A23187 or periodate were observed at valinomycin concentrations of 10^{-8} M and 10^{-7} M. The same concentrations of valinomycin when added to cell cultures prior to stimulation resulted in almost complete inhibition of [^{3}H]TdR uptake, demonstrating the inhibitory effect of valinomycin on mitogen-induced lymphocyte transformation. The inhibitory effect of valinomycin on labeled nucleoside incorporation by the lymphoblasts and resting lymphocytes may result from (a) impairment of nucleoside uptake or transport in the cell or (b) interference of subsequent reactions leading to DNA or RNA syntheses.

The results presented in this report suggest that caution should be used when interpreting some of the results in earlier studies [1, 2] using valinomycin to examine the role of K^+ in PHA-induced lymphocyte transformation. This is based on (a) our inability to overcome the inhibitory effect by valinomycin of lymphocyte transformation by raising the external K^+ concentration and (b) interference with RNA synthesis in resting lymphocytes and DNA and RNA syntheses in maximally stimulated lymphocytes as judged by depressed incorporation of [^{3}H]TdR and [^{3}H]uridine. The actual degree of interference of lymphocyte transformation by valinomycin is difficult to assess because of these previously unknown "side effects" of valinomycin on lymphocyte metabolism.

Valinomycin has also been shown to affect mitochondrial function in intact, resting peripheral blood lymphocytes. Exposure of cells to the ionophore results in decreased cellular ATP and increased oxygen consumption [8–10]. These studies were performed using valinomycin concentrations of 10^{-6} M and greater. The effect of valinomycin in the concentration range used in our study on these parameters of cellular metabolism remains to be established before any relationship between decreased cellular ATP, depressed incorporation of nucleic acid precursors, and mitogen-induced lymphocyte transportation can be established.

In summary, our experiments support the conclusion that valinomycin interferes with cellular function in resting and proliferating lymphocytes as judged by depressed nucleic acid synthesis. The results obtained using valinomycin in intact viable cells in other biological studies should also take into account the side effects of valinomycin on cellular metabolism described in this report.

Acknowledgements—This work was supported in part by grants from the National Institutes of Health (CA0536 and GM07131). We thank Judy Setzkorn and Jean Martin for their technical assistance.

Department of Pediatrics
University of Wisconsin,
Madison, WI 53792, U.S.A.

JOHN C. SCHULTZ*
NASROLLAH T. SHAHIDI

* Correspondence should be addressed to: John C. Schultz, Ph.D., University of Wisconsin Center for Health Sciences, Room K4/423, 600 Highland Ave., Madison, WI 53792 U.S.A.

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Biochemical Pharmacology, Vol. 33, No. 13, pp. 2154–2158, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
Pergamon Press Ltd.

A non-α-adrenoceptor binding site for [^{125}I]-BE 2254 in guinea pig brain membranes

(*Received* 29 *December* 1982; *accepted* 6 *December* 1983)

There is currently much interest in the new radioligand ^{125}I-labeled BE 2254* which is proving to be a valuable tool for the study of α_1-adrenoceptors in rat cerebral cortex membranes [1–6]. However, when using [^{125}I]-BE 2254 to study α_1-adrenoceptors in guinea pig cerebral cortex membranes, we found a difference between the total amount of bound [^{125}I]-BE 2254 displaced by saturating concentrations of BE 2254 and phentolamine [7], indicating a second binding site for the radioligand. The present investigation attempts to characterise this second binding site by using displacement studies with drugs which interact with a wide variety of receptors and transport processes and also attempts to establish whether this binding site is present in cerebral cortex membranes from other animal species. Since [^{125}I]-BE 2254 is being used increasingly to characterise α_1-adrenoceptors, it is important to establish the identity and prevalence of this second site. To study the displacement effects of ligands on the second binding site alone, the α-adrenoceptor was first blocked with a saturating concentration of phentolamine.

* Abbreviations: BE 2254, 2-(β-(4-hydroxyphenyl)-ethylaminomethyl)-tetralone; and PMSF, phenylmethylsulfonyl fluoride.

Methods and materials

Radioiodination. BE 2254 was labeled with ^{125}I (Na ^{125}I carrier-free, 100 mCi/ml, from the Radiochemical Center, U.K.) using a modification of the chloramine-T method of Maguire *et al.* [8], and the iodinated product was purified by ethyl acetate extraction followed by paper and thin-layer chromatography as described previously by Adams and Jarrott [7].

