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# Inhibition of Platelet Serotonin Transport by Propranolol

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SUMMARY

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Transport of serotonin into human platelets is inhibited by the *beta*-adrenergic blocking agent, propranolol. Both D- and DL-propranolol, at a concentration of 1 μM, inhibit the initial rate of serotonin transport by approximately 50%, but do not affect the steady-state level of accumulation. Although most of the serotonin accumulated by intact platelets is stored in intracellular dense granules, this reserpine-sensitive component of platelet serotonin transport is not inhibited by propranolol. In isolated porcine platelet plasma membrane vesicles, DL-propranolol is a competitive inhibitor of serotonin transport with a $K_I$ of 1.03 ± 0.27 μM. Moreover, propranolol displaces imipramine bound to the plasma membrane serotonin transporter. In contrast, propranolol does not inhibit transport of serotonin into membrane vesicles derived from platelet dense granules. These results indicate that propranolol inhibits platelet serotonin transport at the level of the plasma membrane.

## INTRODUCTION

Platelets accumulate serotonin from the external medium to high internal concentrations by a process similar, if not identical, to serotonin reuptake by serotoninergic neurons (for review see ref. 1). This process results from the action of a $Na^+$-dependent transporter in the plasma membrane, which concentrates serotonin inside the cell (2–4). In a subsequent step, intracellular platelet storage organelles (dense granules) further concentrate serotonin by transporting it from the cytoplasm to the granule lumen (5, 6). In intact platelets, these two distinct transport systems are distinguished by their response to imipramine and reserpine, which are competitive inhibitors of the plasma membrane and granule systems, respectively (7, 8). Moreover, the two transport systems may be studied independently in platelet osmotic lysates (9) or in isolated plasma membrane vesicles (4). Plasma membrane vesicles also bind imipramine with high affinity, at the substrate site of the serotonin transport system (10).

Propranolol is widely used in the treatment of hypertension (11) and also in the treatment of conditions such as schizophrenia, narcolepsy, migraine, and essential tremor, which are believed to be due, at lease in part, to disorders in serotoninergic neurons (12–15). Fenfluramine, which inhibits serotonin transport (16), has antihypertensive effects clinically. Grobecker *et al.* (17) demonstrated that propranolol inhibits serotonin accumulation by platelets. In this report, we confirm their findings and, moreover, by using the properties of the two serotonin transport systems described above, we report that propranolol specifically inhibits the platelet plasma membrane serotonin transporter.

## METHODS

*Preparation of platelets.* Freshly drawn human blood, anticoagulated with acid-citrate-dextrose (18), was subsequently diluted with an equal volume of eight parts platelet buffer (66 mM NaCl containing 25 mM disodium phosphate, 6 mM KCl, 6 mM D-glucose, and 1 mM $MgSO_4$, adjusted to pH 6.7 with phosphoric acid) and two parts acid-citrate-dextrose at room temperature. The erythrocytes and white cells were removed by sedimentation at 200 × *g* for 10 min. After addition of potassium ethylenediamine *N,N,N′,N′*-tetraacetate (final concentration, 1 mM) to the platelet-rich supernatant layer, platelets were collected by sedimentation at 7500 × *g* for 10 min and resuspended in platelet buffer at a concentration of 0.1–1.0 mg of platelet protein per milliliter.

*Preparation of plasma membrane vesicles.* Platelet plasma membrane vesicles were isolated from porcine blood as described previously (19).

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*Preparation of platelet lysate.* Platelet lysate was prepared from human platelets as described previously for porcine platelets (9).

*Serotonin transport measurements.* Transport was assayed in intact platelets by adding [1,2-$^3$H]serotonin (17,000 cpm/pmole) to 0.2 ml of a suspension of platelets in platelet buffer at 37°. After incubation of the suspension for the indicated period of time, the reactions were terminated by rapid addition of 2 ml of ice-cold 0.1 M NaCl, and filtration through Millipore HAWP filters (nitrocellulose, 0.45-μM pore size), followed by a wash with an additional 2 ml of cold NaCl. Dilution, filtration, and washing were accomplished within 15 sec. The filters were then dried and counted as described previously for membrane vesicles (4).

Serotonin transport was assayed in plasma membrane vesicles and in dense granule membrane vesicles as described previously (4, 9). Imipramine binding was assayed in membrane vesicles by filtration as described by Talvenheimo *et al.* (10).

