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INHIBITION OF LYMPHOCYTE CAPPING AND TRANSFORMATION BY PROPRANOLOL AND RELATED COMPOUNDS

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1 The effects of propranolol on phytohaemagglutinin (PHA)-induced transformation of murine T lymphocytes and capping of anti-IgG on the surface of murine B lymphocytes have been examined.

2 A 50% inhibition of transformation was observed with 10^{-5} M propranolol, whereas a higher concentration, of the order of 10^{-3} M propranolol was required to inhibit capping by 50%. The (+)- and (−)-isomers of propranolol proved equipotent in these respects, and the relative potencies of selected analogues of propranolol (alprenolol, oxprenolol, metoprolol, practolol and sotalol) coincided with their potencies as membrane stabilizers; however, lymphocyte transformation was consistently more sensitive than capping.

3 Similar effects were also seen with quinidine, chlorpromazine and lignocaine, and it was concluded that the inhibition of both lymphocyte functions was due to the membrane stabilizing actions of propranolol.

Introduction

Chlorpromazine and lignocaine have been shown to inhibit capping of anti-IgG molecules on the surface of B lymphocytes and concanavalin A on neutrophil polymorphonuclear leukocytes, (Ryan, Unanue & Karnovsky, 1974; Schreiner & Unanue, 1976) and also transformation induced by both T and B lymphocyte mitogens (Ferguson, Schmidtke & Simmons, 1975; 1976). From these separate studies it appears that transformation was markedly more sensitive than capping, although both effects were probably due to the membrane 'stabilizing' properties of chlorpromazine and lignocaine.

Propranolol at concentrations of the order of 1 mM acts as a membrane 'stabilizer' (Roth & Seeman, 1971; Seeman, 1972), and this action can be systematically characterized and distinguished from β-adrenoceptor antagonism. Thus for example, membrane 'stabilization' is non-stereo specific for the isomers of propranolol, and is produced by quinidine, frequently being referred to as the 'quinidine like' action of propranlol (Howe & Shanks, 1966; Vaughan Williams, 1966; Barrett & Cullum, 1968; Marshall, von Borcke & Malan, 1975; Marshall, von Borcke, Florin-Christenson & Ekins, 1977). Moreover, analogues of propranolol are available with different potencies as membrane 'stabilizers' (Singh & Vaughan Williams, 1970; Hellenbrecht, Lemmer, Wiethold & Grobecker, 1973; Ablad, Borg, Carleson, Ek, Johnsson, Torbjorn & Regordh, 1975). We have therefore investigated the effects of propranolol and related compounds on both lymphocyte capping and transformation processes.

Methods

Preparation of lymphocytes

T lymphocytes from mouse lymph nodes The peripheral and mesenteric lymph nodes were removed from adult, male Balb/C mice (supplied by Banting and Kingman), aseptically. These were placed in sterile Hank's Balanced Salt Solution (BSS).

The lymph nodes were transferred to fresh BSS under a bacterially filtered laminar air-flow. Lymphocytes were released by squeezing the nodes between flame sterilized forceps into a fresh batch of BSS. The lymphocyte suspension was transferred to sterile conical centrifuge tubes, placed in ice. After the large particulate debris had settled the supernatant lymphocyte suspension was transferred to fresh tubes and centrifuged at 800 *g* at 2 to 4°C for 15 min, the supernatant decanted and the cells resuspended in ice-cold fresh BSS. This washing procedure was repeated twice more and the cells finally resuspended in RPMI con-

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taining foetal calf serum (10% v/v), L-glutamine 2 mM and antibiotics (benzylpenicillin, at a final concentration of 2×10^3 units/ml and streptomycin at a final concentration of 75 units/ml). The number of cells in suspension were counted in a haemocytometer and adjusted to a concentration of $1{\cdot}8 \times 10^7$ cells/ml.

B-lymphocytes from mouse spleens Spleens were removed from two month old Balb/C mice, teased and washed ($\times 3$) in Eagle's Minimal Essential Medium (EMEM), and counted by phase contrast microscopy.

Preparation of drug solutions and 'master cultures'

Master solutions were made up in RPMI at a concentration at least twice as great as the maximum required for culture and sterilized by filtration. (All subsequent manipulations were carried out aseptically). Serial dilutions of the master solution for each drug were made such that a convenient volume of each dilution could be added to the 'master culture' to give the required final concentration.

