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Intracellular distribution of GABA in the rat anterior pituitary. An electron microscopic autoradiographic study

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Abstract. We studied the internalization and intracellular distribution of [^{3}H] GABA in rat anterior pituitary cells. Electron microscopic autoradiography of anterior pituitary fragments or dispersed pituitary cells incubated with [^{3}H] GABA showed that lactotrophs and, to a lesser extent, somatotrophs were the only cells that contained radioactive grains. Grain density analysis performed on dispersed pituitary cells after a pulse-chase experiment (10 min pulse and then change to a medium without radioactive GABA for various periods up to 2 h) revealed that GABA internalized by lactotrophs was distributed in various intracellular membranous organelles. Of the cell compartments examined, plasma membrane, Golgi apparatus, mitochondria and secretory granules had different time-dependent labeling patterns. The highest grain density values were associated with plasma membrane (at the first chase time) and the Golgi apparatus. Mitochondria and secretory granules also showed significant grain density values. A similar pattern of distribution was observed when fragments of prolactin-secreting pituitary adenomas were incubated with [^{3}H] GABA. These results provide morphological data on the cellular specificity and intracellular distribution of GABA in anterior pituitary cells. © 2000 Harcourt Publishers Ltd

Introduction

Evidence from several laboratories has demonstrated the role of GABA in the control of prolactin secretion by the anterior pituitary gland. GABA acts at the pituitary level as an inhibitory factor on prolactin release and synthesis (McCann & Rettori, 1986; Loeffler et al., 1986). GABA released from median eminence terminals reaches the pituitary via the hypophysial portal venous system and interacts with specific receptors in the anterior pituitary. Receptor-binding studies have suggested the coexistence of both $GABA_A$ and $GABA_B$ receptors (Apud et al., 1989; Anderson & Mitchell, 1986 (a)) in the anterior pituitary gland. In addition, the expression of several $GABA_A$ pituitary subunit mRNAs has been reported in the gland (Valerio et al., 1992; Berman et al., 1994; Boué-Grabot et al., 1995). Immunocytochemical studies looking for GABAergic innervation in the pituitary, using highly specific antibodies against GABA, have not found any immunoreactivity in the anterior lobe (Sakaue et al., 1988; Rabhi et al. 1987; Pow, 1993). However, central-type benzodiazepine receptors, which are linked to the GABA receptor molecular complex, have been demonstrated by autoradiographic studies in the anterior pituitary gland (Brown & Martin, 1984; De Souza et al., 1985). No reports

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have been published on the localization of these GABA receptors on a specific type of pituitary cells. Previously we demonstrated that the anterior pituitary gland is able to transport and incorporate GABA, which interacts with intracellular particles (Duvilanski et al., 1994). In the present investigation we studied the internalization and intracellular localization of [^{3}H] GABA in rat anterior pituitary and human prolactin-secreting pituitary adenomas by electron microscopic autoradiography.

Material and methods

Male Wistar rats weighing 180–200 g were used. The rats were kept at 20–25°C with controlled lighting (12 h light–12 h darkness) and fed a rat chow diet and water *ad libitum*. They were maintained in accordance with NIH Guide for the Care and Use of Laboratory Animals. Rats were killed by decapitation and the anterior pituitary lobes quickly removed and separated from the neurointermediate lobe. Human prolactin-secreting pituitary adenomas (n=4) were obtained in the operating room. A portion of the tissue was sent for pathologic evaluation, and the remaining tissue was immediately incubated and processed.

Drugs

All drugs were purchased from Sigma Chemical Co. (St Louis, MO) except: [^{3}H]GABA: [2,3–^{3}H] GABA (SA 85.4 Ci/mmol) and Solvable were obtained from New England Nuclear (NEN); Kodak NTB2 emulsion and microdol from Eastman Kodak, Rochester, NY; Medium TC 199 from Difco Laboratories, Detroit, MI.