Transport of propranolol by intact platelets was measured by the same method used for serotonin transport except that 1 μM L-[$^3$H]propranolol (1600 cpm/pmole) replaced [$^3$H]serotonin.

Protein was assayed by the method of Lowry *et al.* (20).

*Materials.* Human blood was drawn from healthy donors who were taking no medication. Porcine blood was obtained at a local slaughterhouse. Labeled serotonin and propranolol were purchased from New England Nuclear Corporation (Boston, Mass.). All other chemicals were reagent grade obtained from commercial sources.

## RESULTS

*Inhibition of serotonin transport into intact platelets.* Figure 1 demonstrates the accumulation of serotonin by intact platelets and its inhibition by propranolol. The reactions were initiated by addition of tritiated serotonin to platelets suspended in buffer as described under Methods. At platelet concentrations approaching those found in platelet-rich plasma, the medium is essentially depleted of serotonin within 10 min (data not shown). At lower platelet concentrations, a steady-state is achieved when serotonin influx is balanced by efflux. At the platelet concentrations used in the experiment shown in Fig. 1, platelets transport serotonin linearly with time during the first few minutes of incubation, and then reach a steady-state at 10 min, when approximately 30% of the radioactivity remains in the medium. DL-Propranolol, at 1 μM, dramatically and reproducibly inhibits the initial rate of transport to less than 50% of the control rate, but changes the steady-state level of accumulation little, if at all.

Table 1 demonstrates that the inhibitory effect of propranolol is nonstereospecific. Over a range in D- or DL-propranolol concentrations from 0.2 to 0.8 μM, serotonin transport is inhibited to the same extent, whether the D-isomer or the racemic mixture is used.

The results shown in Fig. 2 indicate that the steady-state level of serotonin accumulation mostly represents serotonin storage in intracellular granules rather than the serotonin gradient across the plasma membrane. In this experiment, platelets were incubated with labeled serotonin in the presence or absence of reserpine, an alkaloid which inhibits amine transport into a variety of intracellular organelles including platelet dense granules (9, 21). At the time indicated by the *arrow*, the ionophore gramicidin was added. Gramicidin uncouples the plasma membrane serotonin transporter from the $Na^+$ and $K^+$ gradients which drive serotonin across the plasma membrane (4), and is expected to cause rapid efflux of cytoplasmic serotonin. The data in Fig. 2 demonstrate that only when the dense granules are inhibited by reserpine is such rapid efflux observed. Thus, in the absence of reserpine, most of the intracellular serotonin is probably not cytoplasmic, but stored within dense granules, as first proposed by DaPrada and Pletscher (5).

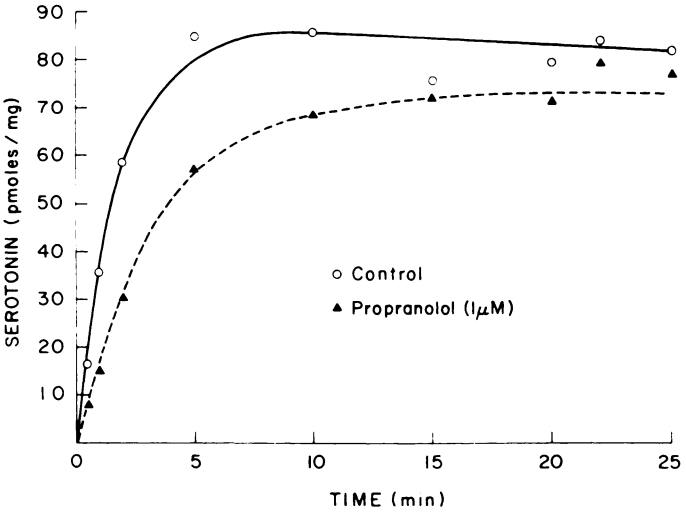


FIG. 1. *Effect of propranolol on the time course of serotonin transport into intact platelets*

Transport assays were performed as described under Methods using a final concentration of 0.9 mg of platelet protein per milliliter and 0.1 μM [$^3$H]serotonin, in the presence (▲- - -▲) and absence (○——○) of 1 μM DL-propranolol.

