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Adenosine Tetraphosphate, Ap_4, a Physiological Regulator of Intraocular Pressure in normotensive rabbit eyes

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Running title: Ap_4 regulates IOP

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Abbreviations:

Ap_4, adenosine 5' tetraphosphate; Ap_4A, diadenosine tetraphosphate; ATP-γ-S, adenosine 5´-3-O-thiotriphosphate; α,β-me ATP, α,β-methylene-adenosine 5' triphosphate; β,γ-meATP, β,γ-methylene-adenosine 5' triphosphate; CNS, central nervous system; HPLC, high performance liquid chromatography; IOP, intraocular pressure; PPADS, pyridoxal phosphate 6-azophenyl-2´,4´-disulphonic acid; RB-2, reactive blue 2.

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ABSTRACT

Adenosine 5' tetraphosphate, Ap_4, is a natural nucleotide present in many biological systems. This nucleotide has been found as a constituent of the nucleotide pool present in the aqueous humor of New Zealand rabbits. HPLC analysis confirmed its identity and calculated its concentration levels to be 197 ± 21 nM. When applied topically to the rabbit eyes this mononucleotide produced a reduction in the intraocular pressure, which was dose-dependent. The pD_2 value calculated from the dose-response curve was 7.28 ± 0.47, which is equivalent to 52.48 nM. The time-course of such intraocular pressure reduction presented a maximal decrease of IOP to 75.1 ± 2.3 % compared to the vehicle control value (100%), and the effect lasted for more than 2 hours. Cross-desensitisation studies demonstrated that Ap_4 effect was mediated via a P2X receptor in this system. P2 receptor antagonists suramin, PPADS and reactive blue 2, showed that only the latter was able to revert the effect of Ap_4. Antagonists of adrenoceptors and cholinoceptors were able to partially reverse the effect of this nucleotide, this might indicate a connection with the neural mechanisms that control the intraocular pressure.

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Adenosine 5' tetraphosphate, Ap_4, is a natural nucleotide present in neurosecretory vesicles together with other nucleotides and classical transmitters (Gualix et al., 1996). Although it has been described in many biological systems, it is the least investigated for its physiological/pharmacological actions when compared to other nucleotides. Compounds such as adenosine 5' triphosphate, ATP, uridine 5' triphosphate, UTP or diadenosine polyphosphates, Ap_nA, have been investigated for their physiological effects and their receptor function (Hourani et al., 1998; Pintor et al., 2000b), but Ap_4 has not been given as much attention.

Ap_4 was identified first from horse muscle (Lieberman,1955), and also in other tissues such as rabbit muscle and platelets, yeast spores and rat liver (Small and Cooper, 1966; Lobaton et al., 1975; Van Dyke et al., 1977). This nucleotide is present and releasable from neurochromaffin cells (Winkler and Carmichael, 1982; Gualix et al., 1996), and its synthesis is carried out by a transphosphorylation process involving ATP (Gualix et al., 1996). When stored in the vesicles it can be released to the extracellular space together with other transmitters. Once at the extracellular space, this nucleotide can activate both ionotropic P2X and metabotropic P2Y receptors. In this sense, the effect of Ap_4 on the vascular system is to reduce blood pressure by means of the activation of a P2Y receptor, however in stress situations, such as in an haemorrhage, it produces vasoconstriction by the activation of smooth muscle P2X receptors (Lee et al., 1995; Lewis et al., 2000). Very recently, Ap_4 has been identified in human myocardial tissue. The application of this nucleotide in isolated rat heart can produce vasodilatation (Westhoff et al., 2003).

It is only recently that the nucleotides are considered as active compounds in the eye (Pintor, 1999). Processes in which extracellular nucleotides can modify ocular physiology include tear secretion, intraocular pressure and retinal fluid reabsorption (Pintor et al., 2002; Yerxa 2001). Mononucleotides, such as ATP and its

synthetic analogues, can modulate intraocular pressure (IOP). There is a clear difference in the way they can modify IOP, since those nucleotides with a P2Y agonistic profile tend to increase IOP while P2X agonists reduce it (Pintor and Peral., 2001). Recently, it has been demonstrated that diadenosine polyphosphates can modify IOP. Diadenosine tetraphosphate (Ap_4A), induces a decrease in IOP while the other members of this family produce an increase in the rabbit's intraocular pressure (Pintor et al., 2003).

