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Histidine-419 plays a role in energy coupling in the vesicular monoamine transporter from rat

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Abstract Vesicular monoamine transporters (VMAT) catalyze transport of serotonin, dopamine, epinephrine and norepinephrine into subcellular storage organelles in a variety of cells. Accumulation of the neurotransmitter depends on the proton electrochemical gradient across the organelle membrane and involves VMAT-mediated exchange of two lumenal protons with one cytoplasmic amine. It has been suggested in the past that His residues play a role in H^+ movement or in its coupling to active transport in H^+-symporters and antiporters. Indeed VMAT-mediated transport is inhibited by reagents specific for His residues. We have identified one His residue in VMAT1 from rat which is conserved in other vesicular neurotransmitter transporters. Mutagenesis of this His (H419) to either Arg or Cys completely inhibits [^{3}H]serotonin and [^{3}H]dopamine accumulation. Mutagenesis also inhibits other H^+-dependent partial reactions of VMAT such as the acceleration of binding of the high affinity ligand reserpine, but does not inhibit the [^{3}H]reserpine binding which is not dependent on H^+ translocation. It is concluded that His-419 plays a role in energy coupling in r-VMAT1.

Key words: Neurotransmitter transport; H^+ transport; TEXANS; Drug resistance; Antiporter; Histidine; Energy coupling

1. Introduction

Classical neurotransmitters are stored in synaptic vesicles and storage organelles of secretory cells. Transport of the monoamines serotonin, dopamine, norepinephrine, epinephrine, and histamine into storage organelles in a variety of cells is catalyzed by a family of vesicular monoamine transporters (VMATs). Accumulation of the neurotransmitter depends on the proton electrochemical gradient generated by the vesicular H^+-ATPase and involves the VMAT-mediated exchange of two lumenal protons with one cytoplasmic amine [1–4].

VMATs are known for their substrate promiscuity and they share substrates and inhibitors with multidrug transporters from bacteria and higher animals [4]. In addition, VMATs show a distinct homology to a class of proteins present in bacteria and yeasts which are known for their ability to confer resistance to drugs and antibodies. It has been suggested that members of this family (the TEXANS) may play a role in detoxification at the cellular level also in higher organisms by removal of toxic compounds away from their target into acidic subcellular organelles.

Reserpine is a potent competitive inhibitor of VMAT which binds at the site of amine recognition and dissociates very slowly if at all from the transporter, even after solubilization [5]. Thus it has been used to label the transporter and follow its separation through a variety of procedures [6]. Changes in [^{3}H]reserpine binding rate occur upon imposition of a transmembrane H^+ gradient [7,8]. They probably reflect changes in the availability of reserpine binding sites, and translocation of a single H^+ generates the binding form of the transporter [5]. The energy invested in the transporter by H^+ flux is released by ligand binding and is converted into vectorial movement of a substrate molecule across the membrane or directly into binding energy. In the case of a substrate, a second conformational change results in the ligand binding site being exposed to the vesicle interior, where the substrate can dissociate. The second H^+ in the cycle, may be required to facilitate the conformational change or to allow for release of the positively charged substrate from the protein [5].

A clue for the molecular basis of some of these processes was obtained using phenylglyoxal (PG) and diethyl pyrocarbonate (DEPC), two reagents relatively specific for Arg and His residues, respectively. They both inhibited VMAT-mediated serotonin accumulation in chromaffin granule membrane vesicles in a dose-dependent manner (IC_{50} of 8 and 1 mM, respectively) [9,10]. The inhibition by DEPC was specific for His groups since transport could be restored by hydroxylamine [9]. Neither PG nor DEPC inhibited binding of reserpine, indicating that the inhibition of transport was not due to a direct interaction with either of the known binding sites. Interestingly, however, the acceleration of reserpine binding by a transmembrane H^+ gradient was inhibited by both reagents [9]. The results suggested that either proton transport or a conformational change induced by proton transport is inhibited by both types of reagents.

