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GAMMA-AMINOBUTYRIC ACID UPTAKE AND LOCALIZATION IN BOVINE CHROMAFFIN CELLS IN PRIMARY CULTURE

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Abstract—γ-Aminobutyric acid (GABA) uptake was studied in bovine chromaffin cells maintained in primary culture. Uptake was found to be dependent on Na^+, but not on K^+ and Ca^{2+} ions; it was found that 2 Na^+ ions were necessary for each molecule of GABA transported. 2,4-Dinitrophenol, ouabain and vanadate inhibited GABA uptake showing the energy dependency of the system. Two affinity sites were demonstrated, a high affinity site and a low affinity site with K_m values of 10 μM and 170 μM, respectively. While the low affinity site did not show large variations with culture age, the K_m of the high affinity site increased from 1 μM in freshly isolated cells to 10 μM in 3–9 day-old cells. GABA uptake was unaffected by glutamic acid, aspartic acid, glycine and catecholamines, while taurine, beta-alanine, nipecotic acid and L-2,4 diaminobutyric acid inhibited GABA uptake. Nipecotic acid and L-2,4 diaminobutyric acid acted as competitive inhibitors modifying K_m values of the high affinity site. Subcellular studies performed on [^{3}H]GABA-loaded chromaffin cells showed that GABA was not in secretory granules but was recovered in the 100,000 *g* soluble fraction. The GABA uptake process associated with chromaffin cells may be an important mechanism for regulating the modulation of catecholamine secretion. In addition, the presence of GABA in the cytosol indicates that this molecule may be an effector of chromaffin cell activity in addition to modulating catecholamine secretion.

The neuromodulatory role of GABA† is to regulate hormonal secretion in numerous endocrine tissues or smooth muscle contraction in various muscular tissues. In most of these tissues, the presence of the major biosynthetic and metabolic pathways for GABA, as well as the existence of low and high affinity uptake systems for GABA have also been established [1–5]. The low affinity uptake system, with apparent K_m values ranging from 10^{-4} to 10^{-3} M has been described in rat thyroid, guinea pig gallbladder, human blood platelets and retina. A high affinity system with apparent K_m values of 5×10^{-5} M or less, is observed in sympathetic and sensory ganglia, retina, parasympathetic ganglia and endocrine organs including adrenal medulla [see Ref. 6 for a review].

In bovine adrenal medulla, the presence of GABA, GABA- synthesizing and metabolizing enzymes and GABA receptors has been demonstrated [7–12]. Although the functional role of GABA in the regulation of the secretory process in adrenal medulla is not clear, an effect on the basal and evoked catecholamine secretion in adrenal glands and chromaffin cells has been shown [12–14]. Thus GABA should be added to the list of putative neuromodulators of adrenal medulla secretion, together with dopamine [15], adenosine [16], prostaglandins [17] and peptides such as substance P, somatostatin and VIP, all of which are present in adrenal glands [18–22].

We have previously reported the existence of a GABA uptake system in slices from bovine adrenal medulla. In this paper we examine GABA uptake processes in bovine chromaffin cells maintained in primary culture, since these cells have been proven to be a suitable model to study the electrical, ionic, biomedical and pharmacological events occurring at the level of the plasma membrane. Here we describe the characteristics of GABA uptake in chromaffin cells with regard to Na^+-dependence, ionic and energetic requirements, kinetics, specificity, and influence of cellular age. In addition, the localization of accumulated GABA in chromaffin cells has been examined.

MATERIALS AND METHODS

Isolation and culture of chromaffin cells. Chromaffin cells were isolated from bovine adrenal glands by collagenase (EC 3.4.24.3) treatment and purified by centrifugation on Percoll gradients to remove contaminating erythrocytes and cell debris [23, 24], carefully collected and washed with Dulbecco's modified Eagle's medium (DMEM). Cells were suspended in DMEM containing 10% foetal calf serum, 10 μM cytosine arabinoside and 10 μM 5-fluorodeoxyuridine and grown in 16-mm 24-well Costar plates, at a density of 5×10^5 cells/well. Culture medium was replaced by fresh medium every 3 days.

