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The Role of a Transmembrane pH Gradient in Uptake and Release of Imipramine and Haloperidol in Synaptosomes

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SUMMARY

By using the methylamine method, the influence of a transmembrane pH gradient on the distribution of the antidepressant imipramine and the neuroleptic haloperidol was assessed in synaptosomes. Resting synaptosomes concentrated methylamine 8- to 9-fold at pH 7.4, as compared with a 400- to 600-fold concentration of the lipophilic weak bases imipramine and haloperidol. Ignoring compartmentation, the methylamine ratio is consistent with an internal pH of 6.5. Weak acid partition yielded an internal pH of 7.1. In agreement with the pH partition hypothesis, elevation of the internal pH by NH_4Cl reduced whereas internal acidification enhanced imipramine and haloperidol uptake. Release of both drugs could also be elicited with excess K^+ or veratridine. The underlying mechanism is a depolarization-induced rise of the internal pH by 0.12 to 0.25 units. In polarized synaptosomes, about 70% of the uptake of imipramine and haloperidol depended on the transmembrane pH gradient. A predominant localization in synaptic vesicles *in situ* is derived from the proportional release of methylamine, imipramine, and endogenous dopamine by nigericin. It is concluded that the acidic internal pH plays an important role for the passive concentration of lipophilic bases like imipramine and haloperidol in the brain, thereby profoundly influencing the extra- and intracellular free concentrations. Binding to cellular constituents contributes to internal accumulation. Especially high concentrations are attained in acidic vesicles.

The influence of a transmembrane pH gradient on the distribution of weak electrolytes can be utilized to estimate the pH in cells and vesicles too small to have electrodes introduced (1). The method rests on the assumption that cell membranes are freely permeable only to the uncharged form of a weak electrolyte. At equilibrium, equal concentrations of the uncharged compound will be present on either side of the membrane, whereas the charged species is distributed according to the pH gradient. Bases will concentrate on the more acidic side of the membrane and acids on the more basic side. In contrast, lipophilic ions that exist only in the charged form, such as tetraphenylphosphonium cation, distribute according to the membrane potential across lipid bilayers.

When internal pH is acidic relative to outside, weak bases are generally more suited as pH_i indicators than acids. The most commonly used base is methylamine, introduced by Rottenberg *et al.* (2) to monitor ΔpH in chloroplasts. By using methylamine partition, $^{31}PNMR$, and optical probes, it has been shown that granules involved in the storage, synthesis, and release of transmitters and hormones are acidic inside (as reviewed in Ref. 3). Because catecholamines are weak bases, it has been proposed that they concentrate in the storage granules by nonionic diffusion and intravesicular protonation, like methylamine itself. This hypothesis was abandoned when it was found that the transport of catecholamines is electrogenic, molecularly selective, and specifically inhibited by reserpine, a drug that does not affect vesicular pH (as reviewed in Ref. 4).

The classical antidepressant imipramine, a tertiary amine, has been reported to accumulate in brain slices relative to the medium (5–7). Because imipramine is a weak base (pK_a 8.6), the hypothesis was tested in the present study that transmembrane H^+ gradients may play a role in its distribution. The cytosolic pH of most cells is around 7.0 at outside pH of 7.4 (1), sufficient for a 2- to 3-fold concentration of a weak base in the cytosol. In addition, nerve endings contain a substantial number of acidic synaptic vesicles, which can be expected to act as a sink for basic drugs. The vesicles are preserved during the preparation of synaptosomes (8). Using [^{14}C]methylamine as a pH_i indicator, I demonstrate that imipramine distribution in synaptosomes is determined by the existence and magnitude of a transmembrane pH gradient. The neuroleptic haloperidol (pK_a 8.3) was included in the study to show that pH-dependent distribution also occurs with other weakly basic drugs. A preliminary account of this work has been presented in abstract form (9).

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ABBREVIATIONS: pH_i, average intrasynaptosomal pH (ignoring compartmentation); ΔpH, transmembrane pH gradient; DMO, 5,5-dimethyloxazolidine-2,4-dione; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

Materials and Methods

Drugs and chemicals. Unlabeled drugs were generously donated by the following companies: imipramine HCl and desmethylimipramine HCl (Ciba-Geigy, Basel, Switzerland); chlorpromazine HCl (Bayer, Leverkusen, FRG); haloperidol (Janssens Pharmaceutica, Beerse, Belgium); amiloride HCl (Merck Sharp & Dohme, Rahway, NJ). [^{3}H] Imipramine HCl (75 Ci/mmol), [^{3}H]haloperidol (20.5 Ci/mmol), and [^{14}C]methylamine HCl (50 mCi/mmol) were obtained through New England Nuclear (Dreieich, FRG). [^{14}C]DMO (54 mCi/mmol), ^3H_2O, and [^{14}C]sucrose (10 mCi/mmol) were from Amersham Buchler (Braunschweig, FRG). The silicone oils were from Wacker Chemie (München, FRG). Nucleosil 5-μm C_{18} was from Macherey-Nagel (Düren, FRG). Veratridine, tetrodotoxin, and nigericin were obtained from Sigma Chemie (München, FRG). All other chemicals were purchased from Merck (Darmstadt, FRG) in analytical or Suprapur grade.