Phytohaemagglutinin (PHA) was reconstituted according to manufacturers directions using reagent grade material, and this solution was then diluted 1:100 and added to the 'master cultures'. Master cultures were prepared in sterile tubes and the lymphocyte suspension was added immediately before dispensing the mixtures to microtitre cell culture plates.

Setting out culture trays

Disposable, sterile, flat-bottomed well microtitre plates with a lid were used in the incubation of the cultures. Aliquots (200 µl containing 1.2×10^6 lymphocytes) from each 'master culture' were used in each well (5 replicates of each drug concentration). In addition control cultures were of lymphocytes alone, and lymphocytes plus PHA and no drug. The wells at the edges of the plates were not used and were filled with 200 µl RPMI only. In addition, the order in which the cultures were distributed in the trays was randomized.

Cultures were incubated in an atmosphere of 5% CO_2 at 37°C for 48 h.

Pulsing with [^3H]-thymidine, harvesting of cultures and counting of [^3H]-thymidine

A measured volume (50 µl) of a thymidine solution containing 1 µCi [^{3}H]-thymidine (sp. act. 5 Ci/mmol) and 2.7 µmol carrier thymidine was added to each well after 42 h culture. The cultures were incubated for a further 6 h and then harvested by filtering on to discs of Whatman glass fibre paper (GF/C) and washed five times with ice-cold phosphate buffered saline. The DNA was precipitated on to the paper by washing with ice-cold 5% TCA followed by ice-cold methanol.

The discs were placed in scintillation counting vials dried at 60°C and the scintillation solution added. Vials were counted in a Hewlett Packard scintillation counter for a minimum of 10,000 counts.

Capping studies

A total of 3×10^7 spleen leucocytes were stained by adding 300 µl of rabbit anti-mouse immunoglobulin fluorescent conjugate diluted from a stock preparation (1:20 dilution in EMEM, giving a final concentration of approximately 30 µg IgG/ml) to the dry pelleted cells. The cells were agitated and incubated on ice for 30 min, washed three times in ice-cold EMEM, and finally, resuspended in 1 ml EMEM, with or without the addition of propranolol or related compounds. These cell suspensions were then gassed with 5% CO_2 and incubated in a water bath at 37°C. After stated incubation periods, 200 µl aliquots were removed and immediately dispersed into ice-cold EMEM containing sodium azide 5 mM: the cells were spun down, fixed with ice-cold 2% paraformaldehyde in phosphate buffered saline (pH 7.5), and then examined for staining and capping the following day using u.v. incident illumination; 80 to 100 B cells per preparation were observed for capping, and the results expressed as percentage cells capped.

Reagents

Rabbit anti-mouse immunoglobulin fluorescent conjugate was prepared from serum of rabbits hyperimmunized with purified mouse immunoglobulin. The immunoglobulin fraction of the serum was purified by double 40% ammonium sulphate precipitation, and then conjugated with fluorescein isothiocyanate.

(+)- And (−)-isomers of propranolol, and (±)-practolol were generous gifts from ICI Ltd, alprenolol from Hassle, oxprenolol and metoprolol from CIBA-Geigy, sotalol from Mead Johnston, and lignocaine from Pharmaceutical Manufacturing Co. Quinidine sulphate and chlorpromazine were purchased from Sigma Chemical Company, and PHA (Reagent Grade) from Wellcome Reagents Ltd. Tissue culture medium, RPMI, and BSS were supplied by Flow Laboratories, U.K.

Results

Inhibition of transformation

The addition of increasing concentrations of propranolol inhibited PHA-induced transformation of T lymphocytes as shown in Figure 1. In the presence of