GABA uptake. Cells were incubated for 1 hr at 37° in 1 ml fresh aminoacid-free DMEM at 37°. After

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† Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; GABA, γ-aminobutyric acid; AOAA, aminooxyacetic acid; DMEM, Dulbecco's modified Eagle's medium; L-DABA, L-2,4-diaminobutyric acid.

removing this medium chromaffin cells were incubated with 1 μCi of [2,3-^{3}H]-GABA (29.3 Ci/mmol, Amersham, Les Ullis, France) and unlabelled GABA to give the final GABA concentration. Uptake was performed in a Krebs–Hepes solution of the following composition: 140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, 0.01 mM EDTA, 0.58 mM ascorbic acid and 15 mM Hepes, pH 7.5. The reaction was terminated by aspirating the incubation medium, washing four times with GABA-free Krebs–Hepes medium and adding 0.5 ml of 0.4 M perchloric acid. Precipitated material was scraped off and centrifuged for 10 min, at 10,000 g at 4°. Radioactivity from the clear supernatant was measured by liquid scintillation spectrometry.

Except as indicated, all GABA uptake experiments were performed in the presence of 1 mM aminooxyacetic acid (AOAA). To study the role of Na^+ on [^{3}H]-GABA uptake, normal Krebs–Hepes solution was replaced with medium in which NaCl was substituted by equimolar quantities of choline chloride. Control values were obtained by incubating cells in a Na^+-free, iso-osmotic Krebs–Hepes medium.

Specific uptake was calculated by subtracting the radioactivity obtained in the absence of sodium from that obtained in the presence of Na^+. Uptake in absence of NaCl was considered to be due to passive diffusion.

Quantification of GABA metabolism. Prelabelled cells were washed four times with 2 ml of Krebs–Hepes solution. Radioactivity was extracted from the cells with 0.4 M perchloric acid. After 10 min centrifugation at 10,000 g, the supernatant was applied to a Dowex 50 × 8 (H^+, 100–200 mesh, 0.5 × 2.5 cm) column. The column was washed with 5 ml of distilled water and then [^{3}H]-GABA was eluted with 10 ml of 3 M NH_4OH.

Subcellular fractionation of cultured chromaffin cells. After loading cells with [^{3}H]-GABA (5×10^{-7} M) for 1 hr and washing with Krebs–Hepes medium (six washes, 10 min each), cultured chromaffin cells (60×10^6) were homogenized in 15 ml of 0.32 M sucrose buffered with 10 mM Hepes at pH 7.2. A low speed pellet was removed by centrifugation at 800 g for 10 min and the supernatant fraction was centrifuged at 11,500 g for 20 min. The resulting pellet which represents the crude chromaffin granule fraction was suspended in 1 ml of 0.32 M buffered sucrose and layered onto 11 ml of a continuous 10 mM Hepes-buffered sucrose gradient, pH 7, ranging from 1.0 M to 2.2 M. The tubes were then centrifuged in an SW41 Beckman rotor at 113,000 g for 90 min. After centrifugation, 500 μl fractions were collected and the radioactivity content measured by liquid scintillation spectrometry.

RESULTS

Time course of [^{3}H]-*GABA uptake by chromaffin cells*

In confirmation of the observation of Kataoka *et al.* [12], we found that bovine adrenal chromaffin cells maintained in primary culture are able to accumulate extracellular GABA. Figure 1 shows the time-course of [^{3}H]-GABA uptake in Krebs–Hepes buffer, the final GABA concentration being adjusted to 10 μM: GABA uptake was linear during the first hour, but lost linearity thereafter. GABA uptake rate for the first 60 min of incubation was calculated to be 833 fmol/min/5 × 10^5 cells. When accumulated GABA was expressed per cell, values were found to be constant at all cell densities tested.

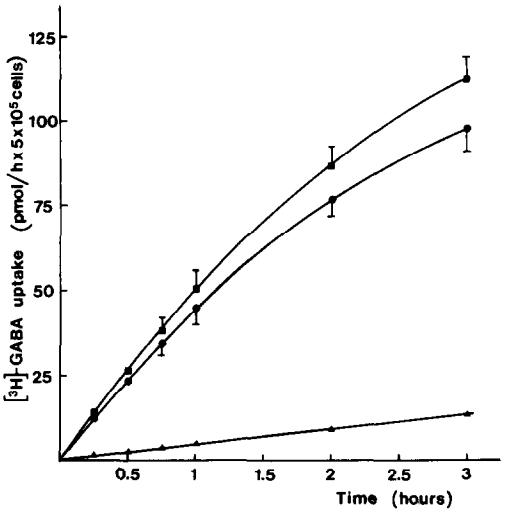

Fig. 1. Time-course of [^{3}H]-GABA uptake in bovine chromaffin cells in culture. Total uptake: squares; Na^+-dependent uptake: triangles; Na^+-independent uptake: circles. The external GABA concentration was 10 μM. Each point is the mean (± SE) of three experiments, each performed in duplicate.

