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Activation of Dihydropyridine Receptors Differentially Regulates Temperature Responses in Rat

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PILLAI, N. P. AND D. H. ROSS. *Activation of dihydropyridine receptors differentially regulates temperature responses in rat.* PHARMACOL BIOCHEM BEHAV **25**(3) 549–554, 1986.—Rats receiving the dihydropyridine Ca^{++} agonist BAY K8644 (0.1–3 mg/kg SC) displayed increasing loss of body temperature. At the highest dose tested (3 mg/kg) rats exhibited decreased motor activity, ataxia, increased vocalization upon handling and increased auditory sensitivity. Nimodipine (1 mg/kg SC) produced antagonism of this response when used as pretreatment at 15 and 30 minutes. The phenylalkylamine, verapamil (5 mg/kg) and the benzothiazepine diltiazem (10 mg/kg) did not alter BAY K8644-induced hypothermia. None of the three Ca^{++} channel antagonists produced changes in body temperature at the antagonist doses used. BAY K8644 (3 mg/kg SC) produced stimulation of Ca^{++}/Mg^{++}ATPase activity by 31% in hypothalamus but not in cortex or cerebellum. This stimulation of enzyme activity was selectively prevented by nimodipine but not verapamil or diltiazem. No changes in enzyme activity were observed when Ca^{++} channel antagonists were used alone. These studies demonstrate that the Ca^{++} agonist BAY K8644 produces receptor mediated hypothermia which is dihydropyridine receptor dependent. Activation of Ca^{++}ATPase in the hypothalamus suggests that activation of dihydropyridine receptors may be coupled to Ca^{++} transport systems in this brain region and may reinforce the Ca^{++} set point theory of thermoregulation.

Dihydropyridine receptors BAY K8644 Thermoregulation Ca^{++}ATPase activity Nimodipine Verapamil Diltiazem

THE role of monovalent and divalent cations in thermoregulation is well documented [30]. Na^{+} and Ca^{++} ions are believed to play opposing roles in the ionic mechanism for temperature set point [31]. For these mechanisms to be maintained, critical communication between anterior and posterior hypothalamus is essential. While communication between the anterior hypothalamus-preoptic area (AH/POA) and posterior hypothalamus (PH) relies on selective activation of neurotransmitter pathways, these steps may in fact be secondary to initial changes in ionic ratio [29]. Thus, availability of Ca^{++} ions either by direct injection into the hypothalamus or by perfusion techniques results in a fall in body temperature in the conscious animal [30].

Recently selective Ca^{++} channel antagonists have become available, which have profound effects of Ca^{++} metabolism in a wide variety of cells [4, 9, 14, 17]. In addition to phenylalkylamines such as verapamil and D-600, newer agents such as diltiazem, a benzothiazepine, and the 1–4 dihydropyridines, such as nitrendipine, nifedipine and nimodipine, are becoming increasingly important in studying the pharmacological actions of Ca^{++} within cells. Radio labeled antagonists have allowed the additional study of receptors for these agents in vascular and intestinal smooth muscle, cardiac and brain tissue [8, 12, 16].

Recent studies have demonstrated that Ca^{++} channel antagonists may produce behavioral effects when administered alone or in combination with other CNS acting drugs. Thus, antagonists such as diltiazem and verapamil potentiated the effects of morphine on thermoregulation and analgesia [2], while nifedipine was successful in preventing sleep induction induced by flurazepam [26]. Nimodipine has been shown to potentiate hypothermia induced by ethanol [15], while Bolger *et al.* [3] have reported that the Ca^{++} agonist BAY K8644 (methyl-1, 4-dihyro-2, 6-dimethyl-3-nitro-4 (2-trifluoromethyl-phenyl)-pyridine-5-carboxylate) induced tremors and anxiogenic like activity in mice, an effect blocked by nifedipine.

Given the behavioral actions of the Ca^{++} channel agonists and antagonists, we were interested to determine whether or not Ca^{++} entry activators or antagonists could alter the thermoregulation given its history of Ca^{++} dependence. We report here the differential effect of dihydropyridine agents on thermoregulation and its possible biochemical correlates.

