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Cloning and functional expression of a tetrabenazine sensitive vesicular monoamine transporter from bovine chromaffin granules

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Abstract

Using oligonucleotide primers derived from the vesicular monoamine transporters sequences, a cDNA predicted to encode the bovine chromaffin granule amine transporter has been cloned (b-VMAT2). Surprisingly, its structure is more similar to the rat brain transporter (VMAT2), than to the rat adrenal counterpart (VMAT1). Unlike rat VMAT1, bovine VMAT2 appears to be expressed both in the adrenal medulla and the brain, as judged by Northern analysis. After modification/deletion of the seven amino acids at the N-terminus of the protein it was expressed in a functional form. The order of affinity of the bovine VMAT2 transporter to substrates is: serotonin>dopamine = norepinephrine>epinephrine. Also, the recombinant bovine adrenal transporter is highly sensitive to tetrabenazine, in sharp contrast to the rat adrenal transporter. The findings indicate, therefore, a clear species variation in which structure and function of the bovine adrenal transporter resemble the rat brain protein, while its tissue distribution is distinct from both types of rat proteins. In addition, the predicted protein sequence is identical to the experimentally determined N-terminus sequence of the purified vesicular amine transporter [Stern-Bach et al. (1992) Proc. Natl. Acad. Sci. USA 89, 9730–9733].

Key words: Vesicular monoamine transporter; Bovine adrenal medulla; Chromaffin granule; Catecholamine; Serotonin; Tetrabenazine; cDNA cloning

1. Introduction

Classical neurotransmitters are stored in synaptic vesicles and storage organelles of synaptic terminals and secretory cells. Transport and storage of the monoamines serotonin, dopamine, norepinephrine, epinephrine, and histamine in a variety of cells are catalyzed by vesicular monoamine transporters (VMATs). In bovine adrenal chromaffin granules vesicular transport has been found to depend on a proton electrochemical gradient generated by the vesicular H^+-ATPase and to involve the exchange of two lumenal protons with one cytoplasmic amine [1–4]. Transport is inhibited by a wide array of compounds, best represented by reserpine and tetrabenazine. The two inhibitors interact with the transporter in distinct ways. Reserpine binds at the site of amine recognition and its binding is accelerated by the imposition of a proton electrochemical gradient [5,6]. In contrast, tetrabenazine binds at a different site since its binding to the transporter is inhibited only by high substrate concentrations [6]. Since transporters from different tissues accumulate the various amines with similar affinities and they display practically identical pharmacology, it has been proposed that either identical or closely resembling proteins catalyze the transport in all the tissues.

Using the approach of cloning by functional expression, the cDNA sequences for two distinct rat amine transporters have been obtained [7,8]. VMAT2 (previously termed SVAT for synaptic vesicle amine transporter and VMAT1) is found predominantly in brain, while VMAT1 (previously termed CGAT for chromaffin granule amine transporter) is found predominantly in the adrenal chromaffin granule. Tetrabenazine has been shown to be roughly 30-fold more effective at inhibiting the bovine adrenal transporter relative to that of the recombinant VMAT1, suggesting an important species difference which should be elucidated.

We have used oligonucleotide primers derived from the VMAT sequences to clone a cDNA predicted to encode the bovine chromaffin granule amine transporter. We find, surprisingly, that its structure is more similar to the rat brain transporter (VMAT2), than to the

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Abbreviations: VMAT2, vesicular monoamine transporter, type 2 (previously known as SVAT and MAT1); VMAT1, vesicular monoamine transporter, type 1 (previously known as CGAT); CCCP, carbonylcyanide-*p*-trichloromethoxyphenylhydrazone.