Membrane preparation. Cerebral cortex, kidney or seminal vesicle tissue was weighed and homogenised in 50 vol. of 40 mM potassium phosphate buffer, pH 7.4, containing PMSF, centrifuged at 45,000 *g* for 10 min,

washed twice, and resuspended in the same buffer, as described previously [7]. The final resuspension was in 200 vol. to study displacement from both sites or 100 vol. to study displacement from the second site alone.

Radioligand receptor assay. Aliquots (50 μl) of membrane suspension were added to polystyrene culture tubes (75 × 12 mm) containing [^{125}I]-BE 2254 at a final concentration of approximately 50 pM together with various concentrations of competing ligands in a total volume of 200 μl of potassium phosphate buffer (40 mM, pH 7.4). To measure displacement from the non-α-adrenoceptor site, phentolamine was included in the incubation volume at a final concentration of 1 μM. Non-specific binding was estimated in incubation tubes containing 1 μM BE 2254. The tubes were incubated in a shaking water bath at 37° for 30 min, and the reaction was stopped by the addition of 2.5 ml of ice-cold incubation buffer. The tube contents were immediately filtered under vacuum through a 12 mm diameter Whatman GF/B filter disc, and the tube was rinsed with a further 2.5 ml of ice-cold buffer which was also filtered. The filter was then washed twice with 5 ml of ice-cold buffer, placed in a flat-bottomed polystyrene tube (100 × 15 cm), and counted in an LKB Multigamma counter with an efficiency of approximately 80%.

Data analysis. Drug displacement measurements were fitted to a four parameter logistic function [9].

Results and discussion

Figure 1A shows the displacement of bound [^{125}I]-BE 2254 from guinea pig cerebral cortex membranes by increasing concentrations of BE 2254 and phentolamine. There was a difference of approximately 35% in the total amount of [^{125}I]-BE 2254 displaced by saturating concentrations of the two drugs, with BE 2254 effectively displacing all of the radioactivity bound to the membranes. Other α-adrenoceptor antagonists and agonists, e.g. prazosin, yohimbine and noradrenaline, gave similar shaped displacement curves to phentolamine. They also failed to displace the extra 35% of bound [^{125}I]-BE 2254 (data not shown) revealing a non-α-adrenoceptor binding site for the radioligand. Membranes prepared from other areas of guinea pig brain, i.e. cerebellum, pons, medulla, hypothalamus, midbrain, hippocampus, striatum and spinal cord, showed no significant difference at the $P = 0.01$ level from cerebral cortex membranes in the proportion of [^{125}I]-BE 2254 bound to the second site (see Table 1).

A similar experiment was then carried out on dog cerebral cortex membranes, and the results are shown in Fig. 1B. In contrast to Fig. 1A, BE 2254 and phentolamine displaced the same total amount of [^{125}I]-BE 2254, the only difference being the IC_{50} values (BE 2254, 1.5 nM; phentolamine, 19 nM). This latter pattern of displacement was observed with membranes prepared from the cerebral cortices of rat, mouse, cat, rabbit, chicken, fish and man as well as membranes from guinea pig seminal vesicle and kidney (see Table 1). These results suggest that the second binding site may be unique to guinea pig brain membranes.

Many different drugs were tested for their ability to displace [^{125}I]-BE 2254 from the non-α-adrenoceptor binding site in guinea pig cerebral cortex membranes, and the results are recorded in Tables 2 and 3. Table 2 lists forty-eight drugs of known binding site specificity, most of which did not displace the radioligand when present at concentrations up to 1 μM. The drugs in part (b) of the table displaced some of the [^{125}I]-BE 2254 bound to the second site and so were tested at higher concentrations to determine the IC_{50} values of their displacement curves. Naloxone had an IC_{50} value of approximately 1 μM and WB 4101 4 μM; ketocyclazocine, methiothepine, xylamidine, danitracin, pirenperone, cyproheptadine, metergoline, mianserin and methysergide all had IC_{50} values of approximately 5 μM. Thus, none of the drugs in Table 2 was very potent at displacing [^{125}I]-BE 2254 bound to the second site, showing that this site cannot be ascribed to any of the neurotransmitter receptors or transport processes associated with these ligands.