Preparation and incubation of synaptosomes. Synaptosomes were prepared freshly from cerebral cortices of male Wistar rats (200–250 g) or bovine caudate nuclei by differential and Ficoll gradient centrifugation (10). The final pellets were stored on ice and were suspended immediately before an experiment. Protein was determined by the Coomassie method (11). Incubations were carried out at 37° in 0.4-ml tubes with an extended tip of low diameter (Sarstedt, Nümbrecht, FRG). The NaCl/Tes medium consisted of 130 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM Tes, pH 7.4 at 37°. To prevent changes of medium pH in experiments requiring changes of the incubation temperature, a phosphate-buffered medium was used, composed as the NaCl/Tes medium except that Tes was replaced by 20 mM phosphate, sodium salt. In some experiments, both $CaCl_2$ and $MgCl_2$ were replaced by a Ca^{2+} buffer to adjust a low external Ca^{2+} activity, as described previously (12). Protein concentration ranged over 0.5 to 0.8 mg/ml.

Determination of imipramine and haloperidol distribution. If not otherwise stated, the reactions were initiated by mixing 100 μl of a synaptosomal suspension with 100 μl of a medium containing [^{3}H] imipramine or [^{3}H]haloperidol. To terminate the reaction, the synaptosomes were centrifuged through silicone oil. Centrifugation was performed either at 4° or at 37° in a refrigerated centrifuge (45,000 × g for 1 min) or at room temperature in the microfuge (10,000 × g for 1 min). Both sediments and supernatants were taken for counting in a scintillation counter. The tips of the tubes were cut off within the silicone oil layer. Pellets containing [^{3}H]imipramine or [^{3}H]haloperidol were solubilized in 50 μl of Protosol and counted in 4 ml of a toluene-based scintillator. The remaining part of the tube with the entire supernatant was counted in 3 ml of Aqualuma (Baker Chemicals, Deventer, Holland). The channels-ratio method was used for automatic quenching correction.

Analysis of imipramine binding data. Plotting of data according to the method of Scatchard (13) yielded a biphasic curve for imipramine binding at 4° (see Fig. 1b), suggesting site heterogeneity. However, accurate determination of nonspecific binding, by definition unsaturable, was difficult, because binding decreased continuously when up to 10 μM imipramine were added. Because the shape of the Scatchard plot will vary artifactually with the amount subtracted from total binding, the nonspecific component was assessed from an analysis of total binding according to Mendel and Mendel (14). These authors have documented that unsaturable 'nonspecific' binding can be treated as binding to a second set of independent sites having lower affinity and higher capacity for the drug than the receptor site. According to their approach, the experimental data were fitted to an equation for the binding of one ligand to two classes of noninteracting sites, derived from the more generalized model of Feldman (15). Calculations were performed by using a commercially available computer program (BDATA; EMF software, Baltimore). The given value represents the best fit in the least squares sense. Increasing the number of sets of binding sites did not significantly improve the fit, as evaluated with the F test (16).

Determination of pellet spaces, pH_i, and $[K^+]_i$. The steady state uptake of [^{14}C]methylamine and [^{14}C]DMO was used to measure pH_i. ^3H_2O and [^{14}C]sucrose served for the measurement of the total water-accessible space and the external space of the pellet, respectively. The protocol was the same as described above. Synaptosomes were equilibrated at 37° in separate tubes with ^3H_2O, 10 μM [^{14}C]sucrose, 2 μM [^{14}C]methylamine, or 9 μM [^{14}C]DMO. After silicone oil centrifugation, the pellets were resuspended in 2% Triton X-100 and counted in 3 ml of Aqualuma. The difference between the ^3H_2O space and the [^{14}C] sucrose space was taken as the intracellular volume. For determination of $[K^+]_i$, pellets were resuspended in 0.4% $HClO_4$ and diluted with 5 mM CsCl (Suprapur grade). K^+ was measured by flame emission spectroscopy.

The principles governing the H^+ gradient-dependent distribution of weak acids and bases have been reviewed by Roos and Boron (1). Methylamine and DMO uptake were corrected for entrapment in the [^{14}C]sucrose space and then referred to the intracellular space. Neither methylamine nor DMO is significantly bound to intracellular constituents (1, 17). Ignoring compartments, the average internal pH (pH_i) was calculated according to Eq. 1 for methylamine ($pK \gg pH_i$)

$$pH_i = pK - \log \frac{[CH_3NH_3^+]_i}{[CH_3NH_3^+]_o} \quad (1)$$

and according to Eq. 2 for DMO ($pK < pH_i$)

$$pH_i = pK + \log \left\{ \frac{[DMO]_i}{[DMO]_o} \left(10^{pH_o - pK} + 1 \right) - 1 \right\} \quad (2)$$

Determination of endogenous dopamine. Synaptosomes were incubated exactly as described above. Immediately after the reaction was terminated by centrifugation, the samples were put on ice. The supernatant was discarded and the pellet was resuspended in 0.2 N $HClO_4$ containing 3,4-dihydroxybenzylamine and N-methylserotonin as internal standards for catecholamines and serotonin, respectively. Norepinephrine, dopamine, and serotonin were determined by high performance liquid chromatography with electrochemical detection (18, 19).

Presentation of results. Results are presented either as the means ± standard deviations of the number of independent experiments specified or as the means ± standard deviation representative of at least three similar experiments. Samples were run in triplicate for high performance liquid chromatography measurements and in quadruplicate for [^{14}C]DMO partition. In general, the other parameters were determined in duplicate only because of the low experimental error (typical variation coefficient less than 2%). Statistical significance was evaluated by the unpaired Student t test (two-tailed). When multiple means were compared, analysis of variance preceded t test. Data were considered to be significantly different at $p < 0.05$. Correlation coefficients (r) were derived from linear regression analysis using standard formulas.