At higher concentrations of serotonin (5 μM), where more of the amine accumulates within dense granules, reserpine noticeably inhibits the steady-state level of serotonin accumulation (Fig. 3). Propranolol has little or no effect on this reserpine-sensitive component of sero-

TABLE 1

*Inhibition of serotonin transport by D- and DL-propranolol*

Initial rates of serotonin transport into human platelets was measured as described under Methods in the presence of the indicated concentration of D- or DL-propranolol. Results are given as picomoles of serotonin transported/mg of protein·min$^{-1}$ ± standard deviation. Figures in parentheses are per cent inhibition due to propranolol.

| Propranolol concentration | Transport rate | |
|---|---|---|
| | Isomer DL- | Isomer D- |
| μM | *pmoles/min/mg* | |
| 0 | 15.9 ± 1.1 (0) | 12.7 ± 0.5 (0) |
| 0.193 | 12.1 ± 0.7 (24) | 10.4 ± 0.5 (17) |
| 0.386 | 9.81 ± 0.25 (38) | 8.48 ± 2.04 (32) |
| 0.771 | 6.8 ± 1.7 (57) | 6.2 ± 1.8 (50) |

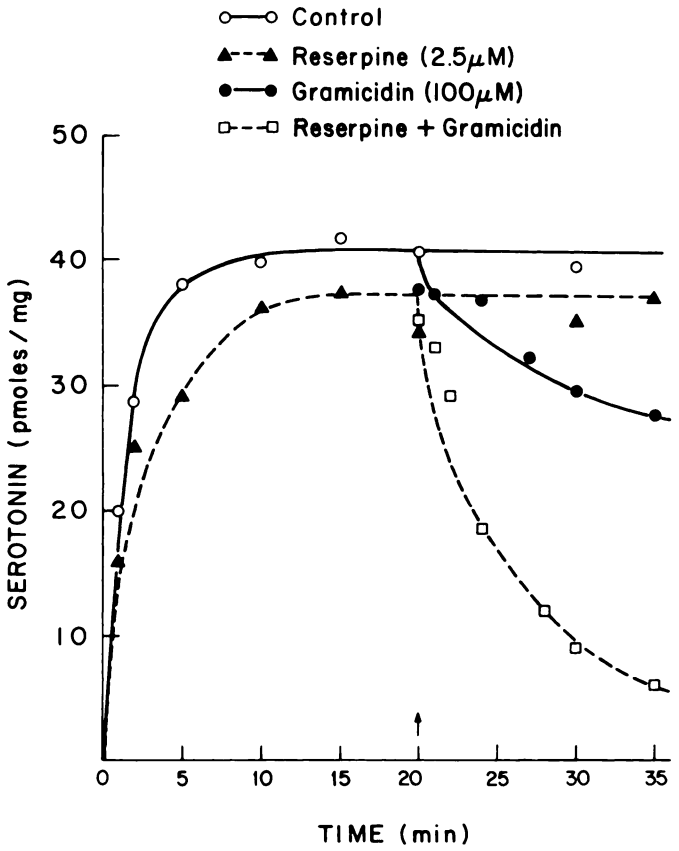


FIG. 2. *Gramicidin-induced serotonin efflux in normal and reserpinized platelets*

Serotonin transport was measured as described under Methods using a final concentration of 1.25 mg of platelet protein per milliliter and 0.1 μM [$^3$H]serotonin. Platelets treated with reserpine (*triangles* and *squares*) were incubated with 2.5 μM reserpine for 5 min at 25° prior to addition of serotonin. This temperature was selected to decrease basal efflux. Twenty minutes after serotonin addition, gramicidin was added to a final concentration of 100 μM (*closed circles* and *squares*) from a 10 mM stock solution in ethanol.

tonin accumulation. In this experiment, propranolol was incubated with platelets for 15 min prior to addition of reserpine and serotonin to ensure that the high concentrations of serotonin and reserpine used would not block the access of propranolol to the dense granules if that were its site of action. The data in Fig. 4 show that little, if any, accumulation of propranolol occurs during this incubation time. The relatively small amount of [$^3$H]-propranolol associated with the platelets even after 30 min, and the small time-dependence of association, as shown in Fig. 4, suggest that it may result mostly from binding of propranolol to the platelets.

