Br. J. Pharmac. (1984), **81**, 201–214

Role of auto-inhibitory feed-back in cardiac sympathetic transmission assessed by simultaneous measurements of changes in ^{3}H-efflux and atrial rate in guinea-pig atrium

J.A. Angus, A. Bobik, G.P. Jackman, I.J. Kopin[1] & P.I. Korner

Baker Medical Research Institute, Commercial Rd., Prahran, Victoria, Australia, 3181

1 Guinea-pig right atria were labelled with [^{3}H]-noradrenaline or [^{3}H]-dopamine before superfusion in a flow-cell. Choice of label did not significantly alter either the relationship between ^{3}H-efflux and number of electrical field pulses or the inhomogeneity of labelling.

2 The relationship between ^{3}H-efflux and frequency of 4 field pulses (0.125–2 Hz) was hyperbolic and similar to the tachycardia-frequency relationship measured simultaneously. No evidence was found for a U shaped ^{3}H-efflux-frequency relationship (Story, McCulloch, Rand & Standford-Starr, 1981).

3 Phentolamine (1 μM) did not alter the ^{3}H-efflux or atrial rate responses to 4 field pulses at stimulus levels that gave 50–60% of the maximum rate response.

4 In the presence of neuronal uptake inhibition (desipramine, DMI 0.1 μM), rate and ^{3}H-efflux responses to 4 field pulses were enhanced at all frequencies and were further increased by phentolamine.

5 In the absence of DMI, prolonged trains of field pulses (8 and 12 pulses) at low frequency (0.25 Hz) were not sufficient to activate auto-inhibitory feed-back. At 2 Hz phentolamine enhanced both ^{3}H-efflux and rate responses at 12 field pulses.

6 We conclude that in guinea-pig right atrium auto-inhibitory feed-back plays little role in the modulation of transmitter release at levels of stimulation that cause 50–60% of maximum tissue response. This is because neuronal uptake normally prevents synaptic concentrations of noradrenaline from activating prejunctional α_2-adrenoceptors. Stimulation sufficient to induce a near-maximal response or the presence of neuronal uptake inhibition are necessary to evoke auto-inhibitory feed-back.

Introduction

The prejunctional α_2-adrenoceptor has been considered to be part of an inhibitory feed-back loop whereby transmitter released from sympathetic nerve endings diminishes subsequent transmitter release (Starke, 1977; Rand, McCulloch & Story, 1980; Langer, 1981). Auto-inhibitory feed-back has been thought to modulate transmitter release from pulse to pulse (Rand, Story, Allen, Glover & McCulloch, 1973). Much of the work on which the hypothesis has been based involves analysis of changes in [^{3}H]-noradrenaline efflux under different conditions.

Angus & Korner (1980) recently challenged the importance of auto-inhibitory feed-back under moderate conditions of sympathetic stimulation. They found in the guinea-pig isolated right atrium that the tachycardia response was not influenced by pretreatment with phentolamine and yohimbine at sympathetic stimulus strengths that raised the rate up to 50% of the maximum response. This was investigated further by Story *et al.* (1981) who measured both ^{3}H-efflux and atrial rate changes over a wide range of stimulus frequencies. They confirmed the

[1]Present address: Intramural Research Program, Building 10, Room 5N-214, National Institute of Mental Health, National Institute of Health Bethesda, Maryland, 20205, U.S.A.

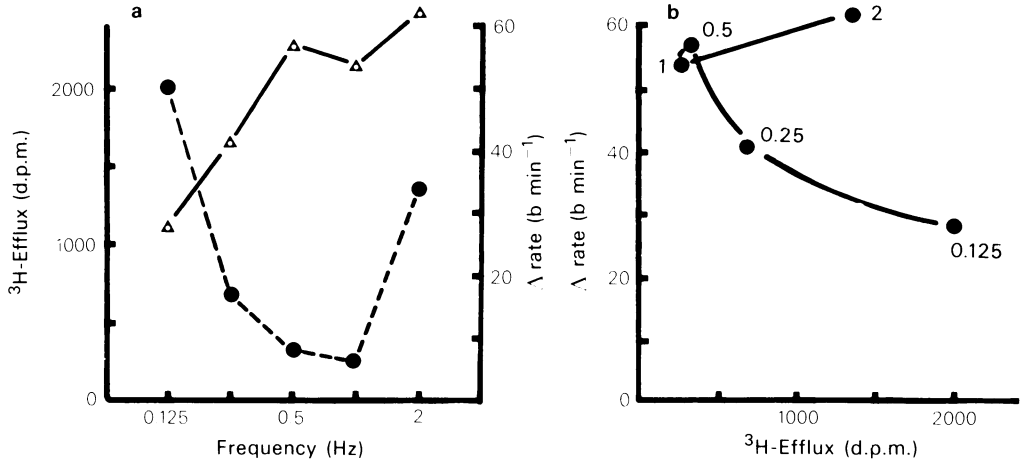

Figure 1 Data redrawn from Story *et al.* (1981) of the relationship between stimulus induced ^{3}H-efflux and tachycardia in guinea-pig atria during trains of 4 electrical field pulses at frequencies 0.125–2 Hz. (a) ^{3}H-Efflux (●---●) and tachycardia (△—△, rate beats min^{-1}) plotted against the frequency of the 4 pulses train. (b) Correlation graph of tachycardia and ^{3}H-efflux responses from the data on the left. Numbers refer to the frequency (Hz) of the 4 pulse train at which the rate and ^{3}H-efflux measurements were made.

absence of auto-inhibitory feed-back under stimulus conditions similar to those employed by Angus & Korner (1980). But they defined two specific conditions under which sympathetic transmission was altered by auto-inhibitory feed-back: (1) a maximum interval between successive stimuli (4–8 s) and (2) an adequate length of stimulus train of at least 1.5 s at higher frequencies (2 Hz).

Surprisingly with stimulation by 4 pulses Story *et al.* (1981) observed a U-shaped relationship between the frequency of stimulation and ^{3}H-efflux, which contrasted with the hyperbolic relationship that they observed between frequency and atrial rate (Figure 1, left). Hence the two effects of sympathetic stimulation (i.e. ^{3}H-efflux and the atrial rate response) were themselves related non-linearly (Figure 1, right). Both responses are functions of the transmitter concentration in the synaptic cleft during stimulation, so that the marked non-linearity between them is unexpected, though no comment about the anomaly was made by Story *et al.* (1981). If true it would suggest a completely different relationship between synaptic concentration of transmitter and the two measures of sympathetic stimulation, which *prima facie* appears unlikely.

The purpose of the present study was to reinvestigate the evidence for or against auto-inhibitory feed-back at various levels of sympathetic stimulation in the guinea-pig atrium. In the experiments of Story *et al.* (1981) the rate and ^{3}H-efflux responses had been determined in separate experiments, rather than simultaneously. In the present investigation the changes in ^{3}H-efflux and in rate were determined simultaneously using a superfused flow-through cell. The superfusion technique has been used to study [^{3}H]-noradrenaline release from brain slices (Baldesserini & Kopin, 1967) and rat atrium (Katz & Kopin, 1969). We compared the effects of phentolamine on the two sympathetic stimulus-response curves in right atria to assess the role of auto-inhibitory feed-back under normal conditions and in the presence of neuronal uptake block induced with desipramine (DMI).

Methods

Guinea-pig atrium preparation

Guinea-pigs (Hartley-English short haired) of either sex,weighing 500–750 g,were killed by cervical dislocation. The heart was rapidly removed and placed in warmed oxygenated solution (see below). The right atrium was carefully cut away from the right ventricle and surrounding vessels and two (2 mm diameter) platinum rings were tied to the tip of the atrial appendage and the cut edge of the inferior vena cava. The atrium was transferred to a 3 ml glass organ bath for ^{3}H-labelling and was suspended between 2 platinum rings on an epoxy (Araldite D) tissue support 3 mm in diameter. This support had 2 platinum field electrodes and 2 fine teflon coated platinum wires for measuring the surface electrogram (see flow cell method). The bathing solution was of the following composition (mM): Na^+ 144, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, $H_2PO_4^-$ 1.2, Cl^- 128.7, SO_4^{2-} 1.2,

HCO_3^- 25, glucose 11 and EDTA 0.04 and was maintained at 37°C bubbled with 95% O_2 and 5% CO_2. To prevent cholinergic responses during field stimulation, atropine 1 μM was added to the bathing solution in all experiments.

