Effects of N-ethylmaleimide on 5-hydroxytryptamine transport and sodium content in rabbit platelets

¹R. Wölfel, T. Halbrügge & K.-H. Graefe

Institut für Pharmakologie und Toxikologie der Universität Würzburg, Versbacher Straße 9, D-8700 Würzburg, F.R.G.

1 The present study analysed the mechanism underlying the inhibitory action of N-ethylmaleimide (NEM) on the 5-hydroxytryptamine (5-HT) uptake by blood platelets.

2 Rabbit platelets suspended in protein-free buffer were first preincubated for 45 min in the absence and presence of NEM (20 to 160 μM) or ouabain (0.5–2.0 μM) and then either analysed for their Na^+ and K^+ content or incubated (15 s) with various concentrations of [^3H]-5-HT (0.13–4.03 μM) to determine K_m and V_{max} for 5-HT uptake.

3 Both NEM and ouabain produced concentration-dependent decreases in V_{max} with IC_{50} values of 52 and 0.58 μM , respectively. Neither drug changed K_m significantly.

4 Both NEM and ouabain increased the Na^+ and decreased the K^+ content of platelets in a concentration-dependent manner.

5 There was a linear correlation between V_{max} (expressed in % of control) and the reciprocal cellular Na^+ content, with the results for both drugs falling onto one and the same regression line ($r = 0.992$; $n = 8$). This regression showed that an increase in Na^+ content by 69% sufficed to reduce V_{max} by 50%.

6 At concentrations that reduced 5-HT uptake by about 60%, neither NEM nor ouabain altered the potency of imipramine for inhibition of 5-HT uptake.

7 Hence, NEM inhibits 5-HT transport by inhibiting the Na^+/K^+ -ATPase and not by a direct interaction with the 5-HT carrier. The consequential increase in the intracellular Na^+ concentration reduces the transmembrane Na^+ gradient and, therefore, hinders 5-HT inward transport. This action of the drug does not affect the ability of the carrier to bind 5-HT or imipramine.

Introduction

Several lines of evidence indicate that [^3H]-imipramine specifically binds to a membrane site which is associated with the 5-hydroxytryptamine (5-HT) carrier present in the plasma membrane of blood platelets and 5-hydroxytryptaminergic neurones (Raisman *et al.*, 1979; Briley *et al.*, 1979; Talvenheimo *et al.*, 1979; Rehavi *et al.*, 1980; Langer *et al.*, 1980; 1981). Since the inhibition by imipramine of 5-HT transport is competitive in nature (Born *et al.*, 1972; Tuomisto, 1974; Marcusson *et al.*, 1986; Wölfel *et al.*, 1988) and since 5-HT competitively displaces [^3H]-imipramine specifically bound to plasma membranes of platelets (Talvenheimo *et al.*, 1979; Marcusson & Tiger, 1988) and brain tissue (Marcusson *et al.*, 1986), it would seem reasonable to assume that imipramine binds to the substrate

recognition site of the 5-HT carrier or else to a carrier site whose occupation mutually excludes 5-HT from entering transport. However, the finding that substrates of the carrier (e.g., 5-HT and tryptamine) slow down the dissociation of bound [^3H]-imipramine (Sette *et al.*, 1983; Wennogle & Meyerson, 1983; Segonzac *et al.*, 1985) led to the suggestion that the site involved in [^3H]-imipramine binding and the carrier protein are distinct, but allosterically coupled entities. This 'two-site hypothesis' of imipramine action, which was challenged by others (Marcusson *et al.*, 1986; Marcusson & Tiger, 1988; Humphreys *et al.*, 1988), states that the site labelled with [^3H]-imipramine represents a receptor which, when occupied, modulates 5-HT transport by an allosteric interaction with the carrier.

Recently, Meyerson *et al.* (1987) reported that N-ethylmaleimide (NEM), an agent known to alkylate

¹ Author for correspondence.

sulphydryl groups, inhibits 5-HT uptake by human platelets in a concentration-dependent manner without affecting [^3H]-imipramine binding to these cells. These results were interpreted as indicating that the 5-HT carrier is more susceptible to alkylation by NEM than is the [^3H]-imipramine binding site and, hence, were taken to support the 'two-site hypothesis' described above. While this is one possible explanation, there is an alternative one. NEM also inactivates the Na^+/K^+ -ATPase and, therefore, reduces Na^+ pump activity (Akera & Brody, 1978). Consequently, NEM may well indirectly inhibit 5-HT transport by reducing the transmembrane Na^+ gradient which is the main force driving 5-HT uptake (Sneddon, 1969; Rudnick, 1977). If the Na^+/K^+ -ATPase were more susceptible to alkylation by NEM than the 5-HT carrier, the results of Meyerson *et al.* (1987) could be explained without the need to postulate a binding site for imipramine other than the 5-HT carrier.