Results

Internal pH of synaptosomes. The intrasynaptosomal pH was estimated from the distribution of the weak base methylamine and the weak acid DMO. In polarized synaptosomes with operating Na^+/H^+ antiporter, the methylamine ratio in 16 independent experiments was 8.5 ± 0.5 at a medium pH of 7.40, corresponding to a pH_i of 6.47 ± 0.02. The intrasynaptosomal water space determined in parallel was 3.8 ± 0.3 μl/mg of protein. DMO partition yielded a much higher pH_i of 7.06 ± 0.03 (the mean of four independent experiments) close to the pH_i of 7.05 of rat brain *in vivo* (pH_o around 7.40) determined by using the same indicator (20). The lower pH_i derived from weak base than for weak distribution is indicative of pH_i inhomogeneity and can be explained by the existence of an enclosed acidic compartment, i.e., the synaptic vesicles. Because of their small volume, the vesicles will exert only an

insignificant influence on the distribution ratio of the weak acid.

Absence of high affinity binding of imipramine at 37°. Synaptosomes in physiological salt solution concentrated imipramine and haloperidol very rapidly. An apparent equilibrium of distribution was obtained within 1 min of incubation. The tissue/medium ratios given on the left ordinate in Fig. 1a were calculated by referring to an intrasynaptosomal volume of 3.8 μl/mg of protein. It should be noted that the distribution ratios were not corrected for binding to cell membranes and proteins.

The concentration and temperature dependence of imipramine partition in synaptosomes is shown in Fig. 1b, in the form of Scatchard graphs. As apparent from the figure, the partition is temperature dependent. The *horizontal line* in the Scatchard plot indicates nonsaturability at 37°. However, high affinity binding was observed when synaptosomes were incubated and centrifuged at 4° (Fig. 1b). At 4°, 0.3 μM imipramine displaced 26% of total binding as compared with only 5% at 37°. An apparent dissociation constant of 1.8 nM and a maximal number of binding sites of 1.1 pmol/mg of protein at 4° were calculated from the data of Fig. 1b using nonlinear least-squares analysis (see Materials and Methods). A similar Scatchard graph for imipramine binding at 0° was obtained using rat brain homogenate and the conventional filtration assay (21). It is concluded from these results that high affinity binding of imipramine is absent under the experimental conditions employed in the present study, i. e. incubation and centrifugation at 37°C. The concentration of 50 nM imipramine chosen for the experiments described below is in the range of therapeutic levels in the cerebrospinal fluid (22).

Effect of internal alkalinization on the distribution of imipramine and haloperidol. Imipramine and haloperidol are tertiary amines with pk_a values of 8.6 and 8.3, respectively. To test whether the high tissue/medium distribution ratios in Fig. 1a depended in part on the acidic pH_i, synaptosomes equilibrated with [^{3}H]imipramine were challenged with NH_4Cl. NH_4Cl is known to dissipate H^+ gradients because it enters the cell as NH_3, which combines with internal H^+ (1). As shown in Fig. 2a, addition of 7.8 mM NH_4Cl immediately released imipramine from synaptosomes. Within 15 sec, the imipramine distribution ratio declined by about 55%. The steep efflux was followed by a slow reuptake of the drug. Haloperidol gradients responded to NH_4Cl in a similar manner as imipramine gradients; within 15 sec, the distribution ratio was lowered by 50% (not shown).

Internal alkalinization was confirmed by monitoring methylamine distribution. The methylamine ratio decreased on addition of NH_4Cl, as shown in Fig. 2b. The increase of pH_i by 0.44 units within 15 sec (the methylamine ratio declined by 64%) was followed by a slow and incomplete recovery. By varying the NH_4Cl concentration between 30 μM and 10 mM, a linear relationship could be established between methylamine gradients and imipramine distribution (Fig. 2c; $r = 0.972$). The results are consistent with the hypothesis that imipramine and haloperidol uptake depend on ΔpH. As shown in Table 1, imipramine uptake was not significantly reduced by 1 μM reserpine, an inhibitor of biogenic amine transport in storage vesicles (4). The inhibition of imipramine uptake at 10 μM reserpine paralleled the dissipation of the H^+ gradient by this drug in synaptosomes (Table 1).

Proportional release of imipramine and dopamine from caudate nucleus synaptosomes. In the experiment depicted in Fig. 3, methylamine and imipramine distribution were determined in the presence of the carboxylic ionophore nigericin, which catalyzes K^+/H^+ and Na^+/H^+ exchange. Nigericin, which has been shown to disrupt ΔpH in chromaffin granules and other granules (3), was also effective in releasing methylamine and imipramine from synaptosomes. Imipramine distribution was linearly related to H^+ gradients imposed by varying the nigericin concentration (Fig. 3a; $r = 0.982$).

