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Depolarization-induced release of propranolol and atenolol from rat cortical synaptosomes

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1 The accumulation and release of [^{3}H]-propranolol and [^{3}H]-atenolol were examined in synaptosomes from rat cerebral cortex.

2 Synaptosomes accumulated 20 pmol propranolol and 0.6 pmol atenolol mg^{-1} protein when incubated at 30°C with radiolabelled drugs (0.1 μM).

3 Exposure of propranolol-loaded synaptosomes to elevated K^+, Rb^+ or Cs^+ evoked a concentration-dependent increase in propranolol efflux. The action of these ions in releasing propranolol was highly correlated with their ability to produce synaptosomal membrane depolarization, as estimated with the voltage-sensitive dye diS-C_3-(5).

4 Elevated K^+ also promoted atenolol release from synaptosomes in a concentration-dependent manner.

5 Veratridine (10 μM) released propranolol and atenolol from synaptosomes and these effects were antagonized by tetrodotoxin (1 μM).

6 Under Ca^{2+}-free conditions, K^+-induced release of propranolol was reduced by 37% and atenolol release was diminished by 68%.

7 The results support the concept that both polar and non-polar β-adrenoceptor blocking drugs may be accumulated by nerve endings for release by membrane depolarization and suggest that neural storage and release of these molecules may influence their concentrations at localized sites of action.

Introduction

Chronic administration of β-adrenoceptor blocking drugs has been shown to diminish the vasoconstrictor response to sympathetic nerve stimulation in a variety of preparations. Lewis (1974) found that practolol pretreatment decreased the pressor response of pithed rats to electrical stimulation. Ljung *et al.*,(1975) described similar observations in the isolated portal vein of spontaneously hypertensive rats administered either metoprolol or propranolol. Propranolol pretreatment has also been observed to attenuate the vasoconstrictor response to nerve stimulation in the perfused canine gracilis muscle preparation (Russell *et al.*, 1983). Taken collectively, these results suggest that chronic treatment with β-adrenoceptor blocking drugs may significantly affect transmission at vascular neuroeffector junctions.

In previous work we have demonstrated that propranolol is released along with noradrenaline (NA) during sympathetic nerve stimulation at several sites. In the heart, activation of the cardiac accelerator nerves or the intravenous administration of tyramine evoked a parallel increase in the overflow of both propranolol and NA into the coronary sinus blood of chronically pretreated dogs (Daniell *et al.*, 1979). Nerve-stimulation induced release of propranolol was also observed in the spleen and the vasculature of the perfused canine hindlimb (Russell *et al.*, 1983). Similarly, in man, treadmill exercise was found to produce a simultaneous elevation of propranolol and NA in the plasma of hypertensive patients and normal volunteers receiving therapeutic doses of drug (Hurwitz *et al.*, 1983). Thus, propranolol release in association with sympathetic nerve stimulation appears to represent a generalized phenomenon at adrenergic neuroeffector junctions and, conceivably, may contribute to pharmacological actions of the drug at vascular and other synapses.

In the present study, the release of propranolol in response to membrane depolarization was examined in synaptosomes from rat cerebral cortex and compared with the release of the more polar, cardioselective β-adrenoceptor blocking drug atenolol. Both

drugs accumulated in synaptosomes and were released by membrane depolarization. However, differences between propranolol and atenolol were observed in terms of the characteristics of their respective accumulation and release processes. The findings are consistent with the concept that neural accumulation and release may be a phenomenon that applies to all β-adrenoceptor blocking drugs and that may have relevance to certain of their therapeutic actions.

Methods

Preparation of synaptosomes

Synaptosomes were prepared from rat cerebral cortex by discontinuous sucrose density gradient centrifugation as described by Gray & Whittaker (1962). All preparative procedures were performed at 4°C and each sucrose solution contained 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) and was adjusted to pH 7.4. Adult male Sprague-Dawley rats (225–275g) were killed by decapitation and whole brains removed. The cerebral cortex was dissected free and homogenized in 10 volumes of ice cold 0.32 M sucrose solution using a glass homogenizer with a loosely fitting Teflon pestle (0.25 mm clearance) at 800 r.p.m. for 10 passes. The resulting homogenate was centrifuged at 1000 *g* for 10 min and the supernatant decanted and retained. The pellet was resuspended with 0.32 M sucrose solution and centrifuged at 1000 *g* for an additional 10 min. The resulting supernatant was added to that from the first centrifugation and centrifuged at 17,000 *g* for 20 min to yield the crude mitochondrial pellet or P_2 fraction. The P_2 fraction was resuspended in 0.32 M sucrose solution, layered over a discontinuous sucrose density gradient consisting of equal volumes of 1.2 M and 0.8 M sucrose solutions, and then centrifuged using a Beckman SW-27 rotor at 53,000 *g* for 120 min. The synaptosome-enriched fraction was collected from the 0.8/1.2 M sucrose interface, diluted with an equal volume of water and centrifuged at 27,000 *g* for 15 min to yield the synaptosomal pellet. The synaptosomal pellet was resuspended in a volume of 0.32 M sucrose solution equivalent to 2 ml g^{-1} wet weight tissue and was then diluted with cold oxygenated modified Krebs-Ringer buffer (pH 7.4) of the following composition (mM): NaCl 145, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1.2, Na_2HPO_4 1.2, glucose 10, HEPES 20. Protein concentration was determined using the method of Lowry *et al.* (1951).