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rat adrenal counterpart (VMAT1). However, its distribution does not resemble either type. Bovine VMAT2 appears to be expressed both in the adrenal medulla and the brain, as demonstrated by Northern analysis. The affinity of the expressed bovine VMAT2 transporter to serotonin, dopamine and epinephrine is similar to those determined in membrane vesicles from bovine adrenal chromaffin granules [9–11] and in proteoliposomes reconstituted with the purified transporter [12]. Also, the recombinant bovine adrenal transporter is highly sensitive to tetrabenazine. In addition, the predicted protein sequence is identical to the experimentally determined N-terminus sequence of the purified vesicular amine transporter [12,13]. Furthermore, modification/deletion of the seven amino acids at the N-terminus of the protein does not impair transport activity.

2. Materials and methods

2.1. Materials

Taq polymerase was purchased from Fisher-Promega and all other DNA modification and restriction enzymes were from New England Biolabs, Gibco-BRL, or Boehringer-Mannheim. Sequenase sequencing kits were purchased from US Biochemical Corp. Blotting media were from either Schleicher & Schuell (nitrocellulose), MSI (Nytran), or DuPont (GeneScreen Plus). All radiochemicals were from NEN. Oligonucleotide synthesis reagents were from ABN. All other reagents were reagent grade or better.

2.2. cDNA cloning, sequencing, and sequence analysis

10 ng of DNA from a bovine adrenal medulla cDNA library (1.5–4.5 kb cDNA inserts) in LambdaZAP [14] was used as template in PCR reactions with primers based on comparisons with the existing rat VMATs cDNA sequences [7,8]; one sense-oriented primer AT61S (5′-TGCTGTACGAGTTTGTGG-3′, starting at nucleotide position number 998) of VMAT2 was used with antisense-oriented primer AT288A (5′-ACATGGTCTCCATCATCC-3′, starting at nucleotide number 1256) of VMAT2. The final PCR amplification product produced a predominant 249 bp fragment. This fragment was sub-cloned into the *Eco*RV site of the Bluescript SK(−) vector and the DNA sequence from several clones was determined. A promising clone whose sequence showed significant identity with the VMAT2 and VMAT1 sequences was then radio-labeled by PCR amplification [15] and used to screen 2.5×10^5 plaques of the same library as above, using standard hybridization conditions [16]. Subsequent to cloning, super-infection with VCSM13 helper bacteriophage was used to excise the Bluescript SK(−) plasmid containing inserts. All DNA sequencing was performed by the dideoxy method as described on double-stranded plasmid DNA. Computer analysis was performed using DNANALYZE [17] and Clone and Align software from Scientific and Educational Software. The bovine amine transporter DNA sequence has been submitted to GenBank and has been given Accession No. U02876.

2.3. Construction of a functionally expressing clone

Two primers were synthesized, based on B11-1D clone, in order to excise the coding sequence of the bovine vesicular monoamine transporter. The sense primer, P77, starting at nucleotide #77 of the B11-1D clone, and contains an *Eco*RI site (5′-ATGGGAATTCTGCTCCGCCGGCTTCAG-3′). The antisense primer, RP1633, starting at nucleotide #1633 and contains a *Sna*BI site (5′-CGTACGTATTAGGTCATTGGAGGACT-3′). Following PCR reaction, the 1550 by-product was purified from the gel by QIAEX kit (Qiagen, Germany), cut by *Eco*RI and *Sna*BI and purified again as above. This fragment was cloned into a pTM1 vector [18] (a generous gift from Orna Elroy-Stein) which was prepared for ligation by *Eco*RI and *Stu*I restriction endonuclease cutting, and purified from agarose gel. Correct ligation yielded a 6.9 kbp vector in which translation of the bovine amine transporter starts at the ATG site of the vector and uses the first 3 amino acids of the vector, and then proceeds to amino acid #8 of the bovine transporter, thus truncating the original 7 amino acids from the amino terminal of the protein.

