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STIMULATION OF PLATELET SEROTONIN TRANSPORT BY SUBSTITUTED 1,4-NAPHTHOQUINONE-INDUCED OXIDANT STRESS

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Abstract—The effect of oxidant stress produced by redox cycling of substituted 1,4-naphthoquinones on the activity of platelet (Na^+-K^+)ATPase and the active transport of serotonin (5-HT) was studied. 2-Methyl-1,4-naphthoquinone (menadione) produced a concentration-dependent (0–100 μM) and time-dependent (2–20 min) stimulation of platelet 5-HT transport. Exogenous superoxide dismutase (250 units) and/or catalase (500 units) failed to block the stimulation. Fluoxetine, an inhibitor of the platelet 5-HT transporter, blocked menadione-induced stimulation of 5-HT uptake as did ouabain, an inhibitor of platelet (Na^+-K^+)ATPase. The structure–activity relationship of select 1,4-naphthoquinones suggested that stimulation was due to redox cycling and not arylation. The kinetics of 5-HT transport revealed that menadione markedly increased the maximal rate of 5-HT transport (V_{max} control = 20.6 ± 2.0 pmol/10^8 platelets/4 min vs V_{max} menadione = 46.4 ± 3.9 pmol/10^8 platelets/4 min) but did not significantly alter the K_m values. The activity of (Na^+-K^+)ATPase was determined by measuring the uptake of $^{86}Rb^+$ into intact platelets. Menadione produced a concentration-dependent and time-dependent stimulation of platelet $^{86}Rb^+$ uptake. These changes in platelet (Na^+-K^+)ATPase activity paralleled the changes observed in 5-HT transport and were inhibited in a concentration-dependent manner by ouabain. The data have shown that the redox cycling of 1,4-naphthoquinones caused an increase in (Na^+-K^+)ATPase activity that resulted in the stimulation of the rate of platelet 5-HT transport.

The active transport of serotonin (5-hydroxytryptamine, 5-HT†) by platelets plays an important role in maintaining the circulating concentration of free 5-HT below the levels required to activate receptors on the platelet plasma membrane and vascular smooth muscle cells [1]. Alterations in platelet 5-HT accumulation have been associated with vascular diseases such as hypertension and cardiac ischemia [2, 3]. In addition, the platelet 5-HT transport process has served as a model for the neuronal reuptake system present on presynaptic serotonergic nerve terminals [4].

The uptake of 5-HT is a complex, energy-consuming process that has an absolute requirement for Na^+ and Cl^- in the medium [5–9]. The transport of 5-HT across the platelet plasma membrane is driven by the transmembrane gradients of Na^+, Cl^- and K^+ which result from the activity of (Na^+-K^+)ATPase [10]. The negative membrane potential generated by this ion pump leads to the exclusion of Cl^- from the cell and thereby generates the Cl^- gradient. The (Na^+-K^+)ATPase enzyme has been localized to the platelet plasma membrane [11] with Na^+ and K^+ activating sites located on the cytoplasmic and extracellular sides respectively [12]. The mechanism of 5-HT transport by platelets has been the subject of several reviews [10, 13–15].

Enzyme-catalyzed redox cycling has been shown to occur in biological systems with diverse chemical compounds [16, 17]. Menadione (2-methyl-1,4-naphthoquinone) is an example of a compound that can undergo redox cycling to generate reactive forms of oxygen which can cause cellular injury [18–21]. The redox cycle of menadione is initiated by the activity of microsomal and mitochondrial reductases that reduce it to the semiquinone radical, which can then be reoxidized to the parent 1,4-naphthoquinone, generating superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) [19, 20]. These reactive forms of oxygen are normally detoxified by cellular antioxidant defense systems [22] which are present in platelets and other cells [23, 24]. Under certain circumstances, however, the concentration of these reactive oxygen species can overwhelm and/or escape these defense systems and produce cellular injury [25]. Earlier work from this laboratory [26] has shown that the extracellular addition of H_2O_2 can stimulate the active transport of 5-HT into platelets. Since platelets are exposed to reactive forms of oxygen generated by redox cycling drugs such as Adriamycin® and nitrofurantoin [16, 17], activated neutrophils [27] and after ischemia-reperfusion [28], this study was undertaken to examine the response of the platelet 5-HT transport system and plasma membrane (Na^+-K^+)ATPase to oxidant stress produced by the intracellular redox cycling of menadione.

