

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF THE VESICULAR MONOAMINE TRANSPORTER FROM CHROMAFFIN GRANULES

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Summary

Prior to secretion, monoamines (catecholamines, serotonin, histamine) are concentrated from the cytoplasm into vesicles by vesicular monoamine transporters (VMAT). These transporters also carry non-physiological compounds, e.g. the neurotoxin methyl-4-phenylpyridinium. VMAT acts as an electrogenic antiporter (exchanger) of protons and monoamines, using a proton electrochemical gradient. Vesicular transport is inhibited by specific ligands, including tetrabenazine, ketanserin and reserpine. The mechanism of transport and the biochemistry of VMAT have been analyzed with the help of these tools, using mainly the chromaffin granules from bovine adrenal glands as a source of transporter.

Although biochemical studies did not suggest a multiplicity of VMATs, two homologous but distinct *VMAT* genes have recently been cloned from rat, bovine and human adrenal glands. The VMAT proteins are predicted to possess 12 transmembrane segments, with both extremities lying on the cytoplasmic side. They possess N-glycosylation sites in a putative luminal loop and phosphorylation sites in cytoplasmic domains.

In rat, *VMAT*₁ is expressed in the adrenal gland whereas *VMAT*₂ is expressed in the brain. In contrast, we found that the bovine adrenal gland expressed both *VMAT*₁ and *VMAT*₂. *VMAT*₂ corresponds to the major transporter of chromaffin granules, as shown by partial peptidic sequences of the purified protein and by a pharmacological analysis of the transport obtained in transfected COS cells (COS cells are monkey kidney cells possessing the ability to replicate SV-40-origin-containing plasmids). We discuss the possibility that *VMAT*₁ may be specifically addressed to large secretory granules vesicles, whereas *VMAT*₂ may also be addressed to small synaptic vesicles; species differences would then reflect the distinct physiological roles of the small synaptic vesicles in the adrenal gland.

Physiological function and characteristics of the transport reaction

Monoamines, such as the catecholamines (dopamine, noradrenaline or adrenaline) and the indolamine serotonin, are secreted by exocytosis from precharged vesicles. In the

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nervous system, these vesicles are the synaptic vesicles that are derived from the endosomal compartment, whereas in endocrine cells larger secretory granules, such as the chromaffin granules of adrenal medulla, are derived from the *trans* Golgi network. In both cases, the monoamines (or their precursors) are concentrated in the vesicles by an active transport system. The biophysical basis of the transport has been elucidated (Kanner and Schuldiner, 1987; Njus *et al.* 1987; Johnson, 1988). The driving force utilized by the vesicular monoamine transporter is the H⁺ electrochemical gradient generated by a vacuolar ATP-dependent H⁺ pump (V-ATPase) located on the same membrane. The vesicular monoamine transporter (VMAT) is sensitive to both components of the proton-motive force $\Delta\mu_{\text{H}}$, the pH gradient and the transmembrane potential. It is very efficient in chromaffin granules, where it generates a catecholamine gradient of five orders of magnitude. This transporter differs clearly from those operating at the level of the plasma membrane, which are driven by the Na⁺ gradient (Amara and Kuhar, 1993).

Competition experiments indicate that the same transporter usually carries all monoamines, although a recent observation reveals heterogeneity in the transport of histamine (Peter *et al.* 1994). In addition to physiological monoamines, this system also transports compounds such a *m*-iodobenzylguanidine (MIBG), a compound used for the scintigraphy of monoaminergic tumours (Gasnier *et al.* 1986), or methyl-4-phenylpyridinium (MPP⁺), a neurotoxin (Daniels and Reinhard, 1988; Darchen *et al.* 1988a). The K_{m} for ATP-dependent uptake of monoamines is in the micromolar range and it is inversely correlated to the pH (Scherman and Henry, 1981, 1983).