METHOD

Animals

Male, Sprague-Dawley rats (100–120 g) were used. The

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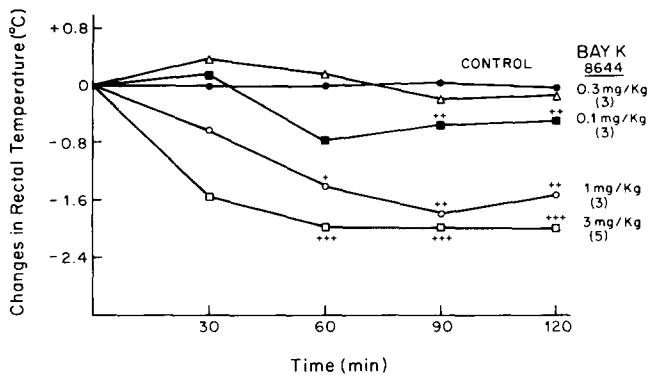

FIG. 1. Effect of different doses of BAY K8644 on the rectal temperature in rats. The figures in parentheses denote the number of experiments. $+p<0.05$, $++p<0.01$ and $+++p<0.001$ vs. control (Student's t-test).

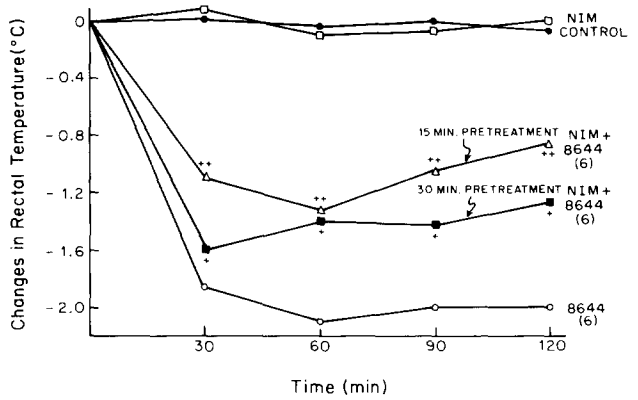

FIG. 2. Effect of nimodipine pretreatment (NIM, 1 mg/kg, SC, 15 or 30 min) on BAY K8644 (8644, 3 mg/kg, SC) induced hypothermia. The figures in parentheses indicate the number of experiments done. $+p<0.05$ and $++p<0.01$ vs. BAY K8644 value. All the values of BAY K8644 were significantly different ($p<0.001$) from control.

animals were housed in the animal room for a few days to acclimatize and had free access to food and water.

Body Temperature

The experiments were done at a room temperature of 24±1°C. The rectal temperature was taken with a telethermometer (Yellow Springs Instrument Co.). The thermometer probe was lubricated and inserted 6.5 cm into the rectum. The temperature reading was taken after a stabilization period of 1 min. During the temperature measurement, the rats were unrestrained and maximum care was taken to avoid any stress to the animals. The basal temperature was the average of two initial readings. Various drug treatments were given between 7:30 and 8:30 a.m. and the temperature readings were taken at 30, 60, 90 and 120 min after the injections. At the end of 120 min, the animals were sacrificed for estimating Ca^{++}ATPase activity in different brain regions.

Preparation of Brain Synaptosomal Fraction

Rats were sacrificed for decapitation and the brains were rapidly removed. The cortex, hypothalamus and cerebellum were separated from each brain. A 10% homogenate of the tissue was prepared in 0.3 M sucrose with a glass homogenizer. The homogenate was centrifuged at 1500 g for 10 min and the supernatant was collected. The pellet was resuspended in 0.3 M sucrose and recentrifuged. The supernatants were pooled and centrifuged at 9000 g for 20 min to yield the P_2 pellet. This P_2 fraction was lysed with a lysing buffer containing Tris (20 mM) and dithioerythritol (DTE, 0.5 mM), pH 8.5, for 1 hr. This suspension was centrifuged at 40,000 g for 20 min. The pellet was suspended in Tris-DTE and used for subsequent experiments.

