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Effect of Bay K 8644, a Calcium Channel Agonist, on Dog Cardiac Muscarinic Receptors

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Abstract □ To investigate further whether the effects of the dihydropyridine (DHP) drugs on calcium channels are related to those of these drugs on muscarinic receptors, the binding characteristics of the DHP calcium channel agonist, Bay K 8644, on muscarinic receptors and calcium channels were compared to those of the DHP calcium channel antagonists, nicardipine and nimodipine in the dog cardiac sarcolemma. Bay K 8644, nicardipine and nimodipine inhibited the specific [^{3}H]QNB binding with K_i values of 16.7 μM, 3.5 μM and 15.5 μM respectively. Saturation data of [^{3}H]QNB binding in the presence of these DHP drugs showed this inhibition to be competitive. Bay K 8644, like nicardipine and nimodipine, blocked the binding of [^{3}H]nitrendipine to the high affinity DHP binding sites, but atropine did not, indicating that the muscarinic receptors and the DHP binding sites on calcium channels are distinct. The K_i value of Bay K 8644 for the DHP binding sites was 4 nM. Nicardipine and nimodipine (K_i : 0.1-0.2 nM) were at least 20 times more potent than Bay K 8644 in inhibiting [^{3}H]nitrendipine binding. Thus, the muscarinic receptors were about 4000 times less sensitive than these high affinity DHP binding sites to Bay K 8644. These results suggest that the DHP calcium agonist Bay K 8644 binds directly to the muscarinic receptors but its interaction with the muscarinic receptors is not related to its binding to the DHP binding sites on calcium channels.

keywords □ *Dihydropyridine, calcium agonist, muscarinic receptor, cardiac sarcolemma*

The calcium antagonists such as verapamil, diltiazem and dihydropyridine (DHP) derivatives which inhibit calcium entry through potential-dependent channels activated by depolarizatioin in many excitable cell[1-3] are a very heterogeneous group of agents with the considerable structural diversity[4,5]. DHPs, the most potent drugs of these types, bind specifically and with high affinity to membrane preparations from cardiac, skeletal, and smooth muscle, and from brain[6-10]. Because of their selectivity to Ca^{2+} channels and their powerful vasomotor effect[11,12], these drugs have great potential for clinical and experimental use. However, at concentrations which are orders of magnitude higher than those required for inhibition of Ca^{2+} influx, the DHP calcium antagonists are known to inhibit the radioligand binding to rat brain muscarinic and α-adrenergic receptors[13].

A potent 1,4-dihydropyridine calcium agonist, Bay K 8644 (1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) acts directly on Ca^{2+} channels to increase Ca^{2+} influx and contractility of cardiac and smooth muscle even though its structure is very similar to the DHP calcium antagonists[14-16]. The binding of [^{3}H]Bay K 8644 to both high and low affinity sites was also identified in the rabbit ventricular microsome and guinea pig brain synaptosome[17]. The high affinity site for this drug has been considered to be a common high affinity binding site for DHP calcium antagonists[17,18]. Therefore, Bay K 8644 has been used effectively to investigate the mechanism of action of calcium antagonists[16,19-21].

The purpose of this study was to examine further

whether the calcium agonist Bay K 8644, like other potent DHP calcium antagonists, inhibits the radioligand binding to the muscarinic receptors and whether there are any relationship between the high and low affinity sites for this drug and the muscarinic receptors in the dog cardiac sarcolemma.

EXPERIMENTAL METHODS

Materials

[^{3}H]Quinuclidinyl benzilate (QNB, 67Ci/mmol) was purchased from Amersham. [^{3}H]Nitrendipine (85Ci/mmol) was from New England Nuclear. Adenosine triphosphate disodium salt (vanadium free) was from Boehringer Mannheim. L-Histidine, nicotinamide adenine dinucleotide (reduced form, NADH), ouabain octahydrate, phosphoenolpyruvate tricyclohexylamine salt, pyruvate kinase/lactic dehydrogenase (suspension) and tris (hydroxymethyl) aminomethane were obtained from Sigma Chemical Co. Dihydropyridine drugs were kindly supplied by the following companies: Yamamouchi Chemical Co. (nicardipine); Miles Laboratories (nimodipine); Bayer AG (Bay K 8644). All other reagents were of reagent grade.