Accumulation studies

To study drug accumulation, each incubation tube contained 50 μl of synaptosome suspension in a total volume of 300 μl Krebs-Ringer buffer (pH 7.4) to give a final protein concentration of 400–600 μg ml^{-1}. After a 5 min equilibration period at 30°C, uptake was initiated by the addition of (−)-[^{3}H]-propranolol, (±)-[^{3}H]-atenolol or (−)-[^{3}H]-noradrenaline to give a final concentration of 0.1 μM. In the case of NA, the incubation medium also contained nialamide (12.5 μM) and ascorbic acid (10 μM). Incubations were terminated at intervals by the addition of 5 ml ice-cold Krebs-Ringer solution, followed by immediate vacuum filtration through Whatman glass-fibre filters (GFB). After an additional 5 ml wash with ice-cold Krebs-Ringer solution for propranolol and NA or two 5 ml washes for atenolol, the filters were dried and transferred to vials containing 10 ml toluene-based scintillation fluor (150 ml l^{-1} SAS solubilizer (Research Products International Corps.) 4 g l^{-1} 2,5-diphenyloxazole and 50 mg l^{-1} 1,4-di-(2-(5-phenyloxazolyl))benzene). The vials were shaken for 15 min and the radioactivity associated with the filters quantitated by liquid scintillation spectrometry. From the total radioactivity, accumulation of [^{3}H]-propranolol, [^{3}H]-atenolol and [^{3}H]-NA was calculated as the parent compound. Propranolol and atenolol were not metabolized by synaptosomes *in vitro* and, under similar conditions to those employed here, 90% of the NA taken up by synaptosomes was retained in the form of the parent molecule (Horn, 1973; Marquardt *et al.*, 1978). The accumulation of [^{3}H]-propranolol and [^{3}H]-atenolol was verified by solvent extraction of preloaded synaptosomes and subsequent chromatography. After incubation with radiolabelled drug, synaptosomes were collected by centrifugation and washed with drug-free Krebs-Ringer solution. The synaptosomes were then resuspended in Krebs-Ringer solution, made alkaline with NaOH, and extracted with 5 volumes of 1.5% isoamyl alcohol in toluene for preparations incubated with [^{3}H]-propranolol (Daniell *et al.*, 1979) or with 10 volumes of methylene chloride for preparations incubated with [^{3}H]-atenolol. Recovery of synaptosome-associated radioactivity was 84% for [^{3}H]-propranolol-loaded synaptosomes and 97% for [^{3}H]-atenolol-loaded preparations. In each case, thin layer chromatography of the extract on silica gel revealed a single peak of radioactivity which migrated as the original drug (i.e. not a metabolite). Solvent systems used were: toluene: ethyl acetate: ammonium hydroxide (0.88): ethanol, 6:2:1:4 (v/v); butanol: acetic acid: water, 4:1:1(v/v); isopropanol: ammonium hydroxide (0.88): water, 20:1:4 (v/v).

Release studies

For the release experiments, synaptosome suspensions were preloaded with [^{3}H]-propranolol (0.1 μM), [^{3}H]-atenolol (1.0 μM) or [^{3}H]-NA (0.1 μM) by incubation at 30°C as described above. In the case of propranolol

and atenolol, aliquots of the preloaded synaptosomes were used directly for release experiments, while synaptosomes loaded with NA were first centrifuged at 12,000 *g* for 5 min, to remove external NA and then resuspended in Krebs-Ringer solution. In all cases aliquots of the prelabelled synaptosomes were further incubated in either control buffer or in test buffer containing elevated concentrations of ions or drugs. In experiments in which the concentration of extracellular potassium or other monovalent cations was increased, the concentration of sodium was decreased to maintain isosmotic conditions. Propranolol and atenolol 'releasing' buffers also contained radiolabelled drug to achieve a final concentration in the external media of 0.08 μM [^{3}H]-propranolol and 0.8 μM [^{3}H]-atenolol. Under these conditions, approximately 5% of the drug associated with the synaptosome was lost spontaneously over the time course of an experiment, typically 2–3 min. At the end of the releasing period, the synaptosomes were collected by vacuum filtration on Whatman glass-fibre filters (GFB). The filters were then rinsed with two 5 ml washes of ice-cold Krebs-Ringer solution in studies with propranolol and NA or with three 5 ml washes in experiments with atenolol. Radioactivity remaining on the filters was then quantitated as described above. Drug or transmitter release from the synaptosomes was generally expressed as a percentage of the total present before depolarization. In all cases, spontaneous or basal release was subtracted from total release observed in the presence of depolarizing stimuli in order to arrive at a figure for net stimulus-induced release.

Determination of changes in synaptosomal membrane potential

Synaptosomal membrane potential was monitored by measuring the fluorescence of the voltage-sensitive dye, 3,3'-dipropyl-2,2'-thiodicarbocyanine, designated as diS-C_3-(5) (Sims *et al.*, 1974). Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer attached to a strip chart recorder. Excitation and emission wavelengths were 622 nm and 670 nm, respectively, and 1 mm slit widths were used. Fluorescence measurements were made after addition of diS-C_3-(5) to a suspension of synaptosomes in Krebs-Ringer buffer containing varying concentrations of potassium or other alkali metal ions. As before, osmolarity was maintained constant by adjustments in the concentration of sodium. Specifically, an aliquot of synaptosome suspension (20 μg protein) was added to prewarmed incubation medium (30°C) to give a final volume of 1.5 ml. The cuvette was placed in the spectrophotofluorometer and allowed to equilibrate for 1 min. Dye solution was then added to give a concentration of 1 μM and fluorescence was recorded. The fluorescence observed was expressed arbitrarily as percentrage of that observed when the external potassium concentration was 100 mM.

