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STIMULATION OF Na^+,K^+-ATPase OF ISOLATED SMOOTH MUSCLE MEMBRANES BY THE Ca^{2+} CHANNEL INHIBITORS, NIMODIPINE AND NITRENDIPINE

MARY PAN and RONALD A. JANIS*
Miles Institute for Preclinical Pharmacology, New Haven, CT 06509, U.S.A.

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Abstract. Nimodipine (0.015 to 1.5 μM) increased Na^+, K^+-ATPase activity by 70–120% in isolated smooth muscle membranes. At 0.015 μM, nitrendipine, but not nifedipine, verapamil or diltiazem, also activated this enzyme. Nimodipine stimulated this Na^+, K^+ATPase three times more than nitrendipine at 15 nM. Marked stimulation of Na^+,K^+-ATPase by nimopidine was seen in membranes from rat and guinea pig aorta and rat vas deferens, but not in membranes from guinea pig heart or brain. Although it is not known whether these results are applicable to intact cells, the results are consistent with the hypothesis that vasodilation produced by nimodipine and nitrendipine may be due not only to inhibition of Ca^{2+} entry but also to the stimulation of the Na^+ pump.

Ca^{2+} antagonists such as verapamil, diltiazem and the dihydropyridines are believed to cause vasodilation by blockade of Ca^{2+} entry through Ca^{2+} channels [1–3], but certain other mechanisms, such as ion pump stimulation, have not been excluded [4–7]. The greater selectivity for smooth muscle of nimodipine and nitrendipine relative to nifedipine and many phenyl-substituted nifedipine analogs [7] might be due to an action at such an additional site of action. Stimulation of the Na^+ pump in vascular smooth muscle could produce vasodilation by two mechanisms. First, increased electrogenic Na^+ extrusion may cause transient hyperpolarization and, thereby, a transient decrease in the tendency for depolarization-induced contraction [8, 9]. Second, decreased intracellular Na^+ activity caused by pump stimulation may produce a steady-state decrease in intracellular Ca^{2+} and, therefore, maintained vasodilation [10]. Activation of the Na^+ pump is a particularly attractive mechanism for vasodilation because of the suggestion that decreased Na^+ pump activity may be involved in the etiology of some types of hypertension [11, 12]. Thus, a drug acting at the Na^+ pump might be acting at the precise site of the derangement that causes some vascular diseases.

One method of estimating Na^+ pump stimulation is to use radioactive cations to examine the rates of ion transport. This measurement is difficult because elevated cation movements in the intact cell would occur only in the brief transient state. In the intact cell, decreased intracellular Na^+ levels would rapidly oppose a maintained increase in the rate of Na^+ extrusion, and the Na^+ pump activity would rapidly return to normal. Reduced steady-state levels of intracellular Na^+ would lead to increased Na^+/Ca^{2+} exchange and, consequently, to reduced intracellular Ca^{2+}. In isolated membranes, the compensatory feedback mechanism would not operate, so that stimulation of Na^+,K^+-ATPase activity in this case may be easier to detect than the stimulation of this activity in the intact cell. We observed that nimodipine and nitrendipine, but not nifedipine or diltiazem, produced marked Na^+,K^+-ATPase stimulation in membranes from smooth muscle but not in those from heart or brain. A preliminary report of these results was published [13].

* Send all correspondence to: Dr. R. A. Janis, Miles Institute for Preclinical Pharmacology, P.O. Box 1956, New Haven, CT 06509.

METHODS

Preparation of aortic microsomes. Male Wistar rats (220–260 g) from Royalhart Laboratory Animals (New Hampton, NY) were killed by a blow to the head, and aortae or vas deferens were rapidly removed and placed in ice-cold homogenizing medium containing 0.25 M sucrose, 1 mM Na_2EDTA and 20 mM Tris-HCl (pH 7.4 at 22°). All preparatory procedures were carried out at 0–5°. Tissues were carefully cleaned of adhering fat, and 1 g of tissue (from twelve to eighteen rats) was then placed in 15 ml of the above homogenizing medium containing 0.05% (w/v) sodium deoxycholate. The tissues were finely minced with scissors and homogenized four times at 10-sec intervals (separated by 10-sec cooling periods) at a setting of 5 with a Polytron (Brinkmann Instruments, Westbury, NY) equipped with a PT 10 probe. The homogenate was then filtered through three layers of cheesecloth and centrifuged at 1300 *g* for 15 min (Beckman SW 40 Ti rotor). The top layer of fat was carefully removed and discarded, and the supernatant fraction was centrifuged in the same rotor at 10,000 *g* for 20 min. The resultant supernatant fraction was centrifuged at 105,000 *g* for 50 min (Beckman 50.2 Ti rotor). Pellets were resuspended in homogenizing medium without deoxycholate.