Incubation of anterior pituitary fragments

Two rat anterior pituitaries or fragments of one human pituitary adenoma were cut into small pieces, then washed with Krebs Ringer bicarbonate buffer, pH 7.4, containing 10 mM glucose, 10 mM Hepes, 0.1% albumin (KRB buffer). The fragments were preincubated for 90 min, to reduce endogenous GABA, in 1 mL of KRB buffer in a Dubnoff metabolic shaker with constant gassing by a mixture of 95% O_2–5% CO_2 at 25°C. At the end of this period, the medium was replaced by a fresh one and rat pituitary fragments were incubated in duplicate for 20 min with 5 μCi/mL [^{3}H] GABA (final concentration 10^{-6} M) with or without 2.4×10^{-5} M aminooxyacetic acid (AOAA), an inhibitor of GABA metabolization, while pituitary adenoma fragments were incubated for 5 and 20 min with [^{3}H] GABA (final concentration 10^{-6} M) with or without cold 10^{-3} M GABA. At the end of the incubation period, the medium was discarded and the fragments were rapidly washed with ice-cold KRB buffer and resuspended in 0.5 mL of KRB buffer. Fragments were immediately fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for ultrastructural studies.

Pulse-chase experiment

Twenty-four rat anterior pituitaries removed in sterile conditions were placed in chambers containing KRB buffer without Ca^{++} or Mg^{++}, with Minimum Essential Medium-aminoacids, washed three times and then cut into 1 mm pieces. The fragments were incubated in the same buffer containing 0.2% trypsin, for 30 min, at 37°C in a humidified incubator gassed with air plus 5% CO_2. They were then treated for 2 additional min with DNAse I. After centrifugation (1 min at 1200 × g) the pellet was resuspended in the same buffer with 0.1% trypsin inhibitor and the cells were dispersed by gentle extrusion through siliconized Pasteur pipetes. Viability of cells, determined by Trypan Blue, was always greater than 95%.

Cells resuspended in Medium TC 199, pH 7.4, containing 10 mM glucose, 10 mM Hepes, 0.1% albumin (TC medium) were preincubated at 37°C, in siliconized tubes, in an atmosphere of air with 5% CO_2 with constant shaking for 24 h. At the end of this period, the cells were centrifuged for 5 min at 400 × g, the incubation medium was decanted and the cells resuspended in 3.6 mL of oxygenated TC medium at 25°C. Three mL of this suspension were incubated for 10 min in the presence of 5.8×10^{-8} M [^{3}H] GABA (5 μCi/mL). At the end of this period an aliquot of the cell suspension (0.3 mL) was taken, diluted to 10 mL with TC medium at 4°C, immediately centrifuged for 5 min at 4°C and fixed by the addition of 2.5% glutaraldehyde in 0.01 N phosphate buffer pH 7.4 (Time-0 post-pulse-chase sample). Simultaneously, the remaining suspension of cells was diluted with TC medium (1:4 v/v) at 25°C and centrifuged (5 min at 400 × g) at room temperature. The cells were resuspended in 8 mL of TC medium and incubated in a vial at 25°C with constant shaking and gassing. An aliquot of the suspension of cells (1 mL) was removed at different periods: 5, 10, 20, 30, 40, 50, 60 and 120 min and processed as previously described for the time-0 post-pulse-chase sample. Also, 0.5 mL of the original suspension of cells were incubated for 10 min with 5.8×10^{-8} M [^{3}H] GABA (5 μCi/mL) and nonradioactive 10^{-3} M GABA and processed in an identical way to that described for post-pulse-chase samples.