The experiments described here were carried out to test this possibility. Ouabain was included in the study as a 'positive' control. This drug was previously shown to inhibit 5-HT uptake by reducing the transmembrane Na^+ gradient (Sneddon, 1971). A preliminary account of the present results, which were obtained in rabbit platelets, was presented to the German Pharmacological Society (Wölfel, 1988).

Methods

Rabbits of either sex (2.5–3.5 kg) were anaesthetized by 0.25 ml kg $^{-1}$ i.m. Hypnorm (Janssen). Blood (20–40 ml) was collected from an ear artery in plastic syringes containing trisodium citrate (final concentration 10.6 mM).

Preparation of washed platelets

Platelet-rich plasma (PRP) was obtained by centrifugation of blood at room temperature (175 g, 15 min). After the addition of prostaglandin E_1 - α -cyclodextrine (PGE_1 ; 30 nM), the PRP was centrifuged (1000 g, 8 min, 25°C). The resulting supernatant was discarded and the cells suspended in buffer of the following composition (in mM): NaCl 140.0, KCl 5.0, MgSO_4 1.2, glucose 10.1, Tris 10.0, PGE_1 0.00003, pargyline 0.01 (to inhibit monoamine oxidase); the buffer was saturated with O_2 and its pH adjusted to 7.4 with HCl. The platelets were kept in buffer for 30 min at 25°C, then centrifuged again (1000 g, 8 min, 25°C) and finally resuspended in buffer. The platelet count in the final platelet-in-buffer suspension (PRB) was determined by use of the Sysmex Platelet Counter PL 100 (Cologra, Lorch, F.R.G.); it amounted to $0.6\text{--}1.3 \times 10^6$ platelets μl^{-1} .

All experiments were carried out with freshly prepared PRB and, unless stated otherwise, at 37°C.

Uptake of tritiated 5-hydroxytryptamine ([^3H]-5-HT)

Samples of PRB were first preincubated for 45 min in the absence and presence of either NEM (20–80 μM) or ouabain (0.5–2.0 μM). Thereafter, the incubation with [^3H]-5-HT was started by diluting 100 μl PRB with 900 μl of buffer containing 33 nM [^3H]-5-HT (specific activity 20.0 Ci mmol $^{-1}$) and unlabelled 5-HT to make up final 5-HT concentrations ranging from 0.13 to 4.03 μM . Uptake of 5-HT was determined as described previously (Wölfel *et al.*, 1988). Briefly, after 15 s of incubation with [^3H]-5-HT, uptake was terminated by rapid dilution (with ice-cold buffer) followed by filtration. The radioactivity remaining on the filter was taken to reflect 5-HT uptake. The carrier-mediated component of uptake was determined from the difference between simultaneous measurements of uptake in the absence and presence of 10 μM imipramine, respectively (Wölfel *et al.*, 1988). To determine K_m and V_{max} for the inward transport of 5-HT, eight substrate concentrations ranging from 0.13 to 4.03 μM were tested in each experiment.

The inhibition of 5-HT uptake by imipramine (5–40 nM) was determined at a [^3H]-5-HT concentration of 30 nM. Platelets were then exposed to imipramine not only during the 15 s of incubation with [^3H]-5-HT, but also during the last 15 min of preincubation.

Determination of Na^+ and K^+ content of platelets

Samples of 750 μl PRB were preincubated for 45 min in the absence and presence of various concentrations of NEM (20–160 μM) or ouabain (0.5–2.0 μM). At 30 min during preincubation, the PRB was thoroughly mixed with 150 μl of a solution containing 250 mM sucrose, 1 mM sorbitol and trace amounts of [^{14}C]-sorbitol. After preincubation, the tubes containing PRB were chilled in a water bath maintained at 2–4°C. All the following steps were done at this temperature. The platelet suspension (750 μl) was transferred into conical 1.5 ml tubes by carefully layering it on top of 200 μl of 10% dextran 40 containing 75 mM sucrose. After centrifugation (7500 g, 3 min), a 100 μl portion of the supernatant buffer layer was analysed for its ^{14}C content, with the rest of the buffer as well as dextran 40 layer being discarded. The platelet pellet was washed with 1 ml of 250 mM sucrose, spun down again (7500 g, 2 min) and finally lysed in 1 ml of deionized water by ultrasonification. The resulting supernatant was then used to determine ^{14}C radioactivity by liquid scintil-

