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ROLE OF IONS AND MEMBRANE POTENTIAL IN UPTAKE OF SEROTONIN INTO PLASMA MEMBRANE VESICLES FROM MOUSE BRAIN

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Abstract—Plasma membrane vesicle preparations from mouse cerebral cortex actively accumulated [^{3}H]serotonin upon the imposition of a K^+ gradient (in > out), a Na^+ gradient (out > in), and the presence of external Cl^-. Maximal stimulation of uptake by internal K^+ occurred at 15 mM and half-maximal stimulation at 2 mM. Internal K^+ did not enhance uptake merely via generation of a membrane potential because simultaneous parallel increases in internal and external K^+ concentration also stimulated uptake. External Cl^- increased serotonin uptake with a K_m of 18 mM and a Hill number of 1.0, suggesting a requirement for one chloride ion for transport. Uptake could not be driven by internal H^+ instead of K^+. Estimation of the membrane potential by the distribution of triphenylmethylphosphonium ion showed a modest effect of valinomycin (1–20 μM) in increasing the potential from −19 to −31 mV accompanied by an increase in serotonin uptake. Proton ionophores prevented this effect of valinomycin and, by themselves, reduced the potential to −6 mV, but did not affect serotonin transport. A model is proposed for serotonin transport in brain plasma membrane vesicles that is similar to the model for porcine blood platelet vesicles as far as electroneutrality and stimulation by K^+, Na^+, and Cl^- are concerned, but that is different in substitution of internal H^+ for K^+.

Uptake of serotonin into plasma membrane vesicles from rodent brain involves a Na^+-dependent component sharing many properties with that observed in synaptosomes, such as a K_m‡ of 0.1 μM, stimulation by external Cl^-, and inhibition by classical blockers of serotonin uptake [1]. Plasma membrane vesicles enable us to study the properties of transport in the absence of storage granules and to control the composition of the internal and external medium [2–5].

The present study explores in more detail the role of ions and the membrane potential in the uptake of serotonin into plasma membrane vesicles from mouse cerebral cortex. With respect to K^+ we address the question as to whether the imposition of a K^+ gradient (in > out) stimulates uptake by the generation of a membrane potential, or by direct interaction of K^+ with the carrier inside the vesicle as has been reported for plasma membranes from porcine blood platelets [3, 6]. Experiments are reported exploring the possibility that serotonin transport can be driven with internal protons instead of potassium ions as described for platelet plasma membrane vesicles [7]. Furthermore, transport is measured as a function of internal K^+ and external Cl^- and an estimate is made of the number of potassium and chloride ions required for optimal transport. Finally, estimates of the membrane potential by the distribution of $TPMP^+$ under various conditions are compared with rates of serotonin uptake in order to assess whether uptake is electrogenic or electroneutral.

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‡ Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; K_m, substrate concentration sufficient to produce a half-maximal transport velocity; and $TPMP^+$, triphenylmethylphosphonium ion.

MATERIALS AND METHODS

Transport of serotonin. Plasma membrane vesicles were prepared from purified synaptosomal fractions from cerebral cortex of male and female BALB/cBy mice, 8–12 weeks of age, and stored in frozen aliquots at −70° as described previously [1]. Aliquots were rapidly thawed at 37° and diluted in 5 vol. of loading solution (at 37°, composition as indicated in tables and figures) resulting in a final protein concentration of approximately 1.8 mg/ml. Except for the pH experiments (see next paragraph), the uptake assay was started by adding 80 μl of the loading suspension to 610 μl of external solution and immediate mixing. The external solution consisted of 540 μl of buffer (composition as indicated in tables and figures), 50 μl of water with or without inhibitor, and 20 μl [^{3}H]serotonin (20–28 Ci/mmol, New England Nuclear, Boston, MA) (final concentration 16–18 nM) at 37°. After 10 sec, 4 ml of ice-cold 0.15 M NaCl was added and the mixture was filtered through a Millipore filter (DAWP 0.65 μm) presoaked in 0.05% (w/v) poly-L-lysine (mol. wt 15,000–30,000, Sigma Chemical Co., St Louis, MO). The filter was washed three times with 4 ml of ice-cold 0.15 M NaCl and placed in a scintillation vial. Determination of radioactivity by liquid scintillation counting and measurement of protein content by the method of Lowry was done as described by us previously [8]. The external K^+ and Cl^- concentrations shown in the figures are final concentrations present during

the uptake assay. Total uptake values were estimated in two ways with the same results: (1) by deducting uptake at zero-time (vesicles added after the stopping solution) or (2) by deducting filter-bound radioactivity observed after filtration of assay mixtures containing loading buffer instead of vesicles. Unless indicated otherwise, nonspecific uptake was defined with 50 μM cocaine (Sigma Chemical Co.). All determinations were performed in triplicate. Filter binding was always equal for total and nonspecific uptake.