2.4. Northern blot analysis

Poly(A)-selected RNA was isolated from flash-frozen adrenal medullae, brain cortex, brainstem and liver as described [14]. RNA was denatured in 50.7% formamide and separated by electrophoresis through 1% agarose gels in 0.22 M formaldehyde. Capillary transfer to GeneScreen Plus membrane was performed for 12 h in a buffer containing 0.025 M $NaPO_4$, pH 6.5. The membrane was baked at 80°C for 2 h, pre-hybridized under standard conditions, and then probed by incubation in the same buffer containing double-stranded DNA probe at approximately 10^6 cpm/ml, 10^8 cpm/μg. The probe was labeled with [^{32}P]dATP by PCR amplification [15]. Hybridization was done at 42°C, in 50% formamide, 10% Dextran-sulfate, 1% SDS, 0.8 M NaCl.

Washing was for 30 min at 55°C in 2 × SSC, 1% SDS, once.

2.5. Growth of cells, transfections and transport assays

The protocol for functional expression of bovine VMAT2 was essentially as described by Erickson et al. [8]. CV1 cells grown in 24-well plates (2.5×10^5 cells per well) were infected with recombinant vaccinia virus encoding bacteriophage T7 DNA polymerase [19] for 30 min and then transfected with the plasmid (1.8 μg DNA) using 6.4 μg Transfection reagent per well (Dotap-Boehringer). After 18–20 h cells were rinsed with uptake buffer containing 110 mM potassium tartrate, 5 mM glucose, 0.2% BSA, 5 mM $MgCl_2$, 1 mM ascorbic acid, 10 μM pargyline and 20 mM PIPES at pH 7.4. The cells were permeabilized for 10 min at 37°C in uptake buffer containing 10 μM digitonin. The medium was removed and replaced with fresh buffer without digitonin containing 5 mM Mg-ATP and the corresponding tracers and/or inhibitors as indicated for the specific experiments.

3. Results and discussion

3.1. cDNA cloning

A 249 bp radiolabeled AT-related PCR product (see section 2) was used to probe approximately 2.5×10^5 plaques from the same cDNA library at high stringency. Thirty-two hybridizing clones were identified and excised from LambdaZAP, and both 5′- and 3′-end DNA sequences checked for each clone. Of these, 21 were found to belong to a family of cDNA's most closely related to the previously-identified VMAT2 sequence [7] at their 5′-ends and termed b-VMAT2 to denote the bovine amine transporter and the higher similarity to rat VMAT2 (SVAT). These clones were grouped into three different sets by virtue of the lengths of their 3′-ends. The clone with the longest 5′-end sequence (11-1D) was used to determine its complete DNA sequence. Of the remaining 20 clones, 19 were found to have identical 3′-ends which were longer than that of clone 11-1D, and the complete DNA sequence was determined for the longest clone from this set, 4-1A. The DNA sequence from this clone was found to be identical to that of 11–1D except for an 871 bp 3′-extension; its 3′-end was identical to that of 18 of the other b-VMAT clones. The last clone, 2-4A, identical in all other respects, was found to have an alternative poly-adenylation site at position 1932.

3.2. Predicted amino acid sequence

The b-VMAT predicted amino acid sequence is pre-

sented in Fig. 1. The 2726 bp cDNA is predicted to encode a protein of 517 amino acids with a pI of 5.34 and calculated molecular mass of 55,690. The sequence is predicted to begin at nucleotide number 56, for the N-terminal sequence previously determined for the amine transporter protein, LSELALLRRLQESRFSRXLILFIVFLXL [12,13]. Thus sequence agrees in all but the one underlined position of the b-VMAT2-predicted amino acid sequence (F→H). This position would also agree well with the predicted starts for both the VMAT1 and VMAT2 predicted proteins. An internal peptide sequence reported at the same time, DPQILIAKG, resembles the peptide sequence running between residues 294 and 302 of the b-VMAT protein sequence except for the underlined positions. Repeated sequencing attempts with multiple clones have confirmed the clonal DNA sequences, suggesting that the discrepancies are either due to peptide sequencing errors or the presence of another protein in the bovine adrenal medulla. When subjected to the Eisenberg moment plot analysis [20], the b-VMAT protein sequence was predicted to contain 12 trans-membrane segments, indicated by overlining in Fig. 2. These regions agree well with the predicted trans-membrane domains for the rat VMAT proteins. Overall identity of the bovine VMAT protein sequence was 87.8% and 62.9% with the rat VMAT2 and VMAT1 protein sequences, respectively. The region between trans-membrane domains I and II shows a much greater sequence divergence with the previously-cloned amine transporter predicted proteins than the rest of the protein (identities of 57.6% for VMAT2 and 30.4% for VMAT1). Because of its higher similarity to rat VMAT2, the bovine clone is defined also as VMAT2 type.