Cloning of VMAT [11,12] made it feasible to try to identify the residue(s) modified by DEPC. In this report we present an analysis of the role of histidines in VMAT carried out by site directed mutagenesis of rVMAT1. There are five His in rVMAT1; out of them, three are not conserved amongst the VMAT family: these are two His (H62 and H95) which reside in the large glycosylation loop, and one (H164) in the loop between transmembrane segment 2 and 3. On the other hand, the two His, H384 and H419, are conserved to varying degrees: H384 in rat VMAT1 is conserved as a Lys in the other VMAT's

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Abbreviations: VMAT, vesicular monoamine transporter; PG, phenylglyoxal; DEPC, diethyl pyrocarbonate; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; $\Delta\tilde{\mu}_{H^+}$, electrochemical potential for H^+.

Site directed mutants are designated as follows: the one-letter amino-acid code is used followed by a number indicating the position of the residue in wild type. The sequence is followed by a second letter denoting the amino acid replacement at this position.

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and H419 is conserved as His throughout all the VMAT proteins known. As much as replacement of H384 with an Arg (H384R) has no effect whatsoever on transport activity, replacement of H419 with either Cys (H419C) or Arg (H419R) completely abolishes transport as measured in permeabilized CV1 cells transiently transformed with plasmids coding for the mutant proteins. Reserpine binding to the mutant proteins in the absence of $\Delta\bar{\mu}_{H^+}$ is at levels comparable to those detected in the wild type. However, $\Delta\bar{\mu}_{H^+}$ does not accelerate reserpine binding to either H419R or H419C proteins. These results suggest that His-419 is associated with H^+ translocation or in conformational changes occurring after substrate binding.

2. Materials and methods

2.1. Growth of cells, transfections and transport assays

The protocol for functional expression of rat VMAT1 was essentially as described by Erickson et al. [12]. CV1 cells grown in 24-well collagen-treated plates were infected with recombinant vaccinia virus encoding bacteriophage T7 DNA polymerase [13] and after 30 min they were transfected with 2 μg of plasmid DNA coding for wt or mutant rVMAT1, 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, 200 μM $MgCl_2$, 1 mM ascorbic acid, 10 μM pargyline and 20 mM PIPES at pH 7.4. 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 MgATP and the corresponding tracers and/or inhibitors as indicated for the specific experiments. At given time periods the reaction buffer was aspirated and discarded and the cells were washed with ice-cold uptake buffer containing 2 mM $MgSO_4$ and no tracers. The cells were then collected with 1% SDS and radioactivity assessed by liquid scintillation. All data presented are mean values of triplicates.

2.2. [^{3}H]Reserpine binding assay

For measurement of [^{3}H]reserpine binding, cells were grown in 6 well plates, infected and transfected with proportionally higher amounts of material. [^{3}H]reserpine binding was measured in lysates prepared from cells 16–20 h after transfection. Cells were collected with a rubber policeman, centrifuged and resuspended in lysis buffer that contains 0.15 M NaCl, 10 mM K-HEPES pH 8.5, 5 mM $MgCl_2$, 5 mM NaEGTA and 1 μg/ml leupeptin. After an additional centrifugation, cells were resuspended in the above buffer (250 μl/10^7 cells) and sonicated in a bath sonicator for 90 s. Unbroken cells were discarded (3,500 × g, 2 min) and the lysate was used to assay binding essentially as previously described [5]. The lysate (containing 60–100 μg protein) was diluted in a solution containing 320 mM sucrose, 10 mM K-HEPES, pH 8.5, 4 mM KCl, 5 mM ATP and 4 mM $MgSO_4$. [^{3}H]Reserpine (20 Ci/mmol) was added to 3 nM or as indicated in the text, and the mixture was incubated at 32°C. After incubation for the time indicated, a 200 μl sample of the suspension was applied to a 3-ml column of Sephadex LH-20 (prepacked in a disposable syringe by centrifugation for 90 s in a clinical centrifuge), centrifuged for 2 min and the effluent was assayed for radioactivity. The assays were performed in duplicates and parallel reaction mixtures, containing 2 μM reserpine were used to subtract nonspecific binding which was typically less than 10% of the binding to membranes from VMAT-expressing cells. All data presented are mean values of duplicates.