For the pH experiments (Table 1), uptake was started by adding 120 μl of the loading suspension (*ca.* 1.2 mg protein/ml) to 600 μl of external solution. The internal pH was taken as the pH of the potassium or lithium phosphate buffer used for the loading solution. The external pH was achieved by dilution of 1 vol. of loading solution with 5 vol. of external solution (0.1 M NaCl, 0.05 M NaH_2PO_4, 1 mM $MgSO_4$) of a pH that was experimentally chosen to give a final pH of 7.2. For instance, in the case of an internal pH of 5.6, the external solution was determined to need a pH of 8.9 in order to give a pH of 7.2 upon mixing of loading and external solutions in the appropriate amounts.

Membrane potential. The uptake of $TPMP^+$ was measured as described above by substituting [^{3}H]$TPMP^+$ (35.4 Ci/mmol, New England Nuclear) in place of [^{3}H]serotonin. The concentration of [^{3}H]$TPMP^+$ was 5 nM, and with the addition of unlabeled $TPMP^+$ a final concentration of 80 μM was attained. The assays were terminated by addition of 4 ml of ice-cold 0.15 M NaCl and filtration through Whatman GF/B filters presoaked with 0.05% (w/v) poly-L-lysine (Sigma Chemical Co., mol. wt 15,000–30,000); the filters were washed twice with 4 ml of ice-cold 0.15 M NaCl. For every condition tested, there was a control consisting of vesicles diluted in external solution of the same composition as the loading solution (zero membrane potential) in order to calculate the concentration of $TPMP^+$ accumulated in the intravesicular fluid (4 μl/mg of protein in our preparation [1]) as a result of the membrane potential. The zero potential control values were not affected by valinomycin or gramicidin; a slight increase was noted, and corrected for, with 2,4-dinitrophenol and CCCP. The membrane potential (E_m) at 37° was computed from $E_m = -61.5 \log (TPMP^+_{in}/TPMP^+_{out})$ [9].

Data analysis. The IC_{50} values were computed with the ALLFIT program developed by De Lean *et al.* [10]. The Hill coefficient for the stimulation of uptake by K^+ or Cl^- was calculated by standard linear regression techniques from a plot of $\log[V/(V - v)]$ versus log[ion] in which V is the maximal uptake. V and the K_m for chloride ion were estimated from the relationship between v and $[Cl^-]$ by nonlinear least square techniques [11]. V for K^+ was taken as the velocity observed at 15 mM $[K^+]_{in}$ (see Fig. 1).