Pharmacology of the vesicular monoamine transporter

Various drugs inhibit monoamine uptake by chromaffin granules by interacting directly with the transporter (Henry and Scherman, 1989). The binding of these drugs to purified chromaffin granule membranes has been characterized. Tetrabenazine (TBZ), which can be considered to be an analogue of dopamine, and its derivative dihydrotetrabenazine have an affinity in the nanomolar range; tritiated dihydrotetrabenazine ([³H]TBZOH) is an excellent ligand of VMAT (Scherman *et al.* 1983). Ketanserin, which was originally developed as an antagonist of serotonergic receptors, binds to the same site with an affinity in the same concentration range at 0 °C (Darchen *et al.* 1988b). Binding to this site is unaffected by the H⁺ electrochemical gradient. In contrast, the binding of reserpine (RES), a natural indolic compound, is greatly accelerated when the ATP-dependent H⁺ pump is energized (Weaver and Deupree, 1982; Scherman and Henry, 1984). Moreover, displacement experiments show that TBZ and RES bind to distinct sites. The RES equilibrium dissociation constant was estimated to 30 pmol l⁻¹ (Darchen *et al.* 1989).

Molecular biology of the vesicular monoamine transporter

Liu *et al.* (1992) cloned by expression the vesicular monoamine transporter from rat pheochromocytoma PC12 cells as a resistance gene to the neurotoxin MPP⁺.

Surprisingly, this gene, which is now called *VMAT₁*, was not expressed in rat brain. Simultaneously, Erickson *et al.* (1992) isolated a different clone from rat brain, *VMAT₂*, also encoding a monoamine vesicular transporter, but which was not present in rat adrenal medulla. A human *VMAT₂* clone was subsequently identified (Surratt *et al.* 1993; Erickson and Eiden, 1993).

Since most pharmacological and biochemical studies of the transporter have been performed on bovine adrenal medulla, we applied a strategy of homology cloning to a cDNA library from bovine chromaffin cells using oligonucleotide probes common to the two rat sequences. We isolated two different clones, which were related to rat *VMAT₁* and *VMAT₂*, and were named accordingly (Krejci *et al.* 1993; D. Botton, E. Krejci, J. Massoulié, J.-P. Henry and B. Gasnier, unpublished results). The degrees of amino acid identity between the deduced rat and bovine sequences are 78 % for *VMAT₁* and 88 % for *VMAT₂*, whereas the two bovine sequences are only 61 % identical.

Our study suggests that the two genes are expressed in the bovine adrenal medulla, although only *VMAT₁* is expressed in the rat adrenal medulla (Liu *et al.* 1992). This difference is supported by *in situ* hybridization experiments. Probes derived from *bVMAT₁* and *bVMAT₂* gave positive signals in bovine adrenal medulla, whereas only *bVMAT₁* gave positive signals in rat adrenal glands (Fig. 1). In contrast, only *bVMAT₂*-derived probes gave positive signals in rat brain (not shown), a result consistent with previous observations (Erickson *et al.* 1992; Liu *et al.* 1992). Analysis of the distribution

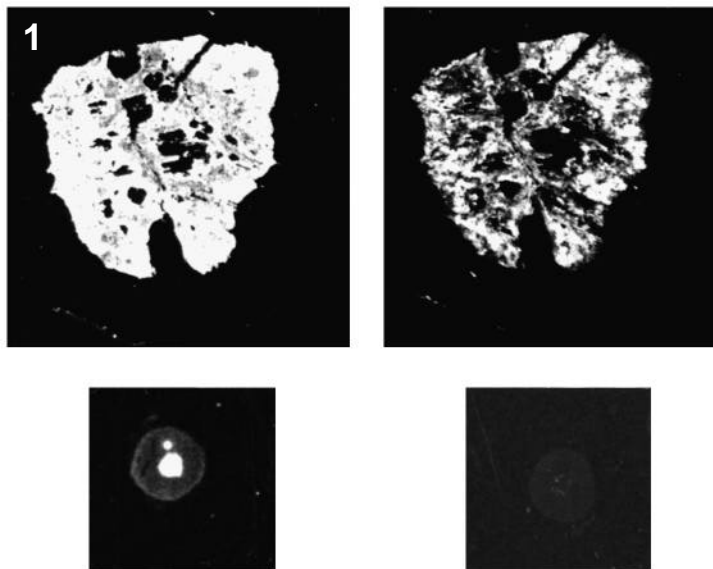


Fig. 1. *In situ* hybridization of *VMAT₁* and *VMAT₂* mRNA in bovine and rat adrenal glands. Adjacent bovine adrenal sections (top) were hybridized with riboprobes derived from *bVMAT₁* (left) or *bVMAT₂* (right). As a control, the same probes were hybridized with rat adrenal section (bottom). The specific radioactivity of the two probes was not identical, so that the levels of the *VMAT₁* and *VMAT₂* signals in bovine adrenal glands are not directly comparable.

of the two isoforms in bovine adrenal medulla at the cellular and subcellular levels might help us to understand the biological significance of the dimorphism of VMAT.