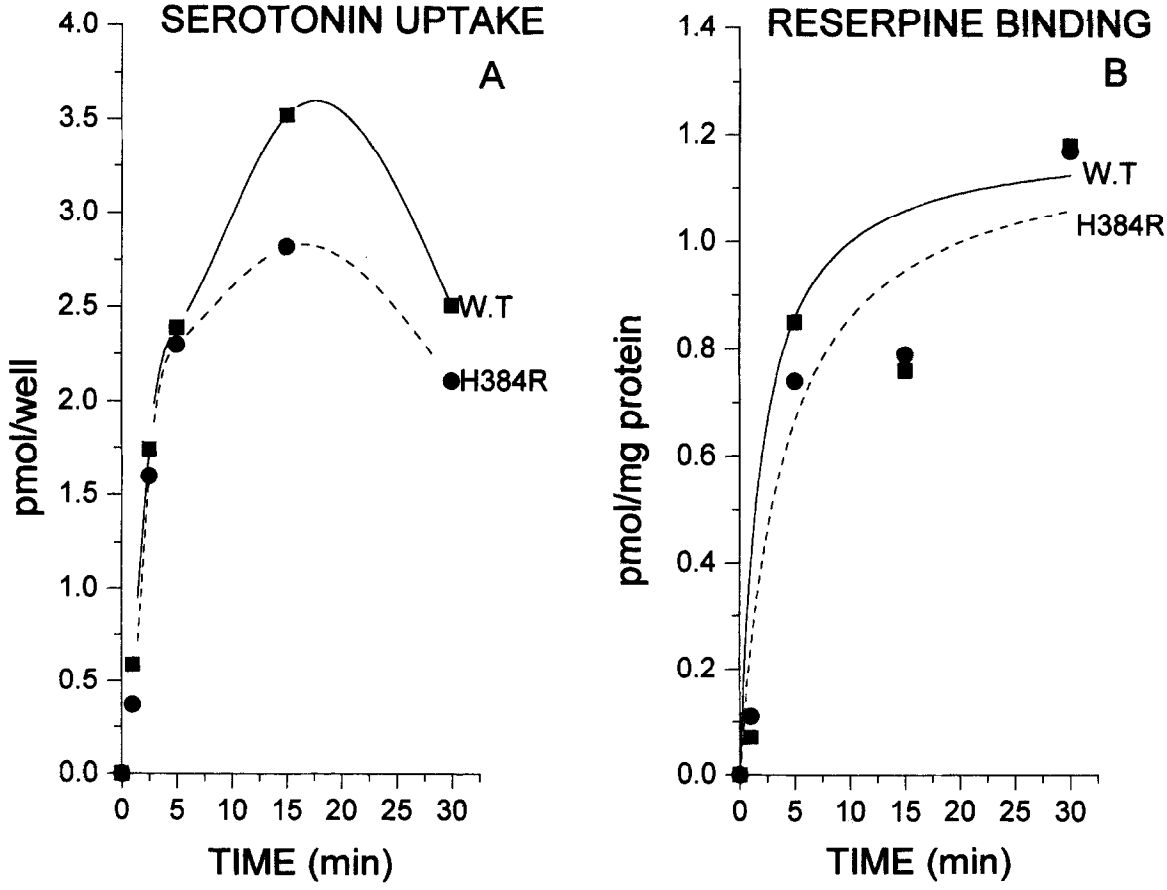

Fig. 1. Histidine-384 is not essential for rVMAT1-mediated serotonin transport and reserpine binding. CV1 cells were infected with vaccinia virus and transformed with the pTM1 plasmid bearing either wild type rVMAT1 (■) or H384R (●) mutant as described in section 2. (A) Transport was assayed in digitonin permeabilized cells after incubation with [^{3}H]serotonin (0.2 μM, 25.7 Ci/mmol). (B) [^{3}H]Reserpine binding was tested in total lysates (70 μg protein) prepared from each type of cell and assayed after incubation with 3 nM of the drug (20 Ci/mmol).

