(–)-Timolol is a more potent antagonist of the positive inotropic effects of (–)-adrenaline than of those of (–)-noradrenaline in human atrium

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- 1 The affinity of (–)-timolol for β_1 - and β_2 -adrenoceptors was determined on isolated atrial preparations from patients undergoing open heart surgery. The times for onset and offset of antagonism of the positive inotropic effects of (–)-adrenaline and (–)-noradrenaline by (–)-timolol were measured.
- 2 The antagonism of the positive inotropic effects of (–)-adrenaline and (–)-noradrenaline by (–)-timolol (0.1–100 nM) was simple competitive in human atrium tissue. The slope of Schild-plots was not significantly different from 1.0 [0.93 ± 0.09 for (–)-adrenaline, 0.97 ± 0.09 for (–)-noradrenaline].
- 3 The inotropic effects of (–)-adrenaline were antagonized significantly more by each concentration of (–)-timolol than those of (–)-noradrenaline. K_B -values ($-\log M$) were 10.10 ± 0.09 against (–)-adrenaline and 9.43 ± 0.07 against (–)-noradrenaline ($P < 0.001$).
- 4 Blocking kinetics of (–)-timolol for the β -adrenoceptor were relatively slow. Half-times for the onset of blockade by 10 times K_B of (–)-timolol were approximately 30 min for both (–)-adrenaline and (–)-noradrenaline; offset times were similar.
- 5 It is concluded that (–)-timolol has a higher affinity for the β_2 -adrenoceptor than for the β_1 -adrenoceptor in human atrium. This property may be beneficial clinically in protecting against the β_2 -adrenoceptor hypersensitivity induced by cardiac β_1 -adrenoceptor blockade, but also explain why severe asthma can occur after administration of very low intra-ocular doses of the drug.

Keywords human atrium timolol β_1 - and β_2 -adrenoceptors noradrenaline adrenaline

Introduction

Timolol was the first topical β -adrenergic receptor antagonist to be used in ophthalmology. Since its introduction in 1977 [1], it has become the first-line drug for medical treatment of glaucoma. Oral timolol is used for the therapy of hypertension and coronary heart disease [2–4]. Even though it is clear that (–)-timolol binds to both β_1 - and β_2 -adrenoceptors (β_1 AR and β_2 AR), its relative affinity for each β AR subtype is still uncertain, especially in humans. The major systemic side-effects reported after ocular instillation of (–)-timolol are cardiac (bradycardia) and pulmonary (asthmatic attack) [5–14], involving probably both

β_1 AR and β_2 AR, respectively. An equal K_i value of (–)-timolol for β_1 AR and β_2 AR was reported in rat heart and lung tissue by Phan *et al.* [15], whereas a two- to threefold higher affinity of (–)-timolol for β_2 AR compared with that for β_1 AR was observed in rabbit lung and rat reticulocyte membrane preparations by Saari *et al.* [16] and a slightly higher β_2 AR occupancy than that of β_1 AR was reported in humans after ocular administration of (–)-timolol by Vuori *et al.* [17]. Our study was designed to compare the relative affinity of (–)-timolol for β_1 AR and β_2 AR in human atrium, which is now recognised to possess functional β -adrenoceptors of both sub-types.

These two subtypes of cardiac β AR can undergo cross

regulation. Kaumann *et al.* [18] and Hall *et al.* [19] found that in patients treated with the β_1 AR selective blocker atenolol, cardiac β_1 AR responses are the same as in untreated patients, whereas β_2 AR responses are sensitized both *in vitro* and *in vivo* [18–20]. To determine whether non-selective β AR blockade can also change the responsiveness of the β AR subtypes upon withdrawal of the blocker, Ferro *et al.* [21] attempted to use (–)-timolol as a model non-selective β AR antagonist and found that 2 days after (–)-timolol withdrawal both β_1 AR and β_2 AR mediated increases in heart rate rise were less than control. This finding contrasted with our previous observations after withdrawal of the β_1 AR-selective blocker, bisoprolol; we found that the β_1 AR mediated increase in heart rate recovered within 3 days, and the β_2 AR-mediated increase in heart rate was enhanced [22]. The question therefore was whether the non-selective β AR-blocker had induced an unexpected hypo-sensitization, or whether there is a marked difference in the dissociation of (–)-timolol from β AR from that predicted by its short plasma $t_{1/2}$ (relative to bisoprolol) [23–25]. A study of the association and dissociation kinetics of (–)-timolol to the β AR subtypes would demonstrate whether (–)-timolol is likely to remain bound to the receptor after withdrawal of the drug. A knowledge of the washout kinetics was also a necessary prelude to determining *in vitro* whether prior (–)-timolol treatment alters cardiac β AR sensitivity, just as atenolol washout was measured as a prelude to the organ-bath studies of Hall *et al.* [19].