Nigericin, by dissipating ΔpH, caused biogenic amines to leak out rapidly from synaptic vesicles of rat brain (23, 24). Therefore, if the nigericin-induced efflux of imipramine resulted from the dissipation of the ΔpH of synaptic vesicles *in situ*, endogenous neurotransmitters should be concomitantly released. Experiments were done on synaptosomes of bovine caudate nucleus because this region contains high concentrations of dopamine (25) but virtually no other amines. As shown

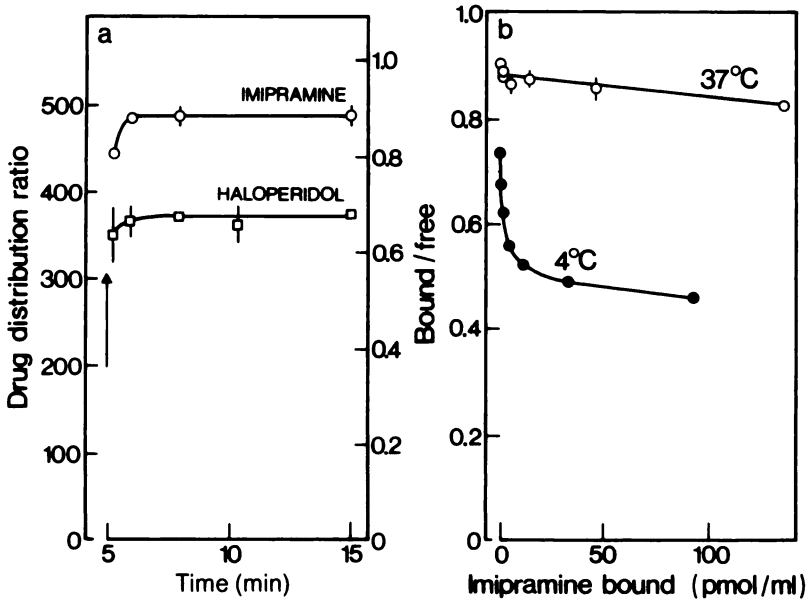

Fig. 1. Uptake of [^{3}H]imipramine and [^{3}H]haloperidol by synaptosomes. a, Time course. Rat cortical synaptosomes were equilibrated in separate tubes in the NaCl/Tes medium at 37°. After 10 min, 10 μl of [^{3}H]imipramine (○) or [^{3}H]haloperidol (□) were added at final concentrations of 50 nM (0.08 μCi/ml) or 6 nM (0.12 μCi/ml), respectively. At the indicated times silicone oil centrifugation was initiated in the microfuge (10,000 × *g* for 1 min). The tissue/medium ratios given on the *left ordinate* in Fig. 1a were calculated by referring to an intrasynaptosomal volume of 3.8 μl/mg of protein. b, Scatchard plot of total binding of [^{3}H]imipramine at 4° and 37°. Rat cortical synaptosomes (0.5 mg of protein/ml) were incubated in the phosphate-buffered medium at pH 7.4 and 37° with [^{3}H]imipramine ranging from 0.3 to 300 nM. After 10 min, half of the samples was subjected to silicone oil centrifugation at 37° (○). The incubation of the remaining samples was continued for an additional 10 min at 4° before silicone oil centrifugation at 4° (●). Bound/free is given in pmol (ml × nM)$^{-1}$.