MATERIALS AND METHODS

Materials. Male Swiss–Webster mice weighing 25–

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† Abbreviations: 5-HT, 5-hydroxytryptamine; H_2O_2, hydrogen peroxide; (Na^+-K^+)ATPase, adenosine triphosphatase, sodium, potassium; $O_2^{\cdot-}$, superoxide anion; and PRP, platelet-rich plasma.

30 g were purchased from Harlan–Sprague–Dawley Inc. (Indianapolis, IN). All animals were fed Purina Rodent Chow 5012 (Ralston Purina Co., St. Louis, MO) and water *ad lib.*, were maintained on a 12/12 light/dark cycle, and were housed for at least 5 days prior to use. 5-Hydroxytryptamine binoxalate [1,2-^{3}H(N)] (27.0 Ci/mmol) and ^{86}RbCl (235–893 mCi/mmol) were purchased from the New England Nuclear Corp. (Boston, MA). Liquid scintillation supplies and fluid (Bio-Safe II) were purchased from Research Products International (Mount Prospect, IL). Catalase (bovine liver), superoxide dismutase (bovine liver), EDTA, β-D-(+)-glucose, menadione sodium bisulfite, 1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, ouabain, and 5-hydroxytryptamine oxalate were purchased from the Sigma Chemical Co. (St. Louis, MO). 2,3-Dimethoxy-1,4-naphthoquinone was synthesized according to the published procedure [19]. Fluoxetine hydrochloride was a gift from the Eli Lilly Co. (Indianapolis, IN). The buffer used in these experiments was composed of 0.1 M Na_2HPO_4 containing 5 mM KCl (pH 7.35) and was found to support 5-HT transport in a manner similar to Tyrode buffer from which divalent cations had been excluded. This simple buffer contained the required ions for 5-HT transport and provided excellent pH control [5–9].

Platelet isolation and preparation. Animals were anesthetized with diethyl ether, the chest cavity was opened, and blood samples (0.8 to 1.0 mL) were withdrawn by cardiac puncture into 1.0-mL disposable syringes containing 0.1 mL of a 3% EDTA solution. Since the active accumulation of 5-HT is not dependent upon the extracellular calcium ion concentration, no alteration of uptake by EDTA or other commonly used anticoagulants (citrate, heparin) would be expected, and this has been confirmed by us and others [29]. Platelet-rich plasma (PRP) was prepared by centrifugation of the samples at 200 *g* for 15 min at room temperature. The PRP from each group was pooled, and the platelet concentration was determined using the Unopette System (Becton, Dickinson & Co., Rutherford, NJ) followed by counting with a Neubauer hemocytometer under a phase-contrast microscope. The PRP was diluted with buffer to give a final platelet concentration of 5×10^8/mL.

Exposure of platelets to 1,4-naphthoquinones. The PRP (0.2 mL, 1×10^8 platelets) was suspended in buffer (0.75 mL) and the specific 1,4-naphthoquinone (0.05 mL, 10–1000 μM final concentration) was added. Samples were incubated at 37° for 2–20 min, rapidly cooled on ice, and centrifuged at 700 *g* for 10 min at 4°. The supernatant was removed by careful aspiration, and the platelet pellet was resuspended in 0.20 mL of cold buffer and maintained in an ice bath until [^{3}H]5-HT uptake was measured.

In certain experiments, superoxide dismutase (250 units/mL), catalase (500 units/mL), ouabain (10–1000 μM), and sodium bisulfite (100 μM) were present in the initial buffer.

Platelet [^{3}H]5-HT uptake. Platelets accumulate 5-HT in a linear manner for 4 min at the concentrations employed, and a 2-min preincubation time has been shown to be optimal [30]. After a 2-min preincubation, [^{3}H]5-HT (0.05 mL, 5×10^{-7} M final concentration, 100 nCi) was added and the incubation was continued for 4 min in 37° in air. The final 5-HT concentration in the kinetic experiments ranged from 8×10^{-8} M to 2×10^{-6} M.