*Inhibition of transport into plasma membrane vesicles.* To define more precisely the site of action of propranolol on platelet serotonin transport, plasma membrane vesicles isolated from porcine platelets were used. In the vesicle system, serotonin accumulates inside in response to transmembrane gradients of $Na^+$, $Cl^-$, and $K^+$ (4, 22). In the experiment shown in Fig. 5, the initial rate of serotonin transport into plasma membrane vesicles was measured as a function of serotonin concentration in the presence and absence of 1 μM DL-propranolol. The results, which are presented according to the method of Hofstee (23) in Fig. 5, demonstrate that propranolol competitively inhibits serotonin transport across the plasma membrane. The absolute value of the slope of the line through the data points equals the $K_m$ in the absence of propranolol, which is 0.37 ± 0.09 μM in this experiment. In the presence of 1 μM propranolol, the apparent $K_m$ increases to 0.73 ± 0.21 μM. In contrast, there is little change in $V_{max}$ values, which are 1.64 ± 0.12 and 1.51 ± 0.18 nmoles/mg of membrane protein·min$^{-1}$ in the absence and presence, respectively, of propranolol. The ability of higher concentrations of serotonin to overcome inhibition by propranolol indicated competitive inhibition, and from the change in apparent $K_m$, a $K_I$ for propranolol of 1.03 ± 0.27 μM was calculated.

*Effect on storage in dense granules.* The transport system responsible for serotonin accumulation by dense granules is expressed in crude platelet lysates under appropriate conditions (9). The experiment shown in Fig. 6 demonstrates the accumulation of serotonin by membrane vesicles derived from platelet dense granules in a human platelet lysate. In the presence of ATP, these vesicles rapidly accumulate approximately 15 pmoles of serotonin per milligram of total membrane protein. This accumulation is completely inhibited in the presence of

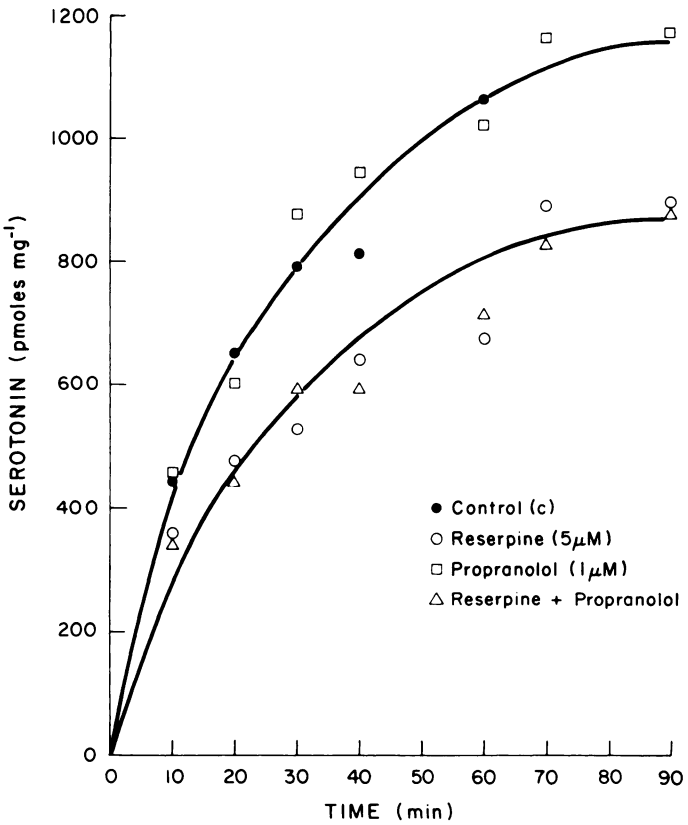


FIG. 3. *Effect of reserpine and propranolol on accumulation of large amounts of serotonin by intact platelets*

Serotonin transport was measured as described under Methods using a final concentration of 0.125 mg of platelet protein per milliliter and 5 μM [$^3$H]serotonin. Platelets were treated with 1 μM DL-propranolol (*squares* and *triangles*) for 15 min at 37° prior to addition of reserpine (*triangles*) or serotonin (*squares*). Reserpinized platelets (*open circles*) and *triangles*) were incubated with 5 μM reserpine for 5 min at 37° prior to addition of serotonin.

