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Ca^{2+}-Channel Blockers and Nucleoside Triphosphate Diphosphohydrolase (NTPDase) Influence of Diltiazem, Nifedipine, and Verapamil

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ABSTRACT. The nucleoside triphosphate diphosphohydrolases (NTPDase; EC 3.6.1.5) are a family of ectonucleotidases associated with vascular endothelial and smooth muscle cells. These ectonucleotidases are involved in the control of vascular tone by regulating the level of circulating ATP. Ca^{2+}-channel blocking agents are currently used for the treatment of hypertension. Considering the external localization of the NTPDase catalytic site and its Ca^{2+} requirement for enzyme activity, a possible interference of calcium antagonists (nifedipine, verapamil-HCl, and diltiazem-HCl and some of its metabolites) could be anticipated. To test that hypothesis, an NTPDase-enriched particulate fraction was used. Our results show that verapamil, diltiazem, and its metabolites all produced a concentration-dependent inhibition of NTPDase, at concentrations greater or equal to 0.1 mM with verapamil and to 0.5 mM with diltiazem and its metabolites, whereas no significant effect was observed with nifedipine. Kinetic studies, carried out to define the mode of action of these drugs, showed a mixed type of inhibition. Based on their respective K_i values (in parentheses, in mM), inhibitory potencies of these molecules were in the following order: desacetyl-*N*-desmethyldiltiazem (M_2-HCl; 0.6) > verapamil (0.76) > *N*-desmethyldiltiazem (M_A; 0.9) > diltiazem (2.4) > desacetyl-*O*-desmethyldiltiazem (M_4-HCl; 3.5) > desacetyl *N,O*-desmethyldiltiazem (M_6-HCl; 3.9). Hence, these calcium antagonists can be considered as weak NTPDase inhibitors. Moreover, based on these K_i values and the range of concentrations found in the blood, NTPDase would not be inhibited significantly *in vivo*. BIOCHEM PHARMACOL **60**;12:1959–1965, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. Ca^{2+} antagonists; NTPDase; apyrase; enzyme inhibition; ectonucleotidases; purine metabolism

In North America, both chronic and acute hypertension are common cardiovascular diseases leading to artherosclerosis and numerous other abnormalities of coronary flow regulation [1]. These include impairments of blood pressure autoregulation, changes in vascular responsiveness, and alterations of endothelial cell functions [1]. To circumvent these pathologies, numerous drugs have been developed, namely Ca^{2+}-channel blockers, which are widely used for the treatment of hypertension. Since endothelial cells possess very active ectonucleotidases which are Ca^{2+}-dependent enzymes, we wondered if Ca^{2+}-channel blockers could interfere with the extracellular purine metabolism [2–6]. This observation could be of great importance since it has been shown that circulating nucleotides cause the release of potent vasodilators such as PGI_2[‖] and NO, via their P2Y purinoceptors [7–9]. The latter are G-protein-coupled receptors that upon activation increase intracellular Ca^{2+} concentration and activate PLA_2 and NO synthase and the release of PGI_2 and NO [7–10]. ATP and its metabolites can also influence platelet aggregation and blood cells involved in inflammatory processes as well as cardiac function [11, 12].

The ecto-NTPDase family (EC 3.6.1.5), previously known as ecto-ATPDase and ecto-ATPase or E-type ATPase, represents the main ectonucleotidases expressed by endothelial and smooth muscle cells of the circulatory system [4, 13]. This family of ectoenzymes converts extracellular nucleoside tri- and diphosphates to their respective monophosphate derivative [14–16]. Several NTPDases have been cloned and sequenced, and their encoding genes were found to correspond to CD39, a lymphocyte marker, and its variants [17–19]. It was shown that transformed COS cells that express NTPDase can inhibit or reverse platelet aggregation induced by ADP, thrombin, and collagen *in vitro* [18]. This observation, together with a previous *in vitro* study [5], confirm the role of this protein as an anti-platelet agent. NTPDases localized in both the intima and media of blood vessels, as shown by immuno-

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‖ *Abbreviations*: PGI_2, prostacyclin I_2; NO, nitric oxide; NTPDase, nucleoside triphosphate diphosphohydrolase; M_2-HCl, desacetyl-*N*-desmethyldiltiazem; M_A-HCl, *N*-desmethyldiltiazem; M_4-HCl, desacetyl-*O*-desmethyldiltiazem; M_6-HCl, desacetyl-*N,O*-desmethyldiltiazem; PLA_2 phospholipase A_2; ATPDase, adenosine triphosphate diphosphohydrolase; and PMSF, phenylmethylsulfonyl fluoride.