2.3. Plasmids

For transient functional expression rVMAT1 was subcloned in the high expression vector pTM1 essentially as described for bVMAT2 [14]. Two primers were synthesized in order to excise the coding sequence of the rat VMAT1 sequence from a Bluescript plasmid carrying the whole sequence of rVMAT1. The sense primer starts at nucleotide 262, which is 2 bp upstream of the ATG initiation codon, and introduces an *Eco*RI site at the 5′ end of the primer. The reverse primer was the M13 commercially available primer (Cat. # 1201 from New England Biolabs Inc.) which is homologous to sequences in the Bluescript plasmid. After PCR amplification, the 2,200 bp fragment was separated on an agarose gel and extracted by the QIAEX gel extraction kit (QIAGEN). The fragment was then cut by *Xho*I and *Eco*RI restriction endonucleases and ligated into pTM1 vector digested with the same enzymes. As a result of such ligation, translation of VMAT1 starts from the ATG initiation codon of the pTM1 vector, and the protein synthesized contains a total of four additional amino acids at its amino terminus (MGIP). Such an addition did not impair the activity in any noticeable way and expression levels were 3–4 times higher than those in Bluescript.

2.4. Mutagenesis

Mutants were obtained by PCR mutagenesis using the overlap extension procedure described by Ho et al. [18]. For each mutation a set of two overlapping oligonucleotide primers containing the desired mutation were constructed. The outside primers were homologous to bp 531–557 and 1664–1628 of VMAT1 in the case of the H419 mutations; in the case of the H384 mutation the reverse primer was the external M13 primer, # 1201. The resulting mutagenised PCR product (1133 and 2000 bp for the H419 and the H384 mutations respectively) were digested with *Bcl*I and *Pfl*mI for H419 and *Sac*I and *SSe*8387I for H384. The H419 mutations were first cloned into Bluescript predigested with the proper enzymes and only later transferred to pTM1. The H384 mutation was cloned directly into pTM1. In each case the mutagenised fragment was sequenced to verify the mutation and to ensure no other mutations occurred during the amplification process. Sequencing was conducted with the Sequenase kit (version 2.0, United States Biochemical).

3. Results

Two histidine residues are conserved amongst various VMATs: His-384 of rVMAT1 is conserved as a positive charge (Lys) in all the other VMATs; His-419 is fully conserved in all five VMAT sequences available [4]. In order to gain insight into the role of these two conserved His in energy coupling and substrate binding, they were mutagenized into either Arg or Cys residues, and activity of the mutated protein was assayed.

3.1. His-384 is not essential for activity

His-384 was replaced with Arg and the activity of the resultant protein was assayed in CV1 cells. Transport of [^{3}H]serotonin into CV1 cells was measured following infection with recombinant vaccinia virus (encoding T7 polymerase) and transfection with the cDNA coding for wild type or H384R rVMAT1 under the control of T7 promoter. Cells were permeabilized with digitonin as described by Erickson and collaborators [12]. When cells were transfected with wild type rVMAT1 or H384R, [^{3}H]serotonin was accumulated in a time-dependent fashion and uptake was maximal at 5–15 min (Fig. 1A). Accumulation decreased after longer incubation periods (> 30 min) due to detachment of the digitonin-permeabilized cells as observed visually. Both the rate and extent of uptake were indistinguishable in cells transformed with a plasmid coding for the wild type protein or for the H384R mutant. In both cases transport was completely inhibited in the presence of 10 μM of the proton ionophore CCCP as expected from the well known properties of VMAT, and by 1 μM reserpine which is a potent competitive inhibitor of monoamine transport (data not shown). Binding of [^{3}H]reserpine to the mutant protein was indistinguishable from that of the wild type (Fig. 1B). In this experiment, membranes from CV1-cells transformed with the wild type VMAT and the H384R mutant were allowed to bind [^{3}H]reserpine for various times, in the presence of ATP. A time-dependent increase was observed with both membranes that reached equilibrium after about ten minutes at levels of about 1.0 pmol/mg protein. The findings described in these experiments demonstrate that His-384 is not essential for transport.

