Binding properties of a novel calcium channel activating dihydropyridine in monolayer cultures of beating myocytes

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Binding characteristics of [^{3}H]BAY K 8644, a new class of pharmacologically potent compounds, the calcium channel activating dihydropyridines (DHP), were demonstrated in cultured myocardial cells. [^{3}H]BAY K 8644 exhibited reversible and saturable binding to myocytes, and specific binding was Ca^{2+}-dependent. The equilibrium dissociation constant, K_d, was 35.2 nM, and maximal binding capacity, B_{max}, was 1.07 pmol/mg protein. Binding of the ^{3}H-ligand was highly specific for various potently displacing DHP derivatives (either the calcium channel activating BAY K 8644, or the Ca^{2+} entry blockers of the nifedipine type) with inhibition constants (K_i values) in the nanomolar range. BAY K 8644, on the other hand, showed very low affinity to other receptors tested in brain and heart membranes. Displacement potency of BAY K 8644 correlated well with data of the functional pharmacology; e.g., the enhanced myocardial contractility. Results from competition studies using [^{3}H]BAY K 8644 and [^{3}H]nimodipine support the conclusion that both the channel activating and inhibiting DHP structures interact with the same specific receptor site that might be associated with the putative Ca^{2+}-channel.

Dihydropyridine *Ca^{2+}-channel activation* *[^{3}H]Nimodipine* *[^{3}H]BAY K 8644* *Radioligand binding* *Cultured myocardial cell*

1. INTRODUCTION

Calcium ions play a central role in myocardial contractility, and it is generally accepted that the intracellular levels of free Ca^{2+} trigger such important cell biological processes as the excitation-contraction coupling or the positive inotropy. Drugs that enhance Ca^{2+} influx, e.g., adrenaline [1] or theophylline (see [2]), are known to exert cardiostimulatory effects. On the other hand, the dihydropyridines, e.g., nifedipine, nimodipine, nisoldipine, reduce Ca^{2+} entry into the cell and have been termed 'calcium antagonists' [3]; they are of considerable potential in the therapy of hypertension and many cardiovascular disorders (see [4,5]).

In 1981, high affinity binding for dihydropyridines (DHPs) was identified for the first time in cardiac membranes [6], and subsequently confirmed also in other tissues [7–10]. Recently, the existence of a functional receptor site for the DHP structure has been reported with the Ca^{2+} entry blocker [^{3}H]nimodipine [11]. The present report documents binding characteristics of [^{3}H]BAY K 8644, a novel type of DHP that has been ascribed to exert Ca^{2+} channel activating effects [12], and further substantiates the regulatory significance of the DHP receptor in modulating transmembrane Ca^{2+} currents.

Abbreviations: BBS, balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; Hepes, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; BAY K 8644, methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate

The work is dedicated to Professor Dr G. Kuschinsky, Pharmakologisches Institut, Universität Mainz, Obere Zahlbacherstraße 67, D-6500 Mainz, on the occasion of his 80th birthday

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2. MATERIALS AND METHODS

[^{3}H]BAY K 8644 (87 Ci/mmol) and [^{3}H]nimodipine (150 Ci/mmol) were synthesized by New England Nuclear (Boston, MA) and their purity was monitored by thin-layer radiochromatography. The ligands were stored in the dark at −30°C under nitrogen gas to prevent radiolysis and oxidation.

Pregnant albino rats (280–350 g) were received 20 days after mating (Wistar, Lippische Versuchstierzucht, Extertal, FRG). They were killed by cervical dislocation and foetuses were removed and cleaned. Embryonic hearts were pooled in buffered saline solution (BSS) containing glucose (5.5 mM) and foetal calf serum (FCS, 10%), minced, and cell suspensions were prepared by a multiple-cycle trypsinization method [13–15]. After complete disaggregation, myocytes were maintained as monolayer cultures in Dulbecco's MEM containing FCS (20%), tryptose phosphate broth (TPB, 10%), Hepes (20 mM), glutamine (6.8 mM) and 8-bromocyclic-AMP (0.1 mM [16]) using an atmosphere of CO_2/air (1:19). After 72 h in culture myocardial cells were removed from tissue culture flasks by trypsinization (0.05%), washed with BSS and seeded onto 24-well tissue culture clusters of costar (Greiner, Nürtingen, FRG) at a density of 1.5×10^5 cells/well. After the 4-h period of attachment the medium was flicked off, and cell cultures were washed twice with cold NaCl (0.9%) containing 2 mM EGTA. Membrane fractions from rat brain cortices were prepared as in [11].