With respect to the bovine clones, only *VMAT*₂ has been expressed at the present time (Gasnier *et al.* 1994; Howell *et al.* 1994). We observed an ATP-dependent noradrenaline uptake in membranes derived from *bVMAT*₂-transfected COS cells. The kinetics of [³H]noradrenaline uptake, the binding properties of [³H]TBZOH and the corresponding pharmacological profiles were similar to those previously reported for bovine chromaffin granules. However, the ratio V_{\max} (for noradrenaline uptake)/ B_{\max} (for TBZOH binding) in transfected cells was 5% of that observed in isolated chromaffin granules (Gasnier *et al.* 1987). This observation may be related to the fact that noradrenaline uptake requires the expression of the transporter in closed structures equipped with an H⁺ V-ATPase, whereas [³H]TBZOH binding does not (Henry and Scherman, 1989). Therefore, the low V_{\max}/B_{\max} ratio suggests that a large fraction of the transporter molecules was not expressed in an adequate compartment of the COS cells.

Recently, a clue to the biological significance of the dimorphism of VMAT was provided by the observation that the two rat proteins, analyzed in the same expression system, exhibit different affinities for the substrates: the K_m of rVMAT₂ for histamine is 100-fold lower than that of rVMAT₁. The two transporters also differ in their sensitivity to pharmacological agents: rVMAT₁ was shown to be TBZ-resistant, although ketanserin-sensitive, and was less sensitive to methamphetamine than was rVMAT₂ (Peter *et al.* 1994).

Purification of the protein from bovine chromaffin granule

The only vesicular monoamine transporter that has been purified to date is that of bovine chromaffin granules. This VMAT has been purified both in a native form, which could be functionally reinserted into liposomes (Stern-Bach *et al.* 1990; Vincent and Near, 1991), and in a detergent-denatured form (Isambert *et al.* 1992). Such progress was made possible for two reasons. First, bovine chromaffin granule membranes can be obtained in large amounts (for instance, 100 mg of protein) and contain 0.3% mg of transporter per milligram of membrane protein. Second, the existence of specific ligands made the assay of the solubilized protein easy.

For structural studies, we have purified the radiolabelled transporter, following photoaffinity-labelling of membranes with the probe 7-azido-8-iodo-azidoketanserin (Isambert *et al.* 1989). The purification used three chromatography steps: (i) anion exchange on DEAE-cellulose at acidic pH, (ii) affinity chromatography on WGA-Sepharose and (iii) chromatography on hydroxylapatite in SDS (Isambert *et al.* 1992).

Recently, a new procedure was devised, based on the observation that VMAT was aggregated by heating solubilized chromaffin granule membrane proteins and that this behaviour was shared by a limited number of proteins. The aggregates were isolated by molecular sieving, freeze-dried and then dissociated by solubilization in anhydrous trifluoroacetic acid. After evaporation of the solvent and solubilization in aqueous solutions, the transporter was easily separated from the other proteins by molecular

sieving. This aggregation property probably reflects the extremely hydrophobic nature of the transporter; it may be possible to extend this approach to other porters (C. Sagné, M.-F. Isambert, J.-P. Henry and B. Gasnier, unpublished results).

Since two genes that potentially encode vesicular monoamine transporters are expressed in chromaffin cells, it was important to determine the relationship between these genes and the purified protein. Amino acid sequence analysis of the material isolated by our techniques showed a unique NH₂ terminus extending over 15 residues and identical to that of bVMAT₂ (Krejci *et al.* 1993 and unpublished data). The same result had been reported by the laboratory of S. Schuldiner for the active reconstituted transporter (Stern-Bach *et al.* 1992). However, in this case, a minor component with a different isoelectric point had been observed (Stern-Bach *et al.* 1990). The purification and the N-terminal sequence of this component and its relationship to VMAT₁ may prove to be interesting.