Preparation of cardiac sarcolemma

Cardiac sarcolemma was prepared by the method of Lee *et al.*[22] Heart was removed from dog and blotted. The atria, connective tissue and major vessels were removed. After weighing ventricle, the tissue was minced with scissors and homogenized twice in 5 volumes of Medium A which contained 10 mM $NaHCO_3$ and 5 mM NaN_3, pH 7.0, with a Polytron (PT-20) at one-half maximal speed for 15 seconds. Samples of the homogenate were taken and analysed for enzyme activities. The homogenate was centrifuged for 20 min at 8,700×g and the pellet was resuspended in 6 volumes of Medium B (10 mM Tris. Cl, pH 7.4) using 4 passes of a 250 m*l* glass-teflon motor driven homogenizer (80% of 140 volts). Microsomes were prepared by centrifugation. Cardiac membranes highly enriched in sarcolemma were then isolated by discontinuous sucrose gradient centrifugation of the microsomal suspension and the final sarcolemma fraction was suspended in a small volume of Medium B using a hand driven glass-teflon homogenizer to give a final protein concentration of 1.5 to 2 mg/m*l*.

Protein was measured by the method of Lowry *et al.*[23] using bovine serum albumin as a standard. The average yield of sarcolemma membranes isolated from 1g of ventricular tissue was 80 μg.

Measurment of ATPase activity

Total and Na^+, K^+-ATPase activities of sarcolemma fraction were measured using a linked-enzyme spectrophotometric assay[24] in a reaction medium containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 0.4 mM NADH, 1 mM phosphoenolpyruvate, 14 units pyruvate kinase plus 20 units lactate dehydrogenase (suspension) and 25 mM L-histidine, pH 7.4, in a total volume of 2.5 m*l*. After equilibration of the medium at 37℃, 10 μg (protein) of sarcolemma was added to the medium to initiate the ATPase reaction. Enzyme activity was determined by continuous monitoring of the decrease in absorbance at 340 nm due to oxidation of NADH. In the absence of inhibitors, this determination is a measure of total ATPase activity. Specific Na^+, K^+-ATPase activity was determined in the same cuvette by measuring the difference between total ATPase activity and activity remaining 30 minutes after addition of 1 mM ouabain (final).

Muscarinic receptor binding assay

[^{3}H]Quinuclidinyl benzilate (QNB) binding to dog cardiac sarcolemma was run in duplicate at 37℃ in medium containing 50 mM Tris·Cl (pH 7.4), 10 mM $MgCl_2$, 20 μg of sarcolemma protein and 50 pM [^{3}H]QNB with or without DHP drugs in a total volume of 5 m*l*. Parallel experiments additionally contained 10^{-6} M atropine in the same medium as above. Incubation mixtures were incubated for 60 minutes and then filtered through GF/B glass fiber filters. The filters were rapidly washed four times with 5 m*l* of ice-cold buffer and placed in scintillation vials containing 8 m*l* of scintillation fluid for count. Specific [^{3}H]QNB binding was defined as the difference between bindings in the presence and the absence of 10^{-6} M atropine.

For Scatchard analysis of [^{3}H]QNB binding, sarcolemma were incubated for 60 min at 37℃ in the same medium as described above and various concentrations of [^{3}H]QNB. The QNB binding site concentration (B_{max}) and affinity (K_D) were estimated from Scatchard plots by linear regression analysis.

Table I. ATPase activity of sarcolemmal fraction prepared from dog ventricle

	ATPase activity(μmole Pi/mg/hr)
Total	119.6± 8.5
Ouabain sensitive	104.3± 6.3
Basal	15.3± 2.3

ATPase activity was measured as described in Methods. Values are the means± SEM of three different preparations.

[^{3}H]Nitrendipine binding assay

Sarcolemma (20 μg) prepared from dog ventricles were incubated for 30 min at 30℃ in 0.1 m*l* medium containing 50 mM Tris·Cl (pH 7.4) and 0.6 nM [^{3}H] nitrendipine with or without various concentrations of unlabeled DHP drugs. The binding was stopped by addition of 5 m*l* of ice-cold, double distilled water and filtration on Whatmann GF/B glass fiber filters. The binding in the presence of 10^{-6} M unlabeled nimodipine was defined as non-specific.

All experiments using DHP drugs were carried out under sodium light in order to prevent photolysis of these compounds.