Assay of Ca^{++}ATPase

The Ca^{++}ATPase activity was assayed according to a modification of the method of Ross and Cardenas [33]. Aliquots of membrane protein (100 μl) were incubated in 1.8 ml incubation buffer (pH 7.4) to contain in final 2.0 ml volume HEPES (20 mM), KCl (100 mM), $MgCl_2$ (150 μM), EGTA (100 μM), sodium azide (10 mM) and ouabain (1 mM) for 5 min at 37°C, with 2.49 μM of free calcium. Mg^{++} and Ca^{++} was omitted in blank assay tubes. The free calcium

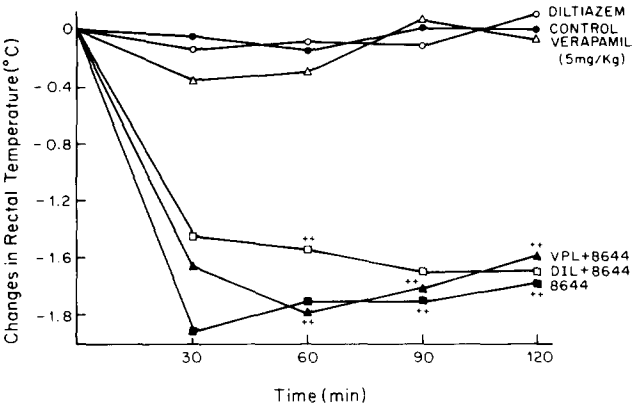

FIG. 3. Effect of diltiazem (DIL, 10 mg/kg) and verapamil (VPL, 5 mg/kg) on BAY K8644 (8644, 3 mg/kg) induced hypothermia. Diltiazem and verapamil were given (SC) 15 min before BAY K8644 (SC). $++p<0.001$ vs. control (n=3).

concentration was determined using the method of Bartfai [1]. After the preincubation, the reaction was initiated by the addition of 150 μM of ATP. After 2 minutes, the reaction was terminated by the addition of 200 μl of 6 N HCl and the assay tubes were immediately cooled in ice. Ca^{++}ATPase activity was measured using the colorimetric method for estimating the hydrolysis of ATP [21]. In brief, 200 μl of the assay mixture was added to 800 μl of malachite green reagent. After one minute, the reaction was quenched by the addition of 100 μl of 34% sodium citrate. The reaction was allowed to stabilize at room temperature for 30 min and the samples were read at 660 nm in a Beckman Model 35 spectrophotometer. Different concentrations of KH_2PO_4 served as standards to determine the Pi release. Ca^{++}ATPase activity is determined as the difference between Ca^{++} + Mg^{++} (total) and Mg^{++} (basal) ATPase. The enzyme activity is expressed as the Pi release nmole per min per mg protein. The protein concentration was determined using the method of Lowry *et al.* [22].

TABLE 1

EFFECT OF BAY K8644 ON REGIONAL BRAIN Ca^{++}ATPase ACTIVITY

	Ca^{++}ATPase nmoles/mg/min ± SEM		Mg^{++}ATPase nmoles/mg/min ± SEM	
	Control	Treated	Control	Treated
Hypothalamus (3)	33.6 ± 0.9	44.0 ± 1.8*	304.2 ± 8.6	323.7 ± 12.7
Cortex (3)	90.8 ± 5.6	81.1 ± 10.6	152.5 ± 1.9	158.1 ± 7.6
Cerebellum (3)	129.3 ± 10.4	110.1 ± 11.7	178.1 ± 9.4	167.8 ± 7.2

Each value denotes the mean ± SEM of 3 duplicate assays. *$p<0.005$ vs. control.

Drugs

The following drugs were used: BAY K8644 (Miles Pharmaceuticals), nimodipine (Miles Pharmaceuticals), verapamil HCl (Knoll AG) and diltiazem HCl (Sigma). All the drugs except BAY K8644 and nimodipine were dissolved in saline. BAY K8644 and nimodipine were dissolved in polyethylene glycol 400 and diluted with saline. The drugs were injected in a volume of 1 ml/kg and the doses refer to their salt forms.