The uptake was terminated by the addition of 1.0 mL of a cold solution of 0.1% EDTA in isotonic saline containing 1 μM fluoxetine hydrochloride, followed by rapid cooling on ice. The platelets were pelleted from solution by centrifugation of the sample at 3500 *g* for 10 min at 4°. The supernatant fraction was removed and the platelet pellet was solubilized in 0.5 mL of 15% hydrogen peroxide by vortexing and incubating the sample for 15 min at 37°. A 0.1-mL aliquot of the sample was added to scintillation vials containing 5 mL scintillation fluid and counted. The tritium counts were quench corrected. Incubation of platelets with 5-HT (8×10^{-8} M to 2×10^{-6} M) at 4° gave blank values which represented 5–28% of control values. Samples were corrected for blank values. In experiments using fluoxetine (1 μM), the drug was present in the buffer used to measure [^{3}H]5-HT uptake.

The kinetic data were analyzed by utilizing an Eadie–Hofstee plot for the determination of K_m and V_{max} values. The passive diffusion component of 5-HT accumulation was determined at 0° by the method of Arora and Meltzer [31] which consisted of preincubating the sample for 2 min at 37°, adding 5-HT (8×10^{-8} M to 2×10^{-6}) and immediately immersing the sample in an ice bath. The passive component was subtracted from the total 5-HT accumulated to obtain the active transport component.

Platelet $^{86}Rb^+$ uptake. The PRP (0.2 mL, 1×10^8 platelets) was suspended in buffer (0–0.05 mL), and menadione (0.025 mL, 10–1000 μM) and $^{86}Rb^+$ (0.05 mL, 1.2 μCi) were added (final volume = 0.3 mL). Samples were incubated at 37° for 2–10 min and the uptake was stopped by placing the samples in an ice bath. The platelets were collected on Whatman GF/F glass microfiber filters and washed with 2×5 mL of ice-cold buffer. The radioactivity was determined in a liquid scintillation counter by measuring the β decay of $^{86}Rb^+$. Blank samples were prepared by replacing PRP with buffer and handling as described above. Samples were corrected for blank values.

Statistical methods. Platelet [^{3}H]5-HT and $^{86}Rb^+$ uptake data are expressed as means ± SEM. Statistical differences were determined using either a one-way analysis of variance followed by Dunnett's test or an unpaired Student's *t*-test with $P < 0.05$ as the limit for significant differences [32]. The platelet kinetic data have been expressed as means ± SD, and the determination of significance was based on the calculation of the 95% confidence band around each plot following linear regression analysis of the data [33].

RESULTS

Effect of menadione on platelet 5-HT transport. Incubation of platelets with increasing concentrations of menadione (0–100 μM) resulted in a concentration-dependent stimulation of 5-HT uptake between 0 and 100 μM (Table 1). The stimulation

Table 1. Concentration–response of menadione stimulation of platelet 5-HT uptake*

Concentration of menadione (μM)	Uptake (pmol/10^8 platelets/4 min)
0	14.2 ± 0.8
0 + Sodium bisulfite (100 μM)	13.8 ± 0.3
0 + Fluoxetine (1 μM)	1.3 ± 0.1†
10	16.5 ± 0.3
25	17.8 ± 1.0†
50	19.9 ± 2.2†
100	29.2 ± 1.3†
100 + Superoxide dismutase (250 units)	27.2 ± 0.3†
100 + Catalase (500 units)	30.2 ± 0.7†
100 + Fluoxetine (1 μM)	1.6 ± 0.2†‡
200	28.9 ± 0.5†
400	28.9 ± 0.5†
800	23.9 ± 0.9†‡
1000	22.0 ± 0.5†‡

* Mouse platelets (1×10^8) in the presence or absence of select drugs/enzymes were incubated for 10 min at 37° after the addition of menadione sodium bisulfite. Platelets were isolated and [^{3}H]5-HT uptake was measured as described in Materials and Methods. Control values for 5-HT uptake were not altered significantly in the presence of superoxide dismutase (250 units) or catalase (500 units). Values are means ± SEM, N = 3.

† Significantly different from control, $P < 0.05$.

‡ Significantly different from 100 μM menadione, $P < 0.05$.

was maximal using menadione concentrations of 100–400 μM and decreased with higher concentrations. The addition of sodium bisulfite to the medium had no effect on 5-HT transport and, thus, the water-soluble sodium bisulfite addition product of menadione was used in all subsequent experiments. The reactive forms of oxygen generated by the redox cycling of menadione exerted their stimulatory effects intracellularly, since the extracellular addition of the oxidant defense enzymes superoxide dismutase and/or catalase failed to prevent menadione-induced stimulation (Table 1).