Pretreatment with 6-hydroxydopamine

Rats were anaesthetized with ether and 20 μl of a solution of 6-hydroxydopamine hydrobromide was injected into the cerebrospinal fluid of the lateral ventricle according to the method of Noble *et al.*, (1967). The 6-hydroxydopamine was dissolved in artificial cerebrospinal fluid containing ascorbic acid (1 mg ml^{-1}) and adjusted to pH 5.0 with HCl. Each rat was injected with two doses of 250 μg 6-hydroxydopamine at an interval of 24 h and killed 16 to 25 days after the last dose. Synaptosomes were then prepared from the cerebral cortex as described. Control animals were injected with equal volumes of vehicle. The effectiveness of 6-hydroxydopamine pretreatment was assessed by measuring the concentration of endogenous NA. For this determination an aliquot of homogenate of the cerebral cortex was acidified with 0.4 N perchloric acid and NA in the supernatant was assayed using the radioenzymatic method of Henry *et al.*, (1975).

Drugs

(−)-[4-^{3}H]-propranolol (sp. act. 22 Ci mmol^{-1}) and (−)-[2,5,6-^{3}H]-noradrenaline (sp. act. 53.5 Ci mmol^{-1}) were purchased from New England Nuclear Corp. (±)-[2-^{3}H]-atenolol (sp. act. 31 Ci mmol^{-1}) was a gift from ICI Pharmaceuticals, Macclesfield, England. Veratridine was obtained from Sigma and tetrodotoxin from Calbiochem.

Statistical analysis

Data are presented as means ± standard error of the mean (s.e.mean). Where appropriate, means were compared by the use of Student's *t* test for paired or unpaired data. The remaining results were analysed by repeated measures analysis of variance, followed by Least Significant Difference analysis.

Results

[^{3}H]-propranolol and [^{3}H]-atenolol accumulation

The time courses for synaptosomal accumulation of [^{3}H]-propranolol (0.1 μM) and [^{3}H]-atenolol (0.1 μM) at 30°C are shown in Figure 1. Accumulation of propranolol was rapid, with 75% of maximal uptake achieved within seconds after addition of labelled drug to the synaptosomal suspension. Equilibrium was attained by 1 min, at which time 20.0 ± 1.5 pmol

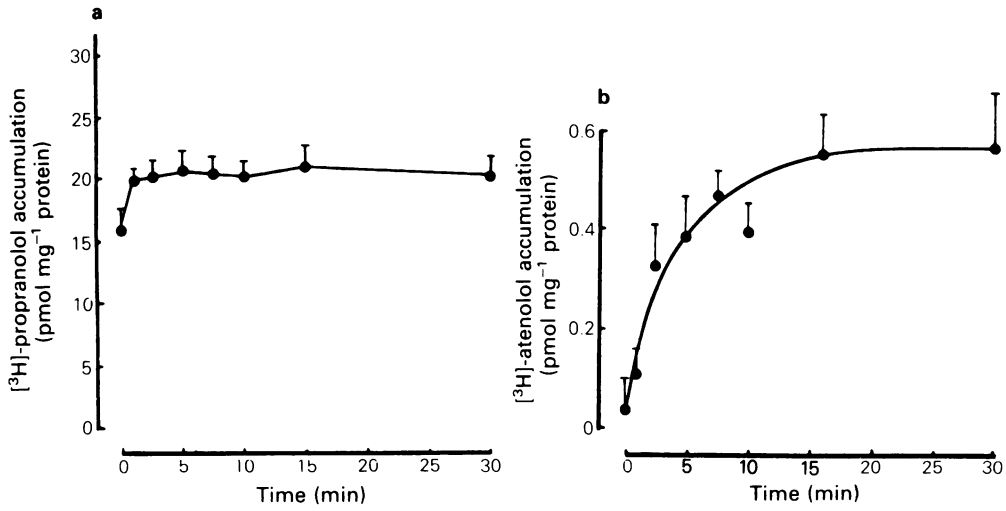

Figure 1 Time course of the accumulation of radioactivity by rat cortical synaptosomes during incubation at 30°C with [^{3}H]-propranolol (a) or [^{3}H]-atenolol (b). A drug concentration of 0.1 μM was used in both cases. Each point represents the mean of 4 to 6 determinations. Vertical lines represent s.e.mean.

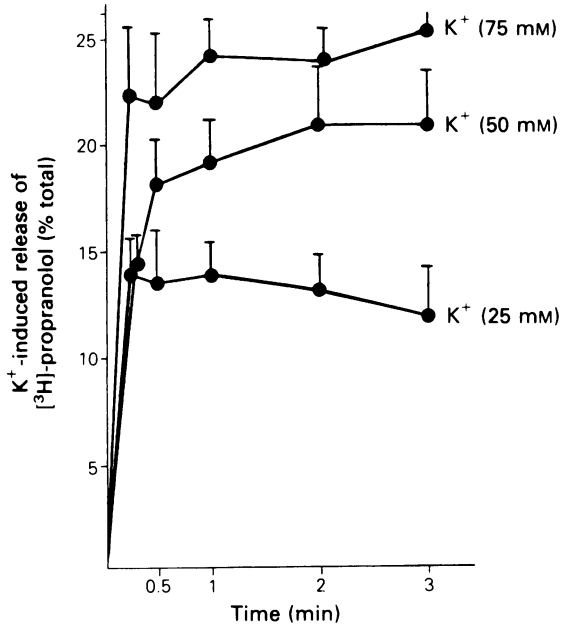

Figure 2 Time course of potassium-induced release of [^{3}H]-propranolol from prelabelled rat cortical synaptosomes at 30°C. Synaptosomes were preloaded by incubation with [^{3}H]-propranolol (0.1 μM) for 10 min. Each point represents the mean of 3 to 15 determinations. Vertical lines represent s.e.mean.