Calculation of dissociation constants (K_i) for unlabeled drugs

The dissociation constants (K_i) of unlabeled DHP drugs for its binding sites were calculated by the following equation:

$$K_i = \frac{IC_{50}}{1 + \frac{\text{radioligand concentration}}{K_D}}$$

Where IC_{50} is the concentration of unlabeled DHP drugs that caused a 50% inhibition of specific radiolabeled drug ([^{3}H]QNB or [^{3}H]nitrendipine) binding and K_D is the dissociation constant of radiolabeled durg for its receptors determined in separate Scatchard analysis.

RESULTS

Na^+, K^+-ATPase activity of cardiac sarcolemma preparation

The relative enrichment of sarcolemma in the sarcolemmal fraction was assayed using Na^+, K^+-ATPase as a membrane marker, which is the membrane enzyme primarily responsible for Na^+ and K^+ transport across the cell membrane. Sarcolemmal Na^+, K^+-ATPase activity was more than 25-fold higher than starting homogenate enzyme activity.

In addition to high Na^+, K^+-ATPase activity, about 13% of total ATPase activity was ouabain insensitive in the sarcolemma fraction (Table I). Although small amounts of non-sarcolemma such as mitochondria, sarcoplasmic reticulum and contractile protein were contaminated in this sarcolemma preparation, sarcolemma enrichment and the restriction of muscarinic receptor to the sarcolemmal membrane make meaningful assessment of binding data possible.

Table II. [^{3}H]QNB equilibrium binding to dog cardiac sarcolemma

	Radioactivity (cpm/20 μg)	[^{3}H]QNB bound (fmol/mg protein)	% from total
Total	2323.3± 327.5	3850.6± 745.6	—
Nonspecific	54.5± 8.7	89.6± 16.1	2.3
Specific	2268.9± 320.9	3761.0± 732.2	97.7

[^{3}H]QNB binding was carried out in the presence of 50 pM [^{3}H]QNB and sarcolemmal protein (20 μg) at 37℃ for 60 min in a final volume of 5 m*l*s as described in Methods. Values are the means± SEM of three different preparations.

[^{3}H]QNB binding to cardiac sarcolemma

Preliminary determinations of the time necessary to reach equilibrium at 50 pM [^{3}H]QNB indicated that the binding reaction had equilibrated by 60 min. When sarcolemmal protein (20 μg) was incubated for 60 min with 50 pM [^{3}H]QNB, total radioactivity was about 2300 cpm. This radioactivity was reduced to about 50 cpm by inclusion of 10^{-6} M atropine to the incubation mixtures (Table II). Accordingly, nonspecific binding under these conditions was less than 3% of total binding, demonstrating that sarcolemmal fraction isolated from dog ventricles in this study is a good preparation for [^{3}H]QNB binding assays.

Inhibition of the [^{3}H]QNB binding by Bay K 8644

The interactions of the DHP calcium channel agonist Bay K 8644 and antagonists nicardipine and nimodipine with the muscarinic receptors of dog ventricular sarcolemma were studied by means of competition experiments with [^{3}H]QNB (Fig. 1).

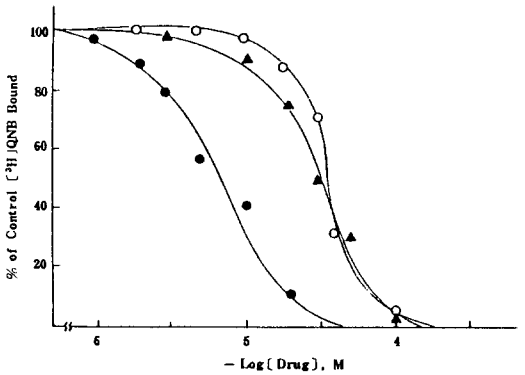

Fig. 1. Inhibition of specific [³H]QNB binding to dog cardiac sarcolemma by the dihydropyridine drugs. Dog cardiac sarcolemmal preparations (20 μg of protein) were incubated with 50 pM [³H]QNB in the presence of the indicated concentrations of Bay K 8644 (○), nicardipine (●) or nimodipine (▲) for 60 minutes and the specific [³H]QNB binding determined.Nonspecific binding was defined as that not displaceable by 1 μM atropine. Each point (means of three separate experiments) represents the percentage decrease of specific [³H]QNB bound at equilibrium displaced by increasing concentrations of unlabeled dihydropyridine drugs (as indicated on the abcissa). IC_{50} values from these plots were about 37 μM, 8 μM and 34 μM for Bay K 8644, nicardipine and nimodipine respectively.