Nothing is known about the role of the nucleotide Ap_4 in the eye, its presence or its physiological behaviour. Hence, this investigation will look at the presence of Ap_4 in the rabbit's aqueous humor and describe its effect on the intraocular pressure.

Materials and methods

Animals

24 normotensive New Zealand White rabbits (males, 2 – 3 kg) were used. The animals were kept in individual cages with free access to food and water, under controlled cycles (12 h/12 h light/dark). Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and the statement of the Association for Research in Vision and Ophthalmology on the Use of Animals in Ophthalmic and Vision Research.

Aqueous humor collection and HPLC procedures

Aqueous humor was collected from anaesthetised rabbits by means of an intracameral injection. These experiments were performed always at the same time, 3:00 pm, when IOP has recovered completely from the night period. Briefly, New Zealand white rabbits were anaesthetised with 1.5 mg/Kg propofol (Abbott Laboratories, Madrid). Aqueous humor (500 µL) was removed with a syringe connected to a 30G needle in the scero-corneal limbus. Samples were stored at -35° C prior to treatment through SEP-Pak Accell QMA cartridges (Waters, Millford, USA). Samples were chromatographed through these cartridges, and the elution of these compound was obtained by applying 500 µL of a mixture containing 0.1 N HCl, 0.2 N KCl. Eluates were neutralised with 10 N KOH prior to HPLC analysis (Rotllán et al., 1991).

The HPLC system consisted of a Waters 1515 Isocratic HPLC pump, a 2487 dual wavelength absorbance detector, a Reodyne injector, all controlled by the software Breeze from Waters. The column used was a NovaPak C18 (15 cm length, 0.4 cm diameter) also from Waters. The mobile phase consisted of 10 mM KH_2PO_4,

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2 mM tetrabutyl ammonium, 17 % acetonitrile, pH 7.5. Detection was monitored at 260 nm wavelength.

In order to confirm the chemical nature of the putative peak identified as Ap_4, enzymatic digestion with alkaline phosphatase (EC 3.1.3.1) 0.3 U/mL (molecular biology grade) was used. Incubation with this enzyme and the putative nucleotide was carried out for 10 min, and the digested products were analysed by HPLC.

Animals used for aqueous humor studies were not used for IOP experiments after 2 weeks of recovery.

Intraocular pressure measurements

Intraocular pressure was measured by means of a Tono-Pen® XL contact tonometer (Mentor Massachusetts Inc., USA). This device has been shown to be the tonometer of choice for measuring intraocular pressures within the range of 3 – 30 mmHg in rabbits (Abrams, Vitale & Jampel, 1996). Because IOP changes from the night to day, all the experiments were performed at the same time, 3:00 pm. At this time, IOP remains more stable and permits an objective comparison with vehicle treatment. All measurements fell within this diapson: the mean baseline value of intraocular pressure was 17.0 ± 0.39 mmHg (n = 100). A topical anaesthetic (Colircusí, Laboratorios Cusí, Spain: 0.1 $mg.ml^{-1}$ tetracaine plus 0.4 $mg.ml^{-1}$ oxybuprocaine in 0.9% saline, diluted 1:3 in 0.9% saline) was applied (10 µL) to the cornea before each measurement of intraocular pressure was made. Ap_4 or saline were applied topically to the cornea in volumes of 10 µL. In order to compare the effect of Ap_4 with other compounds that reduce IOP, Xalatan© (latanoprost 0.005%), 10 µL, was assayed and IOP measured for 6 hours.