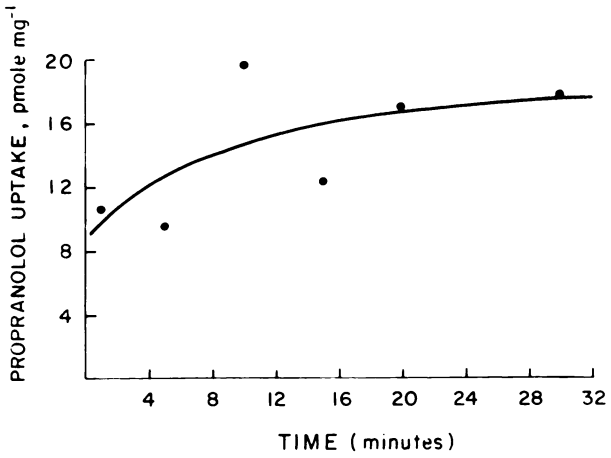


FIG. 4. *Association of L-[$^3$H]propranolol with platelets*

Propranolol uptake was measured as described under Methods using 1 μM propranolol.

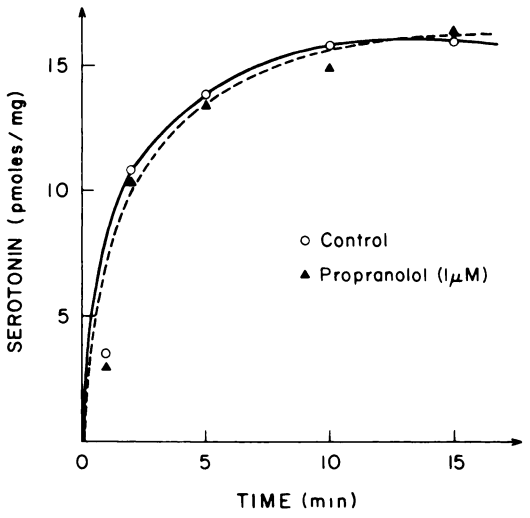


FIG. 6. *Accumulation of serotonin by membrane vesicles derived from human platelet dense granules*

Serotonin transport was measured as described under Methods in the presence (*triangles*) and absence (*circles*) of 1 μM DL-propranolol.

2 μM reserpine (data not shown). In striking contrast to the inhibition by propranolol of serotonin transport across the platelet plasma membrane, 1 μM propranolol has no effect on the dense granule transport system. Thus, the transport inhibition pattern observed with propranolol resembles that of imipramine, which also inhibits only the plasma membrane transport system (9).

*Inhibition of serotonin exchange.* Another characteristic shared by both imipramine and propranolol is inhibition of serotonin exchange. Imipramine inhibits the acceleration of labeled serotonin efflux from plasma membrane vesicles which is induced by external, unlabeled serotonin (10), suggesting that imipramine binds to the substrate site of the serotonin transporter, but is not itself transported. Fig. 7 shows a typical experiment in which propranolol also inhibits serotonin exchange. Vesicles which had accumulated labeled serotonin to high internal concentrations were diluted into medium free of labeled serotonin. In the control, serotonin left the vesi-

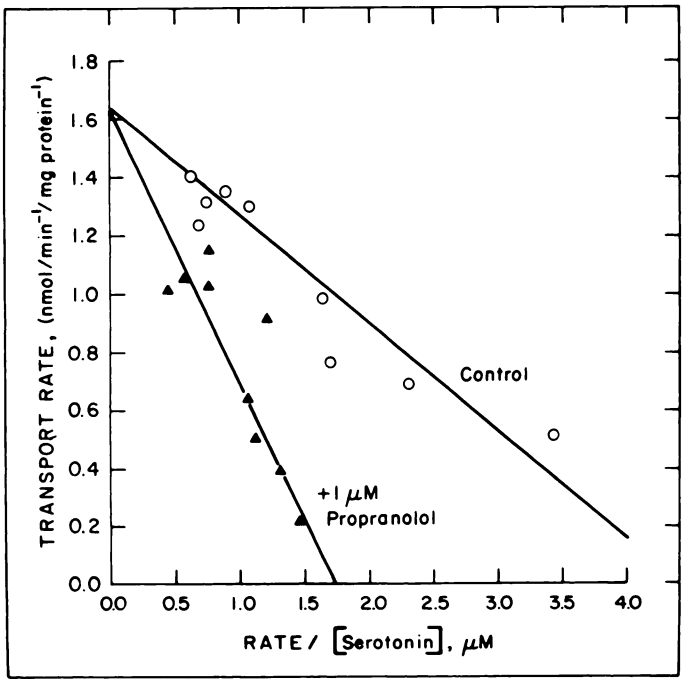


FIG. 5. *Competitive inhibition of serotonin transport into porcine platelet plasma membrane vesicles by propranolol*

Initial rates of serotonin transport were measured as described under Methods in the presence (*triangles*) and absence (*circles*) of 1 μM DL-propranolol. The results are plotted according to the method of Hofstee (23). The *lines* were drawn from weighted least squares analysis of the data before linearization, and calculated using a North Star Horizon II microcomputer.