Microsomes from guinea pig brain and heart were prepared in a similar manner. For guinea pig aortic microsomes, 0.5 g of tissue was put in 14 ml of homogenizing solution with 0.05% (w/v) deoxycholate. Pellets were resuspended in a final protein concentration of 0.2 mg/ml.

Na^+,K^+-*ATPase (EC* 3.6.1.3) *assay*. All experiments were carried out on freshly prepared membranes. The dihydropyridines were dissolved in polyethylene glycol 400 for a 3 mM stock solution and then diluted just before use in water (37°) to give a final concentration of 30 μM. All drug solutions were freshly made for each experiment. Because of possible precipitation of nimodipine and nitrendipine, diluted drug solutions were checked spectrophotometrically. Total ATPase activity was measured by incubating the microsomes in a shaking water bath at 37° in reaction media containing (final mM concentrations): Tris–HCl, 32; Na_2EDTA, 0.2; $MgCl_2$, 3; NaCl, 100; KCl, 10; pH 7.4 (22°). Prolonged preincubation caused decreased activity in the absence of ATP. Therefore, the preincubation time for each sample was exactly 8.0 min. Triplicate or duplicate measurements were carried out. The nimodipine solution was added to the incubation mixture during the preincubation period. Reactions were initiated by the addition of Tris-ATP to give a final concentration of 3 mM. Basal Mg^{2+}-ATPase activity was assayed in the absence of Na^+ and K^+, or in the presence of 7.5 mM ouabain (for rat aorta). Unless noted otherwise, the reaction mixture (1.0 ml) was incubated for 30 min. Na^+,K^+-ATPase was defined as the difference between total ATPase and Mg^{2+}-ATPase activities. The concentration of protein was adjusted to restrict the extent of ATP hydrolysis to 15% or less of the total amount present. Under the conditions of the assay, the rate of inorganic phosphate formation was proportional to both the incubation time and protein concentration. ATPase activity was terminated by addition of 0.5 ml sodium lauryl sulfate (SDS)–EDTA solution [SDS, 10%, w/v, and 90 mM Na_2EDTA]. Inorganic phosphate was assayed by the method of Hegyvary *et al.* [14]. The small absorbance changes measured necessitated the use of a highly sensitive and stable spectrophotometer; a Beckman DU-8 spectrophotometer was used for this study. The specific activity of ATPase was expressed as micromoles of inorganic phosphate released from ATPase per milligram of protein per hour. Assay tubes were prepared at 4°. All procedures involving 1,4-dihydropyridines were conducted under sodium vapor light. Protein concentration was determined by Peterson's [15] modification of the Lowry method.

Na^+,K^+-ATPase was also assayed using γ-labeled [^{32}P]ATP [16, 17]. Assay conditions were similar to those previously mentioned except that 300,000 cpm γ-[^{32}P]ATP was added to each test tube. Total volume was also 1 ml; the enzyme reaction was stopped with 2 ml of an ice-cold 7.5% (w/v) trichloroacetic acid (TCA) solution containing 5 mM KH_2PO_4. The samples were kept on ice for 10–15 min before 1.5 ml of TCA solution containing acid-washed charcoal [5% (w/v) TCA and 20% (w/v) charcoal] was added to each test tube, mixed, cooled on ice for another 15 min, and then centrifuged at 5000 rpm for 30 min. A 0.5-ml aliquot of supernatant fraction was removed and counted in an Aquasol-2 (New England Nuclear)–water mixture that formed a stable gel.

Chemicals. The following chemicals were used: Tris-ATP, sodium deoxycholate, Tris, and ouabain (Sigma Chemical Co., St. Louis, MO); Na_2EDTA (Eastern Kodak Co., Rochester, NY); SDS (sodium lauryl sulfate, Gallard-Schlesinger Chemical, Carle Place, NY); Sucrose Ultra Pure (Schwarz/Mann, Spring Valley, NY); and TCA (Fisher Scientific, Fair Lawn, NJ). All other reagents were of the highest grade commercially available. Nimodipine (batch number 491326), nitrendipine (batch number 576935) and nifedipine (batch number 659751) were from Bayer AG; verapamil was from Knoll A.G., and diltiazem from Marion Laboratories.