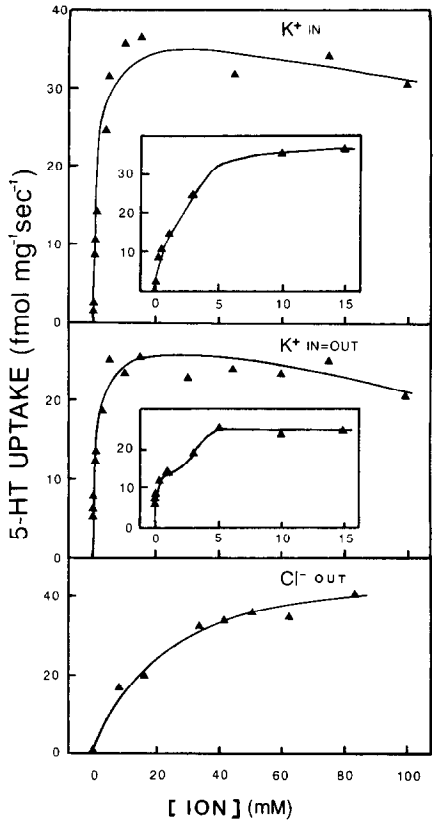

Fig. 1. Effect of varying internal and external K^+ and external Cl^- concentration on specific uptake of [^{3}H]serotonin. *Top panel*: Vesicles, equilibrated in the indicated concentration of KH_2PO_4 with enough sucrose to make the loading solution 0.2 osmolar, pH 6.7, containing 1 mM $MgSO_4$, were diluted into 0.1 M NaCl, pH 6.7, containing 1 mM $MgSO_4$ and 18 nM [^{3}H]serotonin. *Middle panel*: Vesicles, equilibrated in the indicated concentration of KH_2PO_4 with enough sucrose to make the loading solution 0.4 osmolar, pH 6.7, containing 1 mM $MgSO_4$, were diluted into 0.1 M NaCl, pH 6.7, containing the indicated concentration of KCl with enough Tris–HCl to make the external solution 0.4 osmolar, 1 mM $MgSO_4$, and 18 nM [^{3}H]serotonin. Control incubations with $[K^+]_{in} = 100$ mM and no added external K^+ showed that the absolute values in the experiment in this panel can be compared with those in the top panel. *Bottom panel*: Vesicles, equilibrated in 0.1 M KH_2PO_4, pH 7.5, containing 1 mM $MgSO_4$, were diluted into external solution, pH 7.5, resulting in the indicated final concentration of NaCl with enough NaH_2PO_4 to make the total Na^+ concentration 83 mM. The final concentration of $MgSO_4$ was 1 mM and of [^{3}H]serotonin 18 nM. The K_m for Cl^- was estimated to be 18 mM. Nonspecific uptake has been subtracted. Points represent measurements in triplicate obtained in two separate experiments. *Insets*: a more detailed graph of the initial portion of the curves.

RESULTS

Varying internal and external K^+ and Cl^- concentrations. Internal K^+ increased the rate of serotonin uptake (Fig. 1 top panel). Half-maximal stimulation occurred at a K^+ concentration of approximately 2 mM, and the plateau was reached at 15 mM K^+. When the same experiment was carried out in the absence of a K^+ gradient ($[K^+]_{in} = [K^+]_{out}$), the plateau was reached at comparable concentrations of K^+, whereas the maximal uptake rate was lower than observed in the presence of a K^+ gradient [25 and 35 fmol·(mg protein)$^{-1}$·sec^{-1}, respectively, compare Fig. 1 middle and top panel].

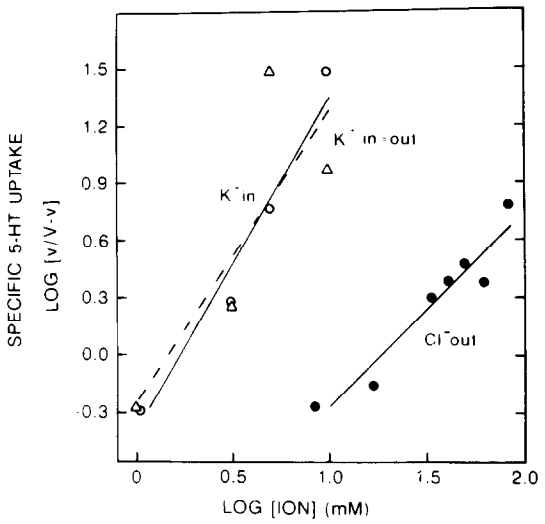

Fig. 2. Hill plot of $\log[v/(V-v)]$ versus log[ion]. V is the maximal uptake, estimated at 36.6 for K^+_{in}, 25.8 for $K^+_{in=out}$, and 48 fmol·(mg protein)$^{-1}$·sec^{-1} for Cl^-. For the K^+ data, K^+_{in}-independent uptake of serotonin (uptake at zero concentration of K^+_{in}, see Fig. 1) was deducted. Straight lines were fitted by standard least square linear regression techniques. The points shown represent measurements in triplicate obtained in two separate experiments.

This is similar to results obtained with porcine blood platelet vesicles, indicating that external K^+ inhibits serotonin uptake but does not prevent it [6]. Estimates of the Hill number for uptake stimulated by K^+_{in} (Fig. 2, open circles) and $K^+_{in=out}$ (Fig. 2, triangles) concentrations between 1 and 10 mM were 1.7 and 1.5 respectively.