Sodium dependence of [^{3}H]-*GABA uptake*

Figure 1 also shows that a low but detectable Na^+-independent GABA uptake is present in cultured chromaffin cells, increasing linearly for at least 3 hr. This uptake represented less than 10% of total GABA uptake. The difference between total GABA uptake and Na^+-independent GABA uptake represents the specific Na^+-dependent uptake (Fig. 1). As shown in Fig. 2a, the GABA uptake was proved to be highly dependent on sodium and the saturation curve to be sigmoidal. V_{max} value for GABA was calculated to be 1.0 pmol/min/5 × 10^5 cells. The hill plot (Fig. 2b) was a straight line which could be described by the equation $Y = -4.3 + 2.34X$ (with $r = 0.99$); the value of 2.34 suggests that 2 Na^+ ions are transported for each molecule of GABA taken up into cells.

GABA uptake did not show significant variations at any of the extracellular K^+ and/or Ca^{2+} concentration tested from 0 to 10 mM.

Energy requirement of GABA uptake

As shown in Table 1, GABA accumulation is inhibited by 2,4-dinitrophenol or by ouabain and vanadate in a dose-dependent manner, indicating that this process is energy dependent. The absence of glucose in the incubation medium did not affect GABA uptake rate (data not shown).

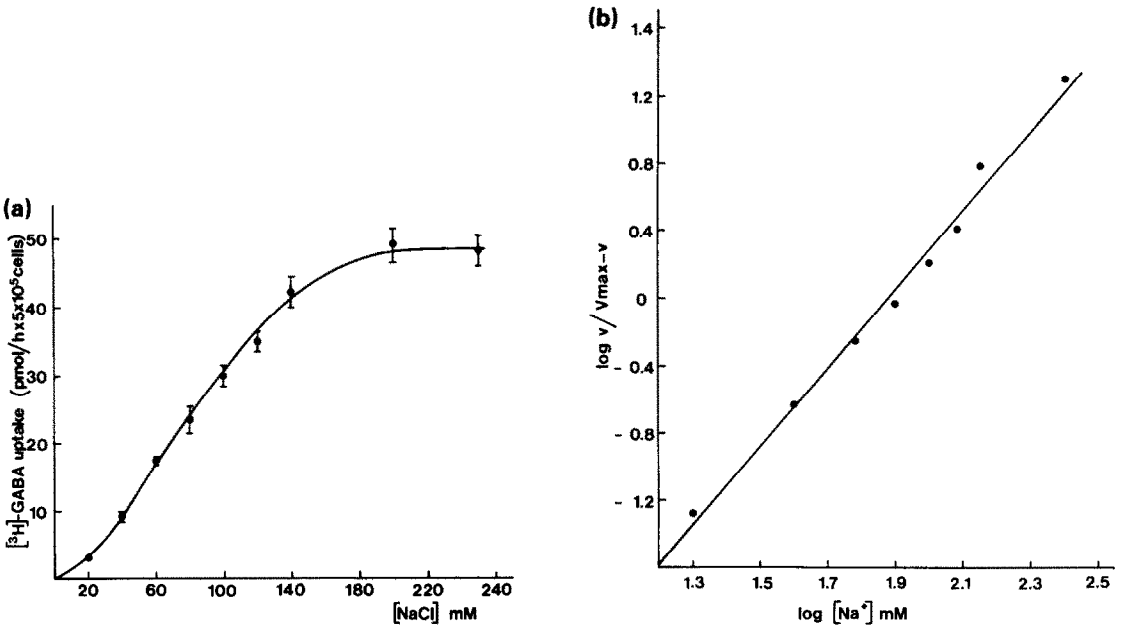

Fig. 2. (a) Sodium dependence of GABA uptake by bovine chromaffin cells in culture. The external GABA concentration used was 10 μM. Each point is the mean (± SE) of three experiments, each performed in duplicate. (b) Hill plot showing $\log[V/V_{max} - V]$ as a function of log[Na^+ concentration]; V indicates initial uptake rates corrected for the insaturable transport component at the different Na^+ concentration. V_{max} was calculated to be 62.2 ± 1.02 pmol/hr/5 × 10^5 cells. The line was fitted to the experimental points by regression analysis ($r = 0.99$) and the slope of the line was 2.34 ± 0.15. Each point is the mean of three experiments, each performed in duplicate.