RESULTS

Nimodipine, nitrendipine and nifedipine were dissolved in polyethylene glycol 400 (PEG) so that the final concentration of PEG was 0.05% (w/v) or less in the experiments reported in this paper. Concentrations of PEG between 1 and 10% were found to inhibit enzyme activity up to 70% in a dose-dependent manner (N = 2). Solvent concentrations of 0.1% did not have a significant effect on enzyme activity (1 ± 0.02% inhibition, N = 7).

The rate of inorganic phosphate release was found to be linear with time, both in the absence and in the presence of nimodipine (data not shown). The magnitude of the Na^+,K^+-ATPase, as defined by omitting Na^+ and K^+, was equal to ouabain-sensitive ATPase defined by 7.5 mM ouabain (Fig. 1). There was also no difference between control Mg^{2+}-ATPase and ouabain-insensitive ATPase. The values obtained for both Mg^{2+}-ATPase and Na^+,K^+ ATPase are similar to those obtained by others for membranes from smooth muscle ([18–22]; see Ref. 8 for review). In the presence of nimodipine (1.5 μM), Na^+,K^+-ATPase activity was increased significantly, and this increase was inhibited completely by 7.5 mM ouabain. This extremely high concentration

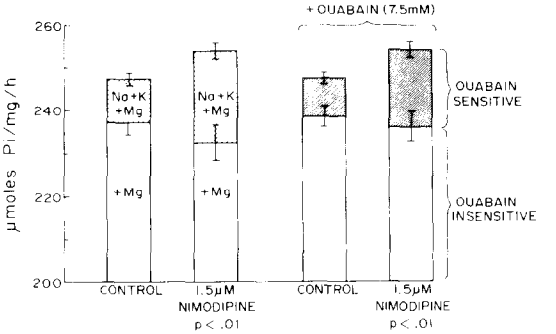

Fig. 1. Effect of nimodipine on Na^+,K^+-ATPase of rat aortic microsomes. Na^+,K^+-ATPase activities were assayed under two conditions: (1) in the presence and absence of 100 mM Na^+ and 10 mM K^+, and (2) in the presence of 100 mM Na^+ and 10 mM K^+ and the presence and absence of 7.5 mM ouabain. The results shown are the mean ± S.E. from four membrane preparations.

was needed to inhibit all of the Na^+,K^+-stimulated ATPase in these rat preparations. Nimodipine (1.5 μM) stimulated Na^+,K^+-ATPase but did not affect significantly Mg^{2+}-ATPase of rat aortic microsomes. The results shown in Fig. 1 are data from four membrane preparations, each prepared from eighteen to twenty-four rats. Since Na^+-K^+-ATPase is ouabain-sensitive ATPase, all other experiments were carried out in the presence and absence of 100 mM Na^+ and 10 mM K^+. A summary of thirty experiments on the effect of 1.5 μM nimodipine confirmed that nimodipine stimulated Na^+,K^+-ATPase of rat aortic microsomes (Table 1).

A number of control experiments showed that nimodipine failed to stimulate the enzyme activity in the presence of either Na^+, or K^+ alone. When choline was substituted for Na^+, no stimulation of the control enzyme activity was observed (data not shown). The results of these experiments indicate that the stimulation of ATPase activity by Na^+ and K^+ in our studies was not due to the associated change in ionic strength.

To determine whether the stimulatory effect of nimodipine would be seen by a method which does not measure other phosphatases, Na^+,K^+-ATPase was assayed using γ-labeled [^{32}P]ATP. There was good agreement between this method and the spectrophotometric method (Table 1) for the percent stimulation of Na^+,K^+-ATPase by 1.5 μM nimodipine. In rat aortic microsomes, the ^{32}P method gave a percent stimulation of 148 ± 29% (N = 4). A similar agreement between the two methods was obtained using guinea pig aortic microsomes. These results measuring ^{32}P release rule out the possibility that the apparent stimulation of Na^+,K^+-ATPase was due to an increased activity of other types of phosphatases. The data with guinea pig aorta also indicated that nimodipine stimulated Na^+,K^+-ATPase of vascular smooth muscle membranes in a species that is more sensitive than the rat to ouabain.