3.3. Tissue distribution

Northern analysis of tissue distribution in rat indicated that VMAT2 was present in brain but not in the adrenal gland, while VMAT1 was present in the adrenal but not in brain [7]. Northern blot analysis with the bovine VMAT2 cDNA indicated that the adrenal medulla contains a cognate mRNA population of about 4.5 kb in size (Fig. 3, lanes D). A related mRNA is detected also in the bovine brain stem and cortex, although in much lower amounts (Fig. 3, lanes B and C). The exposure needed to visualize the brain message is 50-fold higher than that for the adrenal. Under these conditions, a smaller size mRNA of 1700 bp is detected in brain cortex together with a bigger size mRNA (6500 bp) present in adrenal and brainstem. No cross-hybridizing material is detected in liver.

The results described above suggest that the major bovine adrenal transporter is highly similar to the transporter found in the rat central nervous system rather than to the rat adrenal transporter. However, its distri-

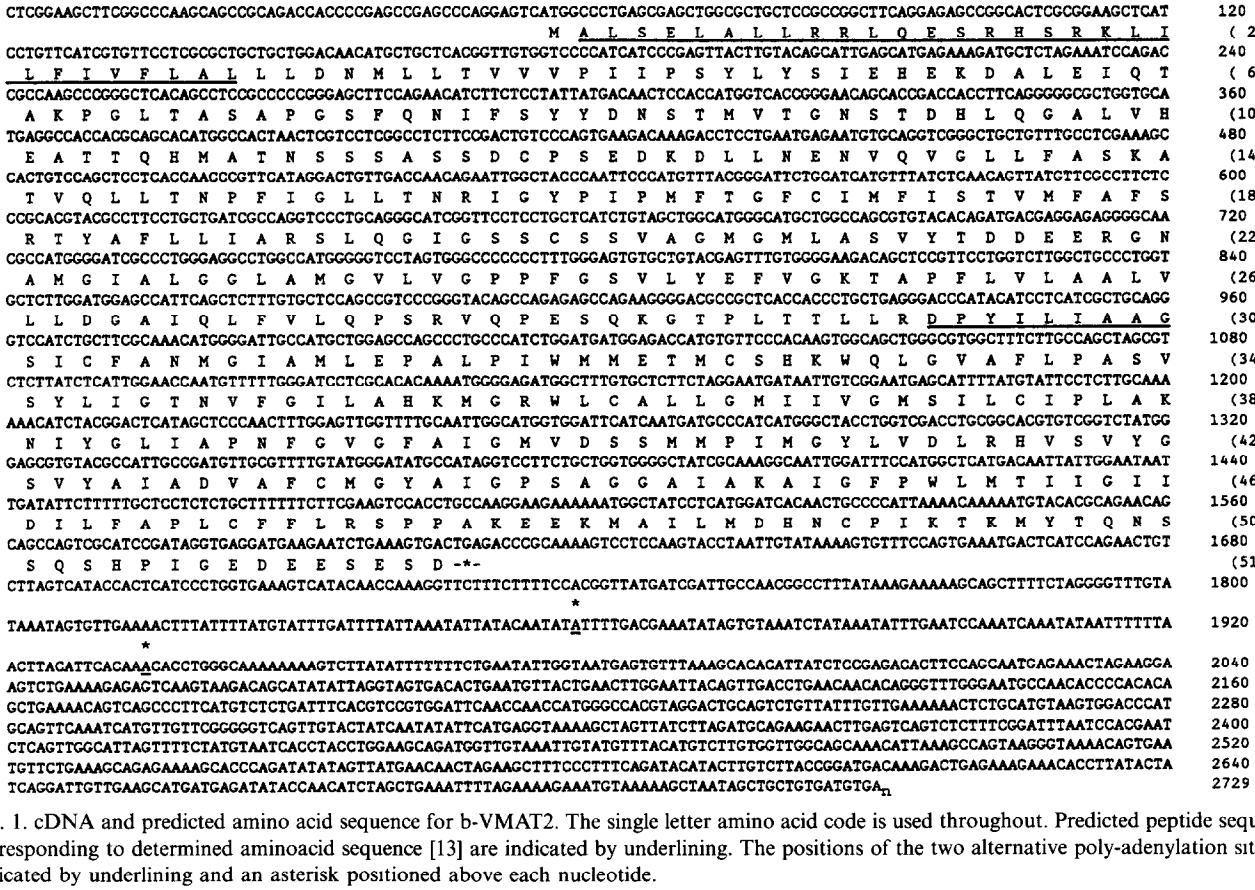

Fig. 1. cDNA and predicted amino acid sequence for b-VMAT2. The single letter amino acid code is used throughout. Predicted peptide sequences corresponding to determined aminoacid sequence [13] are indicated by underlining. The positions of the two alternative poly-adenylation sites are indicated by underlining and an asterisk positioned above each nucleotide.