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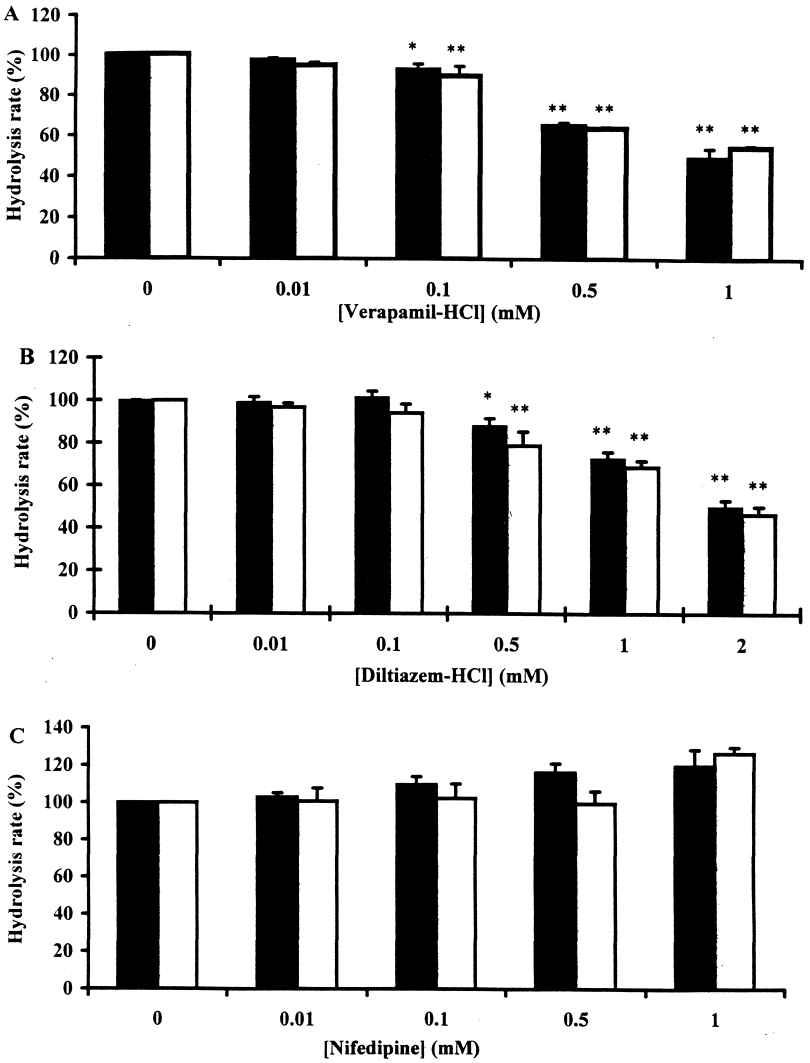

FIG. 1. Influence of verapamil-HCl (A), diltiazem-HCl (B), and nifedipine (C) on ATP (closed bars) and ADP (open bars) hydrolysis by NTPDase. Results are expressed as a percentage of the control and represent the means (± SEM) of three different experiments, each in triplicate. Statistical significance was determined with an ANOVA test; a single asterisk (*) signifies $P < 0.05$ and a double asterisk (**) $P < 0.01$ as compared with the control, where 100% corresponds to ATPase and ADPase activities of 1.8 and 1.3 μmol P_i released/min/mg protein, respectively.

localization, enzymatic assays, and immunohistochemistry, occupy a strategic position in the control of extracellular nucleotide concentrations and, hence, purinergic responses such as modification of vascular tone and platelet aggregation [5, 6, 20–22].

In this study, we measured the influence of three different types of Ca^{2+} antagonists on NTPDase activity: (a) nifedipine (dihydropyridine), (b) diltiazem-HCl (benzothiazepine) and some of its metabolites, and (c) verapamil-HCl (phenylalkylamine).