The atrium was equilibrated in the organ bath for 15 min before adding one of the following ^{3}H-labelled solutions (a) low specific activity noradrenaline (7-[^{3}H]; 1-5 Ci mmol^{-1}, 1 μM) (b) high specific activity noradrenaline (ring labelled -2,5,6,-[^{3}H]; 47.7 Ci mmol^{-1}, 0.1 μM) or (c) 3, 4 dopamine (dihydroxyphenylethylamine (ring labelled -2,5,6-[^{3}H]; 42 Ci mmol^{-1}, 0.25 μM). The tissue was left in contact with the [^{3}H]-noradrenaline for 30 min or 45 min for [^{3}H]-dopamine before replacing the bath solution once with ^{3}H-free solution and transferring the atrium to the flow-cell.

Flow-cell

An 800 μl capacity flow-cell was used to allow the simultaneous collection of ^{3}H-labelled superfusate and measurement of atrial rate (Figure 2). The right atrium was suspended vertically on a perspex leg that was sealed into a water jacketed perspex tube by two O-rings. A double-lumen polyvinyl chloride (P.V.C.) catheter was sealed into the base of the tube; 95% O_2 and 5% CO_2 were bubbled through one lumen whilst warm Krebs solution (see above) was pumped through the other at 4-6 ml min^{-1} using a Watson Marlow roller pump. The gas and superfusate were allowed to escape from a tube inserted in the top half of the leg. The right atrium was lightly stretched between the bottom of the leg and a platinum hook attached to a length of fine monofilament nylon led through a second P.V.C. catheter. This catheter was clamped after adjusting the initial length of the atrium. The bottom half of the perspex leg was cut away to allow the superfusion fluid to come into close contact with the atrium (see Figure 2). The smooth (upper) surface of the atrium was positioned against two short lengths of multistranded Teflon-coated platinum wires (Medwire Corp, New York) that were embedded in the perspex leg. These wires (surface electrodes) were bared at the tips to detect the spontaneous surface electrogram. Following amplification, this signal was used to trigger a microprocessor-controlled period meter, which had an accuracy of better than 2 ms. The cycle to cycle period (interval) was recorded continuously (Angus & Harvey, 1981). To depolarize the intramural autonomic varicosities electrical field pulses (130 mA, 2 ms) were applied across the atrium from the platinum field electrodes (wire diameter 0.8 mm length 1 cm). Because the main experiments called for fixed frequency of field stimulation, the field pulses were not

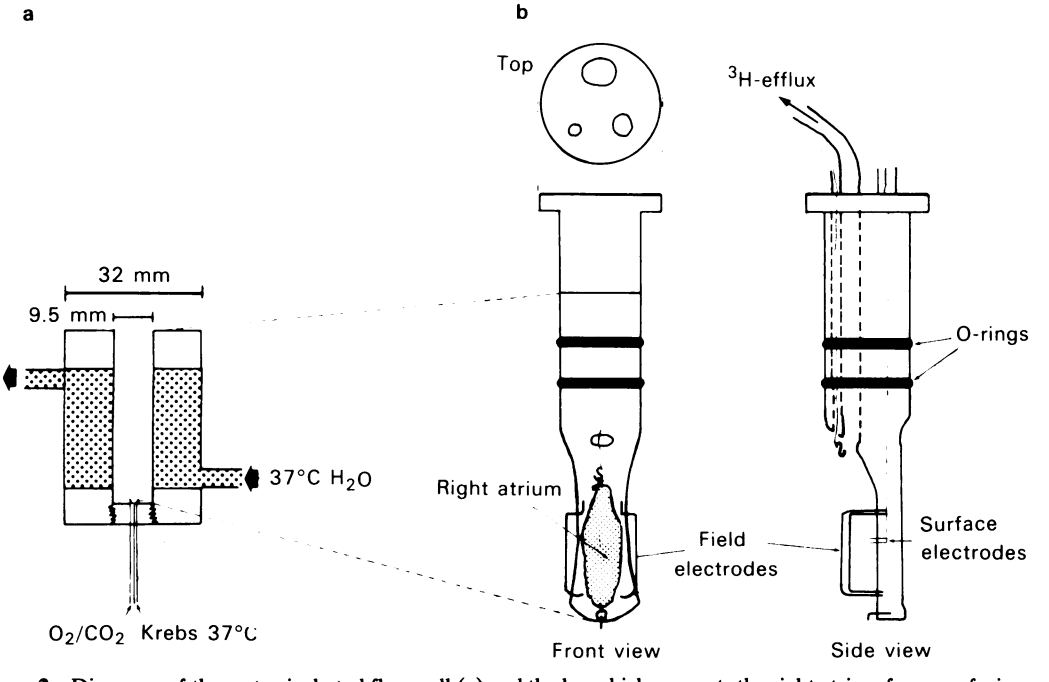

Figure 2 Diagrams of the water jacketed flow-cell (a) and the leg which supports the right atrium for superfusion (b).

specifically programmed to be applied in the refractory period. This method causes some arrhythmias, but is the technique used by Story *et al.* (1981).

Tritium and noradrenaline measurement

Flow-cell effluent (2–3 ml) was vigorously mixed with 10 ml Instagel (Packard Instruments Downers Grove, Ill.) to form a gel before counting in a scintillation counter (Packard Instruments, Model 3375), fitted with an external standard and calibrated for tritium. In experiments where the specific activity of effluxed noradrenaline was also determined, the perfusate was collected into 1 ml of 0.1% Na_2 EDTA containing 20 mg sodium metabisulphite. Noradrenaline and its associated radioactivity was initially purified by adjusting the effluent pH to 8.6 with 1 M Tris HCl, containing 2% EDTA, adsorption onto alumina (100 mg) and subsequent elution with 150 µl of 0.2 M perchloric acid. Noradrenaline was separated from other catechol metabolites by high performance liquid chromatography and measured electrochemically (Jackman, Snell, Skews & Bobik, 1982) with the following modifications: (i) column length was increased to 25 cm, (ii) the electrochemical detector was a model TL-5A with reference electrode RE-3 (Bioanalytical Systems) to permit fraction collection of the column effluent. Noradrenaline specific activity was calculated as the ratio of tritium associated with an absolute amount of noradrenaline measured by the electrochemical detector, previously calibrated with external standards. Atrial noradrenaline content and its specific activity was measured in a similar manner after homogenization in 0.4 M perchloric acid.

Response measurement and statistical analysis

The atrial rate during stimulation was analysed both as absolute peak-rate for different regimens of stimuli and as the change in rate from the resting value before stimulation. Stimulated ^{3}H-efflux was determined as the difference between the sum of two 30 s control periods preceding the stimulus and the following two 30 s periods. This was expressed as a percentage ratio of the average 30 s pre-stimulation control period. Thirty second collections were chosen since the stimulated release was removed from the cell within 1 min. The half-life of the ^{3}H-efflux in these tissues is about 1 h so that control values fall by only 1% between the collections made during control and stimulus periods.

Analysis of variance (ANOVA) was used to compute the average standard error as (error mean square per number of atria)$^{0.5}$ for any stimulus-response relationship by subtracting 'between atrium' and 'between frequency' or 'pulses' sums of squares from the 'total' sums of squares. Comparison of treatments (i.e. phentolamine) were assessed by 'split plot' for between atria or 3-way analysis of variance within atria (Snedecor & Cochran, 1967). Least squares linear regression and asymptotic regression were used for the analysis of ^{3}H-efflux and frequency relationship for 4 pulses. Regression lines were compared by analysis of covariance.

Drugs

Drugs used and their source were [7-^{3}H] (−)-noradrenaline 2.7 or 3.2 Ci mmol^{-1}, [2,5,6-^{3}H] (−)-noradrenaline 47.7 Ci mmol^{-1} and [^{3}H]-dopamine ([2,5,6-^{3}H]-dihydroxyphenylethylamine) 42 Ci mmol^{-1}, New England Nuclear; atropine, BDH; desmethylimipramine (desipramine, DMI), Ciba-Geigy; phentolamine hydrochloride, Ciba-Geigy. The labelled amines were stored at −20°C in acidified solution. Phentolamine solutions were freshly prepared daily.