In a series of experiments the intraocular pressure was measured twice, 30 min apart, before application of a dose of Ap_4 or saline (NaCl 0.9 %, vehicle). In

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establishing dose-effect relationships, measurements of intraocular pressure were made after 30 min and every hour for 6 hours following the application. Maximum responses were observed after 2 hours. One dose was tested per rabbit per day. In experiments where the ATP P2-receptor antagonist, pyridoxalphosphate-6-azophenyl-2´,4´-disulphonic acid (PPADS), suramin and reactive blue 2 (RB-2) were tested, these compounds were applied after the two baseline measurements and 30 min before application of saline or a nucleotide.

Occlusion experiments were carried out in the same fashion as with the antagonists. Briefly, Ap_4, β, γ-meATP and ATP- γ-S (all 100 µM, 10 µL), were applied 30 min prior to the administration of Ap_4 at 100 µM (10 µL).

Cholinergic antagonists (hexamethonium and atropine) and noradrenergic antagonists (yohimbine and ICI-188.581), were assayed at a fixed concentration of 10 µg/µL (10 µL instillation), 30 min before the application of the purinergic compounds (Pintor and Peral, 2001).

Drugs

Ap_4, alkaline phosphatase, PPADS, reactive blue 2 and suramin were purchased from Sigma-Aldrich-RBI (St. Louis, MO, USA). β, γ-meATP and ATP- γ-S were purchased from Roche (Manheim, Germany). Atropine, hexamethonium, ICI 118,851 and yohimbine were from Tocris (Bristol, UK). Xalatan© was purchased from Pharmacia Upjohn (Barcelona, Spain). Other reagents were analytical grade from Merck (Darmstadt, Germany).

Analysis of data

Numerical values are given as mean ± S.E. mean. Means of two groups were compared using Student's *t*-test (unpaired, two tailed, unless stated) with a 5% fiducial point of significance unless stated.

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Results

Presence and quantification of Ap_4 in the aqueous humor

The chromatographic analysis of the samples obtained from rabbit's aqueous humor revealed the presence of various peaks which were identified as mono and dinucleotides. In particular, a peak appeared between the corresponding ATP and diadenosine tetraphosphate, Ap_4A peaks. This peak was tentatively identified as adenosine 5`tetraphosphate (Ap_4) when compared with a nucleotide stardard chromatographed which contained the comercial Ap_4 (figure 1). Since the identification by the retention time and comparison with a standard is not enough to verify the presence of this nucleotide in the aqueous humor of the rabbit, the peak was collected and submitted to alkaline phosphatase treatment as described in methods. The products obtained after the enzyme digestion, with the presence of ATP, ADP and AMP, and the concomitant reduction in the peak of the putative Ap_4, indicated that the nucleotide investigated was Ap_4 (figure 2). The concentration obtained for this compound in the aqueous humor was 197 ± 21 nM (n=8) which is smaller than the one obtained for other mononucleotides (Pintor et al., 2002).

Effect of Ap_4 on intraocular pressure

Since Ap_4 is present in the rabbit aqueous humor, we wanted to know if there was any change in the intraocular pressure (IOP) values when this nucleotide was topically applied. Graded doses of Ap_4 ranging from 10^{-2} M to 10^{-11} M were instilled as described previously, in order to construct a dose-response curve. As it is shown in figure 3, Ap_4 was able to reduce intraocular pressure in rabbits in a concentration dependent manner. IOP was reduced to 75.1 ± 2.3 % compared to control the values (100 %). The analysis of the curve permited the calculation of the pD_2 value which was 7.28 ± 0.47, which is equivalent to an EC50 of 52.48 nM (n=8).

The time course of Ap_4-induced IOP changes was examined. A single dose of 100 µM Ap_4 produced a reduction of IOP, from 17.1 ± 0.6 mm Hg to 12.5 ± 1.5 mm Hg, which was maximal 2 hours after the application of the nucleotide (figure 4). The effect was sustained for about 2 to 3 hours and returned to normal values within 6 hours after (n=6). In order to compare the effect of the nucleotide with other substance which is known that reduce IOP, the commercial hypotensive compound Xalatan (latanoprost) was topically applied to the rabbit eyes. 10 µL of 0.005% Xalatan, produced a marked reduction in normostensive rabbit's IOP, from 16.9 ± 0.8 mm Hg to 11.3 ± 0.1 mm Hg, as observed in figure 4.