MATERIALS AND METHODS

Materials

ATP, diltiazem-HCl, nifedipine, verapamil-HCl, tetramisole (2,3,5,6-tetrahydro-6-phenylimidazo[2,1-*b*]thiazole), PMSF, malachite green, Tween 20 (polyoxyethylene [20] sorbitan monolaureate), BSA fraction V, and DMSO were obtained from the Sigma Chemical Co. Bradford reagent was obtained from Bio-Rad Laboratories, and ADP from Roche. Other reagents were of analytical grade and were bought from Sigma. Diltiazem metabolites (M_A-HCl, M_2-HCl, M_4-HCl, and M_6-HCl) were synthesized following the general procedure of Li *et al.* [23]; the resolution was carried out using α-phenethylamine salt formation and crystallization, as described by these authors.

Isolation of Particulate Fractions

Bovine spleens were obtained from a local slaughterhouse. Isolation of the particulate fraction, enriched in NTPDase, was carried out as previously described [6]. Briefly, the bovine spleen was homogenized with a Polytron in 10 vol. of 45 mM Tris (pH 7.6), 95 mM NaCl, 0.1 mM PMSF, and 20 μg/mL of soybean trypsine inhibitor (SBTI). The homogenate was filtered through cheesecloth and centrifuged for 15 min at 600 *g*. The supernatant was centrifuged for 90 min at 22,000 *g*. The pellet was homogenized in 0.1 mM PMSF and 1 mM sodium bicarbonate buffer (pH 10.0), with a Potter–Elvehjem homogenizer. The homogenate was loaded onto a 40% sucrose cushion, and centrifuged for 90 min at 100,000 *g*. The fluffy layer recovered on the cushion was rinsed twice with the bicarbonate buffer, and suspended