Labelling

The method of ^{3}H-labelling was as follows: the atrium was incubated for 30 min in the 3 ml organ bath; after washing (by replacing the bathing solution with ^{3}H-free Krebs solution) a 30 min rest period was followed by a 30 s stimulation at 1 Hz, using the field electrodes. This is similar to the method described by others in the field (e.g. Langer, Adler-Graschinsky, Almeida & Diniz, 1975; Medgett, McCulloch & Rand, 1978; Story *et al.*, 1981). In the main experiments the atrium was then transferred to the flow cell, but in preliminary experiments we also studied efflux changes in the organ bath (see Results).

Results

Specific activities of tissue and released noradrenaline

In early experiments atria were labelled with low specific activity [^{3}H]-noradrenaline (1 µM; 2.7 Ci mmol^{-1}). In the flow-cell the atria were given a prolonged train of refractory period field pulses (1 field pulse per refractory period for 5 min) that gave maximum tachycardia. During this stimulation period efflux was collected. The observed tissue content and overall specific activity of [^{3}H]-noradrenaline ([^{3}H]-NA) are in agreement with those previously found in the heart and other tissue (Langer, 1970; Farnebo & Malmfors, 1971; Rorie, Muldoon & Tyce, 1980) and indicate that trace labelling of tissue noradrenaline had been accomplished (Table 1). However, the specific activity of the noradrenaline released on nerve stimulation was

Table 1 Analyses of tissue and superfusates from 3 guinea-pig right atria

	mean ± s.e.mean
Fraction of tissue ^3H as [^3H]-NA	93 ± 4.5%
Specific activity of tissue [^3H]-NA	0.19 ± 0.03 Ci mmol^{-1}
Specific activity of label [^3H]-NA	3.19 Ci mmol^{-1}
Background NA efflux	0.30 ± 0.12 pmol min^{-1}
*Stimulated NA efflux	2.78 ± 1.60 pmol min^{-1}
Specific activity of released NA	1.03 ± 0.24 Ci mmol^{-1}
Fraction of released ^3H present as NA	56 ± 33%

NA, noradrenaline.
*Stimulation was 1 field pulse per refractory period for 5 min.

much higher (1.03 Ci mmol^{-1}). This is in agreement with earlier findings that noradrenaline subject to neuronal uptake is preferentially released (Rorie *et al.*, 1980). In addition this neuronal pool is not tracer labelled since at 1.03 Ci mmol^{-1}, 32% of NA is due to the labelling procedure. Further, this preliminary experiment showed that the release of total NA (6–12 fmol per field pulse) is low and hence is beyond the sensitivity of currently available electrochemical detectors used with h.p.l.c.

We also used [^3H]-dopamine ([^3H]-DA) labelling (0.25 μM) in 3 atria to see whether tissue labelling became more uniform during stimulation. Less than 1% of tissue tritium was DA and [^3H]-DA was not detected in the superfusates, showing that it had been efficiently converted to NA by the tissue as reported previously (Hope, Majewski, McCulloch, Rand & Story, 1979). However, during field stimulation (1 Hz, 30 s), the efflux again contained NA of higher specific activity (15.3% of label specific activity) than that of the tissue (2.1%). In 3 other experiments where the atria had been labelled by [^3H]-NA (0.1 μM), the specific activity of NA in efflux was 9.5% compared with the specific activity of 1.1% in the tissues. Thus labelling with DA to generate NA through the metabolic pathway in the nerve terminals did not improve the homogeneity of labelling.

Fractionating the ^3H in the superfusate before and following a strong stimulus (4 Hz, 30 s) indicated that [^3H]-dihydroxyphenylethylene glycol (DHPG) was a major component of the ^3H in the control (prestimulation) superfusate and [^3H]-NA the major component of the stimulated efflux (Table 2). Labelling with [^3H]-DA did not alter this relationship but the degree of labelling was much lower than with [^3H]-NA (0.1 μM). These experiments suggest that NA which was newly synthesized from [^3H]-DA and that which accumulated during uptake of [^3H]-NA are stored at similar sites and readily released during field stimulation.

To evaluate the stimulus-efflux relationship with different labelling procedures, three atria were labelled with [^3H]-NA, [^3H]-DA (0.1 μM) or [^3H]-DA (0.25 μM). Two, 5 or 10 field pulses were applied at 1 Hz. The average relationship for ^3H-efflux and number of pulses at 1 Hz was similar with all 3 labelling methods (Table 3). With high specific activity NA and DA there was less variance in ^3H-efflux than for low specific activity DA. In each case stimulated release increased linearly with field pulse number. In all subsequent experiments atria were ^3H-labelled with high specific activity (47.7 Ci mmol^{-1}) ring labelled (−)-noradrenaline (0.1 μM) for 30 min. The atria were then transferred to the flow-cell for a further 30 min superfusion before giving any field pulses.

Preliminary experiments in flow cell and organ bath

The tachycardia responses evoked by 4 pulses in the flow-cell were similar to those observed in the organ

Table 2 [^3H]-noradrenaline (NA) and [^3H]-dihydroxyphenylethylene glycol (DHPG) content in 60 s perfusates before and after field stimulation (4 Hz, 30 s) in 3 atria labelled with [^3H]-NA (0.1 μM) or [^3H]-dopamine (DA) (0.25 μM)

		[^3H]-NA efflux (d.p.m./60 s)		*[^3H]-DHPG efflux* (d.p.m./60 s)	
Label	*Atrium No.*	*Control*	*Stimulation*	*Control*	*Stimulation*
[^3H]-NA	(1)	0	11,200	2,674	3,650
	(2)	0	20,150	5,810	5,740
	(3)	352	9,690	4,260	3,440
[^3H]-DA	(4)	0	3,582	585	1,624
	(5)	0	728	442	432
	(6)	170	7,190	1,660	1,760

Table 3 Effect of 3 different labelling procedures on the ^{3}H-efflux response to low numbers (2–10) of field pulses (P) at 1 Hz

	^{3}H-efflux (% mean increase)			
Label	*2P*	*5P*	*10P*	*Average s.e.mean*
[^{3}H]-NA 0.1 μM	3.6	11.9	25.4	0.98
[^{3}H]-DA 0.25 μM	0.62	8.3	19.6	1.77
[^{3}H]-DA 0.1 μM	2.06	8.8	22.8	0.87

Average s.e.mean is calculated from 2 way ANOVA (see Methods). Each labelling procedure was performed in a single atrium where the 3 field pulse trains were given 5 times.

bath (Figure 3). But there were acute changes in atrial rate when the organ bath was drained (Figure 3) for making ^{3}H-efflux measurements in the usual way. These fluctuations in rate have forced previous workers to examine ^{3}H-efflux and rate responses to field stimulation in separate experiments (Langer, Adler-Graschinsky & Giorgi, 1977; Story *et al.*, 1981). These fluctuations were absent in the superfused flow-cell (Figure 3).

The labelling procedure was always performed in the organ bath, and was in principle the same as that of Story *et al.* (1981) and Langer *et al.* (1977). After ^{3}H-labelling in 8 atria the peak atrial rates reached after 30 field pulses at 1 Hz were closely similar in the organ bath (261 min^{-1}; Δ 86 beats min^{-1}) or in the flow-cell (266 min^{-1}; Δ 112 beats min^{-1}).

But the changes in ^{3}H-efflux were different depending on whether the 30 s stimulation was performed in the organ bath or in the flow-cell. In the organ bath the increase in ^{3}H-efflux was 13,200 ± 3,800 d.p.m. If the atrium was transferred to the flow-cell after the 30 min rest period (see labelling, Methods), 30 s stimulation at 1 Hz produced an efflux of only 1320 ± 280 d.p.m. despite the similarity in the rate responses. It made no difference whether the first stimulation was performed in the organ bath or the flow-cell; stimulated ^{3}H-efflux from the flow-cell was about $\frac{1}{10}$ of that from the organ bath. The resting efflux from the organ bath in the absence of a previous period of stimulation was also somewhat higher (3910 ± 1090 d.p.m.) than from the flow-cell (2700 ± 580 d.p.m.), though the difference was not statistically significant. Tritium did not appear to be lost on passage through the flow-cell. In two experiments 0.2 μCi [^{3}H]-noradrenaline was given into the perfusion line just before the flow-cell. The ^{3}H-efflux (measured until counts were constant) was 101% and 108% of a similar injection of 0.2 μCi placed directly into counting vials. This is not significantly different from 100% considering the problem of directly transferring the small volume of high activity label to the counting vial.