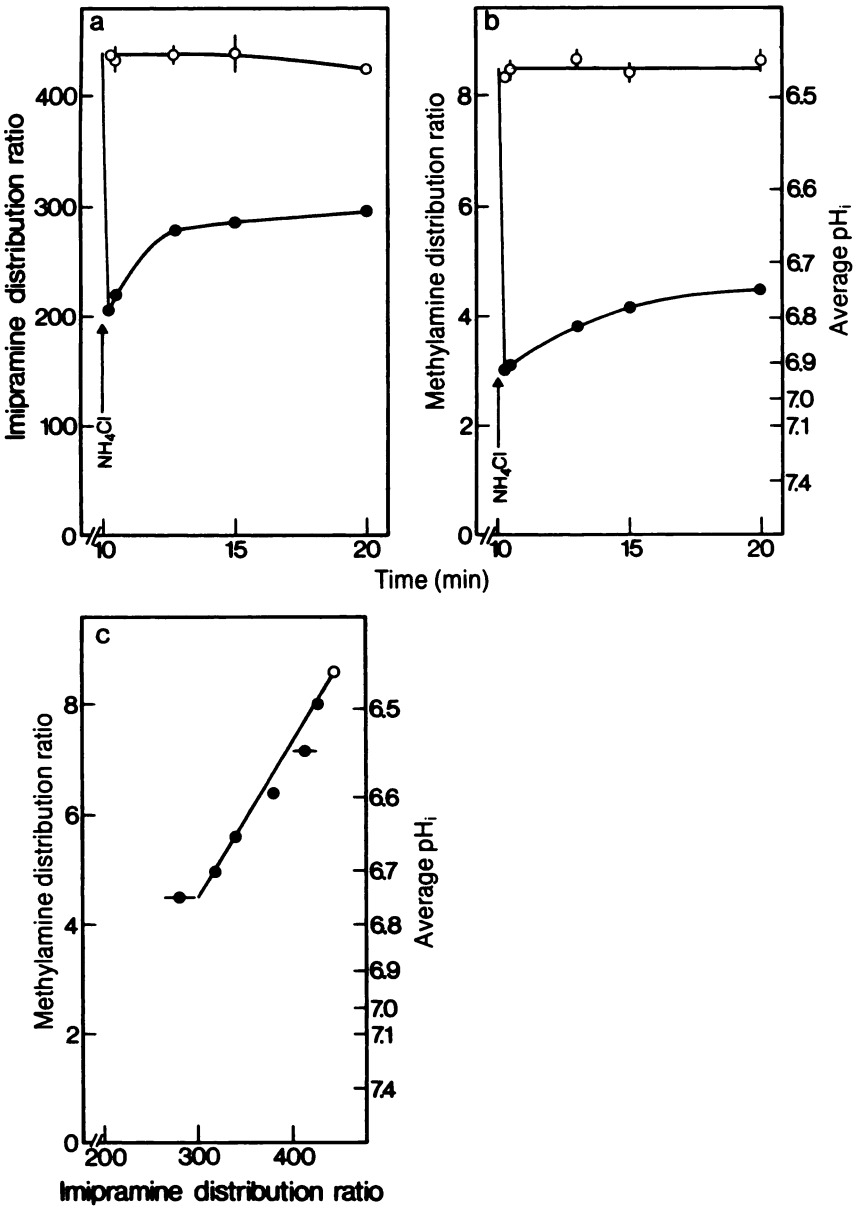

Fig. 2. Effect of NH_4Cl on the distribution of imipramine and methylamine. Rat cortical synaptosomes were equilibrated with 50 nM [^{3}H]imipramine (a) or 2 μM [^{14}C]methylaine (b) in the NaCl/Tes medium for 10 min. At the time indicated by the *arrow*, 10 μl of NH_4Cl was added (●) (final concentration 7.8 mM) and the efflux of imipramine and methylamine was monitored. Controls (○) received saline only. The starting points of the falling curves were derived by extrapolation of the control curves to the time of NH_4Cl addition. c, Synaptosomes were incubated with [^{14}C]methylamine or [^{3}H]imipramine for 10 min in the presence of 30 μM to 10 mM NH_4Cl. ○, Samples lacking NH_4Cl.

TABLE 1

Effect of reserpine on the uptake of methylamine and imipramine

Rat cortical synaptosomes were incubated in the NaCl/Tes medium with reserpine in concentrations as indicated. Controls received 0.1% dimethyl sulfoxide (final concentration) as present in the samples exposed to reserpine. After 5 min at 37°, 10 μl of [^{14}C]methylamine or [^{3}H]imipramine in saline were added per 190 μl of synaptosomes and the incubation was continued for an additional 10 min. Results are the means ± standard deviation from three independent experiments.

Reserpine	Distribution ratio	
	Methylamine	Imipramine
μM		
0	8.4 ± 0.4	498 ± 35
1	8.6 ± 0.3	474 ± 27
10	6.6 ± 0.3*	377 ± 15*

* Significantly different from controls, $p < 0.001$.

in Fig. 3b, imipramine and endogenous dopamine were proportionally released by various concentrations of nigericin (r = 0.975).

Effect of intracellular acidification on imipramine distribution. In order to see whether intracellular acidification enhances the uptake of imipramine and haloperidol, H^+ accumulation was provoked by incubating Na^+-loaded synaptosomes in a Na^+-free medium. The outwardly directed Na^+ gradient established by this procedure reverses the normal direction of the Na^+/H^+ exchange (17). Results are presented in Fig. 4. Synaptosomes gained H^+, as follows from the rise of methylamine gradients shown in Fig. 4. Imipramine and haloperidol distribution ratios increased in parallel. Distribution ratios were about 3-fold higher than in Na^+-based media. Addition of amiloride, an inhibitor of Na^+/H^+ antiport, or of Na^+ to the medium impaired both the formation of H^+ gradients and imipramine or haloperidol uptake. When Na^+/H^+ exchange was artificially enhanced with nigericin or monensin, the uptake of imipramine and haloperidol was further stimulated (Fig. 4).

Effect of depolarization on imipramine distribution. Addition of 10 μM veratridine to synaptosomes preequilibrated with [^{3}H]imipramine or [^{3}H]haloperidol induced a rapid efflux of both drugs (Fig. 5, a and b). Within 20 sec, the accumulation

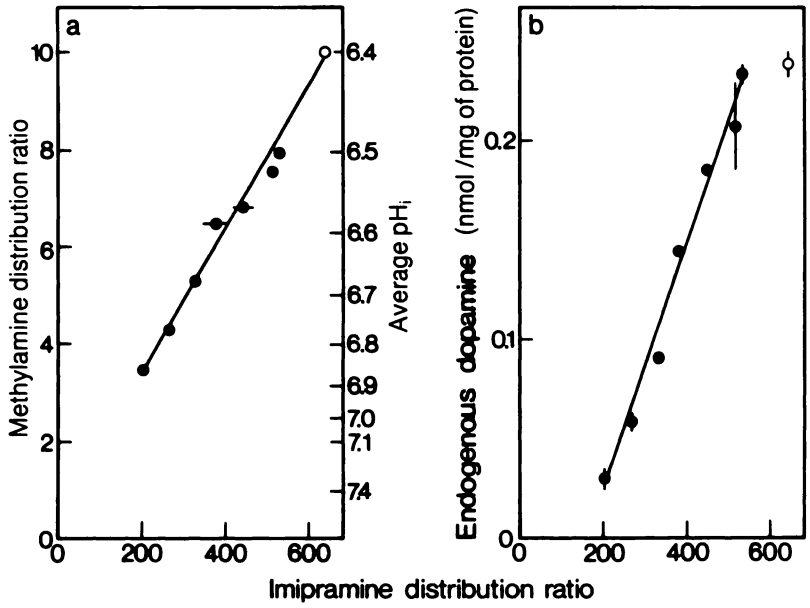

Fig. 3. Proportional release of imipramine, methylamine, and dopamine by nigericin. Synaptosomes of bovine caudate nucleus were exposed to 1 nM – 1 μM nigericin in the NaCl/Tes medium for 10 min. Methylamine and imipramine distribution (a) and endogenous dopamine (b) were determined in samples incubated in parallel. ○, Samples lacking nigericin.