Autoradiography

Anterior pituitaries were fixed in 2.5% glutaraldehyde in 0.01 M phosphate buffer (pH 7.4) at room temperature for 2 h for dispersed cells or eight hours for fragments. The pituitary material was post-fixed in 1% OsO_4 for 60 min, dehydrated in ethanol and embedded in maraglass. Pale gold sections were coated with Kodak NTB2 emulsion using the loop method (Caro, 1969), exposed for 30 days, developed in microdol for 4 min (Kopriwa, 1975) and subsequently stained with uranyl acetate and lead citrate. The autoradiograms were observed in a Zeiss EM 9^a electron microscope. In order to achieve random selection, every cell sectioned through the nucleus in a given grid square was analyzed. In estimating the percentage of radiolabeled cells,

a cell that contained at least 3 silver grains was considered labeled. In each group at least 30 cells were analyzed. In the conditions used in this study, the effective resolution or half distance (HD) of ^3H was 0.38 μm (Salpeter et al., 1976). The percentage of grains in a given compartment was determined by dividing the total grain centers in the compartment by the total grain centers in all the compartments (Salpeter & Mc Henry, 1973). The percent area of each compartment was determined by the point counting method as described by Weibel & Bolender, 1973. The percent area occupied by a compartment was computed by dividing the total area points over a given compartment by the total area points over all of the compartments. Finally, the grain density (GD) of each compartment was determined by dividing the grain percent by the area percent values. A grain density greater than 1 was considered significant labeling (Duello et al., 1983). The cells were identified on the basis of morphological criteria, such as size and shape of the cells and the secretion granules, as previously described (Moriarty, 1973; Pérez & von Lawzewitsch, 1984). These morphological criteria by electron microscopy correlated with light microscopy immunohistochemical studies (Yamaji et al., 1992)

Results

Internalization of [^{3}H] GABA in rat anterior pituitary fragments

The autoradiography of the cells from the pituitary fragments incubated with 10^{-6} M [^{3}H] GABA showed that the silver grains were mainly located in lactotrophs and to a lesser extent in somatotrophs. The silver grains were found close to the plasma membrane and intracellularly distributed in different organelles, such as Golgi complex, mitochondria, secretory granules (Figs. 1a and 1d) and in the cellular prolongations of lactotrophs (Fig. 1b). A similar distribution pattern of the grains in the autoradiograms was observed when anterior pituitary fragments were incubated with [^{3}H] GABA in the presence of AOAA (Fig. 1c).

Pulse-chase experiment with [^{3}H] GABA in dispersed anterior pituitary cells

Immediately after the incubation of dispersed cells with [^{3}H] GABA, approximately 44% of cells were labeled, these being only lactotrophs and somatotrophs. All the lactotrophs were labeled, while approximately 20% of the total number of somatotrophs showed grains of radioactivity. The background was usually very low and other cell types were rarely labeled; when labeled, they never contained more than one silver grain.

The number of labeled cells decreased rapidly after chase (Fig. 2). Five min after chase, 30% of cells contained radioactivity, while at 30 min, only 10% of cells were labeled.

Intracellular distribution of [^{3}H] GABA in dispersed anterior pituitary cells

The quantitative study of the lactotrophs 5 min after chase showed that the highest concentration of silver grains corresponding to [^{3}H] GABA was associated with the plasma membrane, with a grain density (GD) of 9.2 (Table 1, Fig. 3a). The Golgi apparatus showed the second highest level of labeling (GD:2.6). The other compartments contained a GD of less than 1. A marked reduction in the GD of the plasma membrane was observed at 10 and 20 min after chase (Fig. 3b). During the same time interval a marked increase in GABA accumulation in the Golgi complex was observed (Table 1, Fig. 3b). A moderate increase in the GD of the secretory granules and mitochondria was also noted. At 30 min after chase, the picture was similar to that after 20 min (Fig. 3c). At all times the endoplasmic reticulum, lysosomes and nucleus showed the lowest level of labeling. A similar pattern was observed in somatotrophs (Fig. 3d). Neither lactotrophs nor somatotrophs showed silver grains when cells were incubated with [^{3}H] GABA and non-labeled 10^{-3} M GABA (data not shown), ensuring the specificity of the GABA internalization mechanism in anterior pituitary cells [Duvilanski et al. 1994].

Internalization of [^{3}H] GABA in human pituitary adenoma fragments

After 5 min of incubation, the silver granules indicating the presence of [^{3}H] GABA were observed near the plasma membrane and also internalized in the cytoplasm, the Golgi region and in the rough endoplasmic reticulum (Fig. 4 a,b,c). After 20 min, a distribution of silver granules similar to those observed after 5 min incubation were detected. No grains were observed when pituitary adenoma fragments were incubated with [^{3}H] GABA and cold 10^{-3} M GABA for 20 min (data not shown).