3.2. Replacement of His-419 with either Cys or Arg completely abolishes transport catalyzed by rVMAT1

Strikingly, however, when His-419 was replaced with either Arg or Cys the effect on [^{3}H]serotonin transport activity was very dramatic. Fig. 2 shows the transport activity of cells transformed with H419, H419R, H419C or a mock transformation with vector that contains no insert (pTM1). Cells transformed with wild type (H419) rVMAT1 showed a time-dependent reserpine-sensitive accumulation of [^{3}H]serotonin. Cells transformed with either H419R or with H419C showed no measurable activity above the background levels as measured in mock-transformed cells. Although the data are not shown, essentially the same results were obtained when [^{3}H]dopamine, another substrate of rVMAT1 was used (15 nM, 32.2 Ci/mmol) or when the concentration of [^{3}H]serotonin was increased tenfold to test

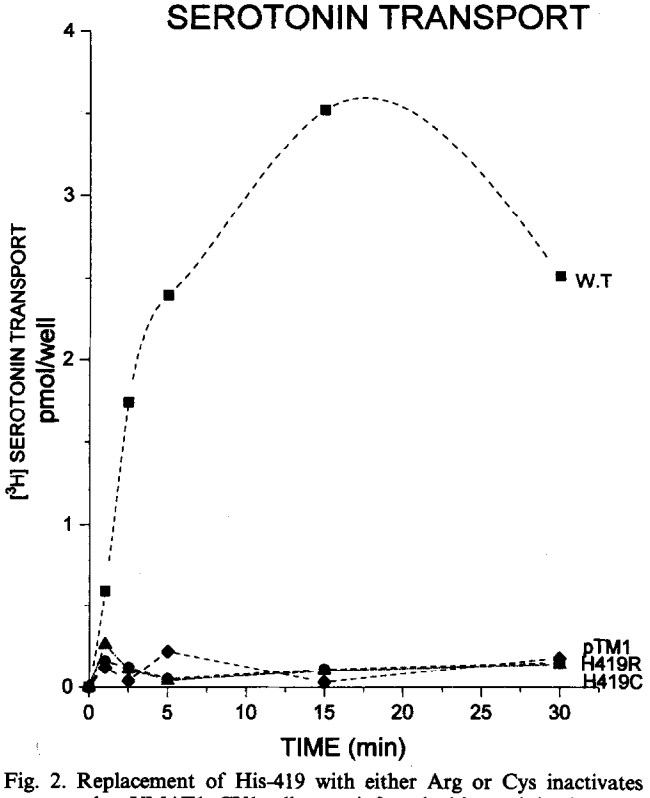

Fig. 2. Replacement of His-419 with either Arg or Cys inactivates transport by rVMAT1. CV1 cells were infected with vaccinia virus and transformed with pTM1 plasmid bearing either wild type rVMAT1 (■), or H419R (●) and H419C (▲) mutants or mock transformed with the plasmid with no insert (◆) as described in section 2. Transport was assayed in digitonin permeabilized cells after incubation with [^{3}H]serotonin (0.2 μM, 25.7 Ci/mmol).