The stimulation of Na^+,K^+-ATPase was found to be concentration dependent (Fig. 2). Due to the limitation of drug solubility, the concentration of nimodipine, nitrendipine and nifedipine could not be further increased without an undesirable solvent-induced inhibition of enzyme activity.

The Na^+,K^+-ATPase activities reported above were obtained from deoxycholate-treated microsomes; non-treated microsomes were also tested to determine if the effect of nimodipine is present in the absence of detergent. Microsomes from rat aorta, rat vas deferens, and guinea pig heart and brain were examined. Na^+,K^+-ATPase activity in non-treated microsomes was 3- to 4-fold lower than the deoxycholate-treated microsomes, but the percent stimulation of Na^+,K^+-ATPase by 1.5 μM nimodipine was the same as in deoxycholate-treated membranes. Thus, the stimulatory effect of nimodipine is not dependent on the presence of detergent, although in rat aortic membranes the Na^+,K^+-ATPase activity was nearly undetectable in non-detergent-treated membranes.

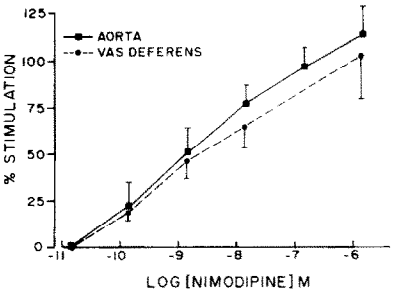

Fig. 2. Nimodipine stimulation of ouabain-sensitive Na^+,K^+-ATPase of rat microsomes and aorta and vas deferens. Na^+,K^+-ATPase activities were measured as the difference between total ATPase and Mg^{2+}-ATPase. Total ATPase and Mg^{2+}-ATPase were measured in the presence and absence of 100 mM Na^+ and 10 mM K^+. The results shown are from eight and six membrane preparations for aortic and vas deferens, respectively.

The potencies of the calcium antagonists nitrendipine, diltiazem and nifedipine on Na^+,K^+-ATPase from rat aortic microsomes were compared. Of these, nimodipine was the most effective and potent stimulant of Na^+,K^+-ATPase; nitrendipine was only about half as potent as nimodipine (Fig. 3). Verapamil did not stimulate this enzyme activity at concentrations of less than 100 μM (data not shown). A comparison of the effect of 1.5 μM nimodipine on Na^+,K^+-ATPase from various tissues is summarized in Table 2. Nimodipine stimulated Na^+,K^+-ATPase of smooth muscle microsomes from rat aorta, rat vas deferens and guinea pig aorta. The results obtained with guinea pig aortic micosomes were independently confirmed by Joseph F. Hoffman. Ventricular

Table 1. Effect of 1.5 μM nimodipine on Na^+,K^+-ATPase and Mg^{2+}-ATPase of rat aortic microsomes*

	Enzyme activities (μmoles · mg^{-1} · hr^{-1})		
	Total ATPase	Mg^{2+}-ATPase	Na^+,K^+-ATPase
Control	210 ± 5.4	191 ± 6.0	19.5 ± 2.3
Nimodipine	227 ± 6.1	187 ± 6.1	40.0 ± 4.2
Stimulation (%)	8.2 ± 0.01	−1.8 ± 0.01	123 ± 10

* Total ATPase and Mg^{2+}-ATPase were assayed in the presence and absence of 100 mM Na^+ and 10 mM K^+. Na^+,K^+-ATPase was measured as the difference between total ATPase and Mg^{2+}-ATPase. The percent stimulation is calculated on the percent change for the individual experiments, not for the pooled data. Data are expressed as means ± S.E.M. of thirty experiments.

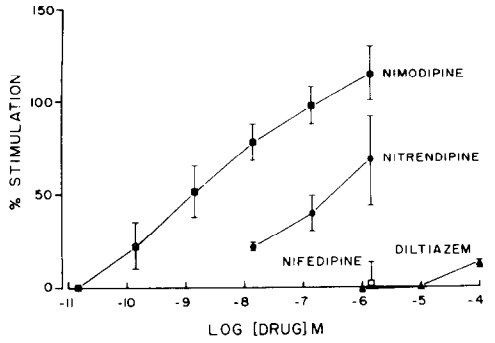

Fig. 3. Comparison of the effects of nimodipine, nitrendipine, nifedipine, and diltiazem on Na^+,K^+-ATPase of rat aortic microsomes. Data for nimodipine are from Fig. 2. The number of membrane preparations studied was three for diltiazem, four for nitrendipine and nifedipine and eight for nimodipine.

membranes from swine and guinea pig exhibited only a very small stimulation (17–21%), and those from guinea pig brain exhibited no stimulation (N = 3). The enzyme from brain was insensitive to nimodipine independent of whether the membranes were treated with 0.01% or 0.05% deoxycholate, or were untreated.