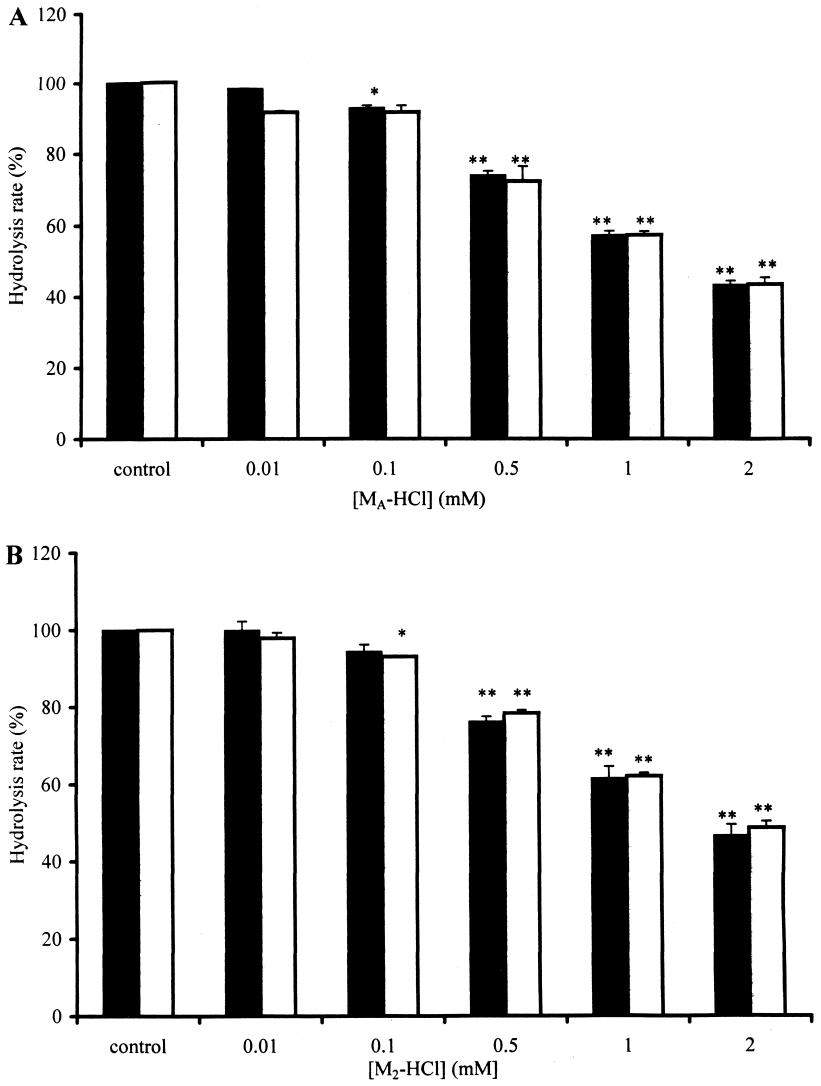

FIG. 2. Influence of the diltiazem metabolites M_A-HCl (A) and M_2-HCl (B) on the ATPase (closed bars) and ADPase (open bars) activities of NTPDase. Results are expressed as a percentage of the control (0 mmol/L of metabolites) and represent the means (± SEM) of two different experiments, each in triplicate. Statistical significance was determined with an ANOVA test; a single asterisk (*) signifies $P < 0.05$, and a double asterisk (**) $P < 0.01$, as compared with the control, where 100% corresponds to ATPase and ADPase activities of 1.2 and 1 μmol P_i released/min/mg protein, respectively.

in 5 mM Tris (pH 8.0) and 7.5% glycerol and stored at −70°. The enzyme preparation had a specific ATPase activity of 4.6 ± 0.4 μmol P_i released/min/mg protein and an ADPase activity of 3.5 ± 0.3 μmol P_i released/min/mg protein. As previously shown, this type of preparation is devoid of any other nucleotidase activities [24]. It is noteworthy that bovine and human NTPDase show very similar biochemical properties [6, 15].

NTPDase assays

Enzyme assays were carried out, with 1.5 μg protein, at 37° in 1 mL of: 8 mM $CaCl_2$, 5 mM tetramisole, 50 mM imidazole, and 50 mM Tris (pH 7.6). Diltiazem-HCl and diltiazem metabolites were tested at concentrations ranging from 0 to 2 mM. Nifedipine was solubilized in DMSO, and 0.5% BSA was added to the incubation medium to stabilize it. Controls for these assays were run with DMSO and 0.5% BSA. In contrast to diltiazem and its metabolites, verapamil-HCl and nifedipine could not be used at concentrations exceeding 1 mM because of their limited solubility. After a 60-min preincubation period at 37°, reaction was started by adding a 200 μM concentration of either ATP or ADP and stopped with the malachite green reagent. Inorganic phosphorus was measured according to the malachite green method as described by Baykov *et al.* [25]. Protein concentration was measured by the Bradford microplate method with BSA as a standard of reference [26]. K_i values were estimated from Dixon replots, which contrary to Lineweaver–Burk plots allowed us to directly visualize the K_i values from the figures and thereby confirm the type of inhibition observed.

RESULTS

Ntpdase Inhibition by Ca^{2+}-Channel Blockers

High concentrations of verapamil-HCl inhibited NTPDase in a concentration-dependent manner (Fig. 1A). At 0.1 mM, ATPase and ADPase activities of NTPDase were reduced by 7 and 10%, respectively. At 0.5 mM, hydrolysis rates of Ca^{2+}-ATP and -ADP were 65 and 64% those of the control and at 1 mM, 49 and 55% those of the control,