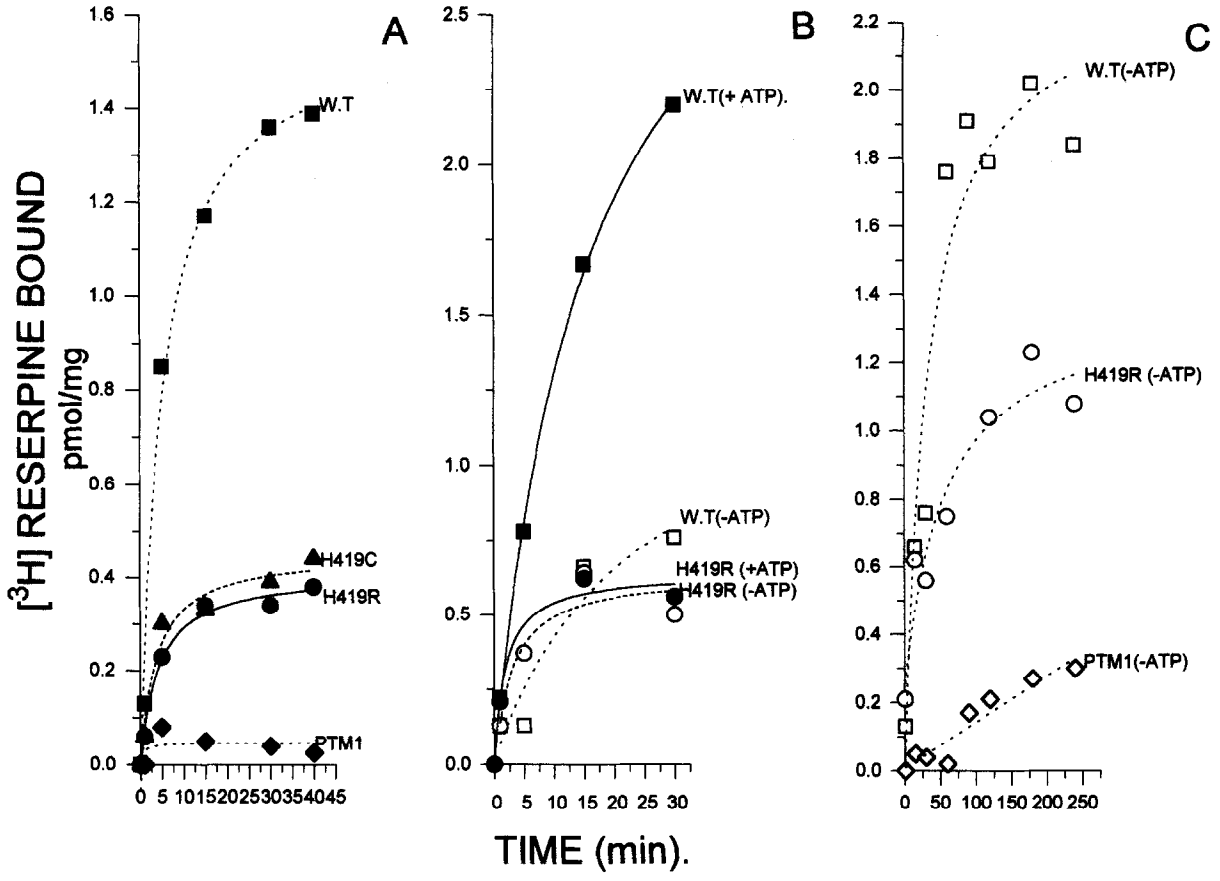

Fig. 3. Effect of $\Delta\tilde{\mu}_{H^+}$ on [^{3}H]reserpine binding by the mutant protein H419R. [^{3}H]Reserpine binding (3 nM, 20 Ci/mmol) was measured in lysates prepared from CV1 cells transformed with wild type plasmid (■ and □), H419R (● and ○), H419C (▲) or pTM1 with no insert (◆ and ◇). (A) All the reactions were carried out in the presence of ATP. (B) Wild type and H419R in the presence (closed symbols) and in the absence (open symbols) of ATP. C) All the reactions were carried out in the absence of ATP.