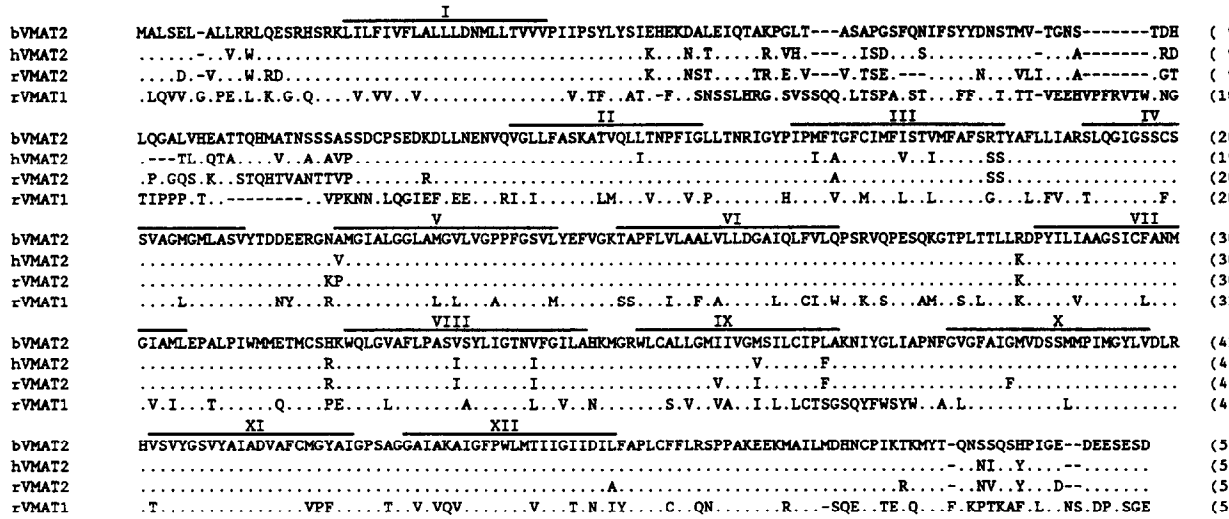

Fig. 2. Alignment of the bovine, rat, and human vesicular monoamine transporters predicted protein sequences. b-VMAT2 = bovine chromaffin cell amine transporter; rVMAT2 = rat 'synaptic vesicle' amine transporter [8]; rVMAT1 = rat 'chromaffin granule' amine transporter [7]; hVMAT2 = human 'synaptic vesicle' amine transporter [25,26]. Identities in the sequence are indicated by periods, differences by the indicated residue, and gaps by hyphens. Overlining indicates the positions of predicted transmembrane segments.

bution resembles neither of them. This is a surprising species difference and its basis will be studied in depth. The possibility that there are two types of transporters in the adrenal gland has been suggested from previous biochemical studies in which two isoforms with different pI values were identified [12]. We have performed repeated screens and failed to find alternative VMAT forms, but we cannot rule out at present the possibility that a VMAT1-type of transporter is present in the bovine adrenal medulla at very low frequency.