The stimulation of platelet 5-HT accumulation involves only the active transport component. The addition of fluoxetine, a specific inhibitor of the platelet 5-HT transporter [34], completely blocked the active transport component of 5-HT accumulation in menadione-stimulated platelets (Table 1). No change in the passive diffusion component of 5-HT accumulation was observed between control and menadione-treated platelets.

The stimulation of platelet 5-HT transport by menadione exhibited a linear time-dependence for at least 20 min (Fig. 1). Adequate stimulation of 5-HT transport occurred at 10 min and all subsequent experiments used a 10-min incubation time.

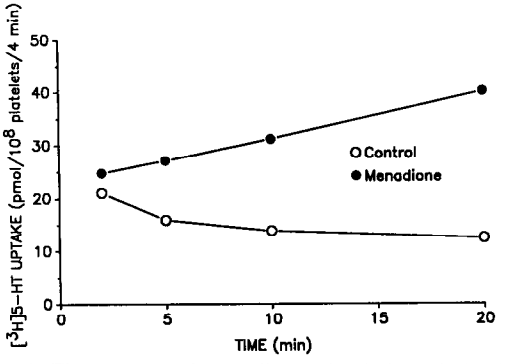

Fig. 1. Time–course of menadione stimulation of platelet 5-HT uptake. Mouse platelets (1×10^8) were incubated for 2–20 min at 37° after the addition of menadione sodium bisulfite (100 μM final concentration). Platelets were isolated and [^{3}H]5-HT uptake was measured as described in Materials and Methods. Values are means, N = 3. The average standard errors were 2.4% of the mean values. At all points menadione-treated platelets were significantly different from control, $P < 0.05$.

Structure–activity relationships of 1,4-naphthoquinones and platelet 5-HT transport. Since 1,4-naphthoquinone compounds are able to generate reactive forms of oxygen by redox cycling and can also arylate cellular sulfhydryl groups, the role of redox cycling in the stimulation of the platelet 5-HT transport system was evaluated through the use of 1,4-naphthoquinones having known reactivities. 2,3-Dimethoxy-1,4-naphthoquinone, which redox cycles but is unable to arylate sulfhydryl groups due to its substitution pattern, caused a significant stimulation of platelet 5-HT uptake (Table 2). In contrast, 1,4-naphthoquinone, which only arylates cellular sulfhydryl groups, caused a marked inhibition of 5-HT transport. The response of platelets to menadione which can redox cycle but has some arylation activity towards sulfhydryl groups resulted in a marked stimulation of platelet 5-HT transport. 2-Hydroxy-1,4-naphthoquinone which does not arylate cellular sulhydryl groups and which is a poor substrate for the cellular one-electron reductases

Table 2. Effects of 1,4-naphthoquinones on stimulation of platelet 5-HT uptake*

Compound	Uptake (pmol/10^8 platelets/4 min)
Control	16.6 ± 0.3
2-Methyl-1,4-naphthoquinone (menadione)	34.3 ± 0.4†
2,3-Dimethoxy-1,4-naphthoquinone	25.5 ± 0.4†
1,4-Naphthoquinone	2.5 ± 0.1†
2-Hydroxy-1,4-naphthoquinone	19.6 ± 0.1†

* Mouse platelets (1×10^8) were incubated for 10 min at 37° after the addition of specific 1,4-naphthoquinones (100 μM). Platelets were isolated and [^{3}H]5-HT uptake was measured as described in Materials and Methods. Values are means ± SEM, N = 3.

† Significantly different from control, $P < 0.05$.