Compared with (–)-adrenaline, the relative pharmacological potency and the affinity of (–)-noradrenaline are lower for β_2 AR than for β_1 AR in human heart [18, 26]. Either β_1 AR or β_2 AR stimulation can cause maximal inotropic activation when the other one of these two receptor populations are blocked [19, 27]. Non-selective β AR-blockade produces an equal shift of the dose-response curves for (–)-adrenaline and (–)-noradrenaline; on the other hand a β_1 AR-selective or β_2 AR-selective blocker will be recognised because they cause a greater shift in the dose-response curves for (–)-noradrenaline or (–)-adrenaline, respectively [19, 28–30]. We therefore compared the blocking potency of (–)-timolol against the effects of (–)-adrenaline (mediated through both β_1 AR & β_2 AR) and (–)-noradrenaline (mediated mostly through β_1 AR) in human atrial tissues.

Methods

Patients

Atrial tissues were obtained from patients undergoing routine cardiac surgery (coronary bypass grafts, valve surgery, or both) at Papworth Hospital. Patients were treated with a variety of drugs (angiotensin converting enzyme inhibitors, diuretics, nitrates, calcium antagonists, β -adrenoceptor blocker, warfarin and aspirin). For the β AR-blocker, except for one patient who was taking nadolol, the others were receiving atenolol (which is

rapidly washed out from atrial strips during the processes of tissue preparation [19]) treatment. Anaesthesia was induced with midazolam and thiopentone, maintained with trichloroethylene and fentanyl with atracurium used as a muscle relaxant. Tissues were randomised to be used for as many of the experiments (described below) as permitted by the size of the tissue.

Preparation of strips of atrial myocardium

The right atrial appendage was excised immediately before the institution of cardiopulmonary bypass. Tissues were immediately placed in a sealed vial with oxygenated modified Krebs solution of the following composition (mm): Na^+ 125, K^+ 5, Ca^{2+} 2.25, Mg^{2+} 0.5, Cl^- 98.5, SO_4^{2-} 0.5, HCO_3^- 32, HPO_4^{2-} 1 and EDTA 0.04.

Dissection and setting up of the tissues were started as soon as the tissues were transported to the laboratory (about 45 min after surgical removal). Strips with a thickness <1 mm were prepared to facilitate diffusion of oxygen and drugs. Each atrial appendage yielded two to six strips. The tissues were mounted in a 50 ml organ bath at 37 °C, containing a modified Krebs solution as above supplemented with (mm) Na 15, fumarate 5, pyruvate 5, L-glutamate 5, and glucose 10 and constantly bubbled with 95% O_2 -5% CO_2 . Water for the solution was double glass distilled.

Atrial muscle strips were attached to strain-gauge transducers and driven at 1 s intervals with square-wave pulses of 5 ms duration and of just over threshold voltage. A length-force curve was determined, and the length was set at 50% of the length associated with maximum developed force in order to avoid the continuous decay of resting tension throughout the experiment and thereby optimise the signal stimulated by catecholamines [28].

The tissues were incubated for 2 h with 5 μM phenoxybenzamine to inhibit uptake of catecholamines and to block irreversibly myocardial α -adrenoceptors [28].