for a possible K_m effect. To assess whether the mutants are able to catalyze partial reactions of the catalytic cycle, [^{3}H]reserpine binding to membranes prepared from transformed cells was measured in the presence of ATP. Under these conditions (3 nM reserpine), the wild type protein bound [^{3}H]reserpine in a time-dependent manner and reached an apparent equilibrium after about 20 min reaction (Fig. 3A). [^{3}H]Reserpine binding to the H419R and the H419C proteins was lower than binding to the wild type protein (0.38 pmol as compared to 1.4 in the wild type). Binding to the mutant proteins, although lower, was significant and easily detectable and could be inhibited by addition of unlabelled reserpine (2 μM, not shown) to the levels observed in membranes from mock-transformed cells. As previously reported, [^{3}H]reserpine binding is accelerated in the presence of ATP or an artificial $\Delta\tilde{\mu}_{H^+}$ [7,8]. Therefore, addition of uncouplers such as CCCP reduced the binding levels significantly. Surprisingly, however, although the binding to the wild type protein and to mutant H384R was inhibited by CCCP, no effect on the binding to the mutant protein H419R was detected (Table 1). This finding was further investigated by analyzing the effect of ATP on the binding to the mutant proteins. As expected, binding to the wild type transporter was accelerated several fold in the presence of $\Delta\tilde{\mu}_{H^+}$ (Fig. 3B). Binding in the absence of $\Delta\tilde{\mu}_{H^+}$ was slower but reached equilibrium at levels comparable to those observed in the presence of ATP after longer incubation period (Fig. 3C). When the wild type and H419R were compared in the absence of ATP, there was practically no difference in the rate of binding (Fig. 3B) and only a 40% decrease in the equilibrium level after 4 h was detected (Fig. 3C). In agreement with the fact that CCCP, which collapsed the $\Delta\tilde{\mu}_{H^+}$, did not decrease the binding level of the mutant, also ATP, that is hydrolyzed by the V-ATPase to generate a $\Delta\tilde{\mu}_{H^+}$, had no effect on the rate of binding of the mutant protein (Fig. 3B).

It was previously reported that only the binding rate but not the apparent affinity of the transporter to reserpine or the number of binding sites were modified by $\Delta\tilde{\mu}_{H^+}$ ([8] and Fig. 4]. Binding was biphasic with two apparent affinities, both in the absence and presence of $\Delta\tilde{\mu}_{H^+}$. The results summarized in the Scatchard plots shown in Fig. 4A and B demonstrate that the

Table 1
Effect of CCCP on [^{3}H]reserpine binding to wild type and mutant proteins

	Control (pmol/mg protein)	+ CCCP (pmol/mg protein)
Wild type	1.35 ± 0.07	0.54 ± 0.06
H384R	1.66 ± 0.09	0.68 ± 0.06
H419R	0.53 ± 0.07	0.46 ± 0.07

[^{3}H]Reserpine binding was measured in lysates prepared from CV1 cells transformed with wild type plasmid or the indicated mutants. Reaction was carried out for 30 min and CCCP concentration was 5 μM.