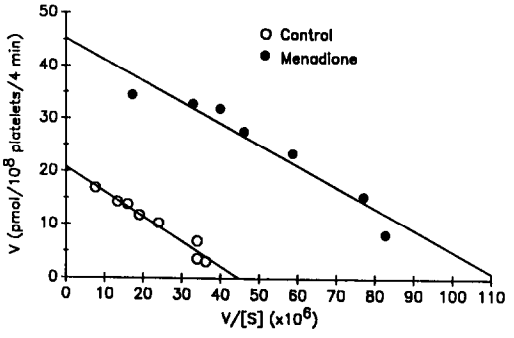

Fig. 2. Eadie–Hofstee plot of the active component of 5-HT uptake by control and menadione-treated platelets. Mouse platelets (1×10^8) were incubated for 10 min at 37° after the addition of menadione sodium bisulfite (100 μM final concentration). Platelets were isolated and [^{3}H]5-HT uptake was measured as described in Materials and Methods. Values are means, N = 3. The average standard errors were 2.2% of the mean values. Linear regression analysis of the data gave kinetic values of K_m control = $4.1 \pm 0.1 \times 10^{-6}$ M, K_m menadione = $4.3 \pm 0.1 \times 10^{-6}$ M and V_{max} control = 20.6 ± 2.0 pmol/10^8 platelets/4 min, V_{max} menadione = 46.4 ± 3.9 pmol/10^8 platelets/4 min.

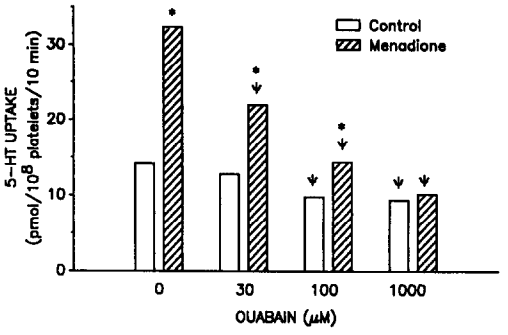

Fig. 3. Ouabain inhibition of menadione stimulation of platelet 5-HT uptake. Mouse platelets (1×10^8) in the presence of ouabain (0–1000 μM) were incubated for 10 min at 37° after the addition of buffer (control) or menadione sodium bisulfite (100 μM final concentration). Platelets were isolated and [^{3}H]5-HT uptake was measured as described in Materials and Methods. Values are means, N = 3. The average standard errors were 1.9% of the mean values. Key: (*) significantly different from the untreated (control) sample, $P < 0.05$; and (Ψ) significantly different from untreated (control) and menadione-treated samples, respectively, at 0 μM ouabain, $P < 0.05$.

required for effective redox cycling was nevertheless able to moderately stimulate the platelet 5-HT transport system. This compound can undergo a two-electron reduction to form the hydroquinone, which rapidly auto-oxidizes back to 2-hydroxy-1,4-naphthoquinone with the formation of H_2O_2.

Effect of menadione on platelet 5-HT transport kinetics. Treatment of platelets with menadione resulted in a significant increase in the maximal rate of 5-HT transport (Fig. 2) when compared to untreated control platelets (V_{max} control = 20.6 ± 2.0 pmol/10^8 platelets/4 min vs V_{max} menadione = 46.4 ± 3.9 pmol/10^8 platelets/4 min). No significant change was observed in the K_m values (K_m control = $4.1 \pm 0.1 \times 10^{-6}$ M vs K_m menadione = $4.3 \pm 0.1 \times 10^{-6}$ M).

Effect of ouabain on menadione-induced stimulation of 5-HT transport. Ouabain is an inhibitor of (Na^+-K^+)ATPase which generates the ion gradients across the platelet plasma membrane that are required for 5-HT transport. This inhibitor was studied to determine the role of ouabain-sensitive (Na^+-K^+)ATPase in the stimulation of 5-HT transport observed with menadione. When ouabain was used at concentrations of 30 and 100 μM, there was a significant reduction in the ability of menadione to stimulate platelet 5-HT uptake (Fig. 3). This stimulation was inhibited completely using a ouabain concentration of 1000 μM. These data suggest that activation of ouabain-sensitive (Na^+-K^+)ATPase by menadione was responsible for the increased platelet 5-HT transport. Untreated, control platelets showed a significant reduction in 5-HT uptake only at the two highest ouabain concentrations employed.

Effect of menadione on platelet $^{86}Rb^+$ uptake. The uptake of $^{86}Rb^+$, a congener of K^+, was used to measure (Na^+-K^+)ATPase activity in platelets. The addition of menadione (0–1000 μM) to platelets caused a concentration-dependent stimulation of $^{86}Rb^+$ uptake between 0 and 100 μM that was

Table 3. Concentration–response of menadione stimulation of $^{86}Rb^+$ uptake by platelets*

Concentration of menadione (μM)	Uptake (pmol/10^8 platelets/10 min)
0	27.2 ± 0.4
10	27.6 ± 0.3
25	29.4 ± 0.5†
50	30.2 ± 0.3†
100	33.1 ± 0.3†
200	31.1 ± 0.3†
400	31.6 ± 0.6†
1000	32.8 ± 0.9†

* Mouse platelets (1×10^8) were incubated for 10 min at 37° after the addition of menadione sodium bisulfite and $^{86}RbCl$. Platelets were isolated by filtration and $^{86}Rb^+$ uptake was measured as described in Materials and Methods. Values are means ± SEM, N = 6.
† Significantly different from control, $P < 0.05$.