Figure 1 Effects of ouabain on the kinetic parameters for 5-hydroxytryptamine (5-HT) uptake (a) and the Na^+ and K^+ content (b) in rabbit platelets. Ordinates: (a) V_{max} in pmol per 10^8 platelets min^{-1} (●) and K_m in nM (○) for 5-HT uptake; (b) cellular Na^+ (○) and K^+ (●) content in nmol per 10^8 platelets. Abscissae: concentration of ouabain in μM during 45 min of preincubation. Shown are geometric means of 14 (a) and 12 (b) observations each; vertical bars show s.e.mean. Asterisks indicate significant differences from control values (H-test according to Kruskal and Wallis followed by Nemenyi's procedure): ** $P < 0.01$.

lation counting (LKB Rackbeta, Freiburg, F.R.G.) and to measure the Na^+ and K^+ content with an Eppendorf flame photometer (Hamburg, F.R.G.). After correction for cation contamination due to trapping of extracellular Na^+ and K^+ (estimated from the [^{14}C]-sorbitol space), the Na^+ and K^+ content of the platelets was expressed in nmol per 10^8 platelets.

Calculations

To determine values of K_m and V_{max} for 5-HT uptake, a least squares non-linear regression analysis

was used to fit a logistic function equivalent to Hill's equation to the saturation curves relating initial rates of uptake to the concentration of 5-HT (Wölfel *et al.*, 1988). The saturation curves obtained under the present experimental conditions exhibited mean apparent Hill coefficients that did not differ from unity. IC_{50} values for inhibition of 5-HT uptake by imipramine were obtained by fitting Hill's equation for multisite inhibition to inhibition curves (i.e., uptake in % of control vs. imipramine concentration) as described previously (Schömig & Bönisch, 1986). Regression lines ($y = a + bx$) were calculated according to conventional procedures (Sachs, 1984).

Statistics

Results are given as geometric means $\pm$ s.e.mean or, when stated in the text, together with their 95% confidence limits. The significance of differences between means was assessed by non-parametric procedures: Wilcoxon's signed rank test for paired observations; Friedman's test followed by the procedure of Wilcoxon and Wilcox for multiple comparisons of dependent observations; H-test according to Kruskal and Wallis followed by Nemenyi's procedure for multiple comparisons of independent observations (Sachs, 1984). Differences were taken to be statistically significant when $P < 0.05$.

Materials

The following were used: 5-hydroxytryptamine creatinine sulphate, pargyline hydrochloride, N-ethylmaleimide and ouabain octahydrate (Sigma, München, F.R.G.); imipramine hydrochloride (Ciba-Geigy, Basel, Switzerland); 5-[1,2- $^3\text{H}(\text{N})$]-hydroxytryptamine creatinine sulphate (NET 498; NEN, Dreieich, F.R.G.); dextran 40 (Rheomacrodex 10%; Schiwa, Glandorf, F.R.G.); Hypnorm (Janssen Pharmaceutica, Beerse, Belgium), prostaglandin E_1 - α -cyclodextrine (Schwarz Pharma, Monheim, F.R.G.).

Results

Effects of NEM and ouabain on K_m and V_{max} for 5-HT uptake and the cellular Na^+ and K^+ content

Preincubation of the platelets with ouabain (0.5–2.0 μM) caused a concentration-dependent decrease in the V_{max} of 5-HT uptake ($\text{IC}_{50} = 0.58 \mu\text{M}$), whilst leaving the value of K_m for 5-HT unchanged (Figure 1a). Qualitatively similar results were obtained after

Figure 2 Effects of N-ethylmaleimide (NEM) on the kinetic parameters for 5-hydroxytryptamine (5-HT) uptake (a) and the Na^+ and K^+ content (b) in rabbit platelets. Ordinates: (a) V_{max} in pmol per 10^8 platelets min^{-1} (●) and K_m in nm (○) for 5-HT uptake; (b) cellular Na^+ (○) and K^+ (●) content in nmol per 10^8 platelets. Abscissae: concentration of NEM in μM during 45 min of preincubation (note the difference in scale between a and b). Shown are geometric means of at least 14 (a) and 17 (b); except at 160 μM NEM, where $n = 7$) experiments each; vertical bars show s.e.mean. Asterisks indicate significant differences from control values (H-test according to Kruskal and Wallis followed by Nemenyi's procedure): * $P < 0.05$; ** $P < 0.01$.

preincubation with NEM (20–80 μM ; IC_{50} for inhibition of $V_{max} = 52 \mu\text{M}$), even though the K_m value tended to rise with increasing NEM concentration (Figure 2b). At a NEM concentration of 160 μM , initial rates of uptake were too low to get reliable estimates of K_m and V_{max} .