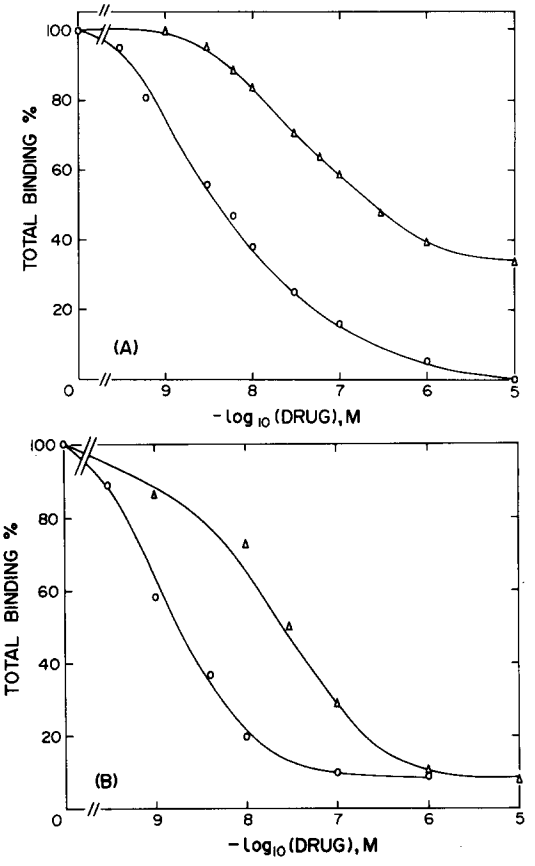


Fig. 1. (A) Displacement of [^{125}I]-BE 2254 bound to guinea pig cerebral cortex membranes by increasing concentrations of BE 2254 (○) and phentolamine (△). For details of experimental conditions, see Methods and Materials. The points are the mean of triplicate determinations from one representative experiment. (B) Displacement of [^{125}I]-BE 2254 bound to dog cerebral cortex membranes by increasing concentrations of BE 2254 (○) and phentolamine (△). For details of experimental conditions, see Methods and Materials. The points are the mean of triplicate determinations from one experiment.

As BE 2254 was the only drug which potently displaced [^{125}I]-BE 2254 from the non-α-adrenoceptor site, we used close structural analogues of the ligand to investigate further the stereochemical requirements for binding to this site. Table 3 shows the inhibition constants and Hill coefficients of BE 2254, BE 2631 and BE 2247 for binding to the second site and both sites together. The K_i values were determined from the IC_{50} of the displacement curves with K_D values of 0.481 ± 0.061 nM (mean ± S.E.M.)* for the second site alone and 0.230 ± 0.024 nM (mean ± S.E.M.)* for the combined sites, using the Cheng and Prusoff equation [10]. We were not able to evaluate the inhibition constants for the α_1 site alone, in this tissue, as we have not found a ligand which selectively blocks the non-α-adrenoceptor site. BE 2254 is the most potent of the three tetralone derivatives studied since the potency decreases with movement of the hydroxyl group to the tetralone ring (BE 2247) and is reduced even further by inclusion of an oxygen atom in the aliphatic chain (BE 2631).

* A. Adams and B. Jarrott, manuscript in preparation.

The finding that only close structural analogues of BE 2254 bind potently to the second site, and its confinement to guinea pig brain membranes, suggests that this site is not of fundamental pharmacological importance. Hence, we conclude that [^{125}I]-BE 2254 remains a reliable probe for the study of α_1-adrenoceptors provided BE 2254 and its congeners are not used to define the level of non-specific binding.

In summary, we found in guinea pig cerebral cortex membranes that [^{125}I]-BE 2254 was bound to both the α_1-adrenoceptor and an additional site from which it was not displaced by phentolamine. We have shown that the residual binding, which represents about 35% of total bound radioligand when labeling with 50 pM [^{125}I]-BE 2254, was reduced to about 5% in the presence of 1 μM BE 2254 and two analogues BE 2631 and BE 2247. By contrast,

Table 1. Differences between percent non-specific binding using phentolamine and BE 2254