RESULTS

Hypothermic Effects of BAY K8644 and Antagonism by Nimodipine

Administration of BAY K8644 (0.1–3.0 mg/kg SC) to rats produced a concentration-dependent decrease in body temperature (Fig. 1). No significant changes were seen at 0.1 and 0.3 at 30 min. However, significant depression of body temperature occurred following 1 and 3 mg/kg at 30, 60, 90 and 120 min. In addition, rats appeared ataxic with some slight rigidity at the higher doses. Gentle handling of treated but not control rats produced vocalization in greater than 75% of the animals. Preliminary experiments with earlier time points have demonstrated changes in temperature at 10 and 15 min of −0.7 and −0.9°C, respectively. Ca^{++}ATPase activity was affected to an even greater extent with a 12% and 24% increase.

In order to determine the best pretreatment schedule for Ca^{++} channel antagonists, nimodipine was administered alone and in combination with BAY K8644 (3 mg/kg) at different time intervals (Fig. 2). As seen in this experiment, nimodipine alone (1 mg/kg) was not significantly different from control. Nimodipine, when used as pretreatment for 15 or 30 min prior to BAY K8644, produced significant antagonism of the hypothermic response. Higher doses of nimodipine were not studied due to the observation of Isaacson *et al.* [15] who reported that while nimodipine (5 mg/kg) did not alter body temperature in mice, this dose potentiated the effects of ethanol on hypothermia.

Effects of Nondihydropyridine Ca^{++} Antagonists on BAY K8644-Induced Hypothermia

Diltiazem and verapamil are two nondihydropyridine Ca^{++} antagonists which can alter Ca^{++} transport in a variety of tissue [17]. Although they block Ca^{++} transport, they appear to interact with dihydropyridine binding sites in an allosteric fashion. Verapamil, while inhibiting Ca^{++}-dependent ^{3}H-dihydropyridine binding does so by acting through an allosteric mechanism [6]. Diltiazem is believed to bind to a site close to the DHP site to reduce the rate of dissociation of ligand receptor complex and enhancing ^{3}H-DHP binding. This enhancement is seen due to a decrease in K_D with no apparent change in B_{max} [37].

It was of interest to study both Ca^{++} antagonists in view of recent reports which demonstrate their behavioral involvement with other Ca^{++}-dependent systems [2]. Verapamil (5 mg/kg) and diltiazem (10 mg/kg SC) were evaluated for their ability to antagonize BAY K8644-induced hypothermia. Each compound was administered as a 15 min pretreatment prior to BAY K8644 injection. As seen in Fig. 3, neither verapamil nor diltiazem alone produced any significant effect on temperature at each time of evaluation. When verapamil or diltiazem was used as pretreatment to BAY K8644, no significant protection against BAY K8644-induced loss of temperature could be observed. Higher doses of verapamil and diltiazem were not tested. However, the doses of verapamil and diltiazem used here were effective in reversing the hyperthermic effects of morphine (Pillai and Ross, unpublished observation). Additionally, high concentrations of verapamil have been suggested to affect sodium channel activity and alpha adrenergic receptor binding. Therefore, we chose the doses based on Benedek and Szikszay's earlier studies [2].

Effects of BAY K8644 on Ca^{++} and Mg^{++}ATPase Activity in Brain Regions

The flux of Ca^{++} ions across membranes has been reported to be involved in the regulation of temperature set point within the hypothalamus [29,31]. Continued Ca^{++} efflux is necessary along with Na^+ accumulation in maintaining temperature set point. Thus, any change in the balance of Ca^{++} entry or Ca^{++} efflux could alter the set point for temperature regulation. Myers [30] has outlined the biochemical and physiological requirements for Ca^{++} and temperature and concludes that increasing [Ca^{++}] produces a reduction of temperature.