Serotonin inhibited the Na^+-dependent uptake of [^{3}H]serotonin in the presence of internal K^+ concentrations of 100 and 3 mM with IC_{50} values of 42 ± 14 and 19 ± 3 nM respectively (mean ± range for two independent experiments) (data not shown).

External Cl^- increased the rate of serotonin uptake (Fig. 1 lower panel). The plot of uptake versus concentration of Cl^- showed the hyperbolic curve expected if one Cl^- were involved in optimal uptake of serotonin. Indeed, the Hill number was 1.0 (Fig. 2 closed circles). Maximally stimulated uptake was estimated to be 48 fmol · (mg protein)$^{-1}$·sec^{-1}, and the K_m for Cl^- was 18 mM.

Varying internal and external pH. In comparing various conditions (Table 1), brain vesicles loaded in 0.1 M KH_2PO_4, pH 7.5, and diluted in an external medium of 0.1 M NaCl, pH 7.5 (condition A), or vesicles loaded with pH 7.2 and diluted in 0.1 M NaCl, 0.05 M NaH_2PO_4, pH 7.2 (B), showed the highest cocaine-sensitive uptake of serotonin. When NaH_2PO_4 was used instead of KH_2PO_4, no uptake was observed because of the absence of internal K^+ and the absence of a Na^+ gradient ($[Na^+]_{in} = [Na^+]_{out}$) (C). Likewise, with LiH_2PO_4 instead of KH_2PO_4 at pH 7.2, uptake was extremely low (D). With lithium phosphate instead of potassium phosphate as the loading solution for the vesicles, lowering the internal pH from 7.2 to 6.7 (E) and 6.2 (F) did not result in appreciable uptake, indicating that H^+ cannot replace K^+ to enable uptake. In the absence of a ΔpH, with $pH_{in} = pH_{out} = 5.6$, transport of serotonin was minimal, both in the presence (G) and absence (H) of internal K^+. Without a ΔpH, and with internal K^+, uptake was observed at pH 6.7 (I) and increased further by raising the pH to 7.2 (B) and 7.5 (A). Again, lowering the internal pH from 7.2 to 5.6 while holding the external pH at 7.2 reduced uptake of serotonin into K^+-loaded vesicles (B,J,K).

Na^+-dependent serotonin uptake was not reduced by (+)-lysergic acid diethylamide (L) or spiroperidol (N) at concentrations (0.1 and 1 μM, respectively, Table 1) that fully block serotonin receptors [12, 13]. In contrast, 50 μM cocaine (M), a classical inhibitor of serotonin uptake [14], virtually eliminated the Na^+-sensitive uptake of [^{3}H]serotonin defined by substituting LiCl in place of NaCl in the external medium.

Varying membrane potential. Different vesicle preparations showed somewhat different distributions of $TPMP^+$. Figure 3 presents an average obtained with three different vesicle preparations. In the absence of valinomycin, the ratio of the intravesicular $TPMP^+$ over external $TPMP^+$ concentration rose from 1.4 at 10 sec to 2.1 at 2 min, was 2.2 at 5 min, and declined to 1.7 at 20 min. These values correspond to membrane potentials of approximately −9 mV, −20 mV, −21 mV and −14 mV respectively. Valinomycin, an ionophore relatively selective for K^+, at 2.5 μM resulted in a marked increase in the accumulation of $TPMP^+$ during the initial 2-min period corresponding to a membrane potential of approximately −35 mV; at later times the values in the presence of valinomycin returned to those measured in its absence (Fig. 3). In subsequent experiments, the 2-min interval was chosen to study the effect of the proton conductors 2,4-dinitrophenol and CCCP on the valinomycin-induced increase in the membrane potential (Table 2). The effect of valinomycin varied somewhat between different batches of vesicle preparations; in the batch shown in Table 2, valinomycin (1 μM) gave a modest increase in the concentration ratio of $TPMP^+$ from 2.0 to 2.6. Co-presence of 2,4-dinitrophenol prevented the valinomycin-induced increase, whereas 2,4-dinitrophenol alone reduced the concentration ratio to 1.2 (Table 2). In parallel experiments with CCCP, valinomycin at 2.5 μM was as effective as at 1 μM, and CCCP had effects similar to 2,4-dinitrophenol (Table 2). A relatively greater effect on the membrane potential was noted with 20 μM valinomycin, and again the effect was reversed by CCCP. Collapsing ion gradients with gramicidin, an ionophore for both K^+ and Na^+, resulted in an internal $TPMP^+$ concentration equal to the external concentration. The presence of the SCN^- anion in addition to Cl^- did not increase the membrane potential, as observed by Rudnick and Nelson [3] in porcine platelet vesicles.