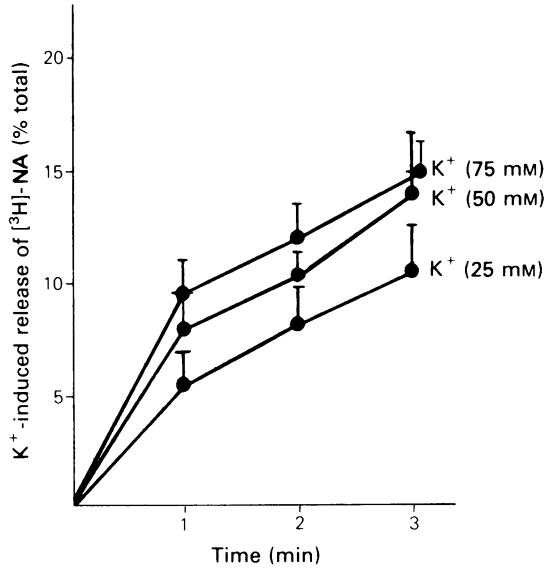

Figure 3 Time course of potassium-induced release of [^{3}H]-noradrenaline (NA) from rat cortical synaptosomes at 30°C. Synaptosomes were preloaded by incubation with [^{3}H]-NA (0.1 μM) for 10 min. Each point represents the mean of 5 determinations. Vertical lines represent s.e.mean.

propranolol was accumulated per mg synaptosomal protein. In comparison, atenolol was accumulated at a slower rate, reaching a steady-state concentration of 0.6 ± 0.1 pmol mg^{-1} protein after 15 min of incubation. On the basis of an intrasynaptosomal water space of 4.0 to 6.2 $\mu l\ mg^{-1}$ protein (Deutsch & Rafalowska, 1979; Wood & Wylie, 1983), the equilibrium distribution of propranolol between tissue and incubation medium was estimated to represent a 33 to 50 fold synaptosomal concentration of drug. However, the corresponding distribution ratio for atenolol was in the range 1.0 to 1.4. Because of the relatively low steady-state concentration of atenolol achieved under these conditions, 1.0 μM [^{3}H]-atenolol was subsequently utilized to preload synaptosomes for the release experiments.

[^{3}H]-propranolol release

Exposure of synaptosomes preloaded with [^{3}H]-propranolol to elevated concentrations of external potassium produced a concentration-dependent increase in drug efflux (Figure 2). At 75 mM external potassium, approximately 26% (5.1 ± 0.9 pmol mg^{-1} protein) of the total propranolol accumulated by the synaptosomes was released. The potassium-induced release of propranolol was rapid and was complete within 15–30 s for all concentrations of the ion examined. Spontaneous efflux of drug over the 3 min time-course of these experiments was 5.0 ± 1.1% of the tissue total. For comparison, the time course of potassium-induced [^{3}H]-NA release from synaptosomes is illustrated in Figure 3. At all concentrations of external potassium, the release of NA was continuous for the 3 min examined and appeared to be linear between the 1 and 3 min time points. After 3 min exposure to 75 mM potassium, approximately 15% (1.1 ± 0.2 pmol mg^{-1} protein) of the NA taken up by the synaptosomes had been released.

To determine if the release of propranolol in response to elevated potassium was related to membrane depolarization, the ability of a series of alkali metal ions to promote propranolol release was examined and compared with the relative actions of these ions to depolarize synaptosomal plasma membranes. Rubidium was equipotent to potassium in

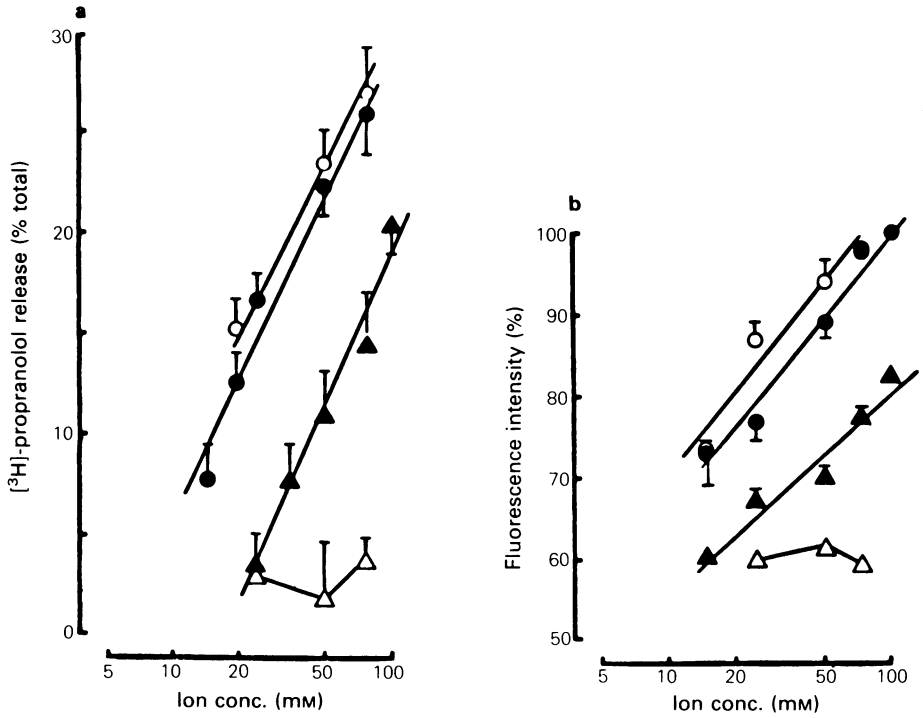

Figure 4 Effects of alkali metal ions in promoting [^{3}H]-propranolol release (a) and depolarizing synaptosomes (b). (○) Rubidium, (●) potassium, (▲) caesium and (△) lithium. [^{3}H]-propranolol release was measured for one minute. Membrane depolarization is reflected as an increase in the intensity of dye (diS-C_3-(5)) fluorescence, expressed as a percentage of the fluorescence observed at 100 mM external potassium. Each point represents the mean of 2 to 10 determinations. Vertical lines represent s.e.mean.

producing propranolol release (Figure 4a). Caesium, however, was less potent to the extent that a three fold greater concentration of caesium was required to elicit release equivalent to that observed with potassium or rubidium. The concentration of ion required to release in one minute 15% of the propranolol accumulated by the synaptosomes was 21 ± 3 mM for potassium, 20 ± 2 mM for rubidium and 61 ± 4 mM for caesium ($P < 0.01$, when compared to potassium). For these three ions, propranolol release was linearly related to log external ion concentration over the range examined. Lithium ion, in concentrations up to 75 mM, had no effect on propranolol efflux.