Table III. K_i values of Bay K 8644, nicardipine and nimodipine for cardiac muscarinic receptors

Drug	K_i (μM)	Maximal inhibition(%)
Bay K 8644	16.7± 1.4	100
Nicardipine	3.5± 0.5	100
Nimodipine	15.5± 1.3	100

K_i values were calculated from IC_{50} values indicated in legends of Fig. 1 using the equations described in Methods. Values are the mean± SEM of three different preparation.

Bay K 8644, nicardipine and nimodipine inhibited the [³H]QNB binding to the cardiac muscarinic receptors in a concentration-dependent manner with the IC_{50} values of 37 μM, 8 μM and 34 μM, respectively. The maximal degree of inhibition by all of the three DHP drugs corresponded to 100 percent of the specific [³H]QNB binding, which indicates that the binding of the DHP drugs was saturable.

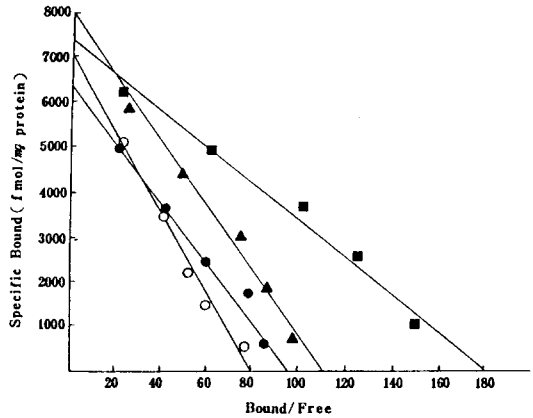

Fig. 2 Scatchard plot of specific [³H]QNB binding to dog cardiac sarcolemma. [³H]QNB binding at various [³H]QNB concentrations was measured as described in Methods, in the absence (■) of unlabeled dihydropyridine drug and in the presence of 40 μM Bay K 8644 (○), 5 μM nicardipine (●), or 30 μM nimodipine (▲). The added concentration of [³H]QNB was corrected to free (F) by subtraction of bound concentration. Intercepts on Y-axis are not statistically different. Each point denotes mean from three separate preparations.

Table III shows the K_i values of these drugs calculated from the IC_{50} value. Nicardipine having K_i value of approximately 3.5 μM was 5 times more potent than nimodipine and Bay K 8644 which had K_i values in the range of 15-17 μM in inhibiting [³H]QNB binding to the muscarinic receptors.

Effects of Bay K 8644 on the [³H]QNB binding parameters

To analyse the mechanism underlying this inhibition of [³H]QNB binding by Bay K 8644, the saturation binding assays for [³H]QNB in the presence and the absence of Bay K 8644 of a concentration close to the IC_{50} value were carried out. As shown in the Scatchard plot of Fig. 2, [³H]QNB binds to a single class of the muscarinic receptor sites in dog ventricular sarcolemma with the apparent dissociation constant (K_D) of approximately 40 pM. Bay K 8644 increased apparent K_D of the receptor for [³H]QNB but did not significantly alter the maximum number of [³H]QNB binding sites (Table IV). Similar results were obtained with the DHP calcium antagonists nicardipine and nimodi-

Table IV. Effects of Bay K 8644, nicardipine and nimodipine on the [^{3}H]QNB binding parameters

Drug	Conc. (μM)	K_D (pM)	B_{max} (fmol/mg)	nH
Control	–	41.2± 4.8	7617.0± 603.3	1.07± 0.03
Bay K 8644	5	52.0± 8.5	8338.1± 785.6	0.99± 0.02
	40	85.9± 8.1*	6896.8± 727.6	1.06± 0.07
Nicardipine	5	70.5± 7.0*	6337.2± 790.4	1.05± 0.01
Nimodipine	30	70.8± 8.7*	7848.8± 750.2	1.07± 0.01

K_D and B_{max} were estimated from Scatchard plots shown in Fig. 2 by linear regression analysis. Hill coefficient (nH) is the regression coefficient of the Hill plots. Values are the mean± SEM of three different preparations. *Significantly different ($p<0.01$) from corresponding value of control.