Table 1. Effect of 2,4-dinitrophenol (DNP), ouabain and vanadate on [^{3}H]-GABA uptake by bovine chromaffin cells in culture

Condition	pmol/hr/5 × 10^5 cells	Inhibition (%)
Control	60.0 ± 7.0	—
+ 2,4-DNP		
2.5 mM	43.5 ± 1.6	28
1.0 mM	58.5 ± 1.4	4
+ Ouabain		
10^{-4} M	32.5 ± 1.6	47
10^{-5} M	51.0 ± 1.7	15
+ Vanadate		
10^{-4} M	43.0 ± 2.4	28
10^{-5} M	59.0 ± 6.0	2

Initial uptake rates were determined in absence or presence of inhibitor at the indicated concentration. [^{3}H]-GABA concentration used was 10 μM. Data are mean (± SE) of two experiments, each performed in triplicate.

Transport kinetics of GABA uptake

The quantity of GABA accumulated in cultured chromaffin cells for 1 hr was determined with extracellular GABA concentrations increasing from 0.1 μM to 1.0 mM and the data plotted. V vs V/S Eadie–Hofstee plots (Fig. 3) revealed the existence of both low and high affinity systems, referred to as sites 1 and 2, respectively. The kinetic constants obtained with the two-system model were the following in cultured cells 3 days old: K_{m_1} = 142 ± 34 μM, V_{max_1} = 639 ± 154 pmol/hr/5 × 10^5 cells and K_{m_2} = 11 ± 3 μM, V_{max_2} = 87 ± 20 pmol/hr/5 × 10^5 cells for the low and high affinity systems, respectively (mean ± SE, N = 8).

As shown in Fig. 4, the kinetic parameters for the high affinity sites increased dramatically during the first 5 days of culture (K_{m_2}, increased from 1 μM in freshly isolated cells to 10 μM, while V_{max_2} increased from 25 to 100 pmol/hr/5 × 10^5 cells), while the V_{max_1} and K_{m_1} low affinity uptake sites were more stable from day 1 to 7. No sodium-dependent uptake could be measured for the low affinity site 1 in freshly isolated cells, possibly as a consequence of membrane damage produced by collagenase treatment [25]. Curiously, at day 7, high affinity uptake constants decreased dramatically; this was shown to be due to an increase in the Na^+-independent transport component, a possible consequence of cellular aging.

Effects of different compounds on GABA uptake

Glutamic acid, glycine and aspartic acid, 5-hydroxytryptamine and noradrenaline at concentrations ranging from 10^{-6} and 10^{-4} M did not affect GABA uptake. In contrast GABA uptake by chromaffin cells was strongly inhibited by taurine, β-alanine, nipecotic acid, L-2,4-diaminobutyric acid and GABA itself with IC_{50}s (the concentration of compounds producing 50% effect, as determined by log-probit analysis at an extracellular GABA concentrations of 10 μM) of 87 ± 12 μM, 95 ± 4 μM, 0.2 ± 0.05 mM, 1.2 ± 0.3 mM and 10 ± 1 μM,

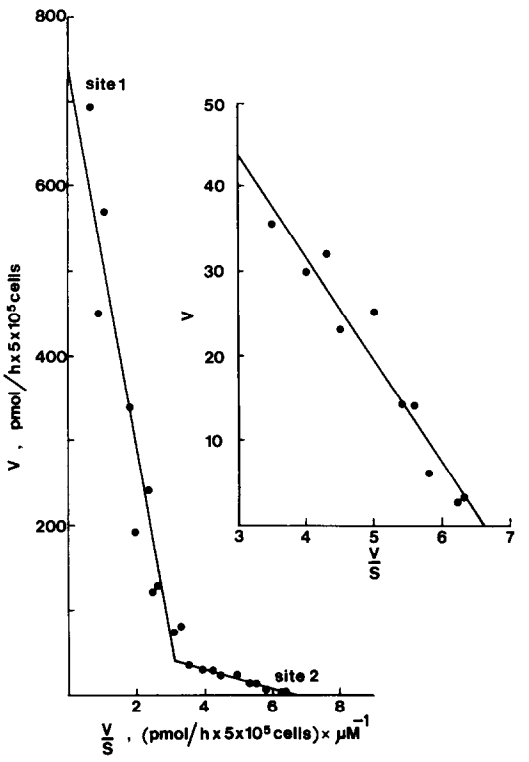

Fig. 3. Eadie–Hofstee plots of GABA uptake by bovine chromaffin cells in culture. The substrate concentration range was from 0.1 μM to 1 mM. Points represent the mean values of one representative experiment. Standard errors for each point ranged from 1 to 10% of the mean values. Inset shows Eadie–Hofstee plots of site 2 on an expanded scale.

respectively. Aminooxyacetic acid (AOAA) activated GABA uptake with an AC_{50} of 25 ± 5 μM.