The ability of these ions to induce changes in the synaptosomal membrane potential was monitored with the voltage-sensitive fluorescent probe, diS-C_3-(5). Depolarization of the synaptosomes was reflected as an increase in the intensity of dye fluorescence (Figure 4b). An approximately linear relationship was observed between log external potassium concentration and synaptosomal fluorescence, a finding consistent with the proposal that synaptosomal membrane potential approximates the potassium diffusion potential (Blaustein & Goldring, 1975). Rubidium ion was essentially equipotent to potassium in producing membrane depolarization, whereas caesium was approximately 3 times less potent and lithium had no effect on membrane potential. As shown in Figure 5, the ability of each of these ions to depolarize synaptosomes was highly correlated ($P < 0.01$; $r = 0.96$) with their ability to promote propranolol release.

Exposure of [^{3}H]-propranolol loaded synaptosomes to the membrane depolarizing agent veratridine also resulted in an increase in drug efflux (Figure 6). At a concentration of 10 μM, veratridine released over 30% of the propranolol accumulated by the synaptosomes. Maximal release was attained within 1 min. The prior addition of tetrodotoxin (1 μM), in a concentration which inhibited veratridine-induced release of NA by over 90% (Figure 6), reduced the veratridine-induced release of propranolol by 40% but did not abolish the effect. Higher concentrations of tetrodotoxin had no greater effect.

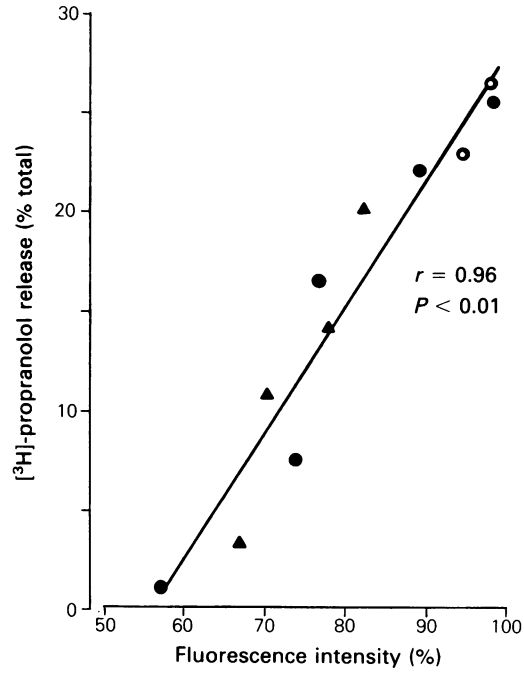

Figure 5 Regression analysis of the relationship between the release of [^{3}H]-propranolol from synaptosomes by the ions K^+, Rb^+ and Cs^+ and their depolarization of the synaptosomal membrane. Membrane depolarization is expressed as the intensity of dye (diS-C_3-(5)) fluorescence as in Figure 4. (●) Potassium, (○) rubidium and (▲) caesium.

[^{3}H]-atenolol release

The effect of increasing concentrations of potassium on [^{3}H]-atenolol release is shown in Figure 7. Elevation of external potassium resulted in a concentration-dependent increase in atenolol efflux from the synaptosomes. Exposure to 75 mM potassium for 2 min released approximately 25% (2.5 ± 0.3 pmol mg^{-1} protein) of the total atenolol accumulated. The time course of atenolol release was slower than that found for propranolol (Figure 2) and, for all concentrations of potassium, was approximately linear after the initial 30s time point. In this respect, potassium-induced atenolol release closely resembles the release observed for NA (Figure 3). Spontaneous atenolol efflux averaged 4.9 ± 0.7% over 2 min. Elevation of external caesium was also observed to release atenolol from synaptosomes, with 75 mM caesium inducing in 2 min the release of 12.3 ± 1.1% of the atenolol accumulated. This release was approximately equal to that produced by 25 mM potassium (15.6 ± 2.0%).

The addition of veratridine to atenolol-loaded synaptosomes also produced an increase in drug efflux. Exposure to 10 μM veratridine for 2 min resulted in the release of approximately 15% (1.0 ± 0.1 pmol mg^{-1} protein) of the total atenolol accumulated. In the presence of tetrodotoxin (1 μM), the veratridine-induced release of atenolol was reduced by approximately 90% (Figure 8).

Calcium-dependence of drug release

To evaluate the importance of extracellular calcium in these release processes, potassium-induced release of [^{3}H]-propranolol, [^{3}H]-atenolol and [^{3}H]-NA was ex-

amined in the presence and absence of the calcium ion. The potassium-induced release of all three compounds was sensitive to extracellular calcium, though to varying degrees. In calcium-free medium supplemented with 2 mM EGTA, potassium-induced release of propranolol was reduced by 37% while atenolol release was decreased by 68% and NA release by 82% (Table 1).