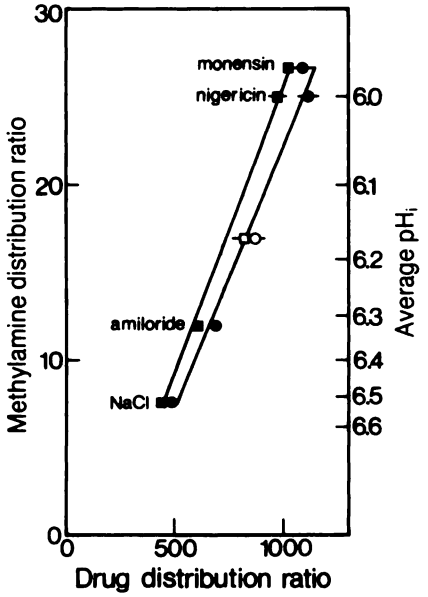

Fig. 4. Accumulation of imipramine and haloperidol in response to internal acidification. Rat cortical synaptosomes were incubated for 8 min in the choline/Tris medium with 2 μM [^{14}C]methylamine or 50 nM [^{3}H]imipramine (●) or 6 nM ([^{3}H]haloperidol (■). When indicated in the figure, one of the following compounds was additionally present: 5 μM monensin, 5 μM nigericin, 0.3 mM amiloride, or 70 mM NaCl. External Na^+ in controls (*open symbols*) amounted to 0.2 mM at the end of the incubation. According to linear regression analysis, the straight lines fitted data points with $r \geq 0.986$.

ratios declined by 36% and 37%, respectively. Veratridine is known to depolarize excitable cells by increasing membrane permeability to Na^+. Because the plasma membrane potential approximates a K^+ diffusion potential (26), changes of the plasma membrane potential can be detected by monitoring K^+ distribution. Addition of 10 μM veratridine to resting synaptosomes caused internal K^+ to decrease from 62 ± 1 mM to 15 ± 0.5 mM, indicating a marked depolarization of the plasma membrane. The effect of veratridine on imipramine distribution and $[K^+]_i$ could be blocked by 10 μM tetrodotoxin (not shown), arguing against a weak base effect of veratridine.

Intrasynaptosomal alkalinization as the underlying mechanism of imipramine and haloperidol efflux was confirmed by monitoring methylamine distribution. Depolarization of synaptosomes by 10 μM veratridine caused a prompt increase of pH_i from 6.50 to 6.75 (not shown). A linear relationship between methylamine gradients and imipramine (haloperidol) partition ratios could be established by varying the veratridine concentration between 0.1 and 30 μM (Fig. 5c; $r \geq 0.990$). As shown in Table 2, the effect of 10 μM veratridine was significantly attenuated when the external free Ca^{2+} concentration was lowered, from 23% to 14% for the distribution of imipramine ($p < 0.01$) from from 37% to 19% for that of methylamine ($p < 0.005$).

A significant efflux of the drugs could also be elicited by elevating the external K^+ concentration. Addition of 34 mM K^+ to the medium lowered the accumulation ratios of imipramine and haloperidol by 15% ($p < 0.01$) and 9% ($p < 0.05$), respectively. According to methylamine distribution, 34 mM K^+ raised internal pH by 0.12 ± 0.02 units (five independent experiments, $p < 0.01$).

Effect of imipramine and haloperidol on pH_i. Provided that imipramine and haloperidol are concentrated by a combination of nonionic diffusion and intracellular protonation, sufficiently high concentrations should overcome the internal buffering capacity and alkalinize the interior of synaptosomes. The results of a representative experiment are depicted in Fig. 6. Lowering of methylamine gradients was significant at an added imipramine concentration of 10 μM. At 100 μM imipramine, pH_i increased by 0.28 units. The antidepressant desmethylimipramine, a secondary amine, and the phenothiazine neuroleptic chlorpromazine were similarly effective in dissipating ΔpH (not shown).

Discussion

The present study provides evidence that the distributions of the antidepressant imipramine and the neuroleptic haloperidol are passively coupled to transmembrane pH gradients in nerve terminals. In agreement with the pH partition hypothesis, dissipation of the existent ΔpH reduced (Figs. 2, 3, and 5)