Cross-desensitisation and studies with P2 antagonists

In order to investigate the P2 purinergic receptor involved in the effect of Ap_4, cross-desensitisation studies were performed by means of ATP-γ-S and β,γ-me ATP, hypertensive and hypotensive nucleotides in this model as previously described (Pintor and Peral., 2001).

Homologous cross-desensitisation of Ap_4 at a maximal dose of 100 µM, allowed the measurement of the first hypotensive effect but not the one due to the second dose of this nucleotide (n= 6, figure 5). A similar behaviour was observed when the P2X agonist β,γ-me ATP was applied before Ap_4. When the P2Y agonist ATP-γ-S, was applied it was possible to measure the hypotensive effect of Ap_4 despite the huge hypertensive effect displayed by ATP-γ-S (n=6, figure 5). These experiments suggest the activation of a P2X receptor which is involved in a reduction of IOP as occur with β,γ-me ATP and diadenosine tetraphosphate (Pintor and Peral, 2001; Pintor et al., 2003).

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Three different P2 antagonists were tested in their ability to reverse the hypotensive effect of Ap_4: suramin, reactive blue 2 (RB-2) and PPADS. As shown in figure 6, only RB-2 was able to significantly reverse the effect of Ap_4. Neither PPADS nor suramin could modify the reduction in IOP produced by Ap_4 in a significant way at the single dose tested (n=6).

Studies with cholinergic and noradrenergic antagonists

Intraocular pressure is a process controlled by the nervous system (Bergmanson, 1982). In order to see if the effect of Ap_4 is coupled to the nervous system, antagonists of the cholinergic and noradrenergic systems were tested.

The application of the cholinergic antagonists hexamethonium and atropine alone produced a clear increase in the IOP (figure 7). When the two cholinergic antagonists were applied 30 min before the instillation of Ap_4, the hypertensive effect of hexamethonium and atropine disappeared and it was possible to observe a reduction in IOP (due to Ap_4), which was not statistically different from the effect of Ap_4 alone (n=6, figure 7).

The application of the adrenergic antagonists yohimbine and ICI 188,551, produced also an increase in IOP (figure 7). When Ap_4 was applied after the previous instillation of these antagonists there was a measurable reduction in IOP, nevertheless there were significant differences between this value and the one by Ap_4 alone (n=6, figure 7).

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Discussion

The nucleotide adenosine 5' tetraphosphate, Ap_4, is a naturally ocurring nucleotide present in rabbit aqueous humor as demonstrated by high performance liquid chromatography. Comparison with a commercial standard, digestion with alkaline phosphatase and analysis of the digestion products, permitted to characterise and identify this compound as a component of the aqueous humor.

Nucleotides and nucleosides have been described in the aqueous humor of different animal models. In this sense, Maul and Sears (1979) described the release of adenosine 5' triphosphate (ATP) after stimulation of the trigeminal nerve of the rabbit eye as an extracelular source of nucleotides. In the same way, Mitchell and co-workers (1998) described the release of ATP from bovine ocular ciliary epithelial cells into the aqueous humor. Also, Greiner et al., (1991) showed the presence of phosphate metabolites in ocular humors as well as the presence of the nucleoside adenosine in rabbits (Crosson and Petrovich, 1999). All these works are principally focused on ATP, which has been generaly considered as the natural agonist of P2 receptors together with ADP, UTP and UDP. Nevertheless, other nucleotides such as diadenosine polyphosphates have been described in the rabbit aqueous humor: Ap_4A and Ap_5A are present at concentrations in the low micromolar range and they can exert differential action on IOP (Pintor et al., 2003). In a recent study, a calculation of the concentrations of mononucleotides in the aqueous humor demonstrated that the values obtained for ATP or ADP were about one order of magnitude higher than the concentration value obtained for Ap_4 in the present study (Pintor et al., 2003). A lower concentration in the aqueous humor is not indicating that Ap_4 is less relevant than other mononucleotides. For example, in terms of extracelular stability, Ap_4 lasts longer than ATP does, as demonstrated by Gomez-Villafuertes et al., (2000). In this way, the rate of hydrolysis of Ap_4 in synaptic terminals is 1.89 % per 2 min, while ATP hydrolyses 24.64 % for the same period of