We applied 4 field pulses at 0.125–2 Hz in both the flow-cell and the organ bath in 8 atria. The order of first stimulation again made no difference to the response. In these experiments there was no clear relationship between frequency of stimulation and ^{3}H-efflux in either organ bath or flow-cell. The efflux

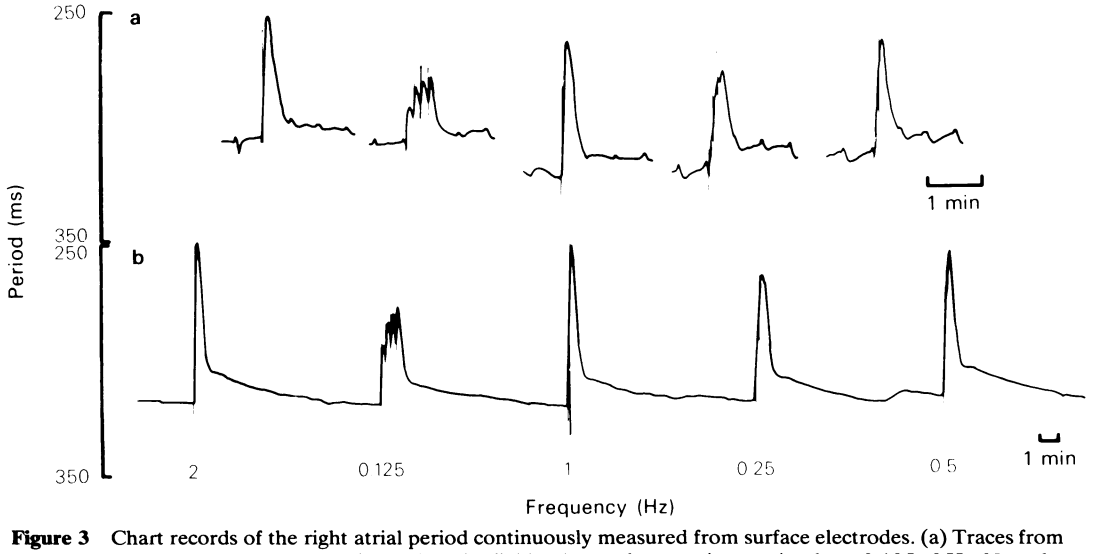

Figure 3 Chart records of the right atrial period continuously measured from surface electrodes. (a) Traces from the atrium in the 3 ml organ bath during trains of 4 field pulses at frequencies ranging from 0.125–2 Hz. Note the baseline changes during the replacement of bathing solution. (b) Traces from the same atrium after being transferred to the flow-cell. The fine lines are period values associated with arrhythmias caused by field pulses delivered outside the refractory period.

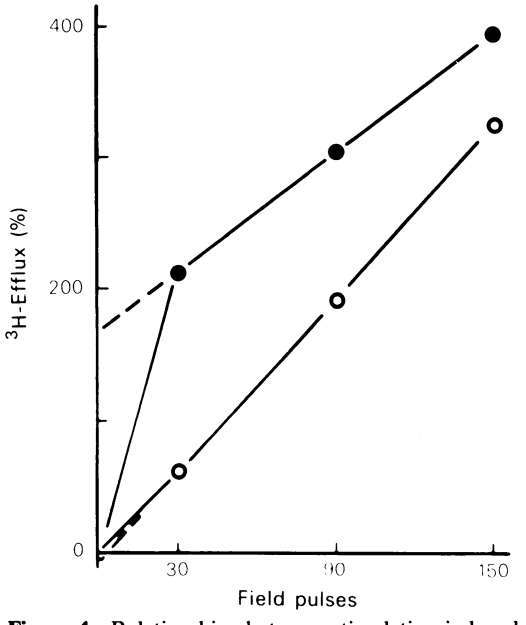

Figure 4 Relationships between stimulation induced ^{3}H-efflux (% of resting) and number of field pulses delivered in 30 s in 3 right atria. Each atrium was given the 30, 90 and 150 field pulses in the organ bath (●) (where it had been ^{3}H-labelled) and again in the flow-cell (○). Points shown are the average values for 3 atria in organ bath (closed symbols) and flow-cell (open symbols). Linear regression lines were $y = 168.3 + 1.52x$ and $y = -7.8 + 2.23x$ for the organ bath and flow-cell respectively.

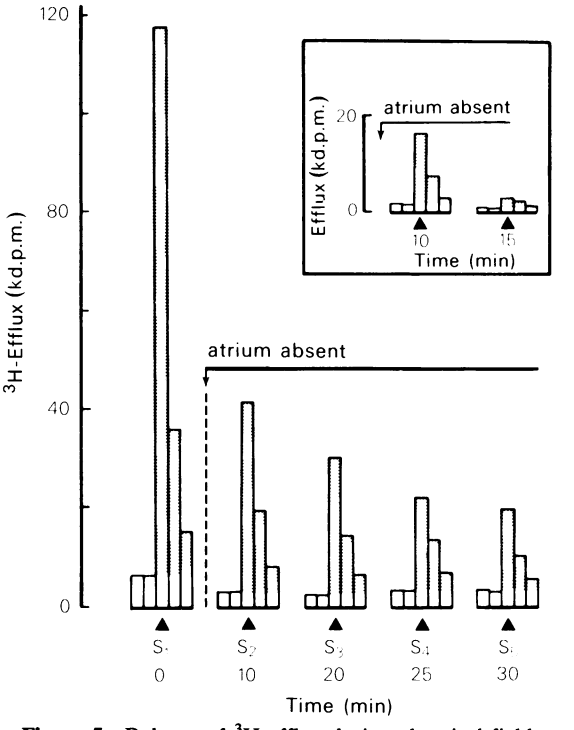

Figure 5 Release of ^{3}H-efflux during electrical field stimulation in the presence and absence of the right atrium. Right atrium ^{3}H-labelled for 30 min in organ bath; washed and given field stimulation for 30 s at 1 Hz (S_1); each bar is ^{3}H-efflux for 30 s collection period. Atrium removed from epoxy tissue holder at arrow (time 0). Field stimulation repeated at S_2-S_5 at times shown. Insert: flow-cell tissue holder (perspex) incubated with [^{3}H]-noradrenaline + ^{3}H-metabolites in organ bath for 30 min then placed in flow-cell for superfusion at 5 ml min^{-1}. Ten and 15 min later field pulses were delivered at S_1 and S_2 (30 s, 1 Hz).

was again substantially greater from the organ bath than from the flow-cell when expressed either as d.p.m. or as a percentage of resting before stimulation. In the organ bath the change in counts for 4 pulses at 0.125–2 Hz was 640 d.p.m. (range 520–930) (36.2% of resting; range 29–45), compared with corresponding values from the flow-cell of 128 d.p.m. (range 40–170) (8.2% of resting; range 4.9–12.5%). The d.p.m. from the organ bath is slightly lower than the values reported by Story *et al.* (1981). This is to be expected as, although we were using the same procedure, we used only the right atrium, whilst Story *et al.* (1981) used both atria.

We obtained a relationship between pulse number and ^{3}H-efflux in both the organ bath and flow-cell by stimulating with 30, 90 and 150 pulses all applied in 30 s (Figure 4). The relationship between pulse number and efflux from the flow-cell was linear and passed through the origin when efflux was expressed either as % of resting (Figure 4), or in absolute d.p.m. By contrast the relationship between pulse number and ^{3}H-efflux relationship from the organ bath was non-linear, though the regression points above 30 pulses per 30 s were approximately parallel to corresponding points from the flow-cell (Figure 4). The variance of the ^{3}H-efflux for the organ bath was significantly greater than in the flow-cell even when the response was expressed as a percentage of its own resting value (residual variance ratio, $F = 6.23$, $P < 0.025$).

