compounds of Kallikrein-Like mRNA in Separate Pituitary Lobes. Using RNA blots, we analyzed the changes in the level of kallikrein, POMC, and actin mRNA in the male neurointermediate pituitaries in response to bromocryptine and haloperidol treatment. Haloperidol increased and bromocryptine decreased the levels of kallikrein and POMC mRNAs (analyzed relative to actin mRNA) (Fig. 3). Similar changes were seen for kallikrein mRNA levels in the neurointermediate lobe of female rats (data not shown). As reported previously (11), neither of these treatments caused any reproducibly detectable changes in the total amount of RNA isolated from the neurointermediate lobe.

In the solution hybrid/nuclease digestion assay, bromocryptine also caused a decrease in the level of kallikrein mRNA in the anterior lobe of female pituitary glands (Fig. 4). The converse effect was seen with haloperidol (Fig. 4). The effects were observed at the earliest time period of treatment analyzed, 1 day, and continued for 5 days. However, in males no changes in kallikrein mRNA levels in the anterior lobe were observed after bromocryptine or haloperidol treatment (Fig. 3). Again, no effect on recovery of anterior lobe total RNA was observed with any treatment.

Transcriptional Regulation of the Kallikrein Gene by Dopaminergic Compounds. To determine if the dopaminergic-mediated changes in kallikrein mRNA levels seen in the female anterior and neurointermediate pituitary were due to changes in kallikrein mRNA synthesis, nuclear transcription run-on assay was performed with female anterior and neurointermediate pituitary cell nuclei. The data are presented in Table 1. In the anterior lobe, haloperidol had little effect on kallikrein or PRL gene transcription 1 hr after injection but had a 2.3- to 2.6-fold stimulatory effect 16 hr after. In the neurointermediate pituitary, kallikrein gene transcription was rapidly stimulated almost 3-fold by haloperidol, and the stimulation was maintained at 16 hr, in parallel with the effects of haloperidol on POMC gene transcription.

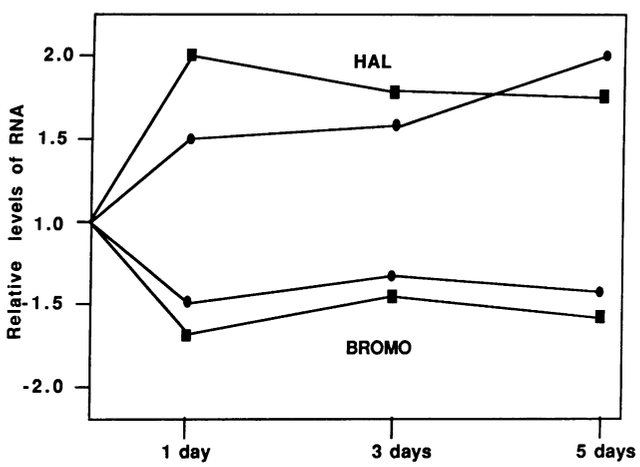

FIG. 4. Analysis of anterior pituitary RNA isolated from haloperidol-treated, bromocryptine-treated, or control female rats. Animals were injected with either haloperidol (HAL) or bromocryptine (BROMO) at 2 mg/kg per day. RNA was isolated, and 1 μg was hybridized to a ^{32}P-labeled single-stranded kallikrein RNA probe or 10 ng of RNA was hybridized to a similarly labeled prolactin probe. After autoradiography, mRNA levels were quantitated by cutting the protected bands from the gel and assaying in a scintillation counter. ■, Kallikrein levels relative to injected controls; ●, prolactin levels for the same animals.

Table 1. Regulation of kallikrein gene transcription

	Transcripts from pituitary lobes							
	Neurointermediate				Anterior			
	POMC		Kall		Kall		PRL	
Treatment	ppm	%	ppm	%	ppm	%	ppm	%
Sacrifice at 1 hr								
Control	2759		593		47		316	
Haldol	8631	313	1544	260	37	79	305	96
Sacrifice at 16 hr								
Control	1172		505		29		208	
Haldol	4503	384	1019	202	68	234	543	261

Animals were injected i.p. with 2 mg of haloperidol (Haldol) per kg of body weight 1 or 16 hr before sacrifice. Results are listed in ppm of specific glandular-like kallikrein transcripts relative to the total amount of nuclear transcripts (28, 29). Data is also presented as the percentage change from the vehicle-injected control animals. Kall, kallikrein; PRL, prolactin.