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time. Also, the time-course for ATP on IOP in these rabbits is different from the one obtained for Ap_4 (result not shown). ATP effect presents a sharp reduction in IOP which rapidly returns to normal values continuing towards an hypertensive effects 2 hours after the application of the nucleotide (Peral et al., 2000; Pintor et al., 2000a). This biphasic effect is probably due to the gradual degradation of ATP to adenosine which has been described as an hypertensive compound when acting through adenosine A2 receptors (Crosson and Gray, 1996). There is, therefore, a difference between the effect of ATP and Ap_4 that suggests that Ap_4 remains stable as an active molecule longer than ATP and therefore may act through purinergic receptors.

Ap_4 present an interesting effect reducing intraocular pressure in normotense rabbits. Other adenosine nucleotides have shown a similar behaviour. ATP and the analogues β,γ-meATP and α,β-meATP can produce a reduction in the IOP. The reduction in IOP is similar in magnitude to the one produced by Ap_4 (Pintor and Peral., 2001). Moreover, it is very probable that in the regulation of IOP by these nucleotides and Ap_4 they are stimulating the same P2X receptor. This conclusion partially supported on the cross-desensitisation studies when using β,γ-meATP and the lack of effect to a second dose of Ap_4. Also because the effect of the hypertensive nucleotide ATP-γ-S (a P2Y agonist in this model) is severely reduced when an application of Ap_4 is used. The blockade by means of the P2 antagonist RB-2 suggests that the action of β,γ-meATP and Ap_4 are performed through the same P2X receptor. It is interesting to compare the differential effect of the P2 antagonists tested. It would be expectable to have a reduction in the effect when suramin is applied, since it is a general P2 receptor antagonist. It needs to be taken into account that all the antagonists have been tested at a single dose and that this does not need to the most effective for all the antagonists. Other possibility is to have different antagonist permeability. The cornea may be selective to the transport of some of the P2 antagonists while not to the others.

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Comparison of the pharmacology for the effect of Ap_4 on IOP with other tissues demand a more detailed pharmacological study, nevertheless, some conclusions can be made. For example, Ap_4 has been demonstrated to activate P2X receptors in areas such as smooth muscle and the CNS (Lee et al., 1995; Gomez-Villafuertes et al., 2000). In rat mid brain synaptic terminals this nucleotide stimulates a heteromeric P2X receptor that present features of a $P2X_2/P2X_3$ purinergic receptor (Gomez-Villafuertes et al., 2000). This receptor is pharmacologically different from the one described in ocular cells/tissues since in the rat synaptic terminals the nucleotide ATP-γ-S is a full agonist while in the rabbit eye is not. This observation is suggesting the existence of a P2X receptor in the rabbit eye which presents a subunit composition that does not match with the one described in the rat brain.

Other locations where Ap_4 stimulates P2X receptors are vas deferens, vascular smooth muscle and mesenteric artery (Bailey and Hourani, 1995; Lee et al., 1995; Lewis et al., 2000). In these models, adenosine 5' tetraphoshate produce vasoconstriction, this nucleotide being specially active on $P2X_1$ receptors (Lewis et al., 2000). It is premature to elucidate which subunits form the P2X receptor involved in the reduction of intraocular pressure according to the preliminary pharmacological studies presented in this work. It is necessary to start with studies at the molecular level with isolated cells from the trabecular meshwork and ciliary processes to fully understand the location and characteristics of this receptor.