We next observed that in the organ bath after the ^{3}H-labelling procedure a 'basal' and 'stimulated' ^{3}H-efflux could be observed even in the absence of tissue in the bath (Figure 5). This was due to retention of tritium by the organ bath tissue holder after the original incubation. This tritium was released only very gradually during subsequent stimulation and washing periods. When the tissue holder from the flow-cell was used in the organ bath there was no increase in ^{3}H-efflux above background during field

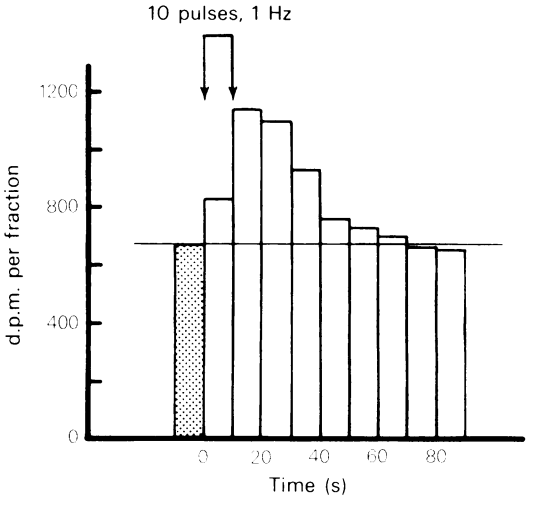

Figure 6 Time course of the washout of ^{3}H-efflux from the flow-cell following 10 field pulses at 1 Hz. The flow rate was 4.5 ml min^{-1}.

stimulation. When the tissue holder of the flow-cell was incubated with [^{3}H]-noradrenaline + ^{3}H-metabolites in the organ bath for 30 min and then placed in the flow-cell, field stimulation again caused an increase in ^{3}H-efflux but to a much lesser extent than for the epoxy tissue holder previously observed in the organ bath (Figure 5). Repeating the field stimulation 5 min later caused very little excess ^{3}H-efflux above background (Figure 5). We have not investigated the exact nature of the efflux from the tissue holder. The epoxy resin clearly takes up tritium and the stimulated release may be due to electrolytic or electrostatic processes.

The organ bath tissue holder was moulded from epoxy resin whilst the flow-cell and tissue holder were made of perspex (polymethyl methacrylate) and the wires were secured by acrylic cement (Vertex, Dentimex Zeist, Holland). The latter appears to be more satisfactory than epoxy resin from the view point of not retaining tritium. These experiments suggest that the use of the same organ bath and tissue holder for ^{3}H-labelling and for the experimental procedure creates problems and that a different organ bath should be used (see Discussion).

In the subsequent experiments therefore we used the flow-cell after previous ^{3}H-labelling in the organ bath with high specific activity noradrenaline. When superfusion was at 4.5 ml min^{-1} the ^{3}H-efflux in response to 10 pulses at 1 Hz cleared the cell in about 60 s (Figure 6).

Experiments in flow cell

Effect of four pulses Trains of 4 field pulses delivered at 0.125–2 Hz caused a frequency-related rise in atrial rate, similar to the findings of Story *et al.* (1981), (Figure 7b, compared to Figure 1a). The responses obtained during the first and second stimulation periods were closely similar even though the resting atrial rate had fallen by 8.6 ± 3.3 beats min^{-1} (4.7%). When neuronal uptake was blocked by pretreatment with DMI (0.1 μM) the relationship between frequency and rise in rate was slightly steeper ($P = 0.75 \pm 0.17$ compared with untreated atria $P = 0.63 \pm 0.25$) (P is the non-linear parameter, Snedecor & Cochran, 1967). The asymptotic maximum increase in rate was significantly greater in the DMI-treated atria (137.4 ± 48 compared with 65.2 ± 16 beats min^{-1}). This was even more marked during the second stimulation period than during the first (C_2 vs C_1 Figure 7b).

Table 4 Resting mean values of atrial rate and ^{3}H-efflux (over 30 s) before field stimulation of 4 pulses at various frequencies

	^{3}H-efflux (× 1000 d.p.m.)		*Rate* (beats min^{-1})	
Series A		*% fall*		*paired t*
C_1	6.73 ± 0.68	0	182.4 ± 8.1	$P<0.05$
C_2	3.87 ± 0.28	42	173.8 ± 7.5	
C_1	6.53 ± 0.47	0	153.8 ± 11.9	$P<0.05$
Phentolamine	4.53 ± 0.31	30	147.2 ± 12.9	
Series B (DMI)				
C_1	6.20 ± 1.10	0	166.0 ± 10.1	NS
C_2	3.97 ± 0.63	35	168.6 ± 9.8	
C_1	7.32 ± 0.78	0	172.2 ± 8.8	$P=0.052$
Phentolamine	4.89 ± 0.60	33	162.2 ± 8.0	

Values are mean ± 1 s.e.mean ($n = 5$). Desipramine (DMI 0.1 μM) was present for 30 min prior to C_1 in series B experiments. Phentolamine (1 μM) was in contact 30 min prior to measurements.

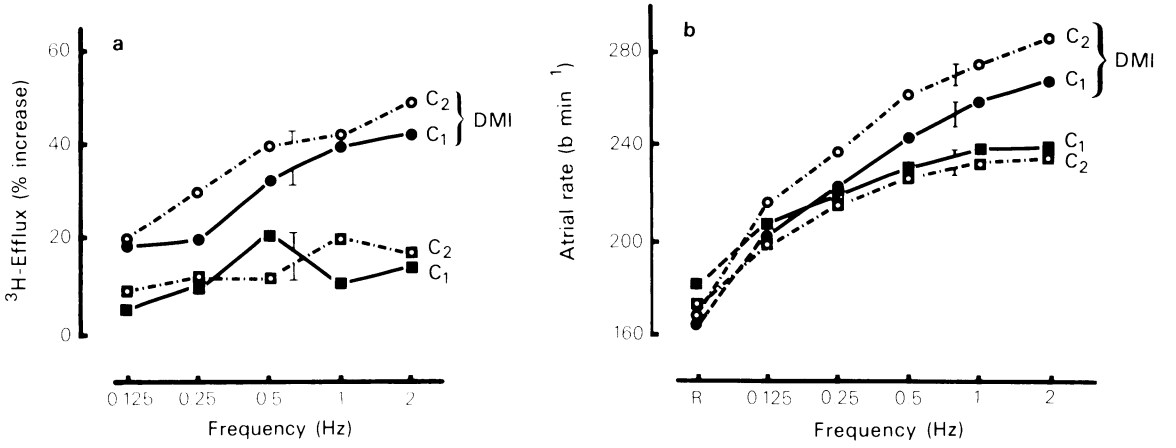

Figure 7 Hyperbolic-like relationships of atrial rate (beats min^{-1}, (b)) and ^{3}H-efflux (% increase, (d) to frequency of 4 field pulse trains. Data are from two series of experiments: no pretreatment (squares) and atria pretreated with desipramine (DMI, 0.1 μM circles). C_1 and C_2 refer to the 1st and 2nd periods of field stimulation separated by 30 min within each tissue. Error bars are average s.e.mean calculated from analysis of variance (see Methods). R refers to resting atrial rate prior to delivery of 4 field pulses.

During field stimulation there was a significant rise above resting in the ^{3}H-efflux responses averaged for all frequencies in the absence of DMI (11.6% ± 2.7) (Figure 7a). During the C_1 stimulus period a frequency-dependence of the efflux could not be demonstrated (slope = 1.88 ± 1.51, mean ± 1 s.e.mean). But during the C_2 stimulus period the regression relationship was significant (slope = 2.35 ± 0.83, $P < 0.05$, d.f. = 23). It was also significant when considering the pooled data of both C_1 and C_2 periods (slope = 2.12 ± 0.85, $P < 0.05$ d.f. = 48). The ^{3}H-efflux at a given frequency was significantly raised in the presence of DMI (C_1 DMI vs C_1 control, F = 9.3, $P < 0.05$). In addition, the regression coefficient relating frequency to efflux was significantly increased compared with atria without DMI (Table 5) ($P < 0.01$ for difference in slope).

Table 5 Linear regression equations and standard error of slope (SE_b) for ^{3}H-efflux data against frequency of delivery of 4 pulses (0.125–2 Hz)

Control		SE_b	*d.f.*
C_1	y = 5.94 + 1.88x	1.51	23
C_2	y = 6.45 + 2.35x	0.83	23
$C_1 + C_2$	y = 6.19 + 2.12x	0.85	48
DMI			
C_1	y = 14.24 + 4.92x	1.63	23
C_2	y = 14.92 + 7.00x	1.59	23
$C_1 + C_2$	y = 14.58 + 5.96x	1.18	48

d.f., degrees of freedom.