For these reasons, we have elected to study the Ca^{++}/Mg^{++}ATPase [33] activity and its response to Ca^{++} entry and antagonist drugs. We have chosen this enzyme for study since it represents a major regulator for cytosolic Ca^{++} levels necessary for synaptic transmission. As seen in Table 1, BAY K8644 (3 mg/kg) produces a 30% increase ($p<0.005$) in Ca^{++}ATPase activity from 33.6 to 44.0 nmoles/mg/min in

TABLE 2

EFFECT OF NIMODIPINE PRETREATMENT ON BAY K8644-INDUCED STIMULATION OF Ca^{++}ATPASE ACTIVITY IN RAT HYPOTHALAMUS

	Ca^{++}ATPase nmoles/mg/min Mean ± SEM	Mg^{++}ATPase nmoles/mg/min Mean ± SEM
Control	32.7 ± 1.5	301.0 ± 17.8
Nimodipine (1 mg/kg, SC) (6)		
30 min pretreatment	34.3 ± 4.2	324.3 ± 21.4
BAY K8644 (3 mg/kg, SC) (6)	43.9 ± 1.6*	304.4 ± 16.1
BAY K8644 + Nimodipine (1 mg/kg, SC) (6)		
15 min pretreatment	35.4 ± 2.1†	304.5 ± 16.8
30 min pretreatment	28.5 ± 2.0‡	275.8 ± 21.5

*$p<0.001$ vs. control; †$p<0.01$ and ‡$p<0.001$ vs. BAY K8644.

TABLE 3

EFFECTS OF Ca^{++} CHANNEL ANTAGONISTS ON Ca^{++}ATPASE IN RAT HYPOTHALAMUS

	Ca^{++}ATPase nmoles/mg/min Mean ± SEM	Mg^{++}ATPase nmoles/mg/min Mean ± SEM
Control (3)	34.9 ± 1.2	330.0 ± 13.0
Diltiazem (10 mg/kg) (5)	34.4 ± 4.6	318.6 ± 20.9
Verapamil (5 mg/kg) (5)	35.3 ± 1.6	297.9 ± 4.0
BAY K8644 (3 mg/kg) (4)	45.7 ± 2.5‡	337.9 ± 21.3
BAY K8644 + Verapamil (5 mg/kg) (5)	40.7 ± 2.1†	325.9 ± 31.0
BAY K8644 + Diltiazem (10 mg/kg) (5)	46.0 ± 2.9‡	342.0 ± 31.8

†$p<0.05$ and ‡$p<0.01$ vs. control (n=3).
The number in parentheses indicates the numbers of experiments.

the hypothalamus with no significant changes in cortex or cerebellum. Mg^{++}ATPase was not affected, as evidenced by enzyme activities for hypothalamus, cortex and cerebellum. While differences in Mg^{++}ATPase activity existed among these three brain regions, no drug-induced differences were seen.

Antagonism of BAY K8644 Stimulation of Ca^{++}ATPase

In order to evaluate degree of receptor specificity, the effect of nimodipine, verapamil and diltiazem on BAY K8644-induced stimulation of Ca^{++}ATPase was studied. As seen in Table 2, (1 mg/kg) nimodipine significantly inhibited BAY K8644-induced stimulation of Ca^{++}ATPase at both 15 and 30 min pretreatment. Nimodipine (1 mg/kg) did not alter Mg^{++}ATPase activity alone or in combination with BAY K8644, neither drug was able to reverse the activation of Ca^{++}ATPase activity seen following BAY K8644 (Table 3). Verapamil and diltiazem were used at concentrations which in themselves did not produce any change in body temperature.

DISCUSSION

The findings presented here confirm the initial observations by Myers and colleagues [29–31] that Ca^{++} ions may play a role in the hypothalamus in determining thermoregulation. Evidence for this lies in the following observations. BAY K8644, an analogue of nitrendipine, promotes Ca^{++} entry and functional activity in a number of systems [19,35]. The hypothermic response (Fig. 1) seen after BAY K8644 administration may be due to facilitation of Ca^{++} entry within certain regions of the hypothalamus. The biochemical nature of this response may also be seen by comparing changes in Ca^{++}ATPase in brain regions. BAY K8644 demonstrated brain region selectively by stimulating Ca^{++}ATPase within the hypothalamus (31%) but not cortex or cerebellum. This is of particular interest since there are significant numbers of high affinity dihydropyridine receptors in both cortex and hypothalamus as determined by both *in vivo* and *in vitro* assay [13, 25, 34]. Cerebellum was not found to have significant numbers of DHP receptors and has been included as an internal control in our study.