Antagonism by (–)-timolol to catecholamine-evoked effects

The tissues were pre-incubated for 2 h to achieve equilibrium blockade with (–)-timolol. After the phenoxybenzamine was washed out and time allowed for basal force to stabilise, a single cumulative concentration-effect curve was determined for (–)-adrenaline or (–)-noradrenaline in the presence and absence of (–)-timolol. The concentration-effect curves were terminated with 0.2 mM (–)-isoprenaline to measure the maximal β AR-mediated response. Finally CaCl_2 (6.75 mM) was added to determine the maximal contractile ability of the atrial muscle.

The degree of antagonism was estimated at EC_{50} -levels and calculated as the concentration ratio (CR) of the chosen catecholamine in the presence and absence of (–)-timolol. Log CR-values were used (see calculations and statistics).

Onset and offset of blockade by (–)-timolol

To determine the time of onset and offset of β AR-blockade by (–)-timolol, atrial tissues were exposed to a (–)-timolol concentration equivalent to around ten or hundred times the equilibrium dissociation constant of (–)-timolol estimated from the experiments of antagonism by (–)-timolol of the catecholamine-evoked effects. The experimental design was according to Kaumann *et al.* [31] and applied to human atrium by Hall *et al.* [19]. Cumulative concentration-effect curves to (–)-adrenaline and (–)-noradrenaline were determined. A concentration of both (–)-adrenaline and (–)-noradrenaline was selected that produced about 80% of the maximum response. The tissue was repeatedly exposed for 10 min periods to this concentration of (–)-adrenaline or (–)-noradrenaline with prewarmed solution washout of the catecholamine between each exposure. The tissue was then exposed to 1 nM or 10 nM (–)-timolol for (–)-adrenaline or 5 nM or 50 nM (–)-timolol for (–)-noradrenaline, and the responses to (–)-adrenaline or (–)-noradrenaline were repeated respectively until equilibrium blockade was seen. (–)-Timolol was then washed out and responses to (–)-adrenaline or (–)-noradrenaline were repeatedly determined until the responses had returned to a new equilibrium. The responses of a time-matched control strip not exposed to (–)-timolol were also determined so as to assess the spontaneous decline of the responses seen with repeated exposure to the catecholamine.

Drugs

(–)-Adrenaline bitartrate, (–)-noradrenaline bitartrate, (–)-isoprenaline bitartrate, (–)-timolol maleate were from Sigma Chemical, Poole, UK. Phenoxybenzamine-HCl was from Smith Kline, Welwyn, UK.

Calculations and statistics

The antagonism by (–)-timolol of the inotropic effects of catecholamines was analysed by the equation of Arunlakshana & Schild [32]. For each log(CR-1) value the corresponding K_B -value ($-\log M$) was calculated assuming a Schild-plot with slope 1.00.

All data are expressed as mean $\pm$ s.e.mean. Significance was determined by the paired *t*-test and by the Mann Whitney *U*-test. Differences were considered significant at $P < 0.05$.

Results

Differential blockade of the inotropic effects of (–)-adrenaline and (–)-noradrenaline by (–)-timolol

(–)-Timolol was five times more potent as antagonist of the effects of (–)-adrenaline than those of (–)-noradrenaline. Both (–)-adrenaline and (–)-nor-

adrenaline induced maximum contraction of human atrium, similar to that caused by 0.2 mM (–)-isoprenaline and 6.75 mM Ca^{2+} . Maximal effects of both catecholamines in the presence of (–)-timolol were not different from those obtained in its absence. (–)-Timolol caused parallel rightward shifts of the concentration-effect curves of both (–)-adrenaline and (–)-noradrenaline (Figures 1a and 1b). These observations and the surmountability suggested competitive inhibition by (–)-timolol. The slope of Schild-plots was not significantly different from 1.0 [0.93 ± 0.09 for (–)-adrenaline, 0.97 ± 0.09 for (–)-noradrenaline, (Figure 1c), supporting a model of simple competitive inhibition. Equilibrium dissociation constants K_B of (–)-timolol against (–)-adrenaline ($pK_B = 10.10 \pm 0.09$) were consistently lower (i.e. higher affinity) than against (–)-noradrenaline ($pK_B = 9.43 \pm 0.07$) ($P < 0.001$).