In parallel experiments, the Na^+ and K^+ content was determined in platelets pre-exposed to various concentrations of ouabain (0.5–2.0 μM) or NEM (20–160 μM). As shown in Figures 1b (ouabain) and 2b (NEM), both drugs caused the Na^+ content of the cells to increase and the K^+ content to decrease. However, while ouabain-induced increases in Na^+ content went always hand in hand with decreases in K^+ content (Figure 1b), preincubation with up to 80 μM NEM induced a rise in Na^+ content without a

Figure 3 Linear correlation between V_{max} (a) or V_{max}/K_m (b) for 5-hydroxytryptamine (5-HT) uptake and the reciprocal cellular Na^+ content. Ordinates: V_{max} and V_{max}/K_m in % of control. Abscissae: reciprocal of the cellular Na^+ content in nmol per 10^8 platelets. Shown are geometric means of at least 12 observations each; vertical and horizontal bars show s.e.mean. Results obtained with ouabain and N-ethylmaleimide (NEM) are shown as (○) and (●), respectively. Control V_{max} was 113 (95% confidence limits: 81 to 156; $n = 16$) and 154 (131 to 181; $n = 22$) pmol per 10^8 platelets min^{-1} in experiments with ouabain and NEM, respectively; the corresponding values of V_{max}/K_m were 0.235 (0.176 to 0.313) and 0.220 (0.172 to 0.283) pmol per 10^8 platelets $\text{min}^{-1} \text{nm}^{-1}$, respectively. The regression lines ($y = a + bx$) obtained from the mean group results ($n = 8$ in both panels) were drawn to fit the following parameters: $a \pm S_a = -24 \pm 4.8$ (a) and -36 ± 4.3 (b); $b \pm S_b = 1118 \pm 59$ (a) and 1237 ± 71 (b). The correlation coefficient was 0.992 in a and 0.990 in b.

statistically significant change in K^+ content (Figure 2b).

When the V_{max} and the ratio of V_{max}/K_m (both being expressed in % of control) were each plotted

against the reciprocal cellular Na^+ content (which, under our experimental conditions, is a measure of the transmembrane Na^+ gradient), linear relationships with similar slopes were obtained (Figure 3). Moreover, irrespective of whether $V_{\max}$ (Figure 3a) or the ratio of $V_{\max}/K_m$ (Figure 3b) was related to the reciprocal of the Na^+ content, the results obtained for both drugs were virtually indistinguishable and fell on one and the same regression line. This finding indicates that NEM, like ouabain, inhibits 5-HT uptake at non-saturating ($V_{\max}/K_m$) as well as highly saturating ($V_{\max}$) substrate concentrations by reducing the transmembrane Na^+ gradient. According to the regression lines of Figure 3, $V_{\max}$ and $V_{\max}/K_m$ were halved when the Na^+ content of the cells had risen by 69% (95% confidence limits: 60 to 79%) and 57% (49 to 66%), respectively. Under control conditions, the cellular Na^+ content was 9.0 (7.4 to 11.0) nmol per 10^8 cells ($n = 34$).

Effect of ouabain and NEM on the potency of imipramine

After pre-exposure of platelets to $0.5 \mu\text{M}$ ouabain or $40 \mu\text{M}$ NEM for 45 min, initial rates of uptake were determined at a $[^3\text{H}]\text{-5-HT}$ concentration of 30 nM, both in the absence and presence of various concentrations of imipramine (5–40 nM). The concentration-effect curves for the imipramine-induced inhibition of 5-HT uptake obtained under these and under control conditions are shown in Figure 4a. Although the inhibitory effect of the highest imipramine concentration tested here (40 nM) was somewhat less pronounced after pre-exposure to NEM than in controls (Figure 4a), the IC_{50} for imipramine was affected neither by NEM nor by ouabain (Figure 4b); it amounted to 19 (16 to 23) nM in the control group of experiments ($n = 10$).