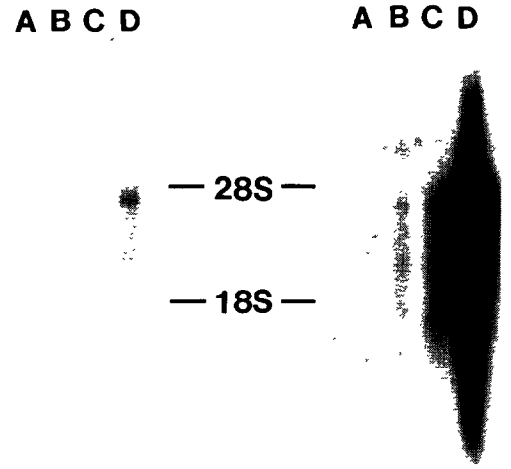

Fig. 3. Northern analysis. Poly(A)-selected RNA extracted from bovine adrenal medulla (A), brain cortex (B), brainstem (C) and liver (D) was separated and a Northern replica of the gel probed with radiolabeled cDNA.

3.4. *Transport catalyzed by bovine VMAT2 is tetrabenazine sensitive*

Transport of [^{3}H]dopamine into CV1 cells was measured following infection with vaccinia virus and transfection with the cDNA coding for VMAT2. Cells were permeabilized with digitonin as described by Erickson and collaborators [8]. Since the original clone B11-1D did not yield significant transport activity, several approaches were taken in order to yield a functional protein. The successful strategy entailed modification of 7 amino acids from the amino-terminal of the transporter: deletion of four amino acids together with replacement of the two amino acids adjacent to the ATG. The modified protein start at the sequence MGILL, as compared to the original cDNA clone B11-1D which predicts the sequence MALSDLVLL. The engineered cDNA (constructed by PCR as described in section 2), was inserted into pTMI vector [18], which was used to transfect CV-1 cells.

When cells were transfected with such a construct, [^{3}H]dopamine is accumulated in a time-dependent fashion and uptake is maximal at 5 min. Transport is practically completely inhibited in the presence of the proton ionophore CCCP as expected from the well known properties of VMAT (Fig. 3A). The findings suggest that, as previously reported for the rat clones expressed in CV1 cells and CHO fibroblasts, the bovine VMAT is also targetted to acid subcellular organelles and exchanges lumenal protons for cytoplasmic monoamine.

The vesicular monoamine transporters are known for their ability to catalyze accumulation of a variety of monoamines. We therefore tested the ability of transfected cells to transport [^{3}H]serotonin. Indeed trans-

fected cells accumulate [^{3}H]serotonin in a time-dependent, CCCP-sensitive fashion (Fig. 3B). Transport of both monoamines is inhibited by reserpine at 5μM (Fig. 3A and B). No accumulation of either monoamine is observed in untransfected cells or cells transfected with the vector alone (data not shown).

Measuring the initial rates of uptake (10 min) into the organelles we have determined [^{3}H]dopamine's K_m and tested the effect of different substrates and inhibitors on [^{3}H]dopamine uptake. Reserpine, tetrabenazine and ketanserin completely inhibit transport in a dose dependent manner with $K_{0.5}$ at 1 nM, 0.06 and 5 μM, respectively. The potency of the three inhibitors is in excellent agreement with previous studies using membranes from bovine adrenal chromaffin granules [12,21–23]. However, the sensitivity to tetrabenazine is in sharp contrast to that reported for the rat VMAT1, the chromaffin granule monoamine transporter. Rat VMAT1 shows a reduced sensitivity to the drug ($K_{0.5}$ = 4 μM).