Binding assays were performed essentially as in [11] under strict sodium light. In a final volume of 0.25 ml, cells were incubated at 37°C in Tyrode solution containing 1 mM $CaCl_2$, the indicated concentration of radioligands and various additives; e.g., drugs as displacers. After the indicated time intervals, the reaction was terminated by rapidly aspirating the incubation fluid and washing the monolayers with 3 ml of ice-cold NaCl (0.9%) solution. Cellular radioactivity was then solubilized by trypsinization (0.05%, 10 min, 37°C), and measured by conventional liquid scintillation spectrophotometry. Protein was measured as in [17] with serum albumin as the standard. Data were calculated and plotted according to Scatchard, and displacement experiments were analyzed with computer programmes.

3. RESULTS AND DISCUSSION

[^{3}H]BAY K 8644 interacted with membrane fractions from heart and brain; however, specific binding in these preparations was low and ranged between 15–30%, only. Thus, intact myocardial cells in monolayer cultures were used for binding studies. As shown by radiochromatography [^{3}H]BAY K 8644 remained stable under all experimental conditions reported here. Specific binding to myocytes was linearly proportional to cell density up to 5×10^5 cells/well (approx. 100 μg of protein); thus, all binding studies were carried out in the linear range using 1.5×10^5 cells/well. Total and non-specific binding were corrected in each experimental value for the amount of radioactivity adherent to the plastic surface. Addition of 10 μM of BAY K 8644 to binding assays displaced 60–75% of the total [^{3}H]BAY K 8644 binding.

[^{3}H]BAY K 8644 bound reversibly and saturably to cultured myocardial cells, and sufficient specific binding for kinetic analyses was dependent on the extracellular Ca^{2+} concentration used. The specific binding of [^{3}H]BAY K 8644 to cultured myocytes reached steady-state values rapidly and showed maximal binding after about 2–3 min (fig.1). The time-course of association yielded a

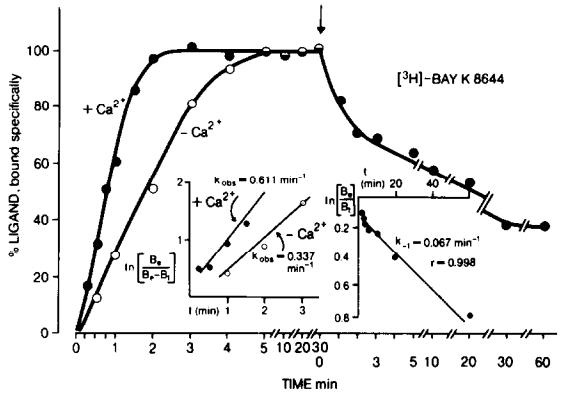

Fig. 1. Time course of [^{3}H]BAY K 8644 specific binding demonstrating reversibility in pulse-chase experiments. The figure shows a representative experiment carried out with cultured myocytes in the presence (1 mM) and absence of $CaCl_2$ in the binding assay. After linear transformation of the data from the association and dissociation (arrow: addition of 10 μM of BAY K 8644) reaction, kinetic constants (insets, $r > 0.966$) were estimated by the K_{obs} method [18].

rate constant K_{obs} [18] of 0.611 min^{-1} (r = 0.966) in the presence of 1 mM $CaCl_2$. In the absence of extracellular $CaCl_2$, binding of [^{3}H]BAY K 8644 was reduced by about 60–70% and association occurred considerably slower with rate constants of 0.337 min^{-1} (r = 0.997), substantiating the efficacy of Ca^{2+} in accelerating [^{3}H]BAY K 8644 binding to the receptor site in beating myocytes.

The specific binding of [^{3}H]BAY K 8644 was saturable and plateaus were achieved between 80 and 100 nM of radioligand (fig.2). Scatchard analyses of the saturation isotherms (fig.1, inset) revealed a straight line indicating the presence of a single binding site. The equilibrium dissociation constant K_d was 35.2 ± 6.53 nM ($r>0.93$; $n=4$), with a number of binding sites (density) B_{max}, equivalent to 1.07 .± 0.122 pmol/mg protein ($r>0.93$; $n=4$). Hill plots of the [^{3}H]BAY K 8644 saturation isotherm (not shown) gave slopes between 0.96 and 1.15 ($r>0.93$) indicating absence of cooperativity.