As shown in Table 2, β-alanine and taurine were competitive inhibitors for low affinity site 1, and could also act as non-competitive inhibitors for the high affinity site 2. By contrast, nipecotic acid and L-2,4 diaminobutyric acid increased the K_m values of high affinity site 2 without affecting V_{max} values, which indicates that these two compounds are competitive inhibitors for the high affinity component of GABA uptake.

Subcellular localization of GABA taken up into chromaffin cells in culture

GABA and its metabolites were examined after accumulation for 1 hr. Results showed that 92% and 96% of GABA stored in cells was authentic GABA when extracellular GABA concentrations were 200 and 10 μM, respectively. These percentages increased to 97% and 98% respectively when aminooxyacetic acid, an inhibitor of GABA transaminase, was present in the incubation medium. Therefore GABA metabolism in cultured chromaffin cells appears to be a slow process.

Subcellular fractionation studies performed on chromaffin cells in culture loaded for 60 min with [^{3}H]-GABA, showed that 93% of GABA taken up was not sedimentable but soluble in the 100,000 *g* cytosol of the cells. Only 7% of the total radioactivity was found in the crude chromaffin granule fraction. Fractionation of this P2 fraction on continuous 1.0–2.2 M sucrose gradient showed that the low radioactivity was not associated with granules, or with mitochondria (<1%) but was totally confined to upper fractions containing cytosol.

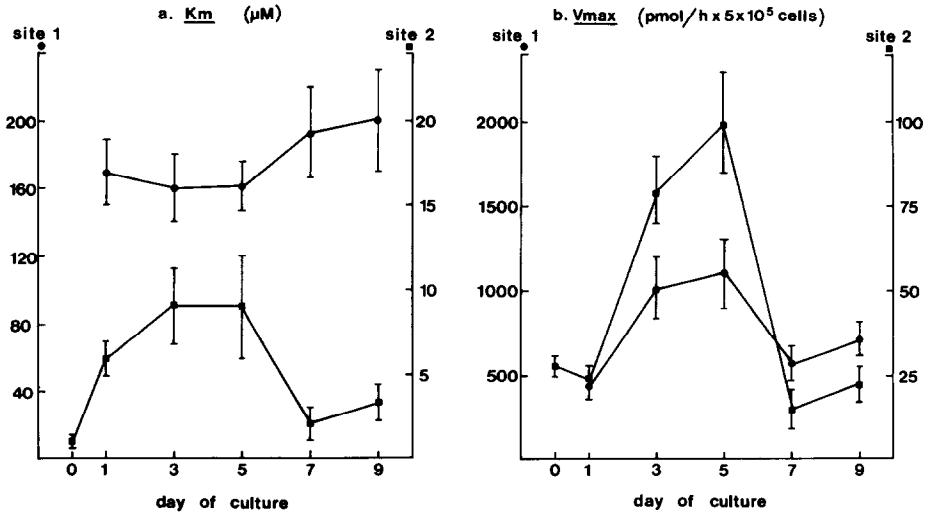

Fig. 4. Evolution of affinity and capacity of GABA transport by cultured chromaffin cells as a function of culture age. Kinetic constants were obtained at different times by Eadie–Hofstee analysis. In (a), K_m values and in (b) V_{max} values of GABA transport are plotted versus culture age of cells. Circles: kinetic values for site 1 and squares: for site 2. Each value is the mean (± SE) of kinetic constants calculated from results of two series of experiments corresponding to two different cultures.

Table 2. Kinetic constants of GABA uptake by chromaffin cells in culture in the presence of GABA uptake inhibitors

Compound	K_m (μm)	V_{max} (pmol/hr/5 × 10^5 cells)
Site 1		
Control	142 ± 34	639 ± 154
β-Alanine	373 ± 139**	590 ± 143
Taurine	266 ± 59**	620 ± 174
Nipecotic acid	166 ± 57	562 ± 196
L-DABA	158 ± 37	526 ± 180
Site 2		
Control	10.8 ± 3.2	86.9 ± 20
β-Alanine	6.5 ± 1.5	28.1 ± 4.7***
Taurine	7.7 ± 2.9	37.0 ± 16**
Nipecotic acid	23.8 ± 5.2***	91.0 ± 9
L-DABA	17.0 ± 2.0**	85.3 ± 6.2

Chromaffin cells in culture were incubated with different concentrations of [^{3}H]-GABA between 0.5 μM and 1 mM in the absence or presence of different compounds at a concentration of 10^{-4} M. The inhibitors were added at the beginning of incubation. Kinetic constants were calculated by Eadie–Hofstee analysis of data and are means (± SE) of four experiments, each performed in duplicate. (**) $P < 0.01$; (***) $P < 0.001$ (Student's t-test).