Discussion

This investigation showed that when anterior pituitary fragments or dispersed cells were incubated with [^{3}H] GABA *in vitro* there was a rapid uptake of this neurotransmitter. Since incubations done with AOAA showed a distribution of silver grains similar to those with GABA alone, the presence of well localized silver grains in anterior pituitary cells may correspond to sites where [^{3}H] GABA has been taken up and retained in an unchanged form.

One of the most remarkable morphological observations was that GABA specifically interacted with lactotrophs and, to a lesser extent, with somatotrophs, and was internalized by them. No other pituitary cell types interacted with or internalized GABA. Dopamine, another neurotransmitter that plays an important role in the intracellular control of prolactin release and synthesis (Lamberts & MacLeod, 1990), is also internalized by lactotrophs (Gudelsky et al., 1980; Rosenzweig and Kanwas, 1982), showing a similar pattern of intracellular distribution to GABA.

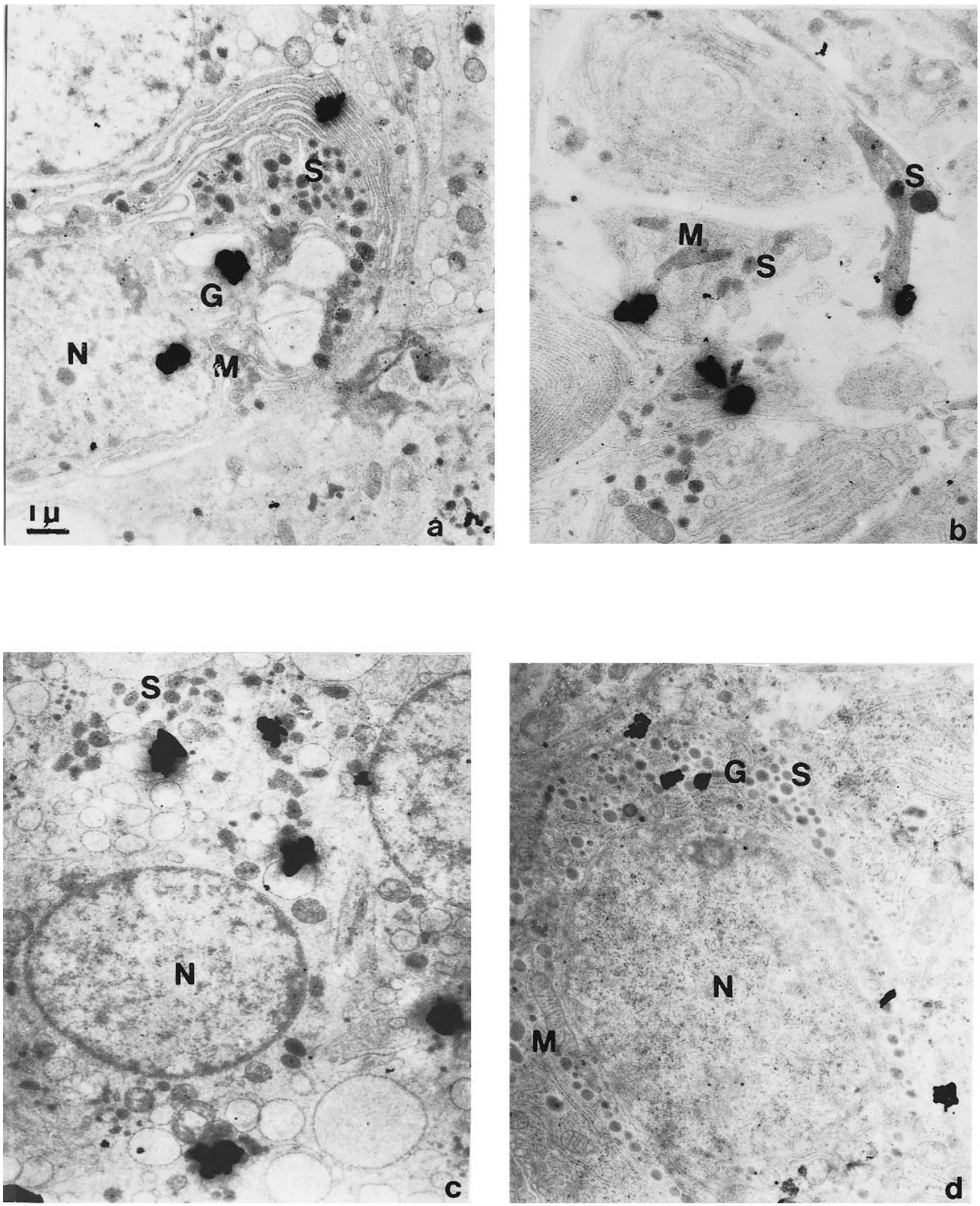

Fig. 1 Autoradiography of rat anterior pituitary fragments incubated with [^{3}H]GABA (a, b, d) or with [^{3}H]GABA and AOAA (c). **a–c** lactotrophs; **b** cellular prolongations of lactotrophs; **d** somatotrophs. Grains are associated with various intracellular organelles. Golgi apparatus (G); secretory granules (S); nucleus (N); mitochondria (M). (X 8,500).

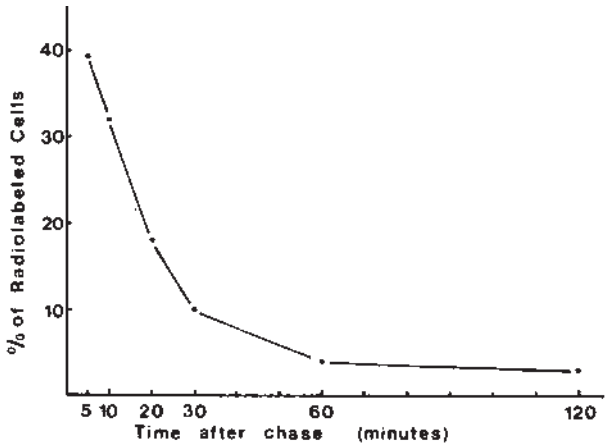

Fig. 2 Loss of internalized [^{3}H]GABA from anterior pituitary cells as a function of time after chase. Dispersed anterior pituitary cells were pulsed for 10 min with [^{3}H]GABA and subsequently chase incubated for different times. The results are the mean of two experiments.