Quaternary and secondary structure of the vesicular monoamine transporter

Expression of cDNA has indicated that most of the monoamine porters, including VMAT, are active as monomers or homo-oligomers. Therefore, the previous hypothesis of a hetero-oligomeric structure, with two subunits of different molecular mass bearing the RES or the TBZ binding site (Henry *et al.* 1987), is not consistent with recent progress in the molecular biology of VMAT. Reconstitution experiments from Schuldiner's group also indicate that full activity is recovered with only one type of subunit, with an apparent molecular mass of 70 kDa (Stern-Bach *et al.* 1990). The hypothesis of a homo-oligomeric structure for the transporter might be investigated by co-expression experiments with both VMAT₁ and VMAT₂, or with homologous isoforms from two different species: we might expect that the resulting hetero-oligomers would differ in their pharmacological or kinetic characteristics from the corresponding homo-oligomers.

Amphipathic plots have been used to predict the organization of the peptidic backbone of membrane proteins in hydrophobic helices crossing the phospholipid bilayer. In the case of the VMAT, 12 putative transmembrane helices have been proposed by Liu *et al.* (1993) using hydrophobic moment analysis. The topological model contains a large hydrophilic loop between transmembrane segments I and II. Putative N-glycosylation sites are found in this loop, suggesting its orientation on the vesicular side of the membrane. Consequently, according to this model, the N and C termini are both located in the cytosol. Although such topological models might be challenged by some experimental data (e.g. Fischbarg *et al.* 1993), this scheme can be considered as a useful working hypothesis. Some features of the model are supported by comparing the different VMAT amino acid sequences: the loop between transmembrane helices I and II, and the N and C termini, coincide with regions of higher divergence (Liu *et al.* 1993). A similar organization, with a shorter loop between the putative transmembrane domains I and II, has been proposed for the *Caenorhabditis elegans* UNC-17 (Alfonso *et al.* 1993) and for the vesicular acetylcholine transporter from *Torpedo californica* (Varoqui *et al.* 1994).

Direct experimental evidence supporting this topology is still lacking. In our laboratory, antibodies have been raised against the cytoplasmic loop and the N terminus

of bVMAT₂, which should be useful tools for exploring its topological organization. It is noteworthy that, for bVMAT₂ (Krejci *et al.* 1993), three potential protein kinase C phosphorylation sites are predicted to exist on the cytosolic side of the membrane on the N terminus (S18) and on the loops connecting transmembrane segments II and III (T156) and transmembrane segments VI and VII (S282). Thus, phosphorylation of intact granules and analysis of the phosphorylated peptides might offer another approach to the study of the topology of the protein.

The transport mechanism

A large number of studies have been devoted to the transport mechanism (Kanner and Schuldiner, 1987; Njus *et al.* 1987; Johnson, 1988). The transporter catalyzes an electrogenic monoamine/H⁺ antiport. Because of the pH-dependence of the transport and of the effect of inhibitors, we initially proposed an R-NH₂/H⁺ exchange (Scherman and Henry, 1981, 1983). However, analysis of the uptake of the quaternary ammonium MPP⁺ convinced us that an R-NH₃⁺/2H⁺ exchange was more likely (Darchen *et al.* 1988a).

The ligands RES and TBZ proved particularly useful for mechanistic studies. On the one hand, the two ligands bound to different sites, with different characteristics (affinity for the substrates, sensitivity to $\Delta\mu_{\text{H}}$) (Scherman and Henry, 1984) and, on the other hand, conditions were found under which the two drugs competed (Darchen *et al.* 1989). To solve this paradox, we propose the existence of two conformations of the protein, each possessing only one site, binding either TBZ or RES. Acceleration of RES binding in the presence of the H⁺ electrochemical gradient may be attributed to an effect on the transconformational equilibrium, favouring the RES binding conformation (Henry *et al.* 1987; Darchen *et al.* 1989). This transconformational equilibrium suggests a model for monoamine translocation (Fig. 2). In the presence of a $\Delta\mu_{\text{H}}$, the protein would be in the RES binding conformation, with a site of high affinity for the substrates directed towards the cytoplasm. After substrate binding to this site, a proton from the matrix would induce a shift to the TBZ binding conformation, with the substrate now being bound to a low-affinity site directed towards the granule matrix. Dissociation of the substrate would allow the system to return to its original state.