Effect of 6-hydroxydopamine pretreatment

To examine the contribution of adrenergic nerve terminals to the accumulation and release of propranolol and atenolol in the synaptosomes, rats were pretreated centrally with 6-hydroxydopamine to destroy adrenergic nerve endings in the brain. Administration of 6-hydroxydopamine significantly reduced the endogenous concentration of NA in the cerebral cortex from 136 ± 5 ng g^{-1} tissue in control animals to 17 ± 9 ng g^{-1} ($P < 0.01$) in treated animals. However, pretreatment with 6-hydroxydopamine did not alter the steady state accumulation or the potassium-induced release of either propranolol or atenolol in the synaptosome preparation (Table 2).

Discussion

Two β-adrenoceptor antagonists, the highly lipophilic drug propranolol and the more hydrophilic and cardioselective agent atenolol, were both accumulated by rat cortical synaptosomes, but there were differences between the two compounds in their rates of accumulation and in the steady-state levels attained.

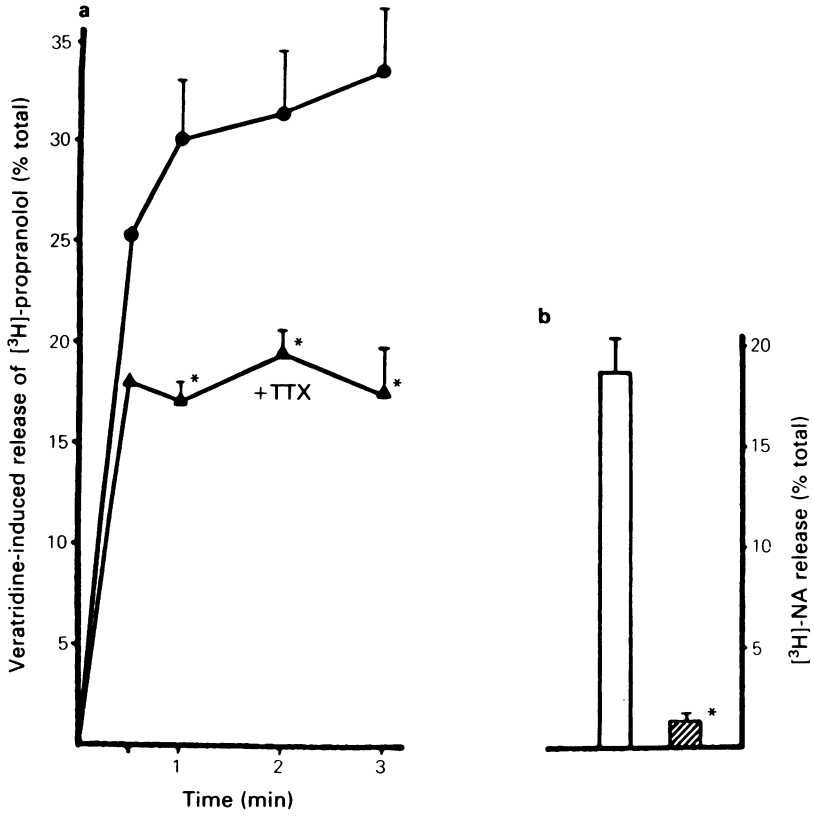

Figure 6 Effect of tetrodotoxin (TTX; 1μM) on veratridine (10μM)-induced release of [^{3}H]-propranolol (a) and [^{3}H]-noradrenaline (NA) (b) from rat cortical synaptosomes. In (a), (●) represents veratridine-induced release and (▲) veratridine-induced release in the presence of TTX. In (b), open column represents veratridine-induced release and hatched column, veratridine-induced release in the presence of TTX. [^{3}H]-NA release was measured for 3 min. Each point represents the mean of 2 to 9 determinations. Vertical lines represent s.e.mean. Statistically significant differences from veratridine-induced release in the absence of TTX are indicated by * $P < 0.01$.

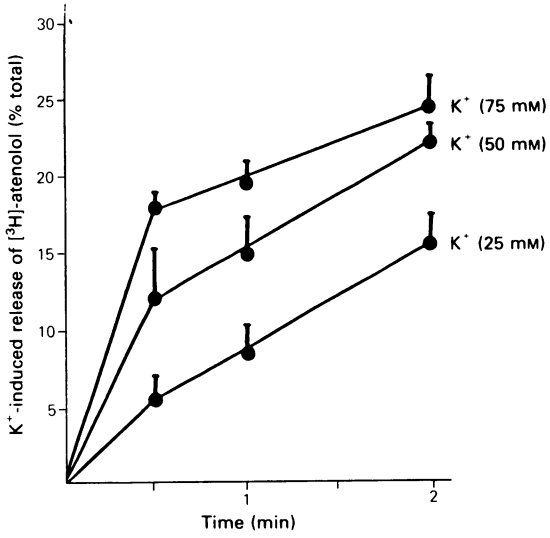

Figure 7 Time course of potassium-induced release of [^{3}H]-atenolol from prelabelled rat cortical synaptosomes at 30°C. Synaptosomes were preloaded by incubation with [^{3}H]-atenolol (1.0 μM) for 20 min. Each point represents the mean of 4 to 11 determinations. Vertical lines represent s.e.mean.

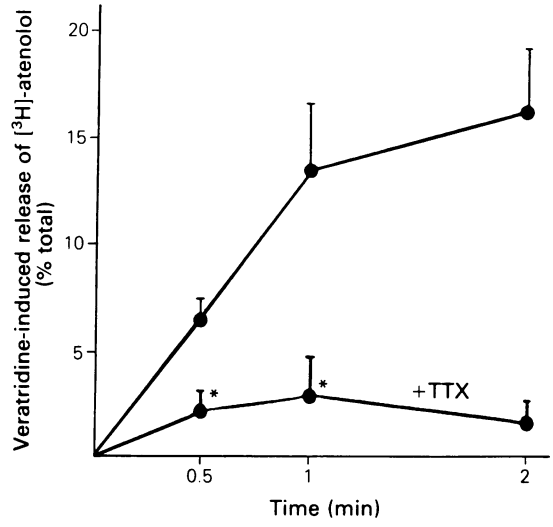

Figure 8 Effect of tetrodotoxin (TTX; 1μM) on veratridine (10μM)-induced release of [^{3}H]-atenolol from rat cortical synaptosomes. Each point represents the mean of 5 determinations. Vertical lines represent s.e.mean. Statistically significant differences from veratridine-induced release in the absence of TTX are indicated by * $P < 0.01$.