While the exact mechanism of enhancement of Ca^{++}ATPase activity is unknown, it may be due to enhanced Ca^{++} entry. Increased cytosolic Ca^{++} in the submicromolar range is known to activate the high affinity Ca^{++}/Mg^{++}ATPase in synaptic membranes [33]. Activation of enzyme activity even after washing and preparation of membranes may involve structural changes in the enzyme micro environment. Thus, activation of Ca^{++}-dependent synaptic membrane phospholipase A_2 may account for the prolonged increase in enzyme activity seen in the present studies [27]. *In vitro* $PPLA_2$ treatment of synaptic membranes greatly enhances Ca^{++}ATPase [11]. Ca^{++} channel antagonists could also alter other intracellular Ca^{++} buffers making more Ca^{++} available for activation of Ca^{++}ATPase. Matlib and Schwartz [24] have recently shown that diltiazem can inhibit Na^+/Ca^{++} exchange in brain mitochondria. Alternatively, BAY K8644 may act directly on the dihydropyridine receptor to increase the activity of Ca^{++}ATPase. Recent studies have demonstrated that Ca^{++} channel antagonists can inhibit calmodulin-dependent enzymes [7, 14, 20, 32]. One recent report suggests that Ca^{++} channel antagonists may not interact with calmodulin but may interact with a separate calcium binding protein which may interact with calmodulin activity [23]. Since Ca^{++}ATPase is a calmodulin-dependent Ca^{++} binding protein [33], the possibility exists that dihydropyridine agonists may interact with the enzyme through a receptor mediated process. In support of this alternative, Movsesian and Adelstein [28] have shown BAY K8644 effects on Ca^{++} efflux mechanisms such as Ca^{++}ATPase and studies are currently underway to investigate this possibility. Alternatively, elevation of cyclic nucleotide levels and subsequent changes in cytosolic Ca^{++} may also underlie events associated with hypothermia [7, 14, 20, 32].

The hypothermic effect of BAY K8644 appears specific for dihydropyridines since hypothermia and subsequent enzyme activation were shown to be inhibited only by nimodipine (Fig. 2, Table 2) but not verapamil and diltiazem (Fig. 3, Table 3). This finding is intriguing since verapamil and diltiazem are known to antagonize the effect of BAY K8644 on stimulation ^{45}Ca-influx under depolarizating conditions [10]. Thus, they can antagonize the Ca^{++} mobilization effected by BAY K8644. The differences may be due to the fact that neuroblastoma cells have always shown a greater

sensitivity to Ca^{++} channel in cell culture and synaptosomes may account for the differences in *in vivo* sensitivity to non-dihydropyridine Ca^{++} channel antagonists. Verapamil, like nifedipine and diltiazem, is a potent Ca^{++} channel antagonist [16]. However, verapamil was shown to be ineffective in preventing BAY K8644 effects on rotored performance [3]. These findings underscore the interaction between dihydropyridine analogues and provide emphasis for receptor mediated antagonism to BAY K8644.

The present studies demonstrate a receptor specific brain region effect of the dihydropyridine agonist BAY K8644 in producing hypothermia. Subsequent activation of Ca^{++}ATPase but not Mg^{++}ATPase seen to parallel with hypothermia suggests that increased Ca^{++} entry may provide the stimulus for development of hypothermia with subsequent activation of Ca^{++}ATPase. These studies confirm earlier observations by Myers [30] that Ca^{++} ions play a critical role in hypothalamic temperature set point and may suggest a new therapeutic role for dihydropyridines in modulating thermoregulation. The behavioral actions of BAY K8644 in producing hypothermia add to the growing list of reports that this compound and other dihydropyridines may possess significant behavioral properties through their actions on the central nervous system receptors [18].

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