Besides mutagenesis, labelling reagents might help to correlate mechanistic studies with the structure of VMAT. The TBZ binding site is covalently labelled by azido derivatives of TBZ (Isambert and Henry, 1985) or ketanserin (Isambert *et al.* 1989). This site is hydrophobic and it is thus likely to be in a transmembrane segment (Scherman *et al.* 1988). The identification of this site has been undertaken by a combination of biochemical and mutational approaches. The monoamine transporter is also inhibited by dicyclohexylcarbodiimide (DCCD), a hydrophobic substance that reacts with carboxylic acids and is known to inhibit various H⁺-translocating enzymes (Gasnier *et al.* 1985). Covalent labelling of the bovine transporter using [¹⁴C]DCCD has been demonstrated by Suchi *et al.* (1991). The involvement of the labelled residue in the transport reaction is suggested by the observation that it is protected from DCCD by TBZ (Gasnier *et al.* 1985). Five acidic residues (D33, D265, D402, D429 and D463 in the bVMAT₂ sequence; see Krejci *et al.* 1993), located in putative transmembrane segments and

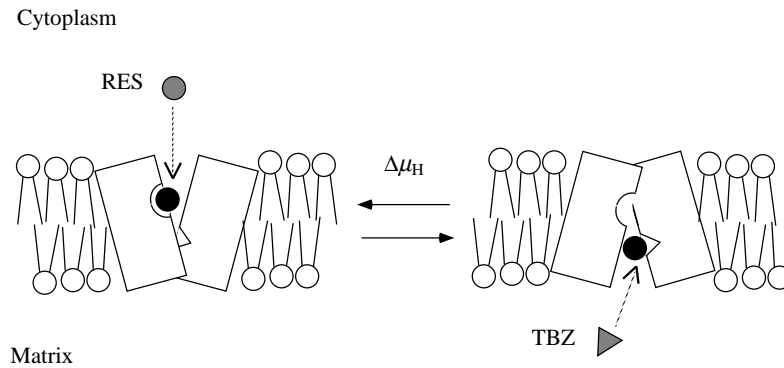


Fig. 2. A model for monoamine translocation. The $\Delta\mu_{\text{H}}$ -dependence of RES binding (Weaver and Deupree, 1982; Scherman and Henry, 1984) and a kinetic analysis of the competition between RES and TBZ binding (Darchen *et al.* 1989) suggest the existence of two conformations of VMAT, binding either RES or TBZ. The model postulates that the inhibitor binding sites also bind monoamines (filled circles) during their translocation. Because monoamines compete with TBZ at much higher concentration than with RES, the sites for RES and TBZ are proposed to bind monoamines from the cytoplasmic and luminal compartments, respectively. A conformational change, which might consist of relative movement of the transmembrane segments, symbolized here by the two boxes, transfers monoamines from the RES to the TBZ binding site. This model emphasizes the monoamine translocation pathway. The proton pathway and its coupling with monoamine translocation are not considered here.

conserved between bovine, human and rat VMAT₂, are likely candidates for the action of DCCD. Transport is also blocked by diethylpyrocarbonate, a substance that reacts with histidine residues (Isambert and Henry, 1981; Suchi *et al.* 1992).

Subcellular localization of the transporter

Neurones possess two classes of secretory vesicles: the small synaptic vesicles, responsible for the storage and secretion of classic neurotransmitters, and the large dense-cored vesicles, mediating the storage and secretion of neuropeptides (Kelly, 1993). Catecholamines can be stored in both classes of vesicles, since sympathetic neurones accumulate noradrenaline in large dense-cored vesicles. Endocrine cells also possess two classes of secretory organelles, the secretory granules and synaptic-like microvesicles (Navone *et al.* 1986), which may be regarded as the counterparts of neuronal large dense-cored and synaptic vesicles, respectively. The two classes of secretory organelles differ in their biogenesis: as shown by studies performed mainly on neuroendocrine cell lines, secretory granules bud off directly from the *trans* Golgi network, whereas synaptic vesicles derive from the endosome and acquire specific proteins *via* constitutive secretion and endocytosis from the plasma membrane (Régnier-Vigouroux *et al.* 1991).