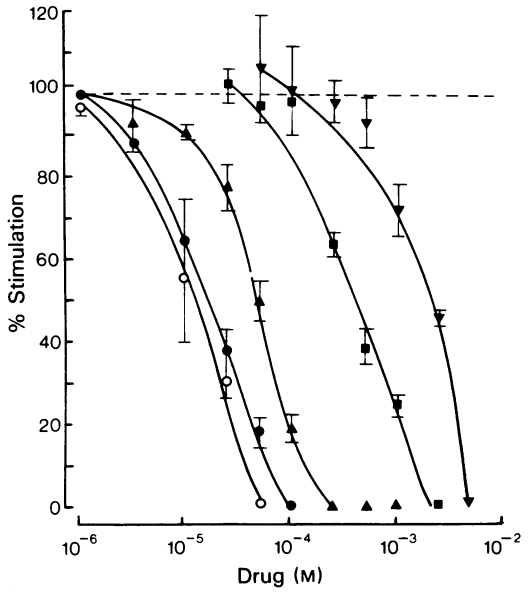

Figure 1 Effect of the (+)- and (−)-isomers of propranolol, and alprenolol, sotalol and lignocaine, on phytohaemagglutinin-stimulated transformation of T lymphocytes: (●) (−)-propranolol; (○) (+)-propranolol; (▲) alprenolol; (■) lignocaine; (▼) sotalol. Results expressed as mean of 5 determinations; vertical lines indicate s.d.

2×10^{-5} M (−)-propranolol 50% inhibition was observed and the (+)-isomer proved equipotent, the slightly greater inhibition seen in the presence of the latter not being significantly different from that observed with the (−)-isomer.

PHA-stimulated transformation of T lymphocytes was also inhibited by other β-adrenoceptor antagonists such as alprenolol, oxprenolol, metoprolol, practolol and sotalol (Figure 1 and Table 1). Alprenolol proved only slightly less potent an inhibitor than propranolol, whereas metoprolol, practolol, and sotalol, analogues of propranolol commonly considered to be virtually devoid of local anaesthetic activity (Singh & Vaughan Williams, 1970; Hellenbrecht, *et al.*, 1973; Ablad *et al.*, 1975) were far less potent, 50% inhibition being achieved at a sotalol concentration of 2.5×10^{-3} M, while no significant inhibition was observed at concentrations below 10^{-3} M. Oxprenolol proved intermediate in its potency, and thus the potencies of this series of β-adrenoceptor antagonists as inhibitors of transformation correlated with their relative potencies as local anaesthetics (Singh & Vaughan Williams, 1970; Lemmer, Wiethold & Grobecker, 1973; Ablad *et al.*, 1975; Marshall *et al.*, 1977).

In confirmation of earlier studies (Ferguson *et al.*, 1975; 1976), chlorpromazine proved a potent inhibitor of lymphocyte transformation, with 50% inhibition observed at a concentration of 5×10^{-6} M, while lignocaine proved less potent, a concentration of 2.5×10^{-4} M being required to inhibit transformation by 50% (Table 1, Figure 1). Moreover, quinidine, a compound which exerts similar membrane 'stabilizing' actions to propranolol, but which like chlorpromazine and lignocaine is inactive as a β-adrenoceptor antagonist, inhibited transformation with an equal potency to propranolol (Table 1).

Table 1 Concentration of selected local anaesthetic and β-adrenoceptor blocking agents required to give 50% inhibition of phytohaemagglutinin-stimulated lymphocyte transformation

Compound	*Local anaesthetic activity*	*β-Blocking activity*	*Concentration at which 50% inhibition observed* (M)	*Partition* coeff. pH 7.0 n-octanol phosphate buffer*
Chlorpromazine	++++	Nil	5×10^{-6}	
Quinidine	+++	Nil	1×10^{-5}	
(+)-Propranolol	+++	Nil	1.2×10^{-5}	5.39
(−)-Propranolol	+++	Yes	2×10^{-5}	5.39
Alprenolol	+++	Yes	5×10^{-5}	3.27
Oxprenolol	++	Yes	1×10^{-4}	0.43
Lignocaine	+	Nil	2.5×10^{-4}	
Metoprolol	Nil	Yes	5×10^{-4}	
Practolol	Nil	Yes	1×10^{-3}	0.009
Sotalol	Nil	Yes	2.5×10^{-3}	0.011

Concentration ranges studied: (i) all except chlorpromazine = 1×10^{-6} M to 5×10^{-3} M; (ii) chlorpromazine = 1×10^{-8} M to 5×10^{-5} M.

* From Hellenbrecht, Lemmer, Wiethold & Grobecker, 1973.