An interesting finding is that the levels found in the aqueous humor for Ap_4 are in the range for promoting IOP decreased, as illustrated in the dose-response experiments presented here. A concentration of 197 nM is near to the EC_{50} value. This suggests that under certain physiological circumstances the humor levels of Ap_4 can oscilate and therefore they may modulate intraocular pressure. We still do not know what factors influence the possible variations in the Ap_4 concentrations, but if

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Ap_4 is released from ocular nerve terminals, its concentrations may be regulated as happens with other neurotransmitters in the eye such as ACh or epinephrine.

It is also possible that Ap_4 is generated from the hydrolytical cleavage of the dinucleotide diadenosine pentaphosphate (Ap_5A). This process carried out by means of an asymmetrical phosphodiesterase is relevant extracellularly and could be an alternative to neural release (Zimmerman, 1996; Mateo et al., 1997). Nevertheless this cannot be the only mechanism for Ap_4 production since the concentrations measured of Ap_5A in the aqueous humor were only 80 nM, insufficient to justify the presence of this mononucleotide as a consequence of Ap_5A cleavage if we consider that 100% of Ap_5A is hydrolysed (Pintor et al., 2003). So far, we do not know which one of these processes is the most likely one or if there are alternative sites for its release such as the ciliary epithelial cells, which can release adenine nucleotides as previously reported (Mitchell et al., 1998).

Concerning the possible side effects the application of Ap_4 can produce we focused rabbit's corneal transparency, corneal thickness and changes in the corneal endothelium (possible polymegatism and changes in cell hexagonality). During the time of the present research, no changes in the parameters measured were observed (results not shown).

In summary, Ap_4 is a mononucleotide present and physiologically active in the rabbit eye. It can modulate intraocular pressure through a mechanism that involves the activation a P2X receptor. This compound may be used for the treatment of those pathologies involved in an abnormal increase in the intraocular pressure.

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Legends to figures

Figure 1.- Presence of adenosine tetraphosphate in rabbit aqueous humor.

Samples analyzed as described under Materials and Methods presented a peak (upper trace) identified tentatively as Ap_4, when compared with a commercial standard (lower trace, 1000 pmol each). Insert, blow-up of the putative adenine tetraphosphate peak. Aqueous humor was taken always at the same time, 3 pm in order to avoid the possible effect of circadian rhythms.

Figure 2.- Alkaline phosphatase treatment and analysis of putative adenosine tetraphosphate. Ap_4 was treated with alkaline phosphatase (molecular biology grade) as described under Materials and Methods. The treatment of the putative Ap_4 (upper trace) completely changed the chromatographic profile with peaks corresponding to AMP and ADP appearing as digestion products (second trace). The lower trace represent a standard of 1000 pmol AMP, ADP and ATP.

Figure 3.- Dose response analysis of adenosine tetraphosphate on rabbit intraocular pressure. Effect of instillation of graded doses of Ap_4 into the rabbit eye on IOP (solid squares) and the effect of vehicle control (open squares). Each point was obtained 3 hours after the application of the nucleotide (maximal effect of Ap_4). Initial IOP values were 17 $\pm$ 0.39 mm Hg while the maximal effect reduced IOP to 12.6 $\pm$ 1.3 mm Hg. All the IOP experiments were started at 3 pm to avoid the possible effect of circadian rhythms. Points represented the mean ± s.e.m. (n=8).

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Figure 4.- Time-course of changes in IOP in response to a single instillation of Ap_4. A single dose of Ap_4 (100 µM ,10 µL), produced a decrease in IOP (from 17.1 ± 0.6 mm Hg to 12.5 ± 1.5 mm Hg), that returned 4 hours after the instillation of the nucleotide (solid squares). The IOP hypertensive compound Xalatan© (10 µL of 0.005% latanoprost) produced a marked reduction in normostensive rabbit's IOP (from 16.9 ± 0.8 mm Hg to 11.3 ± 0.1 mm Hg) (open squares). All the IOP experiments were started at 3 pm to avoid the possible effect of circadian rhythms. Values represent the mean ± s.e.m. of 6 independent experiments.