The effect of valinomycin and CCCP on uptake of [^{3}H]serotonin was measured in the same vesicle preparations used for estimation of the membrane potential (Table 2). The uptake of serotonin was enhanced by valinomycin (2.5 μM), but, in contrast

Table 1. Effects of various internal and external solutions on uptake of [^{3}H]serotonin

Internal medium	External medium	5-HT uptake [fmol·(mg protein)$^{-1}$·sec^{-1}]
A. 0.1 M KH_2PO_4, pH 7.5	0.1 M NaCl, pH 7.5	34.6 ± 1.4 (5)
B. 0.1 M KH_2PO_4, pH 7.2	0.1 M NaCl, 0.05 M NaH_2PO_4, pH 7.2	38.9 ± 4.2 (5)
C. 0.1 M NaH_2PO_4, pH 7.5	0.1 M NaCl, pH 7.5	0 (1)
D. 0.1 M LiH_2PO_4, pH 7.2	0.1 M NaCl, 0.05 M NaH_2PO_4, pH 7.2	6.0 ± 3.5 (3)
E. 0.1 M LiH_2PO_4, pH 6.7	0.1 M NaCl, 0.05 M NaH_2PO_4, pH 7.2	4.1 ± 3.9 (3)
F. 0.1 M LiH_2PO_4, pH 6.2	0.1 M NaCl, 0.05 M NaH_2PO_4, pH 7.2	7.1 ± 5.3 (2)
G. 0.1 M KH_2PO_4, pH 5.6	0.1 M NaCl, pH 5.6	1.0 ± 0.2 (2)
H. 0.1 M LiH_2PO_4, pH 5.6	0.1 M NaCl, pH 5.6	1.1 ± 0.1 (2)
I. 0.1 M KH_2PO_4, pH 6.7	0.1 M NaCl, pH 6.7	18.8 ± 1.6 (2)
J. 0.1 M KH_2PO_4, pH 6.7	0.1 M NaCl, 0.05 M NaH_2PO_4, pH 7.2	40.4 ± 1.8 (2)
K. 0.1 M KH_2PO_4, pH 5.6	0.1 M NaCl, 0.05 M NaH_2PO_4, pH 7.2	22.8 ± 0.8 (2)
L. 0.1 M KH_2PO_4, pH 7.5	0.1 M NaCl, pH 7.5, 0.1 μM (+)-LSD*	34.4 (1)
M. 0.1 M KH_2PO_4, pH 7.5	0.1 M NaCl, pH 7.5, 50 μM cocaine	2.9 (1)
N. 0.1 M KH_2PO_4, pH 7.5	0.1 M NaCl, pH 7.5, 1 μM spiroperidol	35.3 (1)

Specific uptake of [^{3}H]serotonin (5-HT) (16 nM) was determined as described in Materials and Methods. Nonspecific uptake was defined with 50 μM cocaine (A–K) or with LiCl substituted for NaCl in the external medium (L–N). The reaction was started by adding vesicles in internal medium to external medium of the composition shown with the indicated final pH. For conditions L, M, and N, the same results were obtained by preincubating vesicles in the loading solution with the drug for 20 min. All solutions included 1 mM $MgSO_4$. The results are the average of N (indicated in parentheses) determinations, each performed in triplicate, ± SE or range where N = 2.

* Lysergic acid diethylamide.