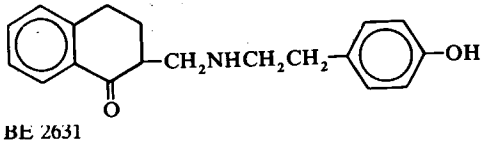
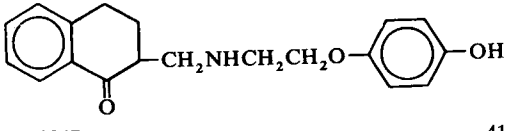
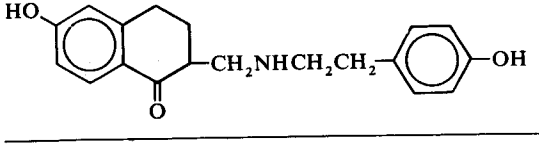
Animal	Tissue region	Differences between percent non-specific binding ± S.E.M.	N
Guinea pig	Cerebral cortex	31 ± 2	15
	Cerebellum	39 ± 3	4
	Pons	37 ± 4	4
	Medulla	33 ± 5	4
	Spinal cord	38 ± 4	4
	Hippocampus	35 ± 5	4
	Hypothalamus	24 ± 3	4
	Midbrain	26 ± 2	4
	Striatum	30 ± 5	4
	Seminal vesicle	4.0 ± 0.06	3
	Kidney	5*	
Rat	Cerebral cortex	6*	
Mouse	Cerebral cortex	5*	
Rabbit	Cerebral cortex	7*	
Dog	Cerebral cortex	0*	
Cat	Cerebral cortex	5*	
Chicken	Cerebral cortex	7*	
Man	Cerebral cortex	3*	
Fish	Brain	4*	

* Means of triplicate determinations from the tissue of one animal.

Table 2. Drugs which either do not displace (A) or partially displace (B) [^{125}I]-BE 2254 bound to the second site, at concentrations up to 1 μM

Drug	Receptor/class of drug
(A) Do not displace	
Phentolamine, prazosin, yohimbine, (−)noradrenaline, (−)adrenaline, clonidine, phenylephrine, 44/549	α-Adrenoceptor
Timolol, propranolol, (−)noradrenaline, (−)adrenaline	β-Adrenoceptor
Mepyramine, diphenhydramine, astemizole, Tiotidine	Histamine H_1 H_2
Diazepam	Benzodiazepine
Baclofen	$GABA_B$
Spiperone, fluphenazine, *d*-butaclamol, *cis*(z)-Fluphenthixol	Dopamine
2-Chloroadenosine, 1-methylisoguanosine	Adenosine
Methohexitone	Barbiturate
Quinuclidinyl benzilate, hexamethonium	Acetylcholine
Spermine	Polyamine
Zimelidine	Monoamine reuptake inhibitor
Clomipramine, amitriptyline, desmethylimipramine	Tricyclic antidepressants/monoamine reuptake inhibitors
Verapamil, gallopamil	Calcium channel inhibitors
Nalorphine, naltrexone, (±)-*N*-allyl-normetazocine	Opiate
Methylaplysinopsin	Serotonin uptake inhibitor
(B) Partially displace	
WB 4101	α-Adrenoceptor
Naloxone, ketocyclazocine	Opiate
Methiothepine, xylamidine, serotonin, danitracin, pirenperone, cyproheptadine, metergoline, mianserin, methysergide	Serotonin

Table 3. Inhibition constants and Hill coefficients for ligands which displace [^{125}I]-BE 2254 bound to both sites in guinea pig cerebral cortex membranes

Drug	Both sites			Second site		
	K_i (nM) ± S.E.M.	Hill coefficient ± S.E.M.	N	K_i (nM) ± S.E.M.	Hill coefficient ± S.E.M.	N
BE 2254 	3.6 ± 0.8	0.68 ± 0.04	3	21 ± 2	0.78 ± 0.02	3
BE 2631 	31 ± 5	0.68 ± 0.08	3	420 ± 90	0.79 ± 0.07	3
BE 2247 	41 ± 5	0.87 ± 0.09	3	160 ± 30	0.7 ± 0.1	3

forty-eight ligands which interact with seventeen well characterized neurotransmitter receptors or transport processes failed to displace potently the phentolamine-resistant bound [^{125}I]-BE 2254. There was no difference in the proportion of [^{125}I]-BE 2254 bound to this second site in membranes prepared from nine different areas of the guinea pig brain. However, in guinea pig seminal vesicle and kidney, as well as cerebral cortex membranes from rat, mouse, rabbit, cat, dog, chicken, fish and man, the phentolamine-resistant binding was barely detectable. The pharmacological importance of this second site remains to be established. The above findings caution against the use of BE 2254 and its close structural analogues in defining non-specific binding of [^{125}I]-BE 2254.

Acknowledgements—This work was supported by a grant from the National Heart Foundation of Australia. We wish to thank the drug companies that provided the compounds listed in Tables 2 and 3 and Dr. L. P. G. Wakelin for valuable discussions and critical evaluation of the manuscript.

University of Melbourne
Clinical Pharmacology and Therapeutics Unit
Austin Hospital
Heidelberg, Victoria 3084
Australia

ADRIENNE ADAMS*
BEVYN JARROTT

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* Author to whom all correspondence should be addressed.