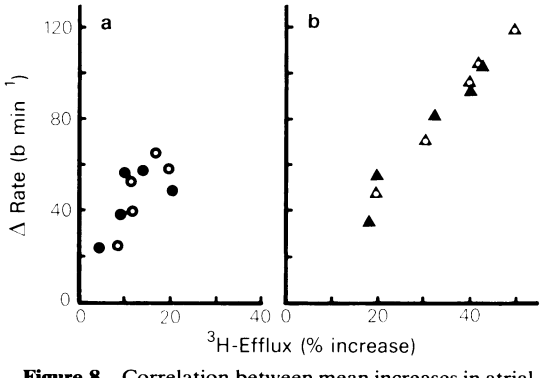

Figure 8 Correlation between mean increases in atrial rate (beats min^{-1}) (ordinates) and ^{3}H-efflux (% of resting efflux) during trains of 4 field pulses delivered at 0.125–2 Hz in series A, no desipramine (DMI) (a), and series B, with DMI (b), experiments. (Data are from Figure 7.) Closed symbols are from the 1st run of field pulses (C_1) and open symbols represent the 2nd run (C_2).

Figure 8 indicates that the two response variables (atrial rate, ^{3}H-efflux) were significantly related, both in the absence and in the presence of DMI. In the absence of DMI the equation of the regression line was y = 21.9 + 1.98x, ((standard error of slope)(SE_b) = 0.72, $r = 0.69$). In the presence of DMI the equation of the line was y = −0.41 + 2.46x ((SE_b) = 0.16, $r = 0.98$).

Effect of phentolamine In these experiments 4 field pulses were delivered at 0.125–2 Hz during the C_1 period. Each atrium was then equilibrated for 30 min with phentolamine 1 μM before the C_2 period of

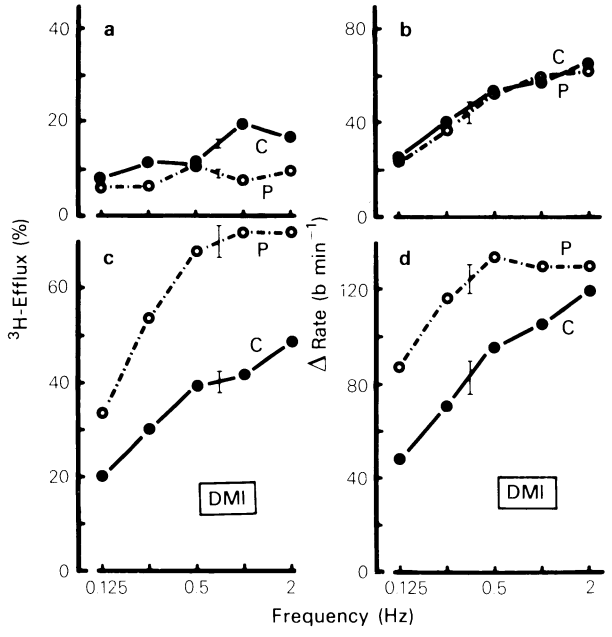

Figure 9 Effects of phentolamine (P, 1 μM) on the relationship between ^{3}H-efflux and frequency (0.125–2 Hz) (a and c) and between increase in rate (beats min^{-1}) and frequency (b and d) measured simultaneously. (a and b) Neuronal uptake present – normal conditions. (c and d) Neuronal uptake inhibited by desipramine (DMI) (0.1 μM). Solid lines and points are data from the 2nd period of stimulation in control atria without phentolamine. Broken lines and open circles are data from separate atria during the second period of stimulation in the presence of phentolamine (P). Error bars are average s.e.mean calculated from ANOVA (see Methods).

stimulation. In normal atria (i.e. no DMI) and in DMI-treated atria there were small time-dependent changes in both ^{3}H-efflux and rate responses between C_1 and C_2 curves (Figure 7). Therefore we assessed the effects of phentolamine from (1) the comparisons of the C_1 and C_2 responses within atria (Figure 10) and (2) from responses obtained during the C_2 periods on one group of atria not given phentolamine and another group of atria pretreated with phentolamine (Figure 9). In both comparisons phentolamine did not significantly alter the rate responses to 4 pulses at any frequency between 0.125 and 2 Hz (Figures 9 and 10). Nor did phentolamine increase ^{3}H-efflux in the normal atria.

Similar comparisons on the effects of phentolamine were made within and between atria after pretreatment with DMI. Phentolamine now significantly enhanced both rate and the ^{3}H-efflux response at every frequency (Figures 9 and 10).

Other stimulation patterns We studied the effects of different pulse numbers on atrial rate and ^{3}H-efflux responses at two frequencies – 0.25 Hz and 2 Hz. In each case we studied the effects of phentolamine in the absence of and after pretreatment with DMI. In normal atria stimulated at 0.25 Hz, atrial rate rose by about 40 beats min^{-1} and the rise was not significantly different at 4, 8 and 12 pulses (Figure 11b). However, ^{3}H-efflux was approximately linearly related to the number of pulses. But neither the rate nor the ^{3}H-efflux responses were significantly altered by phentolamine (Figure 11).

At 2 Hz both rate and ^{3}H-efflux responses of normal atria were significantly related to the number of pulses (Figure 12). Phentolamine treatment increased significantly the slope of the relationship between ^{3}H-efflux and pulse number (comparison of slopes, $F = 7.6$, $P < 0.025$) but a similar comparison for rate responses was not significant ($F = 2.4$). This suggests that auto-inhibitory feed-back was only important with the longer (12 pulse) trains.

After pretreatment with DMI, stimulation at 0.25 Hz increased both rate and efflux responses, with a significant steepening of the slope of the stimulus-efflux response curve (Figure 11). At 2 Hz phentolamine had no effect on the rate response (which was close to maximum) but again there was a marked enhancement of the relationship between

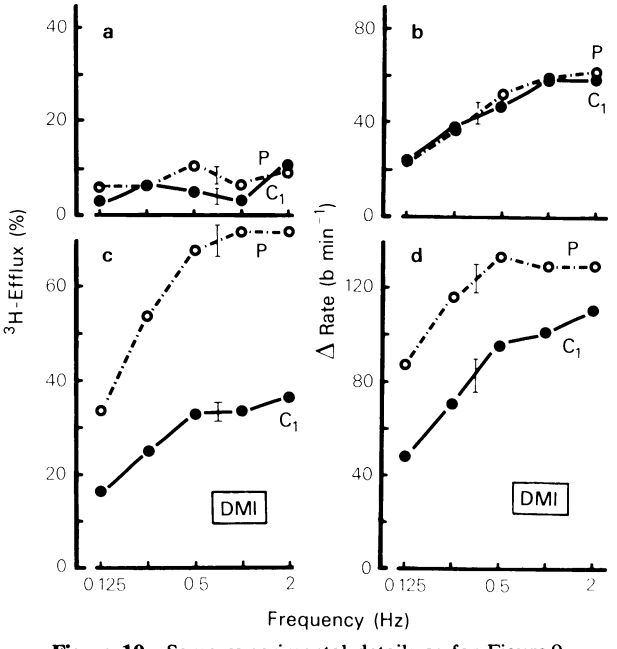

Figure 10 Same experimental details as for Figure 9 except that the control data are for the within atria comparison i.e. the first period of stimulation (C_1) before treatment with phentolamine (P).

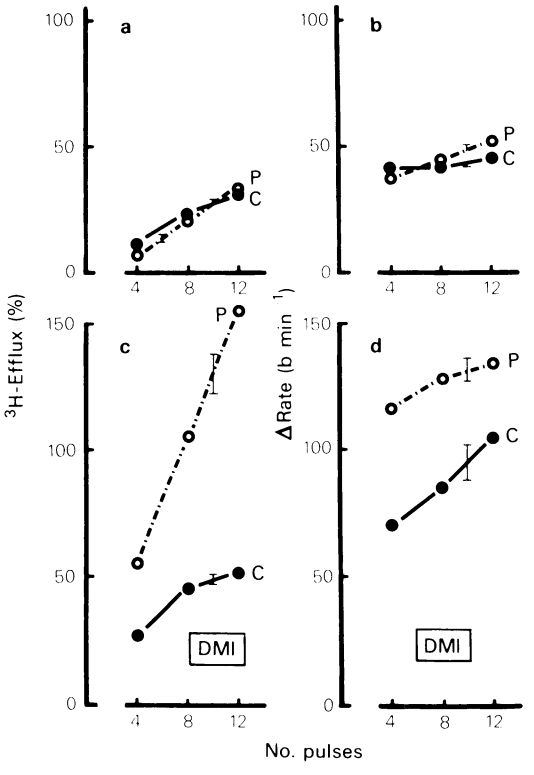

Figure 11 Relationship between ^{3}H-efflux (% increase) (a and c) and increase in atrial rate (beats min^{-1}) (b and d) with the number of field pulses (abscissae) at low frequency (0.25 Hz). Solid symbols and lines are data from normal atria (C, $n = 5$) open symbols and broken lines are from separate atria ($n = 5$) equilibrated with phentolamine (P) (1 μM). (c and d), Experiments from series B where atria were pretreated with desipramine (DMI) (0.1 μM).

pulse number and ^{3}H-efflux with a significant increase in slope.