The mechanism by which GABA is internalized into anterior pituitary cells is not fully clear yet. The internalization of GABA may be due to specific transport mechanisms, such as GABA transporters and/or endocytosis of GABA receptor. GABA transporters have been identified in several neural tissues (Radian et al., 1986, Borden, 1996). Since the anterior pituitary gland can specifically take up and concentrate [^{3}H] GABA by a process independent of receptors (Duvilanski et al., 1994) we can suggest the existence of a GABA transport system in the anterior pituitary cells. It is also possible that GABA could enter the lactotrophs by a receptor mechanism such as endocytosis, which has been observed for haloperidol, a dopamine agonist (Goldsmith et al., 1979). Therefore, GABA receptors (McCann & Rettori, 1986; Apud et al., 1989) and/or a specific GABA transport mechanism (Duvilanski et al., 1994) in anterior pituitary cells may be found mainly in lactotrophs and somatotrophs.

In our study, GABA was associated first with the plasma membrane. Then, the internalized GABA was subsequently distributed into intracellular organelles, especially the Golgi complex. Such a close apparent association with the Golgi complex suggests some participation of GABA in the intracellular processing of prolactin. Mitochondria also presented a low but significant accumulation of silver grains. Internalized GABA associated with mitochondria could be related to GABA metabolization since GABA-transaminase (E.C. 2.6.1.19) is present in mitochondria (Schousboe et al., 1980). Thus, the relatively low accumulation of GABA in mitochondria could be a consequence of the rapid metabolization of GABA by anterior pituitary cells (Duvilanski et al.(a), 1985) and the release of its metabolites. Internalized GABA was also found close to the secretory granules, although grain density was never higher than 1%. On the other hand, other intracellular organelles, such as lysosomes, endoplasmic reticulum and nucleus, did not show a significant accumulation of GABA.