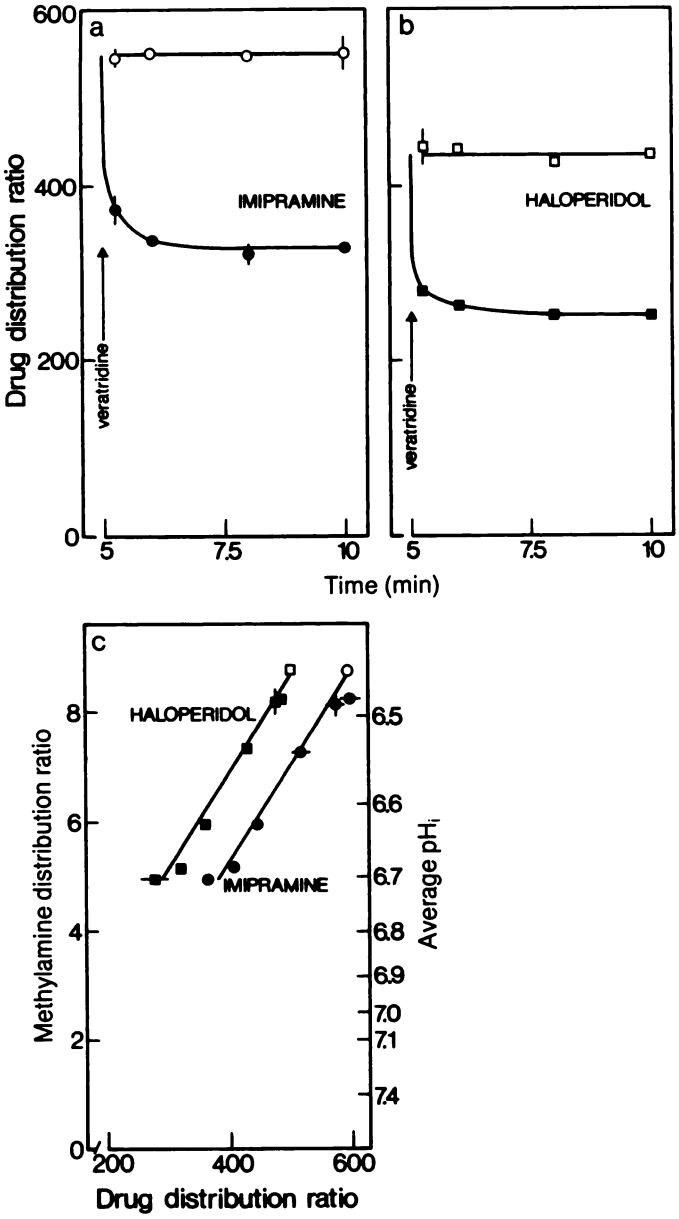

Fig. 5. Effect of veratridine on the distribution of imipramine, haloperidol, and methylamine. Rat cortical synaptosomes were equilibrated with 50 nM [^{3}H]imipramine (a) or 6 nM [^{3}H]haloperidol (b) in the NaCl/Tes medium. After 5 min, each tube received 10 μl of either saline (controls, *open symbols*) or veratridine in saline (final concentration, 10 μM, *filled symbols*). Measuring points refer to the time at which silicone oil centrifugation was initiated in the microfuge (10,000 × *g* for 1 min). The starting points of the falling curves were derived by extrapolation of the control curves to the time of veratridine addition. c, Synaptosomes were equilibrated with [^{14}C]methylamine or [^{3}H]imipramine (●) or [^{3}H]haloperidol (■) for 10 min in the presence of 0.1–30 μM veratridine. Samples lacking veratridine are indicated by *open symbols*.

TABLE 2

Effect of external Ca^{2+} activity on the veratridine-induced release of methylamine and imipramine

Rat cortical synaptosomes in the NaCl/Tes medium were equilibrated with 2 μM [^{14}C]methylamine or 50 nM [^{3}H]imipramine at the external Ca^{2+} activities indicated in the table, in the absence of added $MgCl_2$. After 5 min, veratridine was added and the incubation was continued for an additional 5 min. Controls received saline only.

pCa	Veratridine	Rise of pH$_i$	Imipramine distribution ratio
	μM		
4.9	0		573 ± 7
4.9	10	0.09 ± 0.01[a]	493 ± 3[a]
3.3	0		541 ± 2
3.3	10	0.21 ± 0.01[b]	419 ± 8[b]

[a] Significantly different from controls at pCa 4.9, $p < 0.005$.
[b] Significantly different from controls at pCa 3.3, $p < 0.005$, and veratridine-treated samples at pCa 4.9, $p < 0.01$.

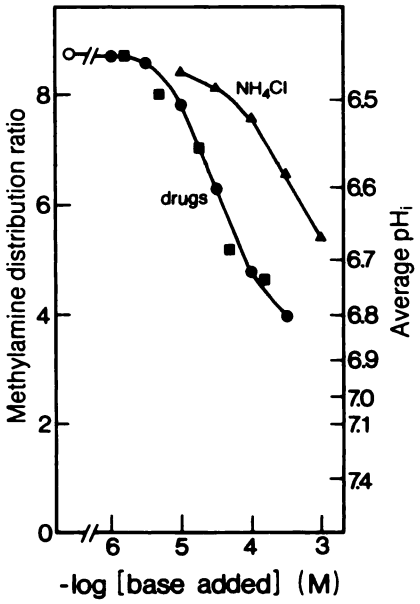

Fig. 6. Effect of imipramine and haloperidol on methylamine distribution. Rat cortical synaptosomes were incubated in the NaCl/Tes medium for 10 min with [^{14}C]methylamine and varying concentrations of unlabeled imipramine (●) or haloperidol (■), as indicated on the *abscissa*. Drug-free controls are indicated by the *open symbol*.

and augmentation of ΔpH enhanced uptake (Fig. 4). The simplest explanation of these findings is that imipramine and haloperidol diffuse in the uncharged form through the membrane. Once inside the cell, most of the uncharged amine combines with protons, the charged form being trapped in the cell. As the pH$_i$ (derived from methylamine distribution) is about 2 pH units below the pK_a of the drugs, only 1% of the entering base will remain uncharged. In accordance with properties of a permeant weak base, accumulation at high concentrations of imipramine and haloperidol raised the internal pH (Fig. 6). The release of dopamine observed at relatively high concentrations of imipramine (5) might be related to a weak base effect of the drug on the ΔpH of enclosed synaptic vesicles. As expected, the distribution of [^{3}H]methylimipraminium, a quaternary analogue of imipramine, did not respond to pH$_i$ changes in the presence of tetraphenylborate that mediated the uptake of the otherwise impermeant compound.[2]