In contrast to the rapid accumulation of propranolol, which reached a steady state level of 20 pmol mg^{-1} synaptosomal protein within 1 min, atenolol was accumulated at a much slower rate and an equilibrium concentration of 0.6 pmol mg^{-1} protein was attained after 15 min of incubation. These findings probably reflect differences in the lipophilicity of the two drugs, propranolol having a log octanol/water partition coefficient of 3.65, while the corresponding value for atenolol is 0.24 (Cruickshank, 1980). Neither propranolol nor atenolol appears to be a substrate for the biogenic amine uptake system since the uptake of these drugs is not stereoselective, is insensitive to inhibition by cocaine and ouabain (Saelens *et al.*, 1977; Street *et al.*, 1984; Street, unpublished observations) and was not reduced by pretreatment with 6-hydroxydopamine. For atenolol, the synaptosome/medium distribution ratio of 1.0 to 1.4 supports this concept and is consistent with the passive equilibration of atenolol between intracellular and extracellular water. For propranolol, a synaptosome/medium distribution ratio in the range 33 to 50 was estimated, suggesting that propranolol is concentrated by the synaptosome, an observation which probably reflects binding of drug to cellular proteins (Barber *et al.*, 1978; Muller & Stillbauer, 1983) and membrane phospholipids

Table 1 Effect of calcium of the potassium-induced release of [^{3}H]-propranolol, [^{3}H]-atenolol and [^{3}H]-noradrenaline from rat cortical synaptosomes

	K$^+$(75mM)-induced release (pmol mg^{-1} protein)		
	Propranolol[a]	*Atenolol*[a]	*Noradrenaline*[b]
Control, 2 mM Ca^{2+}	5.1 ± 0.9	2.5 ± 0.3	1.1 ± 0.2
0 mM Ca^{2+}, 2 mM EGTA	3.2 ± 0.6*	0.8 ± 0.2*	0.2 ± 0.1*
	(↓ 37%)	(↓ 68%)	(↓ 82%)

[a] Release observed for 2 min.
[b] Release observed for 3 min.
* $P < 0.05$ compared to control release; % reduction given in parentheses. Each value represents the mean ± s.e.mean from 4 to 6 observations.

Table 2 Effect of 6-hydroxydopamine on the accumulation and K^+-induced release of [^{3}H]-propranolol and [^{3}H]-atenolol from rat cortical synaptosomes

	Accumulation (pmol drug mg^{-1} protein)		*K^+ (75 mM)-induced release* (pmol drug mg^{-1} protein $2\,min^{-1}$)	
	Propranolol	*Atenolol*	*Propranolol*	*Atenolol*
Control	23.4 ± 4.5	6.7 ± 0.8	6.3 ± 1.7	1.0 ± 0.2
6-Hydroxydopamine treated	27.3 ± 6.8	7.6 ± 1.3	6.9 ± 2.0	1.3 ± 0.3

For accumulation and release experiments, synaptosomes were incubated with [^{3}H]-propranolol (0.1 μM) or [^{3}H]-atenolol (1.0 μM) to achieve steady state concentrations.
Each value represents the mean ± s.e.mean from 4 observations.

(Dachary-Prigent *et al.*, 1979; Surewicz & Leyko, 1981). Conversely, atenolol, a much more hydrophilic molecule, does not bind substantially to proteins (Barber *et al.*, 1978) or membrane phospholipids (Lullmann & Wehling, 1979).

Both propranolol and atenolol were released from synaptosomes by elevated concentrations of potassium or veratridine. The time course of potassium-induced NA release was measured as an index of exocytosis and compared to the time courses of potassium-induced propranolol and atenolol release from the synaptosomes. Release of both atenolol and NA was continuous over the time periods examined and appeared to increase linearly after the initial time point at 30 to 60 s. In contrast, propranolol release was rapid, being complete within 15–30 s. The fractional release of both propranolol and atenolol was within the range of 20–25% at 2 min and the absolute amounts of propranolol, atenolol and NA released ranged from 1–6 pmol mg^{-1} synaptosomal protein. It should be emphasized that in the atenolol release experiments, a loading concentration of 1 μM was used, compared to a concentration of 0.1 μM for propranolol and NA. This is not unreasonable in view of the fact that therapeutic plasma levels of atenolol are as much as ten fold higher than therapeutic levels of propranolol, particularly when the concentrations of free drug in plasma are considered (Amery *et al.*, 1977; Walle *et al.*, 1978).

In experiments designed to determine whether the release of the β-receptor blocking drugs by potassium was indeed a result of membrane depolarization, the relative potencies of potassium and other alkali metal ions to depolarize synaptosomal membranes were compared with their ability to release propranolol. In synaptosomes, changes in membrane potential induced by members of the alkali metal ion series have been monitored as changes in fluorescence of the voltage-sensitive dye, di0-C_5-(3) (Blaustein & Goldring, 1975). Rubidium was found to be equipotent to potassium in depolarizing the synaptosomal membrane and these ions were approximately four fold more potent than equal concentrations of caesium. These results are in close agreement with those of Kamino & Inouye (1978), who demonstrated similar potency ratios for alkali metal ions in the depolarization of isolated synaptic plasma membrane ghosts as measured with the impermeant dye, merocyanine-540. In the present experiments changes in the membrane potential were monitored with the voltage sensitive dye diS-C_3-(5) and the observed ion potency ratios for depolarization of the synaptosomal membrane were in close agreement with those found by the above workers (Blaustein & Goldring, 1975; Kamino & Inouye, 1978). Moreover, a similar potency ratio was observed for the action of these ions on the release of propranolol from synaptosomes. A strong positive correlation was found between the degree of synaptosomal membrane depolarization produced by individual ions and the ability of these ions to promote the release of propranolol. This observation is consistent with the idea that the release of propranolol induced by potassium and other alkali metal ions is the result of membrane depolarization. It is also noteworthy that elevated potassium does not cause the release of propranolol from myelin or mitochondria (Street *et al.*, 1984), the principal contaminants of synaptosome preparations, nor does it depolarize these components (Blaustein & Goldring, 1975).