The differential expression of VMAT₁ and VMAT₂ in rat adrenal medulla and brain, respectively, led Liu *et al.* (1992) to suggest that each isoform is specific for a class of

secretory organelle: according to the previous names of the clones, rVMAT₁ and rVMAT₂ would be localized on secretory granule and on synaptic vesicles, respectively. However, this is clearly not the case in bovine cells, where the N-terminal sequence obtained for the transporter purified from chromaffin granules is related to bVMAT₂ instead of bVMAT₁ (Krejci *et al.* 1993; Stern-Bach *et al.* 1992). This latter observation implies either that the 'one VMAT isoform, one secretory organelle' hypothesis is invalid or that the targeting mechanisms to these organelles are not conserved across mammalian species. Information on the sorting of proteins between the two classes of secretory organelles can be obtained from studies on neuroendocrine cells, where both classes of secretory organelles co-exist (Navone *et al.* 1986). According to such studies, the synaptic-like microvesicles store acetylcholine, but not monoamines, in rat PC12 cells (Bauerfeind *et al.* 1993), whereas these vesicles would be catecholaminergic in bovine chromaffin cells (Annaert *et al.* 1993).

To account for the above results, we propose that VMAT₁ is specifically sorted to secretory granules, whereas VMAT₂ is addressed to both types of secretory organelles, as are other membrane proteins common to the two classes, such as synaptotagmin, SV2 or the proton pump (Fig. 3). This hypothesis predicts that the expression of VMAT₁ is restricted to endocrine cells or to sympathetic noradrenergic neurones. In endocrine cells, the expression of either VMAT₁ or VMAT₂ will determine the nature of the neurotransmitter (monoamine or not) stored in synaptic-like microvesicles. Therefore, the model depicted in Fig. 3 suggests another clue to the biological significance of VMAT dimorphism: the expression of different VMATs in endocrine cells (here, rat and bovine chromaffin cells) would be related to the physiological role of secretion *via* the synaptic-like microvesicles, i.e. probably with the paracrine communication between endocrine cells (Reetz *et al.* 1991).

Regulation of the expression of the vesicular monoamine transporter

Stimulation of neuronal or endocrine cells induces the synthesis of enzymes involved in the biogenesis of the secreted mediator, a phenomenon known as 'trans-synaptic induction' or 'stimulation–secretion–synthesis coupling' (Thoenen, 1974). This regulation has been demonstrated for tyrosine hydroxylase, the first enzyme in the synthesis of catecholamines, both *in vivo* and *in vitro* in cultured bovine chromaffin cells stimulated by nicotinic agonists or K⁺ (Stachowiak *et al.* 1990). More recently, the synthesis of peptides of the chromaffin granule matrix, such as enkephalins, has also been shown to be regulated in the same way (Kanamatsu *et al.* 1986).

Long-term depolarization by 55 mmol l⁻¹ KCl of chromaffin cells in culture induces a clear increase in the number of cellular [³H]TBZOH binding sites (Desnos *et al.* 1992). Significant after 2 days in the presence of KCl, this increase corresponds to a doubling of the number of [³H]TBZOH binding sites after 5 days. This does not result from a nonspecific increase of protein biosynthesis. It is mediated by the Ca²⁺ influx resulting from the depolarization, since it is inhibited by the calcium channel blockers nifedipine and fluspirilene. It is likely to involve second messengers, since it can be mimicked by phorbol esters and by forskolin.