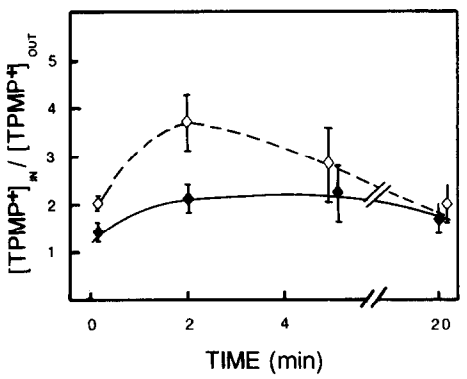

Fig. 3. Effect of valinomycin on distribution of $TPMP^+$. Vesicles equilibrated in 0.1 M KH_2PO_4, pH 7.5, containing 1 mM $MgSO_4$, were diluted into 0.1 M NaCl, pH 7.5, containing 1 mM $MgSO_4$, 80 μM [^{3}H]$TPMP^+$, and 2.5 μM valinomycin where indicated. For each condition there was a control consisting of vesicles diluted into external solution of the same composition as the loading solution in order to compute the amount of [^{3}H]$TPMP^+$ accumulated due to the membrane potential. Key: (◆——◆) without valinomycin; and (◇— — —◇) with valinomycin. Points are the average ± SE (vertical bar) of three to five determinations, each carried out in triplicate. Three different batches of vesicle preparations were used.

to the membrane potential, was not reduced to the control value by the co-presence of CCCP. In addition, CCCP by itself did not decrease serotonin uptake, unlike the effect of CCCP and 2,4-dinitrophenol on the membrane potential (Table 2). The increase in uptake was greater with 20 μM valinomycin than with 2.5 μM valinomycin, but again CCCP did not prevent it. Gramicidin abolished serotonin uptake, as reported previously [1], along with intravesicular accumulation of $TPMP^+$.

DISCUSSION

Uptake: electrogenic or electroneutral? When brain vesicles loaded in 0.1 M potassium phosphate buffer were diluted into 0.1 M NaCl medium, the concentration ratio of $TPMP^+$ indicated a membrane potential of approximately −16 to −23 mV (Table 2). To our knowledge, there are no reports of membrane potentials in plasma membrane vesicles from brain tissue to compare our value to. Plasma membrane vesicles from porcine platelets are known to have no potential, presumably because of their low permeability to K^+ [3]. The value found in our preparation was appreciably less negative than the −58 mV to −87 mV potential observed in freshly prepared preparations of synaptoneurosomes [15], synaptosomes [16, 17], slices [18], and in neuro-

blastoma-glioma hybrid cells [19]. This suggests that the membranes of the plasma membrane vesicles were only slightly more permeable to K^+ and Cl^- than to Na^+ and phosphate ions. Consonant with this is the observation that the increase in the intravesicular concentration of $TPMP^+$ by valinomycin was only transient (Fig. 3). The fall in internal $TPMP^+$ concentration after 2 min could be due to influx of sodium ions offsetting the negative membrane potential resulting from the valinomycin-induced increase in potassium permeability. It has been noted previously that plasma membrane vesicles from brain tissue are not very impermeable to sodium ions [20]. Because the equilibration of $TPMP^+$ takes some time, the actual membrane potential immediately after mixing the plasma membrane vesicles with the external medium is not known. However, the figures in Table 2 can be used as an underestimate in the following manner. After adding the vesicles in loading solution ($5/6 \times 100 = 83$ mM K^+) to the incubation medium (80 μl to 610 μl), the external K^+ concentration is 10 mM. With an extremely small intravesicular volume of approximately 0.6 μl per ml of incubation medium, we can ignore the effect of changes in the intravesicular K^+ concentration on the extravesicular K^+ concentration. At a control membrane potential of -23 mV, the ratio of $[K^+]_{in}/[K^+]_{out}$ can be calculated according to the Nernst equation as 2.4, indicating an internal K^+ concentration of $2.4 \times 10 = 24$ mM. With 20 μM valinomycin, the internal K^+ concentration can be calculated as $3.2 \times 10 = 32$ mM. Because at these K^+ concentrations uptake of serotonin is already maximal (Fig. 1), the increase in serotonin uptake by valinomycin (Table 2) cannot be explained by the increase in internal K^+, demonstrating, in principle, the possibility of distinguishing effects on membrane potential from effects on K^+ gradients in brain membranes that are permeable to K^+. In the present experiments, however, evidence against implication of the membrane potential in uptake of serotonin comes from experiments employing proton conductors (Table 2). CCCP and 2,4-dinitrophenol reduced the normal membrane potential and the valinomycin-induced increase in the potential. CCCP, however, had no effect on serotonin uptake under comparable conditions. These observations suggest that serotonin uptake into plasma membrane vesicles from brain is electroneutral like that in porcine blood platelet vesicles [3]. The present experiments show that internal K^+ did not stimulate serotonin uptake merely because it generated a membrane potential by the K^+ gradient (in > out): increasing the internal K^+ concentration stimulated uptake also when the external K^+ concentration was increased in parallel (no K^+ gradient) (Fig. 1 middle panel). In addition, the increase in serotonin uptake with increasing internal K^+ concentration (Fig. 1, top panel) was not due to the application of a progressively greater K^+ gradient, because the external K^+ concentration was 0.12 times that of the loading solution over the entire concentration range. It should be mentioned also that serotonergic vesicles are only a fraction of the total vesicles to which $TPMP^+$ has access, and that the measured membrane potential is an average for all vesicles. It is therefore difficult to firmly rule out a connection between membrane potential and uptake of serotonin.