That release of propranolol and atenolol is a consequence of membrane depolarization is also supported by the series of experiments with veratridine and tetrodotoxin. Veratridine depolarizes membranes by increasing membrane permeability to sodium (Catterall, 1980), an action which is antagonized by tetrodotoxin (Ohta *et al.*, 1973). In the present experiments, tetrodotoxin, in a concentration which almost totally inhibited the veratridine-induced release of NA, antagonized veratridine-induced propranolol release but did not abolish the response. The tetrodotoxin-sensitive component of propranolol release is presumably related to depolarization, consistent with the observation that release promoted by alkali metal ions was related to their depolarizing

ability. The tetrodotoxin-insensitive component of veratridine-induced propranolol release may represent an interaction between veratridine and propranolol unrelated to depolarization, possibly a displacement of propranolol from membranes by the lipophilic veratridine molecule. Propranolol has been shown to bind to biological membranes and model membrane systems (Huunan-Seppala, 1972; Godin *et al.*, 1976; Lee, 1977; Herbette *et al.*, 1983) and membrane interactions between propranolol and other lipophilic amines have been demonstrated (Dollery & Junod, 1976; Vestal *et al.*, 1980; Hemsworth & Street, 1981; Rudnick *et al.*, 1981).

In experiments with atenolol, the veratridine-induced release of this drug was effectively abolished by tetrodotoxin, suggesting that the release of atenolol by veratridine is principally the result of membrane depolarization. This observation is consistent with the results of the atenolol accumulation experiments which suggest that little binding of atenolol to synaptosomal membranes occurs. Quantitatively, it is also interesting to note that the percentage of atenolol released by veratridine approximates the tetrodotoxin-sensitive component of propranolol release, implying that the magnitude of depolarization-induced release of both β-adrenoceptor blocking drugs was similar.

A comparison of the calcium dependence of potassium-induced release of propranolol, atenolol and NA indicated that the release of all three molecules was affected by extracellular calcium, though to varying degrees. As was the case for NA, the potassium-induced release of atenolol was largely calcium-sensitive. However, approximately 60% of potassium-induced propranolol release was independent of external calcium. The mechanism of propranolol release thus appears complex and it is possible that propranolol is released by a combination of processes. The calcium-dependent component of release may reflect exocytosis, while the release occurring in the absence of extracellular calcium may correspond to a non-exocytotic release mechanism. Alternatively, propranolol may be released solely by a non-exocytotic process which is sensitive to calcium but not entirely dependent on calcium for release to occur. The apparent complexity of synaptosomal propranolol release may reflect the fact that propranolol distributes within multiple compartments of the synaptosome and has been localized to the cytoplasm and synaptic plasma membrane as well as the synaptic storage vesicles (Street *et al.*, 1984). Conceivably, propranolol may be released from any of these sites as a result of perturbations associated with a depolarizing event. It is of interest to note that non-exocytotic release processes have been described for several endogenous substances including taurine (Sieghart & Heckl, 1976), γ-aminobutyric acid (Haycock *et al.*, 1978) and acetylcholine (Tauc, 1982; Meyer & Cooper, 1983). A further consideration is the possibility that the results are complicated by the heterogeneous population of nerve terminals and non-neural elements present in the synaptosome preparation.

While propranolol release from adrenergic neurones has been demonstrated previously (Daniell *et al.*, 1979; Russell *et al.*, 1983), the fact that 6-hydroxydopamine pretreatment failed to alter the release of propranolol or atenolol from synaptosomes suggests that the depolarization-induced release of β-adrenoceptor blocking drugs from this preparation does not require intact adrenergic nerve terminals, which in cortical synaptosomes comprise only 5% of the neuronal population (Iverson & Schon, 1973). Synaptosomes from cerebral cortex appear to be derived principally from cholinergic and γ-aminobutyric acid-containing nerve endings (Iverson & Bloom, 1972; Bradford, 1975) and either one or both types of terminals could represent a primary site for drug release from the synaptosome preparation. A contribution from 5-hydroxytryptamine-containing neurones, which are resistant to 6-hydroxydopamine treatment (Kostrzewa & Jacobowitz, 1974), is also a possibility. In this respect, it is of interest that propranolol has been found to modify tryptaminergic mechanisms within the central nervous system (Middlemiss *et al.*, 1977; Costain & Green, 1978; Green *et al.*, 1983).

In summary, the results of the present study demonstrate that both propranolol and atenolol are accumulated by synaptosomes and are released in response to membrane depolarization. The release of two drugs with such widely differing physiochemical properties but each containing the characteristic β-adrenoceptor blocking side chain supports the concept that neural storage and release may be a phenomenon common to β-receptor blocking drugs as a class. However, it remains to be determined whether the release mechanisms involved are selective for substances with the β-blocking side chain or are operative for other drugs as well. Finally, the finding that propranolol and atenolol appear to be released from non-adrenergic as well as adrenergic neurones suggests that these drugs may have the potential to influence multiple transmitter systems.

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