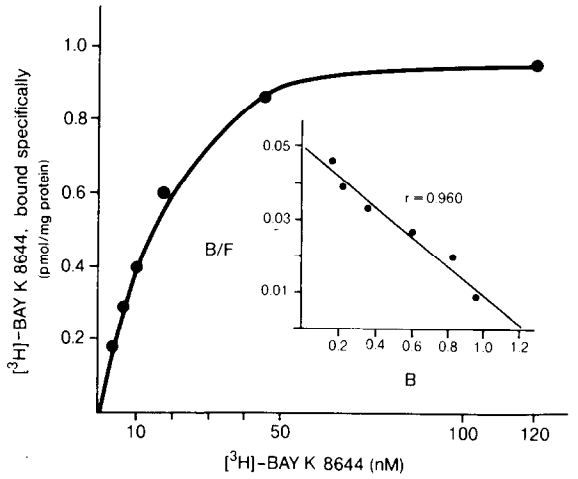

Fig. 2. Saturation isotherm of [^{3}H]BAY K 8644 binding to cultured myocytes. [^{3}H]BAY K 8644, 4.2–120.3 nM, was incubated at least in triplicate with or without excess unlabelled BAY K 8644. Specific binding (●, pmol/mg protein), defined as conditions under which non-specific binding remains linear, was calculated as the difference between total binding and that not displaced by excess (10 μM) BAY K 8644. The latter (non-specific binding) ranged between 25–40% (see text). Inset: Scatchard analysis of specific binding data (B, bound ligand; F, free ligand) revealed linearity and indicated one binding site with an equilibrium dissociation constant K_d = 24.3 nM (reciprocal of the slope value) and maximal binding (B_{max}) of 1.23 pmol/mg protein (r = 0.960, intercept on abscissa). The experiment was repeated several times with different myocyte preparations and yielded identical results ($r>0.95$).

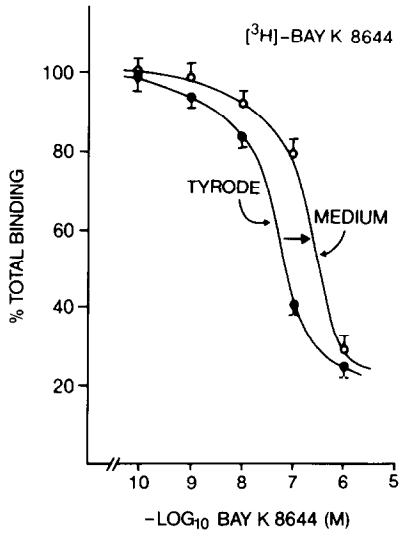

Fig. 3. Displacement experiments using [^{3}H]BAY K 8644 (<30 nM) and the unlabelled compound in salt solution (Tyrode) and serum containing tissue culture medium (Dulbecco's MEM). Total binding to cultured myocytes is plotted against $-\log_{10}$ of BAY K 8644 as displacer concentration (M). Total binding of [^{3}H]BAY K 8644 (set 100%) was reduced by 20–30% in the serum containing Dulbecco's MEM. Data are the means ± SE of 3 experiments using different myocyte cultures.

The pharmacological profile of [^{3}H]BAY K 8644 binding sites was evaluated by displacement studies under various experimental conditions. Fig. 3 shows an example of such a displacement experiment with unlabelled BAY K 8644 performed in Tyrode solution and Dulbecco's MEM. BAY K 8644 in salt solution displayed much higher affinity for the DHP binding site than in nutritional medium that generally reduced total [^{3}H]BAY K 8644 binding by about 20–30% suggesting interaction of the radioligand with additional components in the serum containing medium. Similarly, shifts in the competition curves, and concomitantly changes in the K_i or IC_{50} values toward higher affinities of 1–2 orders of magnitude were obtained by increasing the Ca^{2+} concentration from 0.45 mM up to 3.6 mM in the extracellular fluid.

Calcium channel inhibiting agents with DHP structure, e.g., nifedipine, nimodipine or nisol-

dipine, displaced [^{3}H]BAY K 8644 binding to myocytes with inhibitory characteristics (K_i, or IC_{50} values) close to those reported recently using [^{3}H]nimodipine as the radioligand [11].

The structural specificity of the calcium channel activating BAY K 8644 for the DHP receptor is apparent in the lack of affinity to various receptors measured in brain and heart membranes (table 1). Interaction of BAY K 8644, and certain other DHP derivatives with 10 different receptors – muscarinic cholinergic-, α_1-, α_2-, and β-adrenergic-, benzodiazepine-, dopamine-, γ-aminobutyric acid-, histamine-, opiate- and serotonin receptors – revealed low or even very low displacement activity.