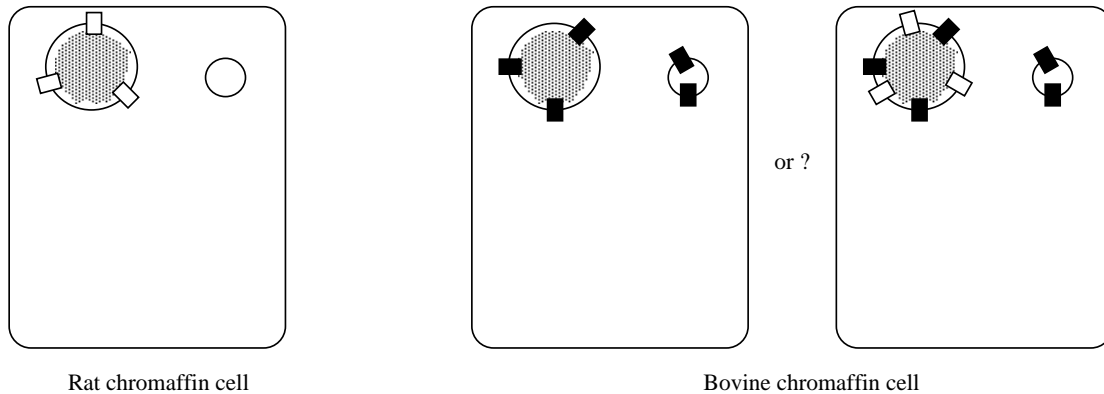


Fig. 3. A model for the subcellular localization of VMAT₁ and VMAT₂. Rat (left) and bovine (right) chromaffin cells are schematically represented with their secretory granule and synaptic-like microvesicle compartments. The model proposes that VMAT₁ (□) is unique to secretory granules, whereas VMAT₂ (■) is shared between the two classes of secretory organelles. Rat chromaffin cells express only VMAT₁ (Liu *et al.* 1992) and do not accumulate monoamines in their synaptic-like vesicles (Bauerfeind *et al.* 1993). The localization of VMAT₂ in secretory granules is supported by N-terminal sequencing of the protein purified from isolated bovine chromaffin granules (Stern-Bach *et al.* 1992; Krejci *et al.* 1993) and suggested by the expression of VMAT₂ in rat basophilic leukaemia cells (Erickson *et al.* 1992), which store histamine in secretory granules. The localization of VMAT₂ in small clear vesicles is suggested by its expression in rat and bovine brain (Liu *et al.* 1992; Erickson *et al.* 1992; Krejci *et al.* 1993) and by the accumulation of monoamines in bovine chromaffin synaptic-like microvesicles (Annaert *et al.* 1993). Both VMAT₁ and VMAT₂ are expressed in the bovine adrenal medulla, but their cellular distribution is not known. On the right, two possibilities are considered: in bovine chromaffin cells, VMAT₂ is expressed either in the same cells as VMAT₁ or in distinct cells.

The increase in [³H]TBZOH binding was blocked in the presence of cycloheximide, an inhibitor of protein synthesis, and of actinomycin D, an inhibitor of RNA polymerase, suggesting an increased biosynthesis originating from transcriptional activation. This hypothesis was directly tested by measuring the levels of bVMAT₂ mRNA in K⁺-depolarized chromaffin cells in culture (Krejci *et al.* 1993). A clear increase was observed in Northern blots and was quantified using the competitive polymerase chain reaction technique. The VMAT₂ mRNA level was increased about threefold after a 4 h K⁺-induced depolarization.

After prolonged stimulation, the number of chromaffin granules per cell was decreased to about 30% of the control value (C. Desnos, M. P. Laran, K. Langley, D. Aunis and J.-P. Henry, unpublished results). This effect might result either from continuous secretion or from a change in granule biogenesis. However, cells could still secrete upon further stimulation. Subcellular fractionation experiments indicated that the large majority of [³H]TBZOH binding sites were present on chromaffin granules, a result consistent with the observation that the increase in the number of [³H]TBZOH binding sites was associated with an increase in vesicular monoamine uptake.

Since the cellular content of [³H]TBZOH binding sites was increased and the number of chromaffin granules per cell was decreased, secretion clearly induced a change in the composition of the secretory granule membrane. We calculated a fivefold increase in the relative transporter content of the membrane, whereas no such change was observed for cytochrome *b*₅₆₁, a major component of this membrane. Since stimulation induced both an increase in the synthesis of tyrosine hydroxylase, and thus of cytoplasmic catecholamines, and a decrease in the number of chromaffin granules, the increase in the amount of monoamine transporter per granule might be physiologically relevant, allowing a more rapid filling of the secretory granules by the transporter operating at its maximal rate.

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