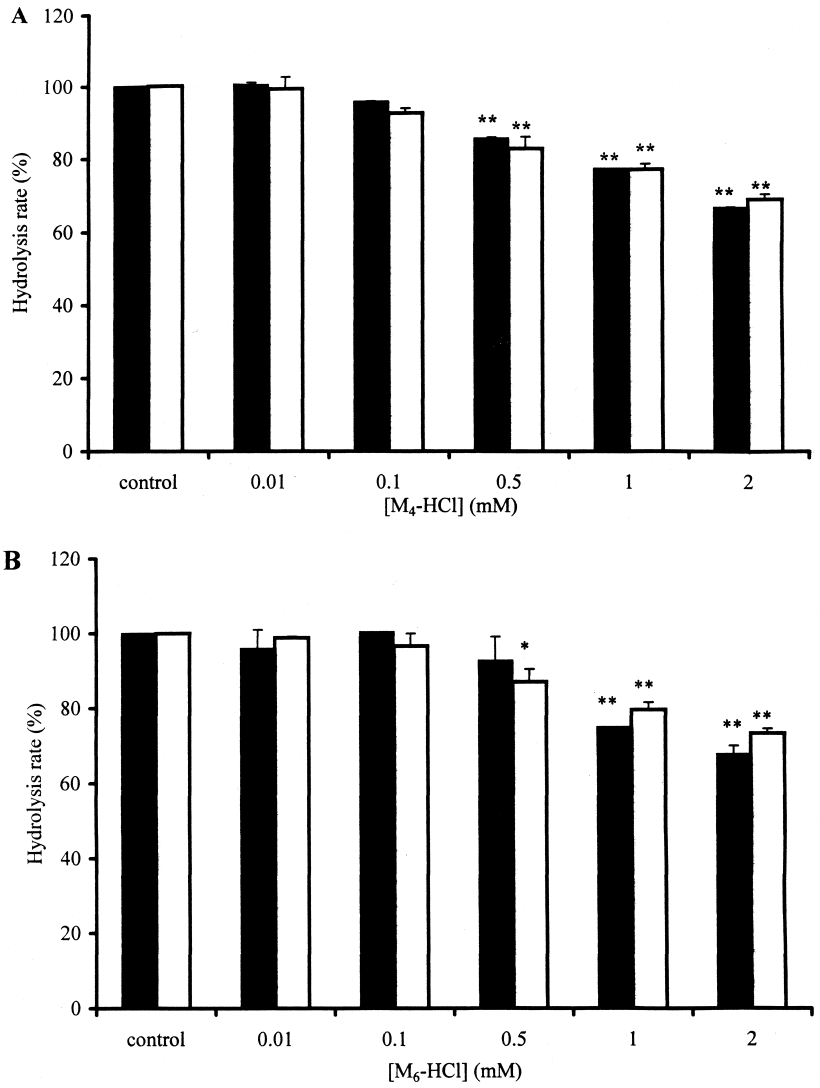

FIG. 3. Influence of the diltiazem metabolites M_4-HCl (A) and M_6-HCl (B) on the ATPase (closed bars) and ADPase (open bars) activities of NTPDase. Results are expressed as a percentage of the control (0 mmol/L of metabolites) and represent the means (± SEM) of two different experiments, each in triplicate. Statistical significance was determined with an ANOVA test; a single asterisk (*) signifies $P < 0.05$, and a double asterisk (**) $P < 0.01$, as compared with the control, where 00% corresponds to ATPase and ADPase activities of 1.2 and 1 μmol P_i released/min/mg protein, respectively.

respectively. Statistical analysis, with a one-way ANOVA test, showed significant inhibition ($P < 0.05$) at concentrations of verapamil equal to or greater than 0.1 mM. Inhibition induced by diltiazem-HCl was also concentration dependent (Fig. 1B). Indeed with 0.5 mM, ATPase and ADPase activities were reduced by 12 and 21%, respectively. At 1 mM, hydrolysis rates were 73 and 69% those of the control and at 2 mM, 50 and 47%, respectively. Comparable analysis showed significant inhibition at concentrations of diltiazem-HCl equal to or greater than 0.5 mM. In contrast, nifedipine (Fig. 1C) had no significant effect on ATP or ADP hydrolysis in the same concentration range.

Inhibition of NTPDase by Diltiazem Metabolites

NTPDase inhibition by the diltiazem metabolites M_A-HCl (Fig. 2A) and M_2-HCl (Fig. 2B) was comparable to that obtained with diltiazem. At 0.5 mM M_A-HCl, hydrolysis rates of ATP and ADP were 74 and 72% those of the control, whereas in the presence of 0.5 mM M_2-HCl the hydrolysis rates were 76 and 78%. At 1 and 2 mM M_A-HCl, hydrolysis rates of ATP and ADP were about 57 and 43% those of the control. Similarly, at 1 and 2 mM M_2-HCl, the hydrolysis rates were 62 and about 47% the rates of the control. The diltiazem metabolites M_4-HCl (Fig. 3A) and M_6-HCl (Fig. 3B) were less efficient inhibitors than diltiazem, M_A-HCl, or M_2-HCl. For comparison purposes, at 2 mM hydrolysis rates of ATP and ADP were 67 and 69% those of the control with M_4-HCl, and 68 and 73% those of the control with M_6-HCl, respectively.