Table 1

Interaction of the calcium channel activating dihydropyridine BAY K 8644 with various receptors measured in membrane preparations from brain and heart

IC_{50} values (M) of drugs on brain receptors	
Receptor and radioligand	BAY K 8644
Dopamine-2, [^{3}H]spiroperidol	$1.9 \pm 0.37 \times 10^{-4}$
ACh-muscarinic, [^{3}H]QNB	$>10^{-2}$
Alpha-1, [^{3}H]prazosin	$2.7 \pm 0.60 \times 10^{-5}$
Alpha-2, [^{3}H]clonidine	$4.9 \pm 2.5 \times 10^{-4}$
Beta, [^{3}H]dihydroalprenolol	$>10^{-2}$
Serotonin, [^{3}H]serotonin	$8.1 \pm 7.1 \times 10^{-4}$
Histamine, [^{3}H]cimetidine	$>10^{-2}$
GABA, [^{3}H]muscimol	$>10^{-2}$
Diazepam, [^{3}H]diazepam	$1.4 \pm 0.22 \times 10^{-4}$
Opiate μ, [^{3}H]naloxone	$6.0 \pm 0.54 \times 10^{-5}$

IC_{50} values (M) of drugs on heart receptors	
Receptor and radioligand	BAY K 8644
ACh-muscarinic, [^{3}H]QNB	$3.6 \pm 1.4 \times 10^{-4}$
Alpha-1, [^{3}H]prazosin	$1.8 \pm 0.32 \times 10^{-4}$
Beta, [^{3}H]dihydroalprenolol	$>10^{-2}$

Displacement experiments were performed with the radioligands (K_d concentrations, approximately) given in parentheses as in [19–28]. Values presented are the means ± SE of at least 4 experiments (in triplicates). Inhibition constants were calculated according to $K_i = IC_{50}/(1 + LC/K_d)$, in which LC is radioligand concentration, K_d its dissociation constant, and IC_{50} the concentration of compound causing 50% inhibition of ^{3}H-ligand specific binding

The interaction of the calcium channel activating BAY K 8644 with the Ca^{2+} entry blocker [^{3}H]nimodipine (fig.4) revealed displacement activity of K_i = 30 nM. The affinities of the potent channel inhibitors nimodipine and nifedipine, on the other hand, were just slightly more affine by about one order of magnitude (NIM, 1.4 nM; NIF, 7.0 nM [11]). Thus, data substantiate the high specificity of the DHP receptor for dihydropyridine derivatives of both the channel activating and inhibiting structures. Slopes of the Hill plot from competition studies with [^{3}H]nimodipine yielded identical results as found with [^{3}H]BAY K 8644 (see above). Interestingly, compounds known to increase intracellular Ca^{2+} concentration – a prerequisite for excitation-contraction coupling – e.g., strophantin, exhibited no affinity to the DHP receptor (fig.4).

Binding and displacement characteristics of [^{3}H]BAY K 8644 in cultured myocardial cells, e.g.,

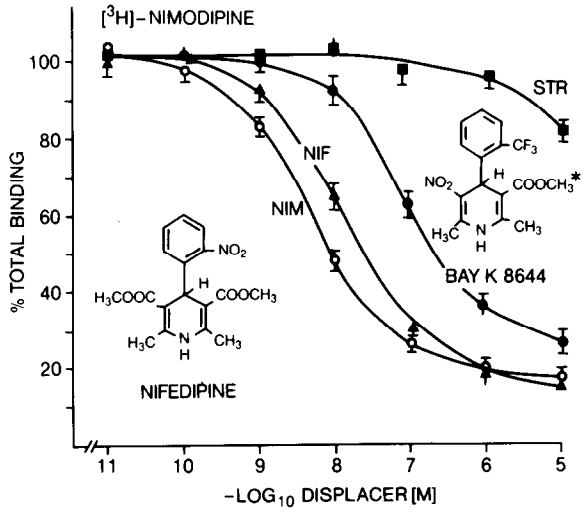

Fig. 4. Displacement experiments using [^{3}H]nimodipine (<1.5 nM) and nimodipine (NIM), nifedipine (NIF), BAY K 8644, and strophanthin. Total binding is plotted against $-\log_{10}$ of displacer concentration (M). The results demonstrate the high affinity of the binding sites for DHP derivatives. Data are the means ± SE of 3–5 experiments using at least 3 different protein preparations of rat brain cortices. Dihydropyridine formulae of both the potent Ca^{2+} entry blocker nifedipine (NIF) and the Ca^{2+} channel activating BAY K 8644 are given (inset), and demonstrate the close similarity of these chemical structures. Locus of tritiation of [^{3}H]BAY K 8644 is indicated by an asterisk.

occupancy of dihydropyridine receptors by one half at 30–40 nM, correlate well with results of the functional pharmacology. The positive inotropic action of BAY K 8644 in the perfused heart (Langendorff), and the calcium agonistic effect in the aortic strip preparation [12] were initiated by 1 nM and exerted maximal effect at about 80–100 nM of BAY K 8644.

In summary, the results of the binding characteristics reported here support the conclusion, that both the channel activating and inhibiting dihydropyridine structures interact with the same specific receptor site which seems to modulate the transmembrane Ca^{2+} fluxes by either inhibiting or enhancing the Ca^{2+} entry via putative calcium channels.

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