Figure 5.- Cross-desensitization experiments with different nucleotides. Homologous and heterologous desensitization experiments for Ap_4 (at 100µM, 10 µl). β,γ-meATP and ATP-γ-S were pre-incubated as described under Materials and Methods at concentrations of 100 µM (10 µL). All the IOP experiments were started at 3 pm to avoid the possible effect of circadian rhythms. Values represent the mean ± s.e.m of six independent experiments. ***p<0.001 *vs* the effect of ATP-γ-S alone.

Figure 6. Effect of P2 receptor antagonists on Ap_4 response. Antagonism of suramin, RB-2, and PPADS (all at 100µM, 10µl) on the hypotensive response induced by Ap_4 (at 100µM, 10µl). All the IOP experiments were started at 3 pm to avoid the possible effect of circadian rhythms. Values represent the mean ± s.e.m. of six independent experiments. *p<0.05 *vs* the effect of Ap_4 alone.

Figure 7.- Effect of cholinergic and adrenergic receptor antagonists on Ap_4 response. The effect of cholinoceptor antagonists (hexamethonium and atropine, 10 µg/µL, 10 µL instillation) and α_2 and β_2-adrenoceptor antagonists (yohimbine

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and ICI 118,551, 10 µg/µL,10 µL instillation) were assayed alone and in the presence of 100 µM Ap_4. All the IOP experiments were started at 3 pm to avoid the possible effect of circadian rhythms. Values represent the mean ± s.e.m. of six independent experiments. *$p<0.05$ *vs* the effect of Ap_4 alone.

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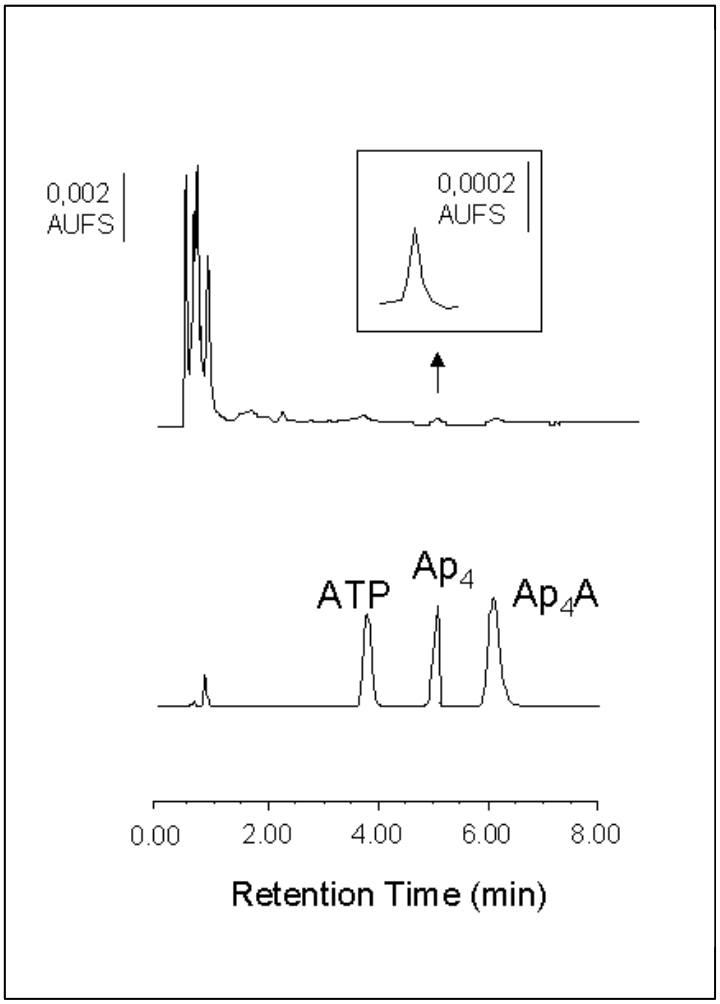

FIGURE 1

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