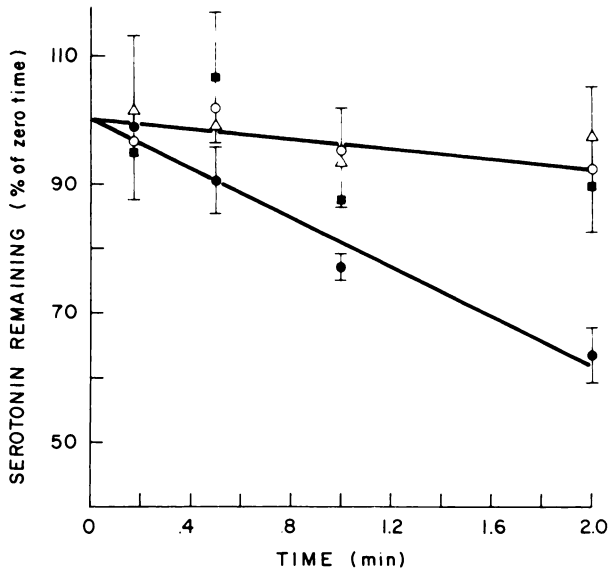


FIG. 7. *Effect of propranolol on serotonin efflux and exchange from porcine vesicles*

Efflux was measured as described by Talvenheimo *et al.* (10). The time after vesicles were diluted into medium free of [$^3$H]serotonin to initiate efflux is shown on the abscissa. Vesicles were diluted into buffer free of serotonin (*open circles* and *filled squares*) or containing 1.5 μM unlabeled serotonin (*filled circles* and *triangles*) in the presence (*triangles* and *squares*) or absence (*circles*) of 100 μM propranolol. Each *point* represents the mean of three determinations with the *error bars* indicating standard deviation.

cles relatively slowly (*open circles*), but external unlabeled serotonin dramatically increased the rate of efflux (*closed circles*). Propranolol (1 μM) in the external medium had little effect by itself (*squares*), but completely blocked serotonin induced efflux (*triangles*). In three other experiments, only the rate in the presence of serotonin alone significantly differed from the control. Thus, propranolol is unable to exchange for serotonin, but inhibits serotonin exchange.

*Inhibition of imipramine binding.* Similarities between the effects of imipramine and propranolol suggested that they might bind at the same site, which is believed to be the substrate site on the plasma membrane transporter (10). The data presented in Fig. 8 demonstrate that propranolol displaces imipramine from this site. Half-maximal inhibition occurs at approximately 1 μM and inhibition is almost complete at 10 μM propranolol. Although data are not shown, nonspecific imipramine binding (observed in the presence of an excess of serotonin or in the absence of $Na^+$) is not displaced by propranolol, strongly suggesting that propranolol also binds to the serotonin site on the transporter.

## DISCUSSION

The data presented in this paper clearly indicate that propranolol inhibits serotonin accumulation into platelets by competitive inhibition of the $Na^+$-dependent plasma membrane transport system. This conclusion contrasts with the proposal of Grobecker *et al.* (17), who suggested that propranolol induced a nonspecific increase in membrane stability. Furthermore, the approach outlined here may be applied to locating the site of action of other inhibitors of serotonin accumulation. In intact platelets, the use of specific inhibitors of plasma membrane and dense granular serotonin transport systems distinguishes between effects at either of those sites, whereas transport measurements using subcellular membrane fractions can confirm and extend such initial results.