Can internal H^+ replace K^+? A physiological role of ΔpH-driven serotonin uptake is probably minor because the internal K^+ concentration is high. However, stimulation of serotonin uptake by internal protons in the absence of internal K^+, as is the case in porcine platelet vesicles, would show that H^+ can replace K^+ in binding to the carrier at the internal face enabling counter-transport of H^+ [7]. The present results show that plasma membrane vesicles from mouse brain differ from those from porcine blood platelets in that internal protons cannot replace potassium ions in enabling uptake of serotonin. The decrease in serotonin uptake as a result of lowering the internal pH (Table 1, B,J,K) would be expected if the protonated form of serotonin were translocated, because it would increase the concentration of protonated serotonin inside. However, it is more difficult to explain the decrease in uptake upon lowering the internal pH and external pH simultaneously (Table 1, A,I,G). Possibly, regardless of what form of serotonin is translocated, there is a component in the transport process in mouse brain vesicles that is pH-sensitive. In this context it is of interest that, also in the presence of internal K^+, a lowering of the internal pH reduced uptake (Table 1, B,J,K), in contrast to the situation in porcine blood platelet vesicles [7]. It is possible that a neutral internal pH is necessary for the binding of K^+ to the carrier before return, or for the transition between the two conformational states of the carrier protein (see below).

Model. The present results for plasma membrane vesicles from mouse brain can be accommodated by the model developed for serotonin uptake into porcine blood platelet vesicles [6]. In this model, the first step in transport is the binding of Na^+, serotonin, and Cl^- to sites on the transporter exposed to the membrane exterior. Then, Na^+, serotonin, and Cl^- are translocated to the interior (with rate constant k_{in}) and dissociate from the carrier. Finally, in the rate-limiting step, the transporter returns (with rate constant k_{out}) to the state with the binding sites for Na^+, serotonin, and Cl^- exposed to the membrane exterior. The major pathway for the return of the carrier is stimulated by K^+; internal K^+ binds to the carrier and is subsequently translocated to the membrane exterior [6]. Our previous results suggest that two sodium ions are translocated with serotonin in mouse brain plasma membrane vesicles [1] as opposed to one sodium in porcine blood platelet vesicles [21]. In the latter case, imipramine binding requires two sodium ions [21]. The carrier for serotonin in brain resembles that for GABA [20] and glutamate [22] that also require two to three and two sodium ions respectively. The present results for the brain serotonin transporter and Cl^- are in agreement with co-transport of one chloride ion (Fig. 2), as is the case for γ-aminobutyric acid transport [20]. Therefore, if serotonin is transported in the protonated form (the predominant one at neutral pH), and if the net transport process is electroneutral, one has to postulate the counter-transport of two potassium ions. In agreement with this is the Hill

Table 2. Membrane potential of and uptake of [^{3}H]serotonin into plasma membrane vesicles under various conditions.