Among the substrates, serotonin shows the highest affinity to bovine VMAT2 ($K_{0.5}$ = 1.2 μM); dopamine displays a lower affinity (K_m = 9 μM; data not shown) very similar to norepinephrine ($K_{0.5}$ = 7 μM) and higher than epinephrine ($K_{0.5}$ = 20 μM). The relative affinities of the four substrates is in very good agreement with those measured in studies using membrane vesicles from bovine adrenal chromaffin granules [9–11] and in proteoliposomes reconstituted with the purified transporter [12].

4. Conclusions

The studies presented in this communication report the cloning and sequencing of a bovine adrenal vesicular monoamine transporter (b-VMAT2) which after modifications at its amino terminal could be expressed in a functional form in CV-1 cells, and its transport properties were characterized. It still remains to be elucidated whether the non-functional form of the original isolated clone is due to a cloning artifact or the presence of sequences upstream of the translation initiation codon, which can negatively affect its expression in CHO and CV-1 cells. In terms of substrate preference, the affinity of the transporter was the highest for serotonin, very similar for dopamine and norepinephrine and the lowest for epinephrine. The affinity for serotonin is practically identical to that reported for the purified transporter [12] and about five times higher than those reported for membrane vesicles [9–11]. The affinities for dopamine and epinephrine are about two to three fold higher than those reported in membrane vesicles. These small differences may just reflect the fact that the protein is expressed in an heterologous cell and that transport is assayed quite differently. On the other hand, since the affinity for serotonin is identical to the one observed using proteoliposomes reconstituted with the purified protein but higher than that observed in membranes it could reflect the possible existence in the native adrenal membranes of two distinct transporters with different affinities.

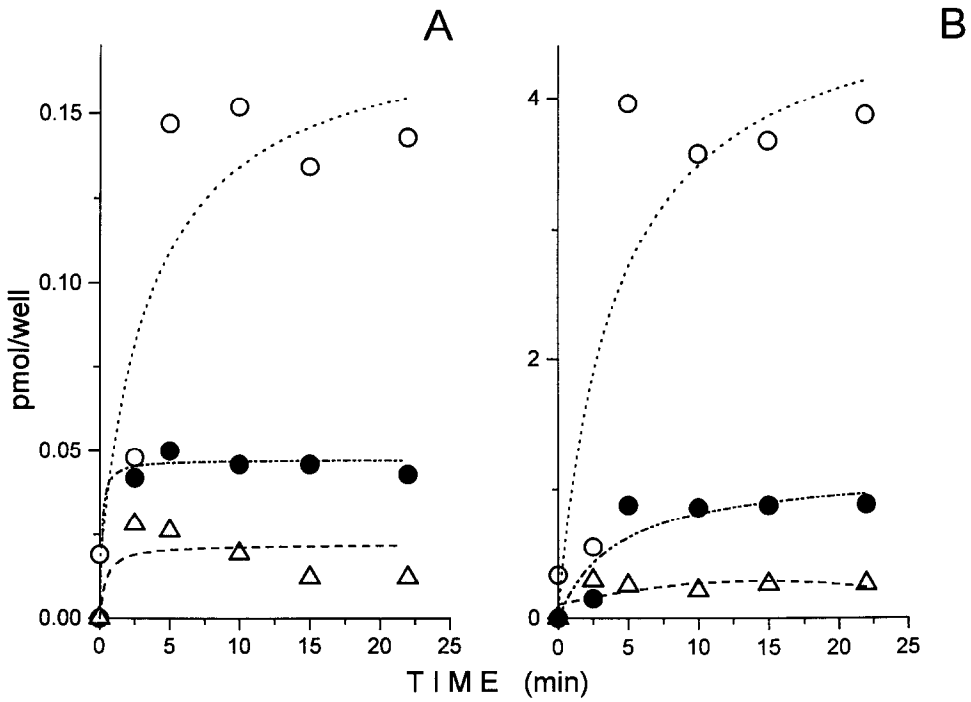

Fig. 4. [^{3}H]Dopamine and [^{3}H]serotonin uptake in permeabilized CV1 cells transfected with b-VMAT2. CV1 cells were treated as described in section 2. Transport was assayed after incubation with [^{3}H]dopamine (15 nM, 32.2 Ci/mmol) (panel A) or [^{3}H]serotonin (0.2 μM, 25.7 Ci/mmol) (panel B) for the indicated time periods. Experiments were performed in the presence of 10 μM CCCP (●), 5 μM reserpine (△) or with no further additions (○).