During the above studies, the cells remained capable of trypan blue exclusion in the presence of all concentrations of propranolol tested, indicating that the drug was not cytotoxic to the murine lymphocytes. Moreover, a high degree of reversibility of the effects of the added drugs was observed, when the lymphocytes were washed once after incubation in the presence of the drug for 24 h, and incubated for a further 24 h in the absence of the drug, (Table 2). For example, in this study, incubation in the presence of

Table 2 Reversibility of inhibition of phytohaemagglutinin (PHA)-induced transformation (% of stimulation by PHA)

Drug	*Conc** M	*After removal of drug*	*Continuous presence of drug*
Chlorpromazine	5×10^{-6}	93.0 ± 4.9	77 ± 4.9
Oxprenolol	10^{-4}	109 ± 10.0	68 ± 4.5
Practolol	5×10^{-4}	109 ± 9.7	81 ± 12.2
Alprenolol	5×10^{-5}	60 ± 10.4	9 ± 0.1
Lignocaine	2.5×10^{-4}	78 ± 9.7	59 ± 5.5
Metoprolol	5×10^{-4}	38 ± 15.0	7 ± 0.7

* Concentration that gave approx. 50% inhibition of transformation by PHA in a previous study (Table 1). Cultures were set up, with drug concentrations shown, as described in the methods section. After 24 h culture, the plates were centrifuged at 500 *g* (15 min, 23°C), and the supernatant aspirated. The cells were washed with RPMI + 1:100 diluted PHA (200 µl/well) once, and finally resuspended in this medium, and incubated in the absence of the drug for another 18 h. Transformation was estimated by addition of [^{3}H]-thymidine during a final 6 h incubation. Parallel control cultures were set up in which the drug was absent throughout or continuously present. Result are expressed as a % of transformation observed with the cells which had not been exposed to the drugs, and are the means of 5 determinations ± s.d.

Table 3 The effect of β-adrenoceptor antagonists and quinidine on rabbit anti-mouse IgG-induced capping of mouse B lymphocytes

	Drug concentration (M)	*% B-cells capped Time (min) of incubation at 37°C* 0	30	90
Experiment I				
Control	0	7.5	79	68
Quinidine	10^{-3}	13	12	15
Propranolol	10^{-3}	10	12	12
Practolol	10^{-3}	12	74	67
Metoprolol	10^{-3}	12	85	74
Experiment II				
Control	0	2	84	—
Propranolol	10^{-3}	—	20	—
Alprenolol	10^{-3}	—	77	—
	5×10^{-3}	—	12	—
Oxprenolol	10^{-3}	—	87	—
	5×10^{-3}	—	11	—
Metoprolol	10^{-3}	—	89	—
	5×10^{-3}	—	87	—
Sotalol	10^{-3}	—	89	—
	5×10^{-3}	—	81	—
Practolol	10^{-3}	—	82	—
	5×10^{-3}	—	88	—

See methods section for experimental details.

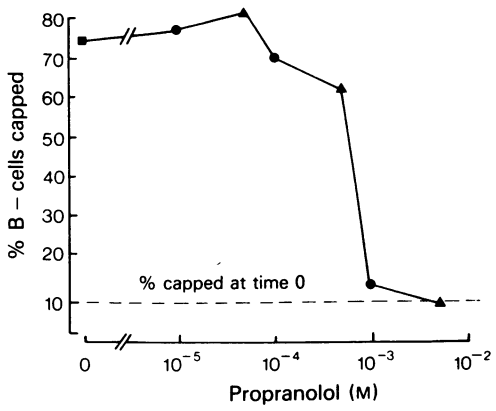

Figure 2 Inhibition of rabbit anti-mouse IgG-induced capping of mouse B-lymphocytes by increasing doses of (+)- and (−)-propranolol: (●) (+)-propranolol; (▲) (−)-propranolol. See methods section for experimental details: % capping was determined after 0 and 30 min at 37°C.

alprenolol (5×10^{-5} M) reduced subsequent PHA-induced transformation to 9% of the level observed in the control incubation in the absence of the drug, whereas washing after 24 h incubation in the presence of alprenolol and subsequent incubation for 24 h in the absence of the drug, resulted in an increased transformation to 60% of the control. The apparent discrepancy in the potency of alprenolol, a concentration of 5×10^{-5} M affecting only 50% inhibition of transformation in the previous study (Figure 1) is probably due to slight variability between different lymphocyte preparations in their sensitivity to the presence of the inhibitors, and illustrates the importance of doing a systematic comparative study such as that shown in Figure 1 with one lymphocyte preparation.