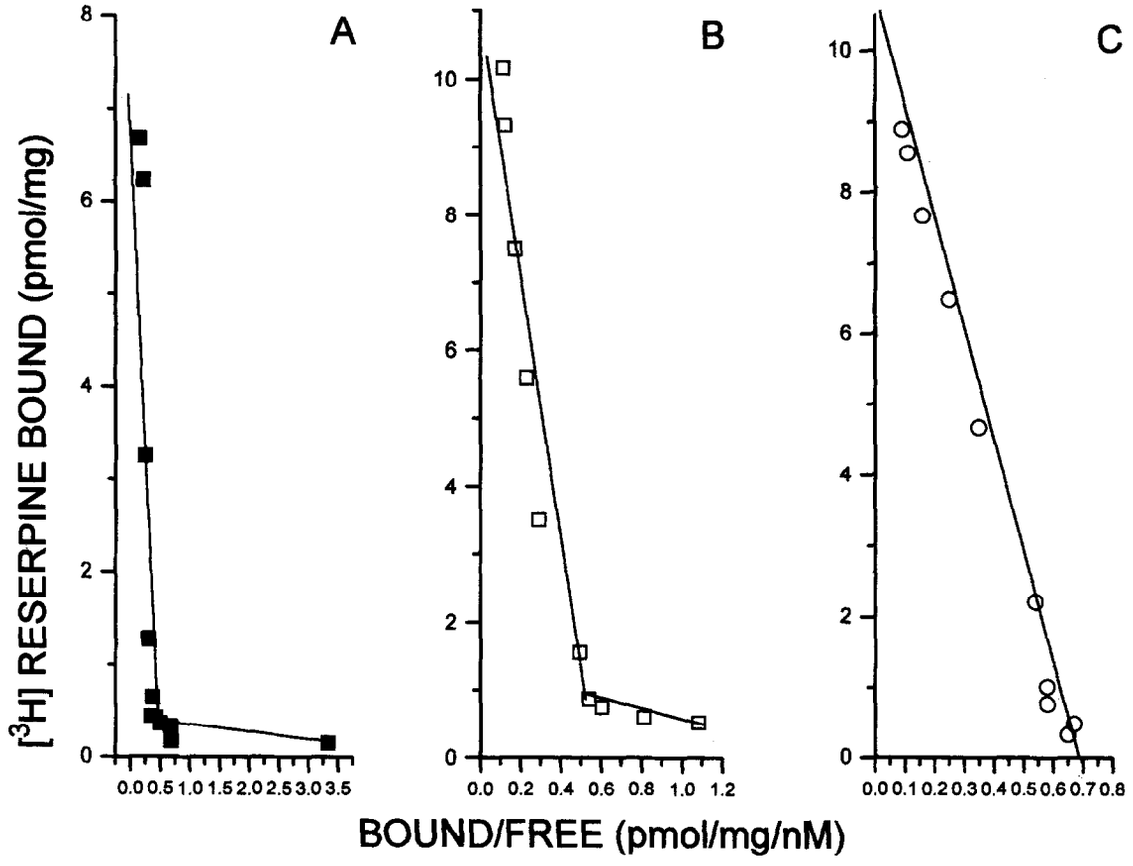

Fig. 4. The number of reserpine binding sites in membranes from wild type and H419R-transformed cells is identical. [^{3}H]Reserpine binding was measured at various concentrations of the drug in lysates prepared from CV1 cells transformed with wild type plasmid (□ and ■) and mutant H419R (○). The assay was performed in the presence (■) or absence of ATP (□ and ○) for 40 and 120 min, respectively.

high affinity binding site (K_d = 0.58 nM) is sparse (0.6 and 1.2 pmol/mg protein, respectively in the presence and absence of ATP) compared to the more abundant (10.9 pmol/mg protein) low affinity site (25–30 nM). Interestingly, in the mutant protein only the low affinity site (15 nM) was detected, with a practically identical site density (10.2 pmol/mg protein) (Fig. 4C).

4. Discussion

In summary, replacement of His419 with either Arg or Cys residues caused a loss in the transport activity of VMAT1 and in its ability to respond to $\Delta\tilde{\mu}_{H^+}$. The rate of [^{3}H]reserpine binding in H419R or H419C was not modified by $\Delta\tilde{\mu}_{H^+}$. The total amount of [^{3}H]reserpine bound to the H419R protein and its affinity were very similar to those observed in the wild type protein except that the high affinity site, which corresponds to the binding of about 10% of the ligand, was not detectable in the mutant. The findings also demonstrate that the expression levels of the various mutants are very similar to those of the wild type as judged by the density of the reserpine binding sites. These findings were corroborated by Western blot analysis of the protein levels of the various mutants using an antibody directed against sequences in the C terminus of rVMAT1 (kindly supplied by R.H. Edwards, UCLA).

The nature of the two types of binding sites, their interconversion and the effect of $\Delta\tilde{\mu}_{H^+}$ on each of the forms is not fully understood yet. The mutants described in this communication may be helpful in understanding the details of the various processes since the different steps can be isolated and studied separately. Moreover, as mentioned above, the H419R mutant protein does not display high affinity binding of [^{3}H]reserpine which may suggest that the His-419 residue plays also a role in substrate binding.

Based on the studies presented in this paper, it is tempting to speculate that histidine 419, an amino acid with a pK in the physiological range may play a direct role in the transport of the proton involved in the translocation cycle. It is also possible, however, that it plays a more indirect role, in the conformational change required for the cycle to function. A role for histidine residues in sensing proton concentration and in coupling of H^+ movements to solute movement has been proposed in several cases in *E. coli*, most notoriously in NhaA, a Na^+/H^+ antiporter and the *lac* permease, a β-galactoside-H^+ symporter [15–17]. Both, in the *lac* permease and in rVMAT1, treatment with DEPC or mutagenesis of the proper His residue yield a very similar phenotype: transport and all the partial reactions dependent on $\Delta\tilde{\mu}_{H^+}$ are inhibited, while ligand binding and reactions which do not require H^+ translocation are not impaired.

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