Discussion

We observed hyperbolic relationships between the level of sympathetic stimulation and the two measures of response i.e. the efflux of labelled transmitter and the rise in atrial rate. The two responses were thus monotonically related and we did not observe the U-shaped relationship shown in Figure 1 from the study by Story *et al.* (1981; their Figure 3). Our experiments thus confirm the findings of Wakade & Wakade (1982) who also observed a hyperbolic relationship between stimulus and ^{3}H-efflux. Our method of labelling was the same as that employed by Story *et al.* (1981) apart from the fact that we employed a label of higher specific activity and lower concentration and used a different cell for labelling and experimental periods. Hence we do not know the reason for the U-shaped relationship that they observed between frequency and ^{3}H-efflux (Figure 1, left), apart perhaps from any imprecision resulting from the absorption of label by their apparatus (see below).

In the present experiments during stimulation at 4 pulses and under normal conditions of neuronal uptake phentolamine was without effect on either the rise in atrial rate or on the increase in ^{3}H-efflux of transmitter (Figures 9 and 10) in agreement with earlier findings (Angus & Korner, 1980; Story *et al.*, 1981). Only after neuronal uptake blockade with DMI did phentolamine increase both the rate and

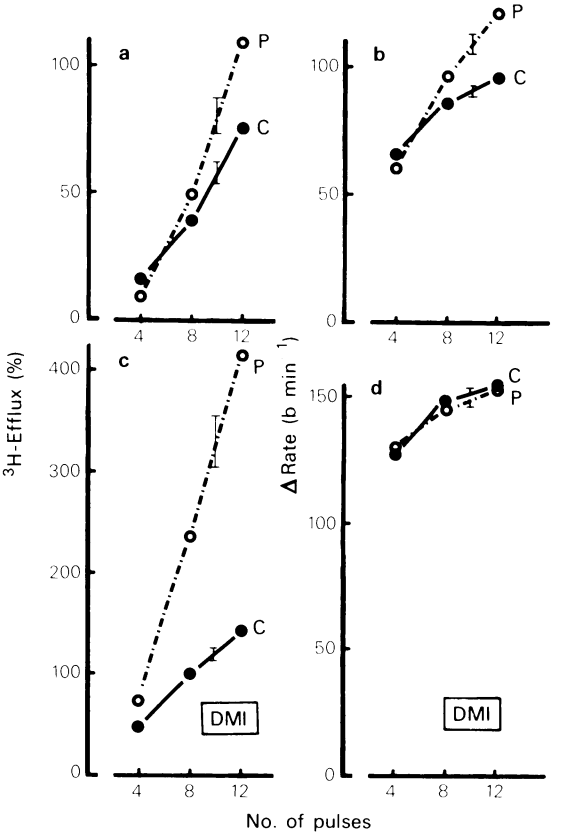

Figure 12 Relationship between ^{3}H-efflux (% increase) (a and c) and increase in atrial rate (beats min^{-1}) (b and d) with the number of field pulses (abscissae) at high frequency (2 Hz). Solid symbols and lines are data from normal atria. C ($n = 5$); open symbols and broken lines are data from separate atria ($n = 5$) equilibrated with phentolamine (P) (1 μM). (c and d), Experiments from series B where atria were pretreated with desipramine (DMI) (0.1 μM).

^{3}H-efflux responses at a given frequency. This suggests that when transmitter concentration over the cardiac cycle is kept low by normal neuronal reuptake processes, there is no activation of the auto-inhibitory feed-back through stimulation of prejunctional α_2-adrenoceptors. Only when the transmitter concentration rises due to blockade of neuronal uptake is there auto-inhibitory feed-back mediated through α_2-adrenoceptors. One explanation of this is provided by our recent suggestion that the prejunctional α_2-adrenoceptors are located some distance from the synaptic cleft so that neuronal uptake or diffusion will limit the transmitter concentration reached in the vicinity of the receptor (Korner, Angus, Lew & Heinzow, 1983).

The experiments in which the number of pulses was varied at a given frequency of stimulation are in agreement with the findings in the 4 pulse experiments at different frequencies. With different numbers of pulses at 0.25 Hz, phentolamine had no effect on either rate or ^{3}H-efflux responses whilst neuronal uptake was normal, and only produced an increase in these responses after neuronal uptake block with DMI (Figure 11). At high frequency stimulation of 2 Hz phentolamine had little or no effect at 4 and 8 pulses in normal atria, but again produced small increases in rate and ^{3}H-efflux response during stimulation with 12 pulses (Figure 12). Presumably only under the conditions of relatively prolonged high frequency stimulation is there an adequate rise in concentration of transmitter close to the prejunctional α_2-adrenoceptor.

Since ^{3}H-efflux is a measure of the average noradrenaline concentration in the synapse, it might have been more appropriate to compare it with changes in the area of rate response curves than with the peak atrial rate responses that we used. However, we have previously found that little is gained from area measurements (Angus & Korner, 1980) and that peak responses provide an adequate index of rate changes. In the present study the ^{3}H-efflux of transmitter and the atrial rate responses were related in an approximately similar manner to the level of the sympathetic stimulus as long as the rate responses were submaximal. Thus the functions describing the two responses were similar under normal conditions and after DMI and both were affected in a similar manner with phentolamine (Figures 8 and 9). This was also the case in the experiments with normal neuronal uptake at 2 Hz with different pulse trains (Figure 12a and b). In the presence of DMI, phentolamine markedly increased ^{3}H-efflux with increasing length of stimulus trains, consistent with the continuing operation of auto-inhibitory feed-back at high synaptic concentrations of transmitter (Figure 12c and d). Failure of the antagonist to affect the rise in rate can be explained by saturation of the tachycardia i.e. attainment of maximum tissue response. It is only under these conditions that changes in ^{3}H-efflux appear to provide a better measure of synaptic concentration of transmitter.

During sympathetic stimulation in the presence of normal neuronal uptake, auto-inhibitory feed-back exerts minimal, if any effects, except at the strongest stimuli. This is in marked contrast to the ready demonstration that clonidine produces concentration-related attenuation of both the tachycardia and ^{3}H-efflux responses (Medgett *et al.*, 1978; Lew & Angus, 1982). The possibility that the α_2-adrenoceptor on which clonidine acts is at an extrasynaptic site was suggested by the absence of significant competition between clonidine and synaptically released noradrenaline for the presynaptic site (Korner *et al.*, 1983).

Some comment should be made about the labelling technique used to measure ^{3}H-efflux in the present experiments. In preliminary experiments we found that we could not measure with adequate accuracy 'cold' noradrenaline efflux in the superfusion fluid using h.p.l.c. with electrochemical detection. The differences in specific activity of transmitter at rest and during field stimulation suggest non-uniform labelling of the different 'pools' of noradrenaline in the tissue. The effect was similar whether labelling was done with ^{3}H-noradrenaline or ^{3}H-dopamine and is in agreement with the concept that, noradrenaline that is taken up or newly synthesized is released preferentially during stimulation (Rorie *et al.*, 1980). These results are consistent with the observations that noradrenaline newly synthesized from tyrosine is preferentially released during nerve stimulation (Kopin, Breese, Krauss & Weise, 1968). But the possibility that preferential uptake of [^{3}H]-NA or [^{3}H]-DA by terminals near the surface results in release of [^{3}H]-NA of higher specific activity than in the whole tissue cannot be excluded.