We observed a similar pattern of distribution of [^{3}H] GABA in a prolactin-secreting pituitary adenoma. These results suggest that lactotrophs of the pituitary adenoma did not loose the ability to bind and incorporate GABA. Supporting these observations, it was demonstrated that GABA application produced membrane hyperpolarization and inhibited electrical activity in the prolactin-secreting tumor cells (Israel et al., 1981).

GABA can modify the secretion of pituitary hormones by exerting direct effects at the pituitary level or by modifying hypothalamic mechanisms involved in pituitary hormone regulation (McCann & Rettori, 1986; Apud et al. 1989). At the pituitary level, GABA inhibits prolactin secretion by acting on specific GABA receptors (Enjalbert et al., 1979; Duvilanski et al., 1985 (b); Apud et al., 1989). The presence of GABA receptors (Anderson & Mitchell, 1986 (a); Apud et al., 1989) and their mRNAs (Valerio et al., 1992; Berman et al., 1994) in the anterior pituitary has been described. Our autoradiographic observations on the interaction of GABA with plasma membrane of lactotrophs support these results and indicate a direct effect of GABA on this cellular type. Regarding the effect of GABA on the

Table 1 Distribution of autoradiographic grains over cell organelles in lactotrophs of anterior pituitary dispersed cells

Compartments	% of total grain (TG) and grain density (GD) (a)							
	5 min chase		10 min chase		20 min chase		30 min chase	
	TG	GD	TG	GD	TG	GD	TG	GD
Plasma membrane	<u>42</u>	9.2	18	3.9	10	2.2	9	2.0
Golgi complex	12	2.6	23	5.0	<u>30</u>	6.5	24	5.2
Lysosomes	6	0.3	6	0.3	7	0.4	<u>15</u>	0.7
Mitochondria	7	0.5	13	0.9	15	1.0	<u>19</u>	1.4
Secretory granules	13	0.7	<u>18</u>	1.0	16	0.9	14	0.8
Endoplasmic reticulum	8	0.4	9	0.4	10	0.5	9	0.4
Nucleus	2	0.09	3	0.1	5	0.2	2	0.09
Cytoplasmic matrix	10	0.9	10	0.9	7	0.6	8	0.7

Anterior pituitary cells were incubated as indicated in pulse-chase experiment (M & M) 5, 10, 20, 30 min after chase aliquots were processed for EM autoradiography. *Underlined* numbers indicate maximum accumulation of grain in compartments that showed changes after chase. (a): includes all grains within 2 HD. Data are the average of two experiments.