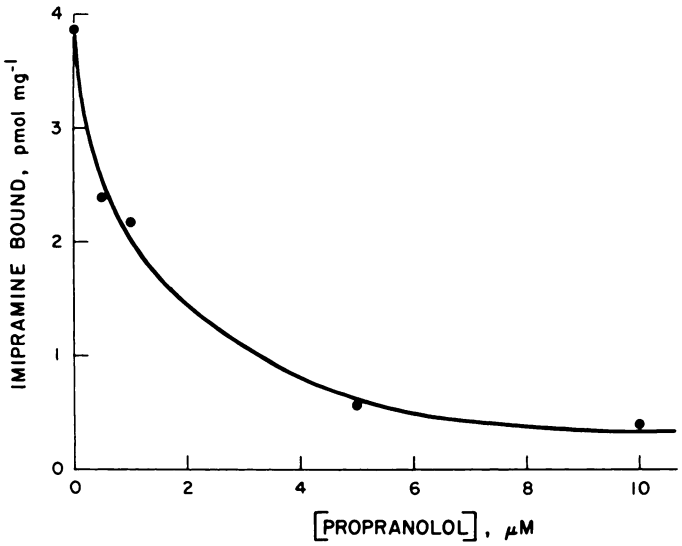


FIG. 8. *Inhibition of imipramine binding to porcine plasma membrane vesicles by propranolol*

Imipramine binding was measured as described under Methods in the presence of the indicated concentrations of DL-propranolol.

We have used plasma membrane vesicles isolated from porcine platelets for measurements of serotonin transport and imipramine binding. Although it is preferable to use membrane vesicles from human platelets, the large volumes of fresh human blood required for membrane vesicle isolation are beyond our resources. However, plasma membrane vesicles prepared from outdated human platelets transported serotonin in a manner qualitatively indistinguishable from that of porcine vesicles, although the rates and extents of transport are lower. We therefore consider porcine platelet plasma membrane vesicles an appropriate model system.

In intact platelets, the initial rate of serotonin uptake is primarily a reflection of the plasma membrane transport system, whereas the steady-state level of accumulation also reflects storage in dense granules. Thus, an inhibitor such as propranolol blocks the initial rate of transport much more than the steady-state level (Fig. 1), but reserpine, which blocks storage of serotonin in dense granules, primarily inhibits steady-state (Fig. 2 and 3). Furthermore, an inhibitor like reserpine renders accumulated serotonin sensitive to uncouplers which deenergize the plasma membrane system by dissipating the $Na^+$ and $K^+$ gradients (Fig. 2), whereas propranolol does not (data not shown). Finally, at high external serotonin concentrations, where reserpine significantly inhibits the steady-state level of accumulation, propranolol does not alter the degree of reserpine inhibition (Fig. 3), supporting the proposal that the two compounds inhibit different sites.

The distinction between inhibitors of the plasma membrane and granule transport systems is more obvious in membrane fractions where each system can be studied independently. Membranes from osmotically lysed platelets contain vesicles derived from dense granules in addition to plasma membrane vesicles. In the presence of ATP at low $Na^+$ concentrations, only the dense granule vesicles transport serotonin, a process inhibited by reserpine but not by imipramine (9). The fact that propranolol does not inhibit this transport process (Fig. 6) confirms the results with intact platelets that reserpine and propranolol have different sites of action. Moreover, propranolol inhibits serotonin transport into purified plasma membrane vesicles in the same way that imipramine inhibits (Fig. 5 and 7 and ref. 10), and also displaces, bound imipramine (Fig. 8).

The similarities between the effects of propranolol and imipramine raise an important question: is propranolol a substrate for the serotonin transporter, or is it, like imipramine, merely bound to the substrate site? Although this question can be answered only by more detailed investigation of propranolol binding and accumulation in membrane vesicles, the results presented here suggest that propranolol is not a substrate for the plasma membrane transporter. The small amount of propranolol associated with platelets and its relative lack of time dependence (Fig. 4) are uncharacteristic of active transport, and the inability of propranolol to exchange with serotonin (Fig. 7) also argues against it being a substrate.

The relationship between serotonin and the control of blood pressure (24, 25) and arrythmias (26, 27) is controversial. Middleniss *et al.* (28) demonstrated that propranolol binds to central serotoninergic receptors with about the same affinity as the serotonin receptor blocker methysergide. Our findings that propranolol produces an inhibition of serotonin transport into platelets suggest an additional site for propranolol action in central serotoninergic pathways. Furthermore, our findings that D- and DL-propranolol are equipotent inhibitors of serotonin transport are consistent with the observation that both D- and DL-propranolol reduce blood pressure when injected into the central nervous system of the spontaneously hypertensive rat and the cat (29, 30). Our results clearly show that propranolol acts at a different site than do other antihypertensives such as reserpine, which inhibits serotonin storage in dense granules and synaptic vesicles. Propranolol inhibits serotonin transport across the plasma membrane. However, the significance of our observations relative to the central effects of propranolol remains to be evaluated.

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