The use of the high specific activity [^{3}H]-noradrenaline labelling technique that we finally adopted came close to tracer labelling and allowed us to detect a linear increase in ^{3}H-efflux in response to 2, 5 and 10 field pulses at fixed frequency (Table 3). However, the stimulation induced excess ^{3}H-efflux following 2 and 5 field pulses was relatively low compared with the basal ^{3}H-efflux before stimulation. We believe that this reflects the low synaptic noradrenaline concentration during the cycle due to efficient neuronal uptake.

In principle therefore our labelling techniques probably labelled the same component of the tissue noradrenaline pool as the method used by others (Story *et al.*, 1981; Langer *et al.*, 1977). It cannot therefore account for the difference in our findings and those of Story *et al.* (1981). The main technical difference between our experiments was that after the initial labelling in the organ bath we used a small

superfused flow-through cell where ^{3}H-efflux and rate changes could be measured simultaneously. For the efflux measurements the time interval was sufficiently short to assume approximately basal 'steady-state' conditions over the periods of measurements. This differs from the use of changes in the S_2/S_1 ratio analysis (i.e. stimulus induced ^{3}H-efflux for the first and second periods of stimulation) which has been used to compare treatment and untreated control periods in different atria by means of Student's unpaired *t* tests in much previous work on the role of prejunctional α_2-adrenoceptors in the guinea-pig atrium preparation, including that used by Story *et al.* (1981) and Langer *et al.* (1977). The use of ratios as the basis of analysis in association with unpaired *t* tests creates greater imprecision in statistical estimation (Snedecor & Cochran, 1967) than the within atria comparison that was used here. The ratio estimation also assumes that the half life of ^{3}H-efflux does not alter between stimulation periods. In addition, the use of the same organ bath for labelling and for the experimental procedures that seems to have been mostly followed by other workers in the field (e.g. Langer *et al.*, 1977; Story *et al.*, 1981) may have added additional 'background' radioactivity due to absorption on the tissue holder. This would add to the difficulty of accurately estimating the experimental effects.

In conclusion, our results suggest that in the guinea-pig atrium auto-inhibitory feed-back normally plays little role in the modulation of transmitter release at levels of stimulation that produce 50–60% of the maximum rate response. This is because of the operation of the very efficient neuronal uptake process of released transmitter. At higher levels of stimulation there is some evidence of a small degree of reduction in transmitter release through prejunctional α_2-adrenoceptor mediated auto-inhibitory feed-back. The original hypothesis of a major physiological self-regulatory auto-inhibitory feed-back thus requires substantial modification. Auto-inhibitory feed-back as a homeostatic mechanism of regulating transmitter concentration would appear to be a 'last resort' mechanism when the level of sympathetic efferent activity has become very high, despite the activity of the body's normal reflex homeostatic mechanisms through the central nervous system. However, prejunctional α_2-adrenoceptors are undoubtedly important in the action of drugs such as clonidine, and possibly in the action of circulating catecholamines in high concentrations.

This work was supported by the National Health & Medical Research Council of Australia. Dr Kopin visited the Baker Institutes as the Edgar Rouse visiting research fellow. We thank Michael Lew for assistance in some experiments, John Baird for making the flow-cell and Susan Weir for secretarial assistance.

References

ANGUS, J.A. & HARVEY, K. (1981). Refractory period field stimulation of right atria: a method for studying presynaptic receptors in cardiac autonomic transmission. *J. Pharmac. Methods*, **6,** 51–64.

ANGUS, J.A. & KORNER, P.I. (1980). Evidence against presynaptic α-adrenoceptor modulation of cardiac sympathetic transmission. *Nature (Lond)*, **286,** 288–291.

BALDESSARINI, R.J. & KOPIN, I.J. (1967). The effect of drugs on the release of norepinephrine-^{3}H from central nervous system tissues by electrical stimulation in vitro. *J. Pharmac. exp. Ther.*, **156,** 31–38.

FARNEBO, L.O. & MALMFORS, T. (1971). ^{3}H-noradrenaline release and mechanical response in the field stimulated mouse vas deferens. *Acta. physiol. scand. (Suppl.).*, **371,** 1–18.

HOPE, W., MAJEWSKI, H., McCULLOCH, M.W., RAND, M.J. & STORY, D.F. (1979). Modulation of sympathetic transmission by neuronally released dopamine. *Br. J. Pharmac.*, **67,** 185–192.

JACKMAN, G., SNELL, J., SKEWS, H. & BOBIK, A. (1982). Effects of noradrenergic neuronal activity on 3,4-dihydroxyphenylethylene glycol (DHPG) levels. Quantitation by high performance liquid chromatography. *Life Sci.*, **31,** 923–929.

KATZ, R.I. & KOPIN, I.J. (1969). Electrical field-stimulated release of norepinephrine-^{3}H from rat atrium: effects of ions and drugs. *J. Pharmac. exp. Ther.*, **169,** 229–236.

KOPIN, I.J., BREESE, G.R., KRAUSS, K.R. & WEISE, V.K. (1968). Selective release of newly synthesized norepinephrine from the cat spleen during sympathetic nerve stimulation. *J. Pharmac. exp. Ther.*, **161,** 271–278.

KORNER, P.I., ANGUS, J.A., LEW, M.J. & HEINZOW, B.G.J. (1983). Characterization of the clonidine receptor site. *Chest*, **83S,** 345S–349S.

LANGER, S.Z. (1970). The metabolism of ^{3}H-noradrenaline released by electrical stimulation from the isolated nictitating membrane of the cat and from the vas deferens of the rat. *J. Physiol. (Lond).)*, **208,** 515–546.

LANGER, S.Z. (1981). Presynaptic regulation of the release of catecholamines. *Pharmac. Rev.*, **32,** 337–362.

LANGER, S.Z., ADLER-GRASCHINSKY, E., ALMEIDA, A.P. & DINIZ, C.R. (1975). Prejunctional effects of a purified toxin from the Scorpion Tityus serrulatus. *Naunyn-Schmiedebergs Arch. Pharmac.*, **287,** 243–259.

LANGER, S.Z., ADLER-GRASCHINSKY, E. & GIORGI, O. (1977). Physiological significance of α-adrenoceptor mediated negative feedback mechanism regulating noradrenaline release during nerve stimulation. *Nature*, **265,** 648–650.

LEW, M.J. & ANGUS, J.A. (1982). Apparent frequency-dependent effect of clonidine on cardiac sympathetic transmission: the role of neuronal uptake. *J. Pharmac. exp. Ther.*, **223,** 540–546.

MEDGETT, I.C., McCULLOCH, M.W. & RAND, M.J. (1978).

Partial agonist action of clonidine on prejunctional and postjunctional α-adrenoceptors. *Naunyn-Schmiedebergs Arch. Pharmac.*, **304,** 215–221.

RAND, M.J., McCULLOCH, M.W. & STORY, D.F. (1980). Catecholamine receptors on nerve terminals. In *Handbook of Experimental Pharmacology*, Vol. 54/1, ed. Székeres, L., ch. 5, pp. 223–266. Berlin: Springer-Verlag.

RAND, M.J., STORY, D.F., ALLEN, G.S., GLOVER, A.B., McCULLOCH, M.W. (1973). Pulse-to-pulse modulation of noradrenaline release through a prejunctional α-receptor auto-inhibitory mechanism. In *Frontiers in catecholamine research.* ed. Usdin, E. & Snyder, S.H., pp. 579–581. New York: Pergamon Press.

RORIE, D.K., MULDOON, S.M. & TYCE, G.M. (1980). The specific activity of retained and released norepinephrine in dog saphenous vein prelabelled with radiolabelled norepinephrine. *Life Sci.*, **26,** 707–714.

STARKE, K. (1977). Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmac.*, **77,** 1–124.

SNEDECOR, G.W. & COCHRAN, W.H. (1967). *Statistical Methods*, 6th edition. Iowa State University Press.

STORY, D.F., McCULLOCH, M.W., RAND, M.J. & STANDFORD-STARR, C.A. (1981). Conditions required for the inhibitory feedback loop in noradrenergic transmission. *Nature*, **293,** 62–65.

WAKADE, A.R. & WAKADE, T.D. (1982). Does presynaptic regulation of sympathetic transmission occur within a limited range of neuronal activity? *Naunyn-Schmiedebergs Arch. Pharmac.*, **321,** 77–79.

(Received August 16, 1983.
Revised September 21, 1983.)