External medium	$[TPMP^+]_{in}/[TPMP^+]_{out}$	Potential (mV)	5-HT uptake [fmol·(mg protein)$^{-1}$·sec^{-1}]
0.1 M NaCl	2.0 ± 0.2 (2)	−19 ± 2 (2)	
0.1 M NaCl + 1 μM valinomycin	2.6 ± 0.1 (2)	−26 ± 1 (2)	
0.1 M NaCl + 1 μM valinomycin + DNP	2.0 ± 0.1 (2)	−19 ± 1 (2)	
0.1 M NaCl + DNP	1.2 ± 0.1 (2)	− 5 ± 0 (2)	
0.1 M NaCl	2.0 ± 0.2 (2)	−19 ± 2 (2)	33.0 ± 1.4 (4)
0.1 M NaCl + 2.5 μM valinomycin	2.6 ± 0.1 (2)	−26 ± 1 (2)	44.8 ± 2.3 (4)
0.1 M NaCl + 2.5 μM valinomycin + CCCP	2.3 ± 0.1 (2)	−22 ± 1 (2)	43.7 ± 2.1 (4)
0.1 M NaCl + CCCP	1.3 ± 0.0 (2)	− 7 ± 0 (2)	30.0 ± 4.9 (4)
0.1 M NaCl	2.4 ± 0.1 (2)	−23 ± 1 (2)	32.4 ± 2.4 (2)
0.1 M NaCl + 20 μM valinomycin	3.2 ± 0.3 (2)	−31 ± 3 (2)	53.9 ± 4.4 (2)
0.1 M NaCl + 20 μM valinomycin + CCCP	2.5 ± 0.3 (2)	−25 ± 3 (2)	47.8 ± 1.2 (2)
0.1 M NaCl	1.9 ± 0.2 (3)	−17 ± 2 (3)	28.4 ± 3.2 (4)*
0.1 M NaCl + gramicidin	0.8 ± 0.1 (3)	2 ± 3 (3)	1.8 ± 1.3 (4)*
0.1 M NaCl	1.8 ± 0.0 (2)	−16 ± 0 (2)	
0.05 M NaCl + 0.075 M NaSCN	1.8 ± 0.1 (2)	−16 ± 1 (2)	

The distribution of $TPMP^+$, the membrane potential, and the specific uptake of [^{3}H]serotonin (5-HT) were determined as described in Materials and Methods. The reaction was started by adding vesicles in internal medium to external medium of the composition shown containing [^{3}H]$TPMP^+$ (80 μM) or [^{3}H]5-HT (17 nM) and stopped 2 min or 10 sec, respectively, later. Gramicidin (50 μM) was present for 20 min in the loading solution before the assays; valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (5 μM), and 2,4-dinitrophenol (DNP) (1 mM) were present in the external medium only. The results are the average of N (indicated in parentheses) determinations, each performed in triplicate, ± SE or range where N = 2. The internal medium consisted of 0.1 M KH_2PO_4.

* Includes two observations in triplicate from O'Reilly and Reith [1].

plot for K^+ concentrations of 1 mM and up (Fig. 2). However, for lower K^+ concentrations the Hill plot deviated from linearity, perhaps because the internal K^+ concentration is somewhat higher than the calculated value because of the contribution of residual endogenous K^+. As a special case of the more general model of Levine *et al.* [23] it can be derived (see also [6]) that

$$K_m = K_S/[1 + (k_{in}/k_{out})],$$

in which K_m is the apparent affinity of the substrate S in the transport process, and K_S the true equilibrium dissociation constant for serotonin binding to the carrier at the outside. The constant k_{out} is, in fact, composed of two terms, k^1_{out} for the return of the carrier in the absence of K^+ and k^2_{out}, a relatively greater rate constant for the return in the presence of K^+. If k_{out} is rate-limiting ($k_{out} < k_{in}$), it follows that $K_m < K_S$, and that an increase in k_{out} will result in an increase in K_m. Thus, internal potassium ions, by accelerating the return of the carrier (increasing the contribution of k^2_{out} to k_{out} making the value of k_{out} greater), are expected to increase the K_m. Indeed, the IC_{50} of serotonin in inhibiting the sodium-dependent uptake of [^{3}H]serotonin was increased from 19 to 42 nM by raising the internal K^+ concentration from 3 to 100 mM. In this context, it should be pointed out that the observed K_m for Cl^- in the transport process (18 mM, Fig. 2) is smaller than the true equilibrium dissociation constant of Cl^-, K_{Cl}, if the return of the carrier is rate-limiting ($k_{out} < k_{in}$) according to the same equation that applies to the affinity of serotonin. More definite evidence on the stoichiometry of the substrate and ions in the serotonin translocation process has to come from future studies measuring fluxes of ions along with the movement of serotonin. However, it may be necessary first to purify the serotonin transporter and to incorporate it into liposomes to prevent influx of ions unrelated to the translocation process [20].

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