Onset and offset of blockade by (–)-timolol

For all tissues the time required to achieve equilibrium blockade by (–)-timolol and dissociation of the drug from the receptor were rather slow (Figures 2 and 3, Table 1). Upon exposure to 10 times K_B of (–)-timolol, half-times for the onset and offset of blockade were both about 0.5 h for (–)-adrenaline and (–)-noradrenaline (Table 1). The speed of onset depends on the concentration of antagonist. Increasing the (–)-timolol concentration to 100 times K_B , the half-times of onset decreased to about 17 min for (–)-adrenaline and 6 min for (–)-noradrenaline (Table 1, Figures 2 and 3).

Discussion

It is well accepted that both β_1 AR and β_2 AR coexist in human heart. In human atria approximately one third of the total β AR population is of the β_2 subtype [33]. (–)-Adrenaline has approximately the same affinity for myocardial β_1 AR and β_2 AR whereas the affinity of (–)-noradrenaline is at least 20 times lower for β_2 AR than for β_1 AR [26]. Studies with highly selective β_1 AR and β_2 AR blockers have shown that (–)-adrenaline, but not (–)-noradrenaline, causes its positive inotropic effects in human atria in part through β_2 AR [18–19, 27–29]. Hall *et al.* [19] found that when either β_1 AR or β_2 AR are blocked selectively, the responses to (–)-adrenaline are either unaffected, because either β_1 AR and β_2 AR stimulation can cause maximal inotropic activation; or, in some patients, blocked only by β_2 AR-blockade. By contrast, the responses to (–)-noradrenaline are not affected by β_2 AR-blockade but a significant shift of the dose-response curve is caused by β_1 AR-blockade. In the presence of both β_1 AR and β_2 AR blockade, the inotropic effects of (–)-adrenaline and (–)-noradrenaline are blocked to the same degree. We therefore expected that a non-selective β AR-blocker would antagonise the inotropic effects of (–)-adrenaline and (–)-noradrenaline to the same degree, while a low concentration of a β_1 AR-selective

Figure 1 Antagonism by (-)-timolol of the positive inotropic effects of catecholamines on human right atrial strips. (a, b) Result are presented as the percentage of 0.2 μM (-)-isoprenaline induced response. a, Responses to (-)-noradrenaline in the absence of (-)-timolol ($\bullet$, $n=13$) and presence of (-)-timolol 1 nM ($\circ$, $n=5$), 10 nM ($\blacksquare$, $n=4$) and 100 nM ($\square$, $n=4$). b, Responses to (-)-adrenaline in the absence of (-)-timolol ($\triangle$, $n=16$) and presence of (-)-timolol 0.1 nM ($\blacktriangle$, $n=4$), 1 nM ($\circ$, $n=4$), 10 nM ($\blacksquare$, $n=6$) and 100 nM ($\square$, $n=4$). c, Schild-plots of the antagonism of the effects of catecholamines by (-)-timolol. Response to (-)-adrenaline (solid line); response to (-)-noradrenaline (broken line). Values are means $\pm$ s.e.mean.

blocker would antagonise only the inotropic effect of (-)-noradrenaline, and a low concentration of a β_2 AR-selective blocker would antagonise only the inotropic effect of (-)-adrenaline. Because (-)-timolol antagonised

Figure 2 Kinetics of the onset and offset of β -adrenoceptor blockade with $10 \times K_B$ (1 nM) (a) ($n=3$) or $100 \times K_B$ (10 nM) (b) ($n=3$) (-)-timolol under repeated challenges with 1 μM (-)-adrenaline. Responses to repeated challenges with (-)-adrenaline at 1 or 10 nM (-)-timolol present ($\bullet$); time-matched control ($\circ$).

the effect of (-)-adrenaline five times more potently than those of (-)-noradrenaline, our evidence is consistent with a higher affinity of (-)-timolol for β_2 AR than that for β_1 AR.