DISCUSSION

Southern blot analysis (data not shown) suggests that there are multiple rat kallikrein-like genes in agreement with the previously published studies of Ashley and MacDonald (4). Although it is not yet certain how many of these genes are expressed and if any are pseudogenes, the neurointermediate and anterior pituitary of the male and female appear to express only one kallikrein-like mRNA. Nucleotide sequence studies, presence of specific restriction endonuclease sites, solution hybridization/nuclease protection studies, and hybridization with true glandular kallikrein-specific DNA oligomers all argue that the true glandular kallikrein gene is the member of the kallikrein gene family that is expressed in the pituitary gland. The low levels of neurointermediate lobe kallikrein-like mRNA may account for its not having been detected in the earlier experiments of Funder and colleagues (29). Recently, however, Funder's group reported that they may have detected kallikrein-like mRNA in neurointermediate pituitary at levels comparable to what we report here, but that they are concerned that the high kallikrein mRNA levels in the female anterior lobe may be the source of the observed intermediate lobe signal (30). Our studies in male rats eliminate this possibility, since kallikrein mRNA levels in anterior and intermediate lobes are the same; thus, it is clear that there is true glandular kallikrein gene expression in the intermediate lobe. Indeed, our preliminary *in situ* hybridization studies support the presence of kallikrein mRNA in the intermediate lobe (data not shown). However, we cannot rule out that other members of the kallikrein gene family are expressed at a level lower than about 5% of that seen for the true glandular kallikrein gene.

Previous studies by Powers and colleagues have shown that dopaminergic agents modulate the kallikrein enzymatic activity in the intermediate lobe (8, 10). However, since these studies only analyzed enzymatic activity, the mechanism of these changes is not clear. Several possible explanations come to mind. First, there could be a change in the synthesis of the enzyme, with a resulting change in the level of the enzyme activity. Second, there could be changes in the amount of the enzyme available because of recruitment of active enzyme from latent forms. Indeed, Powers has shown that the majority of the kallikrein enzymatic activity present in the intermediate lobe of the pituitary is in a latent form (31). This, in fact, may be the reason that other researchers have been unable to detect kallikrein enzymatic activity in the rat intermediate pituitary (32, 33). Third, there could be a change in specific activity of the enzyme because of some type of posttranslational modification. Any one or all of these possibilities (or others) could be involved in the observed dopamine-mediated changes in the enzymatic activity by Powers and colleagues. Our data show that the mRNA encoding the true glandular kallikrein enzyme is regulated by the dopaminergic agents in the same manner as that reported by Powers for the enzymatic activity. Since changes in mRNA levels generally reflect changes in the synthesis of a specific protein, the changes in enzymatic activity should be due, at least in part, to similar changes in the number of enzyme molecules. The transcriptional studies suggest that the change in mRNA level is partly due to a parallel change in the rate of transcription of the glandular kallikrein-encoding gene(s). Thus, we have been able to show that a major component of the mechanism involved in modulation of kallikrein enzymatic activity by haloperidol is an alteration in the transcriptional activity of the gene encoding the enzyme, resulting in a change in kallikrein mRNA levels.

The POMC gene and the glandular kallikrein gene(s) appear to be coordinately regulated by dopaminergic compounds in the neurointermediate lobe at the transcriptional level, setting the stage for parallel changes in synthesis of these two gene products. One interpretation of this observation is that the kallikrein-like enzyme plays a role either in the intermediate lobe-specific processing of the POMC protein to its end-product hormones, or in some other aspect of POMC peptide production and secretion in this tissue. Other enzymes known to be involved in the intermediate lobe POMC processing pathway, such as the amino-terminal acetylating enzyme and the carboxyl-terminal amidating enzyme, have been shown to be coordinately regulated at the enzymatic level with the production of the POMC peptide, insuring that the processing pattern remains constant (12, 13).

It is much more difficult to interpret the data on kallikrein gene expression in the anterior lobe because of the presence of multiple cell types within that tissue. Others have shown that estrogen regulates the level of kallikrein enzymatic activity (7, 28) and of kallikrein mRNA (30) in the female anterior pituitary. These observations would be consistent with the major site of expression of the kallikrein gene being in the lactotroph of the anterior pituitary, as this cell type is markedly stimulated by estrogen. Similarly, data showing that dopaminergic agents modulate the levels of kallikrein mRNA and gene transcription could be explained by the major site of expression of the kallikrein gene being in the lactotroph, as this cell type is also subject to dopaminergic regulation. Whatever the cell type of expression, our data clearly demonstrate that the modulation by dopaminergic agents of kallikrein enzymatic activity is due, at least in part, to changes in kallikrein mRNA levels resulting from changes in transcription of the true glandular kallikrein gene(s). In the male anterior lobe, the lower level of kallikrein gene expression and the lack of regulation by dopaminergic compounds may imply that the site of expression in that tissue is a non-dopamine-regulated cell. However, because of the complexity of cell types in the anterior pituitary, further studies at the single cell and/or isolated cell type level are necessary to resolve this issue.

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