Determination of Kinetic Inhibition Constants for Verapamil, Diltiazem, and Metabolites

To define what types of inhibition are produced by verapamil, diltiazem, and its metabolites (M_A, M_2, M_4, and M_6) on NTPDase, substrate velocity curves were constructed with increasing concentrations of inhibitors, and Ca^{2+}-ADP as the substrate (Figs. 4-6). As shown in Fig. 4, Lineweaver–Burk representation (Fig. 4A) and Dixon plot replots (Fig. 4B), verapamil-HCl produced a mixed type of

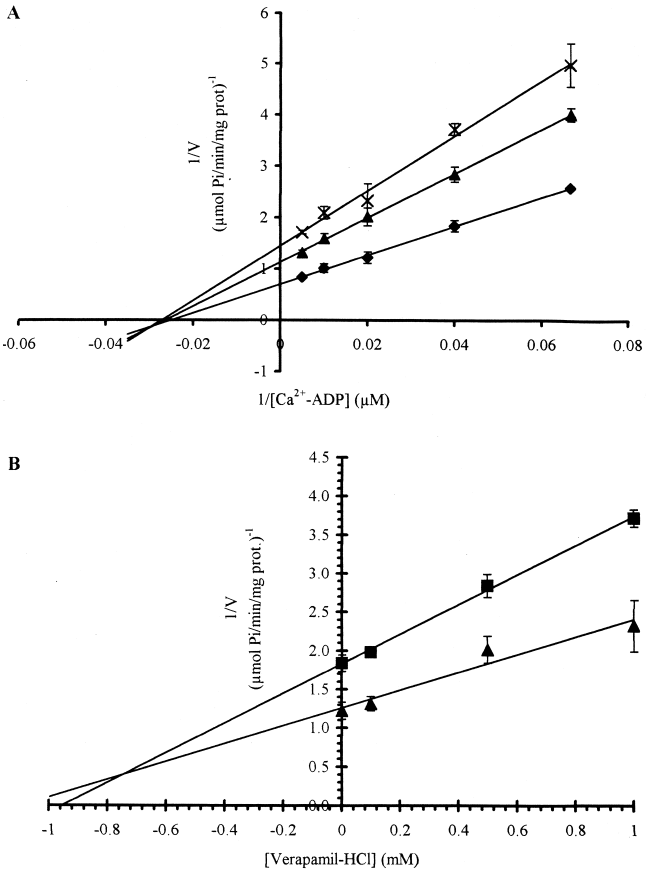

FIG. 4. Inhibition of Ca^{2+}-ADP hydrolysis by verapamil-HCl. (A) Representation of NTPDase inhibition by Lineweaver–Burk plots. Ca^{2+}-ADP concentrations ranged from 25 to 200 µmol/L. Assays were carried out in the presence of verapamil: 0 mmol/L (control: ♦), 0.5 mmol/L (▲), and 1 mmol/L (X). (B) Representation of NTPDase inhibition by Dixon plots. Verapamil concentrations ranged from 0 to 1 mmol/L at ADP concentrations of 25 µmol/L (▲) and 50 µmol/L (■). Each point is the mean (± SEM) of at least three different experiments, each in triplicate. The estimated K_i was 0.76 mmol/L.