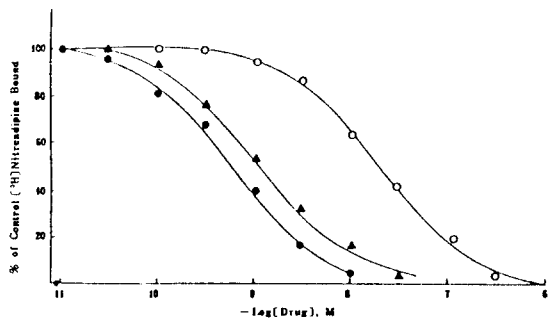

Fig. 3. Effect of Bay K 8644, nicardipine, or nimodipine on the specific [^{3}H]nitrendipine binding.
Dog cardiac sarcolemmal preparations (20 μg of protein) were incubated with 0.6 nM [^{3}H]nitrendipine in the presence of various concentrations of Bay K 8644 (○), nicardipine (●), or nimodipine (▲) for 30 min and the specific [^{3}H] nitrendipine binding determined as described in Methods. Nonspecific binding was defined as that not displaceable by 10^{-6} M nimodipine. Each point denotes mean from three separate preparations.

pine : the K_D increased about twofold with no significant change in the number of binding sits (Table IV). Thus, like the DHP calcium antagonists, calcium agonist Bay K 8644 at high concentrations appeared to act as a reversible competitive inhibitor of the [^{3}H]QNB binding to the muscarinic receptor in ventricular sarcolemma.

Effects of Bay K 8644 on [^{3}H]nitrendipine binding

High affinity binding sites for [^{3}H]nitrendipine have been reported in microsomal and sarcolemmal preparations from heart[8,25]. In this sarcolemmal preparation, [^{3}H]nitrendipine also bound to a single population of high affinity sites with the K_D of 0.14 nM. Bay K 8644, like nicardipine and nimodipine, inhibited specific [^{3}H]nitrendipine binding completely (Fig. 3), suggesting that the DHP calcium channel agonist and antagonists bind to a common high affinity DHP binding site. However, this specific [^{3}H]nitrendipine binding was insenstive to displacement by atropine at 10^{-6} M. The K_i values of these drugs calculated using the IC_{50} data from Fig. 3 and the K_D (0.14 nM) for [^{3}H]nitrendipine are summarized in Table V. Bay K 8644 with the K_i of approximately 4 nM at the dihydropyridine binding sites was about 20 and 40 times less potent than nicardipine and nimodipine respectively. Comparision of these K_i values with corresponding data from experiments with the muscarinic receptors shows that the muscarinic receptors were about 4,000, 32,000 and 74,000 times less sensitive than the [^{3}H]nitrendipine binding sites to Bay K 8644, nicardipine and nimodipine, respectively. These results indicates that the binding sites of Bay K 8644 blocking the muscarinic receptors are distinct sites from those of this drug associated with the calcium activation.

DISCUSSION

The results of this study demonstrate that a dihydropyridine calcium agonist Bay K 8644 at high concentrations competes for [^{3}H]QNB binding to ventricular muscarinic receptors. Although the dihydropyridine (DHP) calcium channel antagonists have received major pharmacologic and therapeutic attention because of their potent and selective ability to inhibit Ca^{2+} entry through potential-dependent Ca^{2+} channels in myocardium and vascular smooth muscle, it has been reported that these drugs at concentrations greatly in excess of those which pro-

Table V. K_i values of Bay K 8644, nicardipine and nimodipine for [^{3}H]nitrendipine binding sites

Drug	K_i (μM)
Bay K 8644	3.89± 0.54
Nicardipine	0.11± 0.03
Nimodipine	0.21± 0.04

The K_i values were calculated from the equation, $K_i = IC_{50}/(1+F/K_D)$. Inhibition of specific [^{3}H]nitrendipine binding by the DHP durgs was measured at [^{3}H] nitrendipine concentration(F) of 0.6 nM. The K_D value for [^{3}H]nitrendipine determined in separate Scatchard analysis was 0.14± 0.02 nM (n=3). Other legends are the same as described in Fig. 3 and Methods. Values are the means± SEM of three different preparations.

duce clinical effects inhibit radioligand binding to rat brain α_1-and α_2-adrenoceptors[13,26], muscarinic[13], serotonin[27], and histamine receptors[28].