Inhibition of capping

Maximum incidence of capped B cells (about 80%) occurred after 30 min incubation at 37°C (Table 3). However, addition of (±)-propranolol (10^{-3} M) immediately prior to the incubation period at 37°C resulted in almost complete inhibition of capping above values seen in time zero controls, at all time periods tested. Patching, observed as dispersion of the initial uniform fluorescence into multiple, randomly distributed, discrete areas leaving other areas devoid of staining was not inhibited by addition of propranolol.

Inhibition of capping was detected at concentrations between 10^{-4} M and 10^{-3} M propranolol (Figure 2). Thus propranolol proved to be a less potent inhibitor of capping than transformation (Figure 1).

The observation that the (+)- and (−)-isomers of propranolol were equipotent in inhibiting lymphocyte capping (Figure 2) is consistent with this action being due to the local anaesthetic and not the β-blocking properties of the drug. Moreover, using analogues of propranolol which also act as β-adrenoceptor antagonists, we observed inhibition of capping with those which exert local anaesthetic activity, alprenolol and oxprenolol, but not with those which are reported to have little or no such effect in other systems, metoprolol, sotalol and practolol (Singh & Vaughan Williams, 1970; Hellenbrecht *et al.*, 1973; Ablad *et al.*, 1975; Marshall *et al.*, 1975; 1977) (Table 3). The concentrations of alprenolol and oxprenolol required to effect inhibition (5×10^{-3} M), were higher than that of propranolol (10^{-3} M), whilst quinidine also inhibited capping when present at 10^{-3} M.

Discussion

Propranolol inhibited both PHA-induced transformation of T lymphocytes, and IgG-induced capping of B lymphocytes. Both T and B lymphocytes have been shown to respond to β-adrenoceptor stimulants (Chisari & Edgington, 1974; Galant & Remo, 1975; Schreiner & Unanue, 1975; Ito, Sless & Parrott, 1977). However, by use of analogues of propranolol with decreasing potencies as membrane stabilizers, both inhibitory effects appeared to be due to the membrane stabilizing actions of propranolol, and not its action as a β-adrenoceptor blocking agent. This conclusion was confirmed by several other criteria, such as the equipotency of the (+)- and (−)-isomers of propranolol, and the effectiveness of other compounds, such as quinidine, lignocaine and chlorpromazine, structurally and functionally unrelated to β-adrenoceptor antagonists, but recognised as acting as membrane 'stabilizing' agents.

The concentrations of propranolol required to inhibit capping, 10^{-4} to 10^{-3} M, were consistent with those which have been shown to modify membrane functions in diverse systems in a manner attributable to the local-anaesthetic properties of propranolol. For example 2.5×10^{-4} M propranolol has been shown to proffer anti-haemolytic protection in 50% of human red cells (Roth & Seeman, 1971), and inhibit TSH stimulation of adenylate cyclase in thyroid membranes by 50% whilst significantly increasing binding of the hormone to the membranes (Marshall *et al.*, 1975; 1977). Moreover, 2×10^{-4} M (+)-propranolol inhibited incorporation of ^{14}C-amino acid into protein in chick embryo liver cells (Schoenfeld & Atsmon, 1977).

However, significantly lower concentrations (between 5×10^{-6} and 10^{-4} M) were required to inhibit PHA-induced transformation. Thus this confirmed that transformation was unusually sensitive to the presence of membrane stabilizing agents, suggested by the previous studies which used chlorpromazine and lignocaine (Ferguson *et al.*, 1975; 1976). Moreover transformation was shown to be consistently more sensitive than capping to all the membrane stabilizers included in this study. Thus, β-adrenoceptor antagonists such as practolol, and sotalol, with low partition co-efficients between *n*-octanol and phosphate buffer (pH 7.0) (Table 1) (Hellenbrecht *et al.*, 1973), and frequently considered to be without detectable membrane stabilising activity were capable of inhibition of transformation at higher concentrations (10^{-3} M).

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