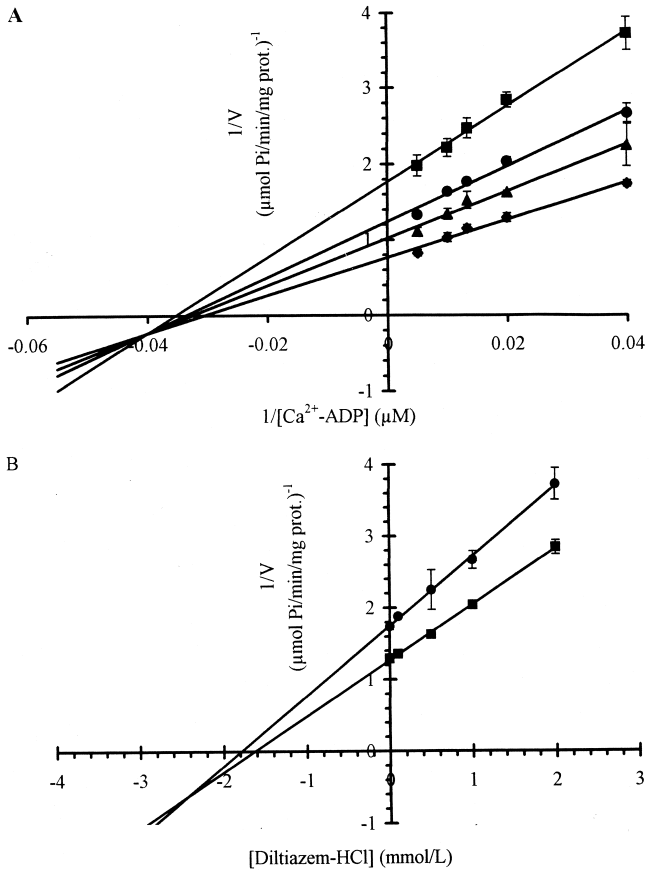

FIG. 5. Inhibition of Ca^{2+}-ADP hydrolysis by diltiazem-HCl. (A) Representation of NTPDase inhibition by Lineweaver–Burk plots. Ca^{2+}-ADP concentrations ranged from 25 to 200 µmol/L. Assays were carried out in the presence of diltiazem: 0 mmol/L (control: ♦), 0.5 mmol/L (▲), 1 mmol/L (●), and 2 mmol/L (■). (B) Representation of NTPDase inhibition by Dixon plots. Diltiazem concentrations ranged from 0 to 2 mmol/L at ADP concentrations of 25 µmol/L (●) and 50 µmol/L (■). Each point is the mean (± SEM) of at least three different experiments, each in triplicate. The estimated K_i was 2.4 mmol/L.

inhibition. Dixon representation allowed us to estimate a K_i of 0.76 mM for the latter molecule. Similar Lineweaver–Burk curves were obtained for diltiazem-HCl (Fig. 5A) and its metabolites (not shown), suggesting that these molecules also act as mixed-type inhibitors, an observation confirmed by Dixon representations. Estimated K_i values were 2.4, 0.9, 0.6, 3.5, and 3.9 mM for diltiazem-HCl (Fig. 5B), M_A (Fig. 6A), M_2 (Fig. 6B), M_4 (Fig. 6C), and M_6 (Fig. 6D) metabolites, respectively. Thus, verapamil-HCl (K_i of 0.76 mM) was a more efficient inhibitor than diltiazem-HCl (K_i of 2.4 mM) or its M_4 and M_6 metabolites (K_i of 3.5 and 3.9 mM, respectively). The inhibitory effect was comparable to those observed with M_A and M_2 metabolites (K_i of 0.9 and 0.6 mM, respectively).

DISCUSSION

In this work, Ca^{2+}-channel blockers belonging to three distinct chemical groups (dihydropyridines, phenylalkylamines, and benzothiazepine) were evaluated for their potential inhibitory action on ectonucleotidases responsible for the conversion of ATP to AMP in blood. Our results demonstrated that millimolar concentrations of diltiazem-HCl (benzothiazepine) or verapamil-HCl (phenylalkylamine) inhibited NTPDase in a concentration-dependent manner and acted as mixed-type inhibitors. In contrast, nifedipine (dihydropyridine) had no effect on the activity. We also evaluated the influence of diltiazem metabolites (M_A-, M_2-, M_4-, and M_6-HCl) considering that Montamat and Abernethy [27] and Yabana *et al.* [28] reported that the latter retain some of the pharmacological activity of diltiazem.

The modalities of NTPDase inhibition by verapamil and diltiazem and its metabolites are not known. The desacetyl-*O*-desmethylation (M_4) and the desacetyl-*N*,*O*-desmethylation (M_6) of diltiazem-HCl appear to attenuate its inhibitory properties, whereas the *N*-desmethylation (M_A) and