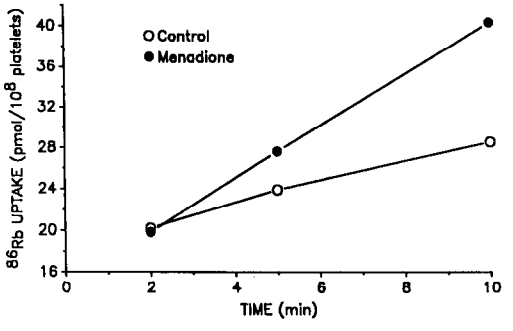

Fig. 4. Time–course of menadione stimulation of platelet $^{86}Rb^+$ uptake. Mouse platelets (1×10^8) were incubated for 2–10 min at 37° after the addition of menadione sodium bisulfite (100 μM final concentration) and $^{86}RbCl$. Platelets were isolated by filtration and $^{86}Rb^+$ uptake was measured as described in Materials and Methods. Values are means, N = 6. The average standard errors were 1.2% of the mean values. At 5 and 10 min, menadione-treated platelets were significantly different from controls, $P < 0.05$.

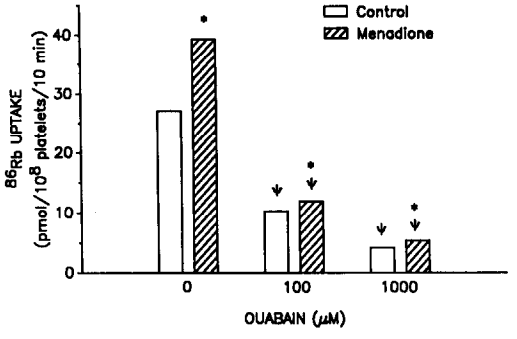

Fig. 5. Ouabain inhibition of menadione stimulation of platelet $^{86}Rb^+$ uptake. Mouse platelets (1×10^8) in the presence of ouabain were incubated for 10 min at 37° after the addition of buffer (control) or menadione sodium bisulfate (100 μM final concentration) and $^{86}RbCl$. Platelets were isolated by filtration and $^{86}Rb^+$ uptake was measured as described in Materials and Methods. Values are means, N = 6. The average standard errors were 4.0% of the mean values. Key: (*) significantly different from the untreated (control) sample, $P < 0.05$; and (Ψ) significantly different from untreated (control) and menadione-treated samples, respectively, at 0 μM ouabain, $P < 0.05$.

maximal at concentrations of 100–1000 μM (Table 3) and similar to that observed for 5-HT uptake (Table 1). The time–course of menadione-induced stimulation of platelet $^{86}Rb^+$ uptake (Fig. 4) was similar to that found for platelet 5-HT uptake (Fig. 1). Further support for the role of (Na^+-K^+)ATPase in menadione-induced stimulation of platelet 5-HT transport was obtained through the use of ouabain, an inhibitor of this enzyme. When platelets were treated with ouabain (100 and 1000 μM), there was a significant decrease in $^{86}Rb^+$ uptake in both untreated (control) and menadione-treated platelets (Fig. 5). A reduction in the magnitude of menadione-induced stimulation of $^{86}Rb^+$ uptake was also observed in the presence of ouabain.