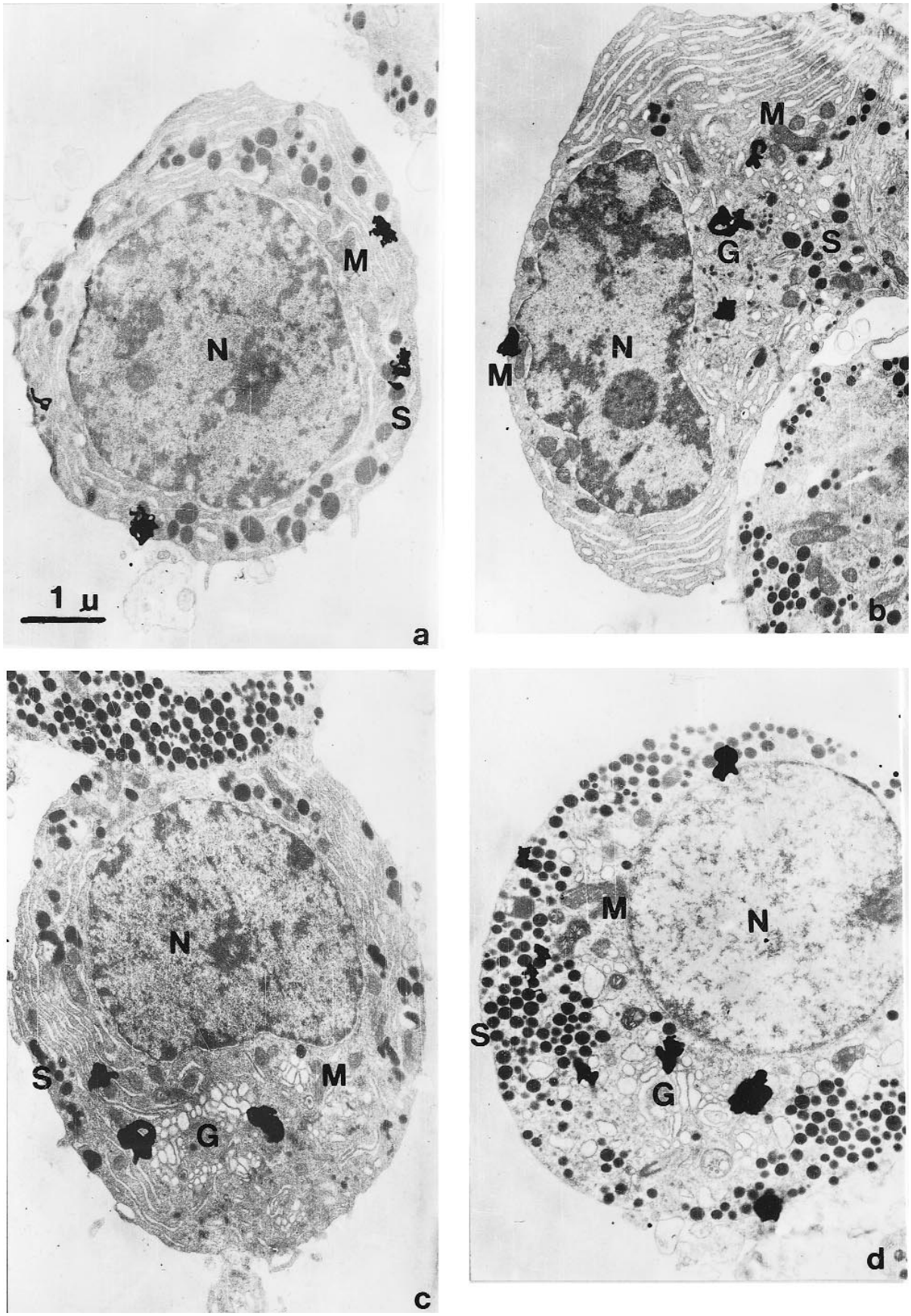

Fig. 3 Autoradiography of a lactotroph (**a, b, c**) and a somatotroph (**d**) that were pulsed with [^{3}H]GABA for 10 min and subsequently chase incubated for 5 (**a**), 20 (**b**), 30 (**c**) and 10 min (**d**). Grains are associated with various intracellular organelles. Golgi apparatus (G), secretory granules (S), nucleus (N), mitochondria (M), lysosomes (L). (X 14,500).