The results of this study also demonstrate that nicardipine and nimodipine competitively inhibit [^{3}H]QNB binding to cardiac muscarinic receptors with the K_i values of 3.5 μM and 15.1 μM respectively. These K_i values are about 32,000 times for nicardipine and about 74,000 times for nimodipine greater than those of these two drugs for the high affinity [^{3}H]nitrendipine binding site. Consistent with these results the specifc [^{3}H]nitrendipine binding was insensitive to displacement by 1 μM of atropine. Thus, depending on the concentrations used, the DHP calcium antagonists are capable of interacting with the cardiac muscarinic receptors in addition to the high affinity dihydropyridine binding sites-calcium channels, but the muscarinic receptor blocking effect of these drugs is not related to the calcium entry blocking effect *via* the binding of these drugs to the high affinity sites.

Bay K 8644, a member of dihydropyridine drugs, was found to have a dose-dependent positive inotropic and chronotropic effect on the heart and to cause vasoconstriction[14,20,29], which are accompanied by an increased influx of Ca^{2+}[2,15,16,30] and blocked competitively by the DHP calcium antagonists including nicardipine and nifedipine[14,31,32], but insensitive to propranolol, phentolamine, atropine, indomethacin, chlorpheniramine and ketanserin[31]. Bay K 8644 was also known to be a competitive inhibitor of [^{3}H]nitrendipine binding at doses that enhance Ca^{2+} influx and produce a positive inotropic response[17,18,33]. Therefore, it is generally accepted that Bay K 8644 interacts directly with the DHP recognition sites.

Although Bay K 8644 and the DHP calcium antagonists bind to the same DHP binding domains in channel receptor complex, there are marked differences in the gating patterns. According to Hess *et al.*[16], the DHP calcium antagonist promotes a mode-0 pattern of Ca^{2+} channel gating in which the channels are not available for opening, whereas Bay K 8644 promotes a mode-2 gating marked by long openings. In this study 4 nM of Bay K 8644, which is concentration corresponded to the K_i value of this drug for the high affinity DHP binding sites, did not affect [^{3}H]QNB binding. These results indicate that the interaction of the DHP calcium agonist with the high affinity DHP binding sites in the agonist binding state (mode-2) is not associated with the muscarinic receptors.

Janis *et al.*[17] demonstrated that [^{3}H]Bay K 8644 binds to both high and low affinity sites with the apparent dissociation constant (K_D) of 2-3 nM and 50-100 nM respectively and that the high affinity binding of this calcium channel agonist is to calcium channels. However, the nature of the low affinity binding sites for Bay K 8444 has not been characterized yet. One electrophysiological study using ventricular myocytes has pointed out that while the low concentrations of calcium channel agonists which increase the inward calcium currents do not change channel structure, the high concentrations of these agonists cause a considerable change in channel structure[34]. Therefore, the low affinity binding sites detected by the high concentrations of [^{3}H]Bay K 8644 might be due to the structural change of calcium channels. We examined whether this low affinity binding state for Bay K 8644 is associated with the muscarinic receptors.

Bay K 8644 inhibited [^{3}H]QNB binding to muscarinic receptors in a competitive manner. The dissociation constant (−17 μM) of the muscarinic receptor for Bay K 8644 in cardiac sarcolemma is at least 4,000-fold larger than the apparent K_i value (4 nM) of this agonist for the high affinity DHP binding sites in the present study, and is also 150-300-fold larger than the K_i value (50-100 nM) for the low affinity binding sites estimated from saturation experiment of [^{3}H]Bay K 8644 binding[17], and is 150-500-fold larger than the ED_{50} values (30-100

nM) for increasing cardiac contractility[14] and calcium current[16,34]. In addition to these results, the facts that 5 μM of Bay K 8644, which is 50 fold larger than the K_i value for the low affinity binding sites, completely inhibited [^{3}H]nitrendipine binding but did not displace [^{3}H]QNB from the muscarinic receptors indicate that both calcium channel structures having high and low affinity for Bay K 8644 are not linked to muscarinic receptors. Our results also indicate that when DHP calcium agonist and antagonist at usual concentrations are used experimentally or therapeutically to understand the pharmacological action of these drugs on calcium channels, these drugs do not affect the muscarinic receptors.

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