The relative selectivity of (-)-timolol for β_2 AR is in agreement with the study by Saari *et al.* [16]. They used rabbit lung as a source of β_1 AR and rat reticulocyte as a source of β_2 AR. The K_i value for the β_1 AR site was 1 nM and for the β_2 AR site was 0.4 nM. The receptor occupancy was also determined from its K_i value after ocular administration of (-)-timolol in patients [17]. The extent of β_1 -adrenoceptor occupancy by (-)-timolol was about 65% and its β_2 -receptor occupancy about 80%. The higher β_2 AR occupancy suggests greater blockade of the β_2 AR and could be related to its respiratory side effect (bronchospasm) after topical administration of (-)-timolol. Clifton *et al.* [34] tested the affinity of β ARs for (-)-timolol in the sinus node and ventricular myocardium of healthy volunteers. They calculated the apparent affinity constant (K_a) from the serum (-)-timolol concentration and the dose ratio of (-)-isoprenaline required to increase either heart rate or Doppler-derived aortic blood flow acceleration by 35%. They found that β ARs in the sinus node have a greater affinity for (-)-timolol (K_a 1.14×10^6 M) than do

Figure 3 Kinetics of the onset and offset of β -blockade with $10 \times K_B$ (5 nm) (a) ($n=3$) or $100 \times K_B$ (50 nm) (b) ($n=3$) (–)-timolol under repeated challenges with $1 \mu\text{M}$ (–)-noradrenaline. Responses to repeated challenges with (–)-noradrenaline at 1 or 10 nm (–)-timolol present (●); time-matched control (○).

receptors in the ventricular myocardium (K_a 7.85×10^5 M). Since human atrium has a higher proportion of β_2 AR population (33–50% of the total β AR) compared with ventricle (20–25% of the total β AR) [35–37], the difference of the affinity of (–)-timolol for sinus node and ventricular myocardium may be explained by differences in the distribution of β -adrenergic subtypes in these tissues. However it is uncertain whether the population in atrium can be extrapolated also to the sinus node.

Our findings suggest some implications for the clinical use of (–)-timolol, both locally in the eye, and systemically. (–)-Timolol is one of the most effective drugs in the treatment of glaucoma, causing a reduction in intraocular pressure. However, systemic adverse effects of ocular (–)-timolol have been reported, especially in the respiratory system and including some serious cases [5–14]. Dunn *et al.* [38] studied 24 asthmatic subjects receiving topical (–)-timolol instillation and found there was a significant impairment of respiratory function 90 min after drug administration. A significant decrease in airflow after (–)-timolol instillation was also found by Ward *et al.* [39]. Provocation of an asthmatic attack has been reported [40–41]. Prince *et al.* [42] reported a 67-year-old man with stable chronic obstructive pulmonary disease who experienced severe dyspnea leading to respiratory arrest within 30 min of the administration of his first dose of (–)-timolol ophthalmic solution. Even in patients without a history of airways disease, (–)-timolol can impair lung function, especially in older people. Diggory *et al.* [43–45] studied a group of elderly glaucomatous patients, without a history of asthma or obstructive airways disease, who were using topical (–)-timolol for control of intraocular pressure. Their topical therapy was changed to either the non- β AR-blocker pilocarpine or the β_1 AR selective antagonist betaxolol. The change was associated with improvement in mean peak flow, mean FEV₁ (forced respiratory volume in one second) and FVC (forced vital capacity). Betaxolol is as effective as (–)-timolol in lowering intraocular pressure, with fewer respiratory complications [46–47]. (+)-Timolol has been found to be only slightly less potent than (–)-timolol at lowering the intraocular pressure, but its affinity for β AR was much weaker [48].

To be suitable for ocular use, a β AR-blocker needs to be potent and long-lasting. Potency (coupled to solubility in the drug vehicle) enables a sufficiently small physical amount of drug to be delivered locally. However potency is also a measure in part of receptor affinity and therefore dissociation constant. It is likely that lipophilic, potent β AR-blockers have a much longer $t_{1/2}$ in the tissues than in plasma because of a local 're-circulation' between receptors and non-specific binding sites on membranes. The main clinical relevance of our finding of a five-fold β_2 AR-selectivity of (–)-timolol is that only one fifth as much (–)-timolol needs to be