DISCUSSION

In these studies, the stimulation of the active transport of 5-HT into platelets in response to the redox cycling of 1,4-naphthoquinones has been demonstrated and has been associated with increased activity of plasma membrane (Na^+-K^+)ATPase. Menadione (2-methyl-1,4-naphthoquinone) was selected for this study and produced a stimulation of 5-HT transport into platelets that was concentration dependent (Table 1) and time dependent (Fig. 1). This time-dependent response contrasted with the rapid response (2 min) observed following the addition of exogenous H_2O_2 [26], but was expected for a compound that required redox cycling in order to generate an effective concentration of reactive forms of oxygen. The decrease in 5-HT transport over time is known as the incubation effect and has been described previously [13]. This decrease in 5-HT transport is not the result of a decrease in

platelet (Na^+-K^+)ATPase activity since platelets preincubated for 0–20 min at 37° accumulated the same amount of $^{86}Rb^+$ over a 6-min incubation period (data not shown). Thus, the decrease in 5-HT transport over time appears to occur at the level of the 5-HT transporter although the mechanism is unknown. The concentration of menadione that produced a significant stimulation of platelet 5-HT uptake was considerably lower than that reported to cause cytotoxicity in isolated hepatocytes after much longer incubation periods [19]. In this study, the optimal menadione concentration (100 μM) and incubation period (10 min) did not produce any observable platelet toxicity. When higher concentrations of menadione were used, there was a partial reversal of the 5-HT transport stimulation (Table 1) with no change in the maximal $^{86}Rb^+$ uptake (Table 3). This suggests that the 5-HT transport system may be more susceptible to the deleterious effects produced by these higher fluxes of reactive forms of oxygen than platelet (Na^+-K^+)ATPase.

The oxidant species that are generated by the redox cycling of 1,4-naphthoquinones and responsible for the stimulation of platelet 5-HT transport have not been identified; however, earlier work using exogenous H_2O_2 or xanthine oxidase generated $O_2^{\cdot-}$ and H_2O_2 indicated that H_2O_2 was the proximate oxidant species [26]. The redox cycling of 1,4-naphthoquinones produces $O_2^{\cdot-}$ which can undergo spontaneous or enzyme-catalyzed dismutation to H_2O_2 [35]. Additional support for a role for H_2O_2 in the stimulation of platelet 5-HT transport by 1,4-naphthoquinones comes from the data obtained with 2-hydroxy-1,4-naphthoquinone (Table 2). This compound is a poor substrate for cellular one-electron reductases, in part because of its very low one-electron reduction potential [36], yet it can produce oxidative stress [37]. It has been suggested that 2-hydroxy-1,4-naphthoquinone produces an oxidative stress in hepatocytes by undergoing a two-electron reduction, to the hydroquinone which is catalyzed by DT-diaphorase [37]. The hydroquinone can rapidly auto-oxidize back to the parent 2-hydroxy-1,4-naphthoquinone which produces H_2O_2. This mechanism for the generation of H_2O_2 by 2-hydroxy-1,4-naphthoquinone appears to occur in platelets and causes a moderate stimulation of 5-HT transport. The redox cycling of 1,4-naphthoquinones could also lead to the formation of hydroxyl radicals by either a Fenton reaction involving H_2O_2 and a transition metal [38] or a metal-catalyzed Haber–Weiss reaction involving $O_2^{\cdot-}$ and H_2O_2 [35, 38]. The role of hydroxyl radicals in this process has not been established.

The stimulatory effect of the reactive forms of oxygen on platelet 5-HT transport occurs intracellularly and does not appear to result from their leakage/escape into the extracellular medium. The presence of superoxide dismutase or catalase in the extracellular medium failed to prevent the observed stimulation of 5-HT transport (Table 1), in contrast to the inhibitory effect of catalase when exogenous H_2O_2 was added or $O_2^{\cdot-}$ and H_2O_2 enzymatically generated in the extracellular medium [26]. This intracellular sensitivity to reactive forms of oxygen is surprising in light of the known abundance of platelet defense enzymes such as superoxide dismutase, catalase and glutathione peroxidase, which protect sensitive intracellular organelles from oxidant injury [23, 24]. Earlier work [25, 26] demonstrated that H_2O_2 is able to effect intracellular platelet sites not protected by these defense enzymes, suggesting that there exist unprotected, sensitive sulfhydryl groups located on the 5-HT transporter and/or at the active site of plasma membrane (Na^+-K^+)ATPase that are highly sensitive to oxidation. Such a shift in sulfhydryl oxidation state may represent a mechanism by which the platelet 5-HT transport system is regulated.