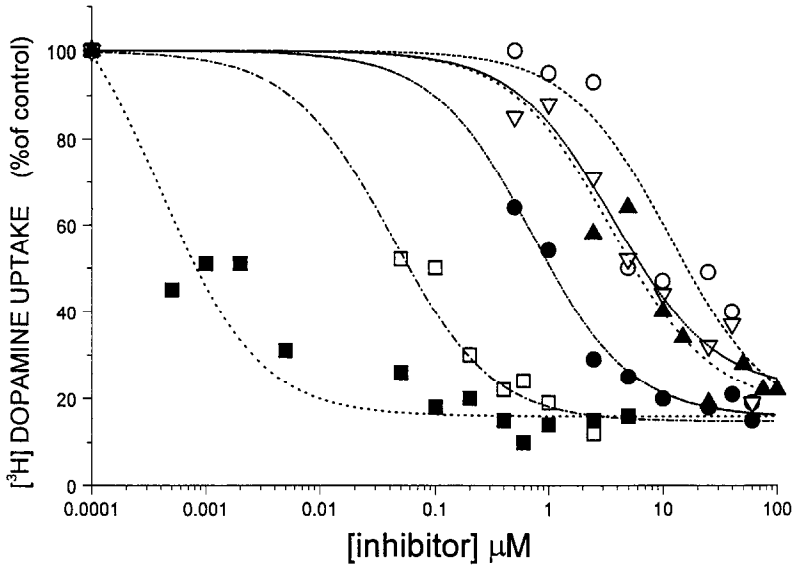

Fig. 5. Effect of inhibitors and transport susbtrates on [^{3}H]dopamine uptake by permeabilized CV1 cells transfected with b-VMAT2. CV1 cells were treated as described in section 2. Transport was assayed after incubation with [^{3}H]dopamine (15 nM, 32.2 Ci/mmol) for 10 min in the presence of the indicated concentrations of reserpine (■), tetrabenazine (□), ketanserine (▲), serotonin (●), norepinephrine (▽) and epinephrine (●). All the experiments were carried on in triplicates and repeated at least twice.

These findings would be in line with the fact that two proteins with distinct pI have been identified during purification of the bovine VMAT [12]. In any case, the one purified is most likely identical to the clone reported in this work since the predicted N-terminus from the cDNA is identical to the experimentally determined one [13]. The purified protein represents at least 60% of the total activity measured in membrane vesicles from bovine adrenal chromaffin granules [12].

Transport catalyzed by b-VMAT2 is sensitive to reserpine, ketanserin and tetrabenazine at concentrations practically identical to those reported in studies of membrane vesicles from bovine adrenal chromaffin granules. This is in contrast to the previous report for the rat adrenal vesicular monoamine transporter (r-VMAT1), which is about 30-fold less sensitive to tetrabenazine [7,24]. In addition, the transporter from bovine adrenal shows a higher similarity to the rat CNS type, VMAT2 (87.8%), rather than to the rat adrenal type, VMAT1 (62.9%). However, while message for rat VMAT2 is not found in the rat adrenal gland, message for bovine VMAT2 is found predominantly in the adrenal and to a lesser extent in the brain. The differences in distribution and in sensitivity to tetrabenazine reflect an important species variation. We do not know yet whether this is an intrinsic difference or reflect some response of the animals to treatment before slaughtering and tissue collection.

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Note added in proof

After this paper was accepted, a report with a practically identical sequence by Krejci et al., appeared in FEBS Letters 335 (1993) 27–32.