Discussion

Pre-exposure of platelets to $40 \mu\text{M}$ NEM for 45 min reduced 5-HT uptake by about 50%, but did not alter the potency of imipramine for inhibition of 5-HT uptake. Since the potency value for imipramine is the functional counterpart of the dissociation constant for imipramine binding, the present results corroborate those of Meyerson *et al.* (1987). However, when the ouabain-like action of NEM is taken into consideration, our findings are clearly against the view of Meyerson *et al.* (1987) who suggested that the 5-HT carrier and the imipramine binding site differ in their susceptibility to alkylation by NEM. In other words, the ability of NEM to impair 5-HT uptake without affecting imipramine binding was taken to support the hypothesis that the

Figure 4 Failure of ouabain and N-ethylmaleimide (NEM) to affect the potency of imipramine in inhibiting 5-hydroxytryptamine (5-HT) uptake. Ordinate scale in (a): uptake of 5-HT in % of control. Abscissa scale in (a) (log scale): concentration of imipramine in nM. Height of columns in (b) (log scale): IC_{50} for uptake inhibition by imipramine in nM. Uptake of 5-HT (30 nM) was determined in the absence and presence of various concentrations of imipramine (5, 10, 20 and 40 nM) both, under control conditions (Con, +) and after pre-exposure (45 min) to either $0.5 \mu\text{M}$ ouabain (Oua, $\circ$) or $40 \mu\text{M}$ NEM (Δ). Shown are geometric means with s.e.mean shown by vertical bars (a) or $\pm 95\%$ confidence limits (b) of 10 dependent observations. Control uptake was 9.5 (Con), 3.7 (NEM) and 3.5 (ouabain) pmol per 10^8 platelets min^{-1} . * $P < 0.01$ for the difference between NEM and control group with respect to the inhibitory effect of 40 nM imipramine (Friedman's test followed by the procedure of Wilcoxon and Wilcox).

5-HT carrier and the imipramine binding site represent distinct plasma membrane proteins. The results of Figure 3 demonstrate that NEM resembles ouabain in reducing the transmembrane Na^+ gradient and that this action fully accounts for the drug-induced inhibition of 5-HT uptake. Hence, it is not

the carrier protein but rather the Na^+/K^+ -ATPase which is highly susceptible to SH-group alkylation by NEM. Therefore, a binding site for imipramine other than the 5-HT carrier need not be postulated to explain the results of Meyerson *et al.* (1987).

Although, within the range of NEM concentrations tested here (20–80 μM), the inhibitory action of the drug on 5-HT uptake was predominantly indirect in nature (i.e., consequent to the impairment of Na^+ pump activity), NEM concentrations higher than 80 μM may well interfere with 5-HT transport by direct inactivation (i.e., alkylation) of the carrier protein. In support of this view it has been reported that NEM concentrations substantially higher than those used here inhibit imipramine binding (Davis, 1984; Meyerson *et al.*, 1987). This may be the explanation for the present finding that 80 μM NEM tended to increase the K_m for 5-HT uptake. Another Na^+ -dependent amine carrier system in which NEM exerts a dual inhibitory action is the noradrenaline carrier associated with the plasma membrane of noradrenergic neurones or PC-12 cells. Schömig *et al.* (1988) found NEM to be 75 times more potent in inhibiting the noradrenaline uptake by intact PC-12 cells than in inhibiting the noradrenaline uptake by plasma membrane vesicles derived from these cells. Since the Na^+ gradient required for noradrenaline uptake to take place is artificially generated in membrane vesicles but brought about through the action of the Na^+ pump in intact cells, these results provide direct evidence for the neuronal noradrenaline carrier being much less susceptible to inactivation by

NEM than the Na^+/K^+ -ATPase. Hence, the noradrenaline and the 5-HT carrier system resemble each other with respect to their liability to be affected by NEM.

Ouabain- and NEM-induced increases in the Na^+ content of platelets reduced the V_{max} of 5-HT uptake but did not change the K_m of the carrier system for 5-HT or the IC_{50} for uptake inhibition by imipramine. As the extracellular Na^+ concentration was held constant in the present experiments, our results can be taken to mean that the Na^+ gradient across the plasma membrane determines the velocity of 5-HT transport at non-saturating (i.e. V_{max}/K_m = rate constant characterizing the unsaturated carrier) and highly saturating (V_{max}) substrate concentrations, while having no influence on the K_m value and the affinity of imipramine binding. The extracellular Na^+ concentration, on the other hand, appears to be the main determinant of the values of K_m for 5-HT and IC_{50} for imipramine. Therefore, the observation of Talvenheimo *et al.* (1983) that the V_{max} of 5-HT uptake increases with increasing external Na^+ concentration was probably due to changes in the transmembrane Na^+ gradient and not the result of changes of the external Na^+ concentration *per se*.

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