A coupling of imipramine transport to H^+ gradients by a carrier mechanism was derived from findings showing that the concentration of imipramine in HeLa cells (27) and cultured neurons from rat cerebellum (28) is sensitive to reserpine, extracellular pH, and monensin. In contrast, in synaptosomes imipramine uptake was not impaired by 1 μM reserpine (Table 1), a concentration that completely blocked biogenic amine transport in synaptic vesicles (23, 24). The inhibition at 10 μM

[2] G. Schmalzing and W. Stelling, unpublished results.

reserpine can be attributed to the dissipation of the transmembrane H^+ gradient by this drug in synaptosomes (Table 1).

Passive distribution in accordance with the pH gradient has previously been noted with other basic drugs, for example nicotine in frog sartorius muscle (29) and procaine in sciatic nerve trunks (30). The 6- to 10-fold concentration of nicotine, atropine, trimethylamine, and procaine in isolated superior cervical ganglia of the rat has been attributed to an enclosed acidic compartment (31). Also, the concentration of desmethylimipramine in cultured human fibroblasts has been speculated to be due to trapping in acidic lysosomes (32).

Heterogeneity of pH in synaptosomes. The different pH_i values derived from weak acid and base distributions (see text under Results) are consistent with pH_i inhomogeneity. In agreement with electron micrographs (8), synaptosomes can be regarded as a system of three membrane-bound compartments consisting of cytosol, enclosed mitochondria, and enclosed synaptic vesicles. Mitochondria, which are alkaline inside, will exclude methylamine but concentrate DMO, whereas the acidic vesicles will do the opposite. DMO and methylamine partition yields only an average pH_i, giving the upper and lower limit, respectively, for the pH of the largest compartment, the cytosol. According to statistical analysis of electron micrographs, the average synaptosome encloses 73 synaptic vesicles (8). If the volume of the vesicles is 4% of the total volume (8) and the cytosolic pH is 7.0 (as in most other cells, see Ref. 1), the vesicular pH is 5.2, as calculated from the methylamine gradient of 8.5. According to this estimate, about 75% of the accumulated methylamine is located in the synaptic vesicles. The proportional release of dopamine and imipramine on dissipation of ΔpH with nigericin (Fig. 3, a and b) is consistent with a predominantly vesicular localization of the accumulated bases.

Contribution of drug binding to distribution. The 50- to 100-fold higher accumulation of imipramine and haloperidol compared with methylamine (Figs. 2–6) can be attributed to additional binding of the drugs to cellular membranes and proteins. It is known that antidepressants and neuroleptics are strongly bound to plasma proteins (33) and readily concentrate in lipid membranes, which can be regarded as solvents for the hydrophobic regions of the drugs. Seeman *et al.* (34) reported synaptosome membrane/buffer partition coefficients of 295 for imipramine and 200 for haloperidol. In contrast, methylamine has consistently been found not to be bound to cellular constituents (1, 17). Extrapolation of the straight lines in Figs. 2c and 5c to an pH_i of 7.4 suggests that the tissue/medium ratios of imipramine are in the range of 150 to 200 in the apparent absence of a ΔpH. Most of the entering drug will become bound and only a fraction of the accumulated drug exists in the free form. The fact that imipramine and haloperidol reduced ΔpH more efficiently than did NH_4Cl (Fig. 6) might also be related to the binding of the drugs to cellular constituents, favoring the influx of additional free base and consumption of H^+. From the increase of pH_i in Fig. 6 and the buffering capacity of synaptosomes of 57 mM/ΔpH (35), it can be calculated that, at a medium concentration of 100 μM imipramine, at least 16 mmol of H^+/1 liter of synaptosomes were consumed.

Irrespective of whether the ratio of unbound to total drug inside the cell remains constant, the internal free imipramine concentration will increase in proportion to the H^+ gradient. At a therapeutic free concentration of 30 nM (22), the free imipramine concentration will attain 75 nM in the cytosol (pH 7.0) and 5 μM in the vesicles (pH 5.2).

Depolarization-induced release of drugs. Depolarization-induced release of imipramine is in accordance with previous studies in brain slices (5–7). Daniels (5) concluded from the Ca^{2+} sensitivity of the release that at least part of the imipramine is from storage vesicles. On the other hand, the insensitivity to Ca^{2+} and reserpine, an inhibitor of vesicular transport of biogenic amines, observed in two other studies argued against an involvement of the vesicles (6, 7).

The present findings strongly suggest that the release of imipramine and haloperidol resulted from an increase of pH_i on depolarization (Fig. 5; Table 2). Exocytosis is a possible explanation for the rise in pH_i, because this process requires the co-release of the acidic contents of the vesicles. The Ca^{2+} dependence (Table 2) is also consistent with exocytosis. On the other hand, preliminary experiments with amiloride analogues suggest that depolarization of synaptosomes is associated with an activation of the Na^+/H^+ exchange system (36).

Passive, ΔpH-dependent concentration and subsequent release from excitable tissues on stimulation appear to occur with other lipophilic bases as well. For example, propranolol and atenolol have been reported to concentrate in synaptosomes and become released on depolarization (37). A passive coupling of the distribution of the β-adrenoreceptor-blocking drugs to ΔpH provides a ready explanation of these results (cf. Fig. 5c).

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