DISCUSSION

The aim of the present study was to characterize further the GABA uptake mechanism in chromaffin cells, a process which has previously been described on adrenal medullary slices [10] and on chromaffin cells in culture [12]. This further characterization was performed on chromaffin cells in culture since these preparations are a good model for the study of phenomena occurring at plasma membrane.

GABA uptake by chromaffin cells in culture proves to be highly dependent on external sodium concentration, an observation also reported by Kataoka *et al.* [12]. Characterization of sodium-dependent kinetics indicates a minimum of two cooperatively-interacting sodium sites in the GABA transport system. The value is similar to the sodium/GABA stochiometry reported for rat brain synaptosomes [26] and synaptosomal plasma membrane vesicles [27, 28]. Moreover the fact that the GABA transport system was affected by inhibitors of energetic pathways and of ATPases indicates that the GABA uptake in chromaffin cells in culture is an active transport process coupled to the sodium gradient. We did not examine Cl^- effect, but it seems likely that chloride is also required as the transporter of GABA in rat brain synaptosomes is coupled to both Na^+ and Cl^- [28].

With respect to kinetic studies, our results have shown the existence in bovine chromaffin cells in culture of a net active transport of GABA with two independent components: one of low affinity and the other of high affinity, each of which obey simple saturation kinetics. The two components seem to have different kinetic characteristics and chemical sensitivities. The low affinity system has apparent K_m values between 10^{-4} and 10^{-3} M and is competitively inhibited by taurine and β-alanine (typical inhibitors of GABA uptake by glial cells) [29, 30] and the high affinity system has apparent K_m values of 10^{-5} M and is competitively inhibited by nipecotic acid and L-2,4-diaminobutyric acid (the main known inhibitors of neural transport of GABA in brain tissue [31, 32]). These results seem to indicate the existence of two different transport systems for GABA, one with high affinity and pharmacological characteristics of neural GABA uptake (site 2) and the other, probably common for other β-amino-acids, with low affinity and chemical sensitivities similar to those found for the GABA uptake by glial cells in different neuronal tissues [33, 34] and other peripheral tissues [35–37]. From these results the question remains whether both uptake transport systems are confined to the chromaffin cell population or correspond to normal contaminants in these cultures, such as endothelial cells from blood vessels [38]. Experiments are now in progress in order to resolve this question.

Studies on the subcellular distribution of labelled GABA taken up by chromaffin cells in culture showed that most of GABA transported by chromaffin cells is stored in the extragranular cytoplasmic compartment of the cell. Adrenal chromaffin cells have been shown to be stained specifically with an antiserum directed against glutamic acid decarboxylase [12], the rate limiting biosynthetic enzyme for GABA, although no mapping at the ultrastructural level was performed. Recently, the subcellular distribution of glutamic acid decarboxylase was determined immunohistochemically in pancreatic islet at the ultrastructural level [39]. This enzyme was found in the insulin-containing cells, and immunoreactivity was confined predominantly in the extragranular cytoplasm, and was not detectable in the insulin-containing granules [39]. The localization of GABA in chromaffin cells and of its biosynthetic enzyme in B-cells as soluble components of the cytosol question the role of GABA in endocrine cells.

Kataoka *et al.* [12] have reported that GABA is released when chromaffin cells are stimulated. From our data, an exocytotic release of GABA from chro-

maffin granules is highly improbable. The significance of GABA release from acetylcholine-stimulated cells is unclear.

Chromaffin cells have been shown to possess GABA receptors on cell membrane surface [12] and it has been suggested that these receptors might modulate catecholamine secretion, as it has been found that catecholamine release from adrenal chromaffin cells [12, 14] and from dog adrenal glands [40] is modulated by GABA. Thus, the efficient GABA uptake system could function to terminate the GABA effect on chromaffin cells. It is thus possible that intracellular GABA may act as an intracellular effector on chromaffin cell activity, as it has been suggested for the activity of pancreatic B-cells [39].

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