Platelets accumulate 5-HT by both active transport and passive diffusion; however, at the 5-HT concentration used in this study (5×10^{-7} M), the contribution of the passive diffusion component was only 9.1%. This passive diffusion component was determined by measuring the extent of 5-HT accumulation in the presence of fluoxetine which inhibits the active transport component of platelet 5-HT accumulation [34] (Table 1). Since reactive forms of oxygen are known to cause alterations in biological membranes through their ability to initiate lipid peroxidation [35], it was possible that the redox cycling 1,4-naphthoquinones were altering the platelet plasma membrane such that the passive diffusion of 5-HT into the platelet increased. This appears, however, not to be the case, since no difference was observed in the passive diffusion component between control and menadione-treated platelets in the presence of fluoxetine (Table 1). Thus, menadione-induced stimulation of platelet 5-HT transport involved only the active component of 5-HT accumulation and was the result of an increase in the V_{max} of 5-HT transport (Fig. 2).

Menadione is known to both redox cycle and arylate peptide and protein sulfhydryl groups [19]. To show that stimulation was due to the redox cycling of menadione and was not the result of sulfhydryl arylation, several 1,4-naphthoquinones with known redox cycling and/or arylation properties [19, 37] were studied (Table 2). 1,4-Naphthoquinone, which is a highly reactive sulfhydryl arylating agent [19], caused a marked inhibition of platelet 5-HT transport while 2,3-dimethoxy-1,4-naphthoquinone, which is a redox cycling compound that is devoid of arylating activity [19], produced a significant stimulation of platelet 5-HT uptake. These data suggest that menadione, which redox cycles but also has arylating activity [18–20], caused the stimulation of platelet 5-HT uptake via its redox cycling ability. Furthermore, menadione may be a more potent redox cycling agent that these data indicate since arylation activity was associated with inhibition of 5-HT transport.

Since the rate of 5-HT transport across the platelet plasma membrane is driven by the transmembrane Na^+, Cl^- and K^+ gradients which result from the activity of (Na^+-K^+)ATPase, increased activity of this plasma membrane enzyme would be expected to result in an increased rate of 5-HT transport. Ouabain, an inhibitor of (Na^+-K^+)ATPase, blocked the menadione-induced stimulation of 5-HT transport

(Fig. 3), indicating that stimulation was due to the increased activity of the ouabain-sensitive form of (Na^+-K^+)ATPase. The activity of this enzyme in intact platelets was analyzed by measuring the uptake of the K^+ congener, rubidium-86 ($^{86}Rb^+$) [39, 40]. The $^{86}Rb^+$ uptake experiments showed menadione concentration–response (Table 3) and time–course (Fig. 4) similar to those observed with 5-HT transport. The increase in menadione-induced stimulation of $^{86}Rb^+$ uptake was less than that observed with 5-HT uptake. This may reflect the fact that small changes in (Na^+-K^+)ATPase activity result in marked changes in 5-HT transport, which supports a role for (Na^+-K^+)ATPase in the regulation of platelet 5-HT transport.

The responses of the basal platelet 5-HT (Fig. 3) and $^{86}Rb^+$ (Fig. 5) uptake systems to ouabain, however, were different. Ouabain (100 and 1000 μM) produced a significant inhibition of basal $^{86}Rb^+$ uptake but exerted a minimal effect on platelet 5-HT transport. The inhibition by ouabain of platelet $^{86}Rb^+$ uptake was consistent with its known mechanism of action on membrane-bound (Na^+-K^+)ATPase [41]. The minimal effect of ouabain on basal 5-HT transport may be the result of several factors. First, short incubation times used in this study may not have been adequate to achieve the complete inhibition of platelet (Na^+-K^+)ATPase. High levels of (Na^+-K^+)ATPase inhibition by ouabain have been reported in platelets when long preincubation times (2–5 hr) were used [39], while shorter preincubation times (<1 hr) have been associated with varying degrees of ouabain inhibition [42–44]. Second, ouabain is known not to completely inhibit platelet 5-HT transport [13], which suggests a ouabain-insensitive component to this process. Therefore, the residual (Na^+-K^+)ATPase activity that results from the incomplete ouabain inhibition combined with a ouabain-insensitive component may be sufficient to support the basal level of 5-HT transport.

In conclusion, this study has shown that redox cycling of menadione caused an increase in platelet 5-HT active transport resulting from stimulation of platelet membrane (Na^+-K^+)ATPase activity. The effects of drugs and environmental agents that redox cycle on platelet 5-HT uptake and the possible regulation of (Na^+-K^+)ATPase activity via sulfhydryl oxidation/reduction require further study.

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