DISCUSSION

These results indicate that nimodipine, at concentrations of 1.5 nM to 1.5 μM, stimulated the Na^+,K^+-ATPase of smooth muscle membranes, but not of membranes from the heart or brain. Verapamil, diltiazem and nifedipine produced little or no activation of this enzyme at concentrations normally used to produce smooth muscle relaxation. Thus, both drug specificity and tissue selectivity were observed in the stimulation of smooth muscle Na^+, K^+-ATPase.

The concentration of nimodipine required to significantly stimulate this enzyme (1.5 nM) was the same as that at which nimodipine and related dihydropyridines relax depolarization-induced contraction of some smooth muscles [1–7, 23]. However, the site at which Na^+,K^+-ATPase was stimulated is unlikely to be the same as the high affinity binding site for [^{3}H]-nimodipine [24] because binding is concentration-dependent over a lower and much smaller range (10–800 pM). The nearly linear stimulation of Na^+,K^+-ATPase by nimodipine over several log units (Fig. 3) indicates that the mechanism of stimulation was not mass-action association of nimodipine with a single population of non-interacting sites. Furthermore, density of Na^+,K^+-ATPase molecules in vascular smooth membranes was 1000-fold greater than the density of high affinity 1,4-dihydropyridine binding sites [25–27].

The mechanism by which nimodipine stimulated Na^+,K^+-ATPase is not known. If it is analogous to that of hydrophobic detergents [28], stimulation could occur by demasking of shielded substrate or activator sites inside of membrane vesicles. Alternatively, stimulation could occur because of decreased binding of inhibitor or repressor molecules. Previous studies have suggested that relaxation of smooth muscle cells may sometimes be caused by a cyclic AMP mediated increase in Na^+ pump activity [8, 29, 30]; it is not known if this mechanism could operate under the conditions used here.

Other evidence has been presented previously that Ca^{2+} channel inhibitors may stimulate ion pumps. For example, very high concentrations (100 μM) of nimodipine and nitrendipine, but not nifedipine, stimulate the Ca^{2+}-ATPase of the cardiac and skeletal muscle sarcoplasmic reticulum [31]. Decreased intracellular Na^+ levels are seen in some blood vessels treated with nifedipine and diltiazem (see Ref. 5 for review). However, this phenomenon may be secondary to the block of Na^+ influx through the Ca^{2+} channel. In any case, these two drugs did not stimulate significantly Na^+,K^+-ATPase under our conditions. Based on an observed hyperpolarization, the suggestion has been made that the nitrendipine stimulates an electrogenic Ca^{2+} pump in vascular smooth muscle [32].

It remains to be determined whether these biochemical studies on isolated membranes indicate that Na^+ pump stimulation occurs in intact vascular smooth muscle cells exposed to nimodipine. The development of drugs that act by the stimulation of ion pumps represents a novel concept for the treatment of many disorders including hypertension, angina and related vasospastic diseases.

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Table 2. Comparison of the effect of nimodipine on Na^+,K^+-ATPase of membranes isolated from various tissues*

	Na^+,K^+-ATPase (μmoles · mg^{-1} · hr^{-1})		
	Control	Nimodipine	% Stimulation
Rat aorta	19.5 ± 2.3	40.0 ± 4.2	123 ± 10 (30)
Rat vas deferens	6.0 ± 1.0	11.0 ± 1.3	101 ± 23 (7)
Guinea pig aorta	1.3 ± 0.2	2.9 ± 0.5	114 ± 27 (5)
Guinea pig brain	29.7 ± 1.4	30.0 ± 1.7	1 ± 0.9 (3)

* Ouabain concentration used in the assay of Na^+,K^+-ATPase for guinea pig tissues was 0.4 mM. For rat aorta and rat vas deferens, Na^+,K^+-ATPase was measured in the presence and absence of 100 mM Na^+ and 10 mM K^+. The number of preparations used is given in parentheses.

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