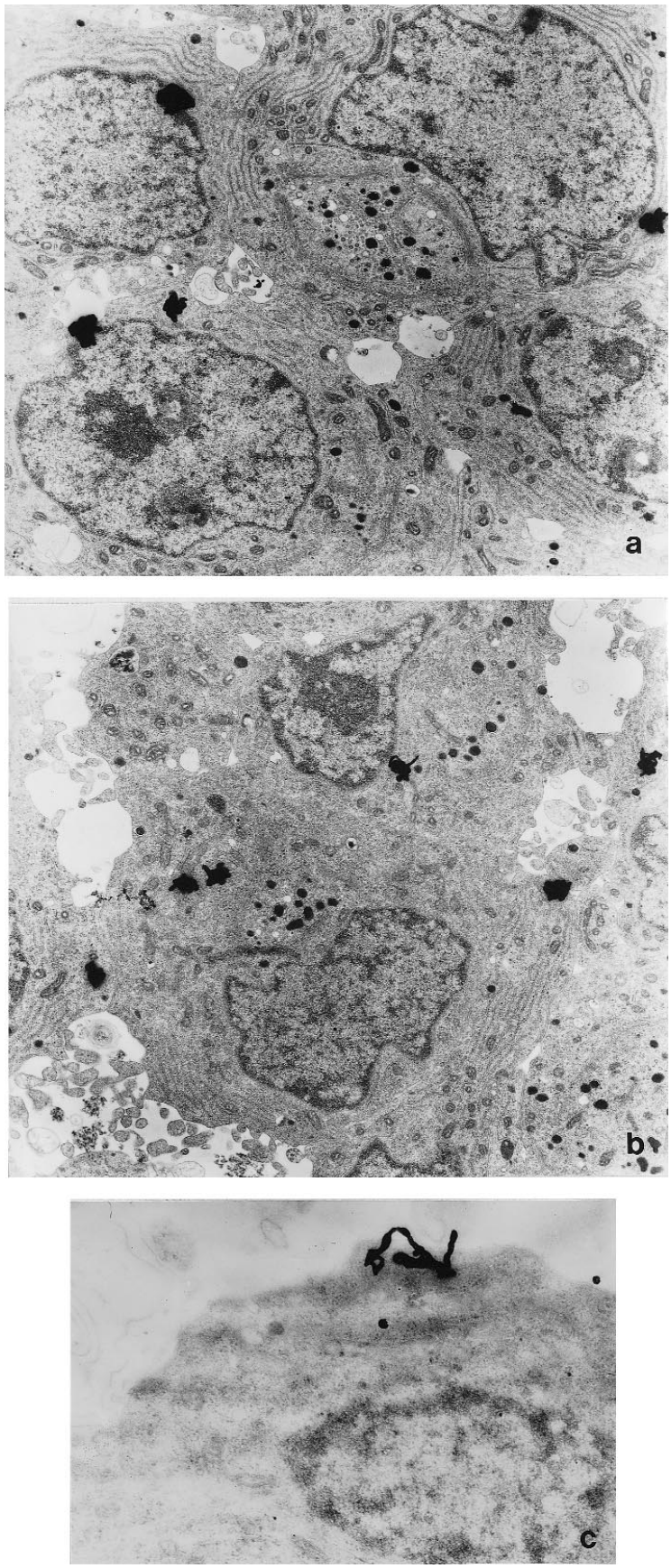

Fig. 4 Prolactin cell from a pituitary adenoma. 5 min after the incubation with [^{3}H]GABA grains can be seen close to the plasma membrane, within the cytoplasm, in the vicinity of the Golgi Complex (G) and mitochondria (M), (a, b), (X 10,000). Higher magnification of prolactin cells with a grain close to the plasma membrane (c), (X 20, 000).

secretion of other pituitary hormones, most information available suggests that these effects of GABA are mediated by changes in hypothalamic hormones or neurotransmitter release rather than by direct pituitary action (McCann & Rettori, 1986). There is some experimental evidence in favor of direct effects of GABA on the secretion of several pituitary hormones (Anderson & Mitchell, (b) 1986; Tapia-Arancibia et al., 1987; Virmani et al., 1990). However, since we could not detect GABA associated with gonadotrophs or corticotrophs, the local effects of GABA on LH and ACTH secretion could result from paracrine interactions between different cell types in the anterior pituitary.

In conclusion, the results of the present investigation demonstrate that GABA interacts almost specifically with lactotrophs. Internalized GABA associated with specific intracellular organelles which are very likely related to the mechanisms involved in prolactin processing and secretion. Therefore, the action of GABA on prolactin cells is not restricted to binding to its receptor at the level of the plasma membrane, but it is also related to the association with specific intracellular organelles. These mechanisms also seem to function in prolactin-secreting cells of human pituitary adenoma.

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