Table 1 Onset and offset of β AR-blockade with (–)-timolol

Patients	Disease	β -adrenoceptor blocker	(–)-timolol	Adrenaline		Noradrenaline	
				t _{1/2on}	t _{1/2off}	t _{1/2on}	t _{1/2off}
				(min)		(min)	
40 years (M)	CABG	Metoprolol	10K _B	26	40	37	18
78 years (M)	CABG	Atenolol	10K _B	30	26	36	34
57 years (M)	CABG	Atenolol	10K _B	30	42		
59 years (M)	CABG	Metoprolol	100K _B	18	54	6	56
62 years (M)	CABG	No	100K _B	19	53	6	26
45 years (M)	CABG	Atenolol	100K _B	14			

CABG, coronary artery bypass graft.

systemically absorbed to cause clinically important blockade of β AR, and that blockade of bronchial β AR is likely to be even longer lasting than expected from heart rate blockade of exercise tachycardia. Saari *et al.* [16] found that 40 μ l of 0.5% (–)-timolol in the aqueous humour occupies the β_1 AR and β_2 AR completely for at least 18 h. They also found that both β_1 AR and β_2 AR occupancies of (–)-timolol in plasma decreased very slowly during the 4 h study period; thus the systemic β AR-antagonistic activity of ocular (–)-timolol may last much longer than 4 h after drug instillation [17]. Our determination of the slow onset and offset of blockade by (–)-timolol on cardiac tissue agrees with the above observations. Even though multiple factors especially diffusion barriers would influence the time course of organ-bath studies which may not be operative *in vivo*, these studies can provide some useful information, particularly in comparisons of β AR-antagonist compounds. Kaumann *et al.* [18] found the half-time of onset in the case of K_B (–)-bupranolol was nearly 20 min, but for K_B ($\pm$)-sotalol was only 1–2 min. Our data are insufficient to compare onset or offset times for (–)-adrenaline and (–)-noradrenaline. Although in theory comparison of receptor offset times should be more precise than onset times because they are independent of antagonist concentration, in practice offset times are harder to quantify because the inotropic response does not return completely to values prior to addition of the antagonist.

A striking contrast is between our present findings and previous experiments with the most hydrophilic β AR-blocker, atenolol. An enhanced β_2 AR sensitivity was observed in atrial tissues excised from patients receiving chronic atenolol therapy [19]. To exclude the masking effect of atenolol on β_1 AR during experiment, the kinetics of onset and offset rate of atenolol were determined. All tissues achieved equilibrium blockade by 10 min after exposure to $10K_B$ atenolol; this blockade was rapidly reversed by washing with atenolol-free Krebs solution and all tissues were free of blockade by 20 min. As described in the Introduction, our studies on the β_2 AR hypersensitivity induced by atenolol led by an indirect route to the present experiments. We showed that 2 weeks of treatment with a more lipid soluble selective β_1 AR-blocker, bisoprolol, induces β_2 AR hypersensitivity in normal subjects present three days after bisoprolol withdrawal [22]. Subsequently we wished to determine whether a non-selective β AR-blocker has the same effect. In contrast with bisoprolol, both exercise induced heart rate increase (β_1 AR-mediated) and salbutamol induced heart rate increase (β_2 AR-mediated) were still significantly reduced 2 days after (–)-timolol withdrawal and no different at 3 days after (–)-timolol withdrawal [21]. Initially we considered whether the non-selective β AR-blocker might have had the opposite effect to selective β_1 AR-blockade on β_2 AR sensitivity. But it is clear now that the effects of (–)-timolol on the heart simply took several days to disappear. Whilst a further study would need to be done to evaluate β_2 AR sensitivity after complete (–)-timolol washout, it is already apparent that both during and immediately after (–)-timolol treatment, protection against

(–)-adrenaline induced tachydysrhythmias will be greater than during and immediately after β_1 AR-selective blockade. The influence of these two types of β AR-blocker have been compared in a prospective, outcome study of high risk patients in East Anglia, and the results will be published separately.

In summary we found that (–)-timolol has a higher affinity for the β_2 AR than for the β_1 AR in human atrium. The speeds of association and dissociation blockade of (–)-timolol were rather slow in comparison with those repeated for other β AR-blockers, such as atenolol. The slow blocking kinetics and the slight selectivity for β_2 -adrenoceptors of (–)-timolol should be taken into account in the clinic.

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