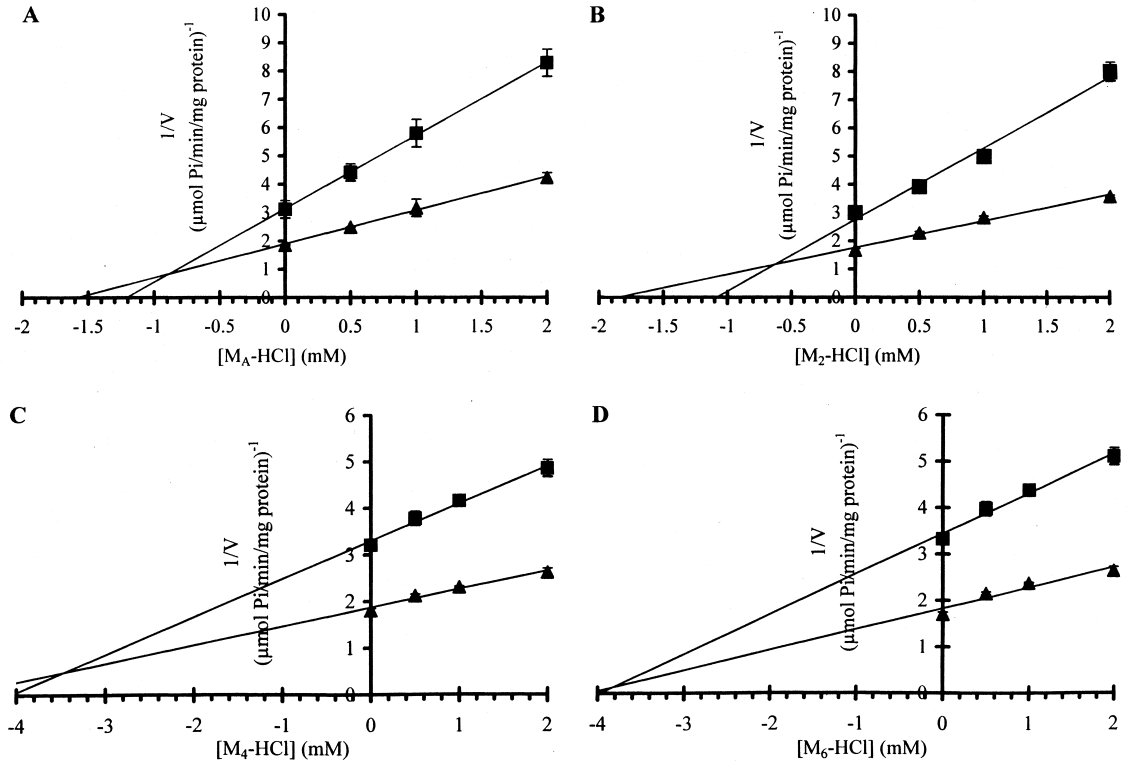

FIG. 6. Dixon representation of NTPDase inhibition by diltiazem metabolites: M_A-HCl (A), M_2-HCl (B), M_4-HCl (C), and M_6-HCl (D). Metabolite concentrations ranged from 0 to 2 mmol/L at Ca^{2+}-ADP concentrations of 15 µmol/L (■) and 50 µmol/L (▲). Each point is the mean (± SEM) of three experiments, each in duplicate. The estimated K_i values were: 0.9, 0.6, 3.5, and 3.9 mmol/L for M_A-HCl, M_2-HCl, M_4-HCl, and M_6-HCl, respectively.

the desacetyl-*N*-desmethylation (M_2) of diltiazem enhances it. Intriguingly, the presence of *O*-methyl groups on the molecules appears to influence their inhibitory properties. Among the three Ca^{2+} antagonists tested, one of them, nifedipine, did not bear an *O*-methyl group, and it was the one that had no significant inhibitory effect. Similarly, the diltiazem metabolites M_4 and M_6, which showed very poor affinity (K_i) for NTPDase, were also poor inhibitors when compared with verapamil and the diltiazem metabolites M_A and M_2. Mixed-type inhibitions induced by these different drugs could possibly be explained by some interactions with the oligomerization of NTPDase subunits, which seems essential for activity [29–31]. Ca^{2+} antagonist binding sites on proteins correspond to specific DNA sequences as defined by Nakayama and Kuniyasu [32]. Analysis of the NTPDase cDNA did not show any significant homology with the sequence of Nakayama and Kuniyasu. Hence, any specific binding of these Ca^{2+} antagonists on NTPDase is improbable.

From a more general viewpoint, some of the Ca^{2+}-channel blockers currently used by clinicians to treat hypertension could act as weak NTPDase inhibitors. However, based on our *in vitro* assays, there is no reason to believe that the function of NTPDase would be impaired by verapamil-HCl, diltiazem-HCl, or its metabolites (M_A-, M_2-, M_4-, and M_6-HCl) at concentrations reached in the blood of patients, which are 60–75, 23–45, 3, and 6–10 ng/mL for M_A-, M_2-, M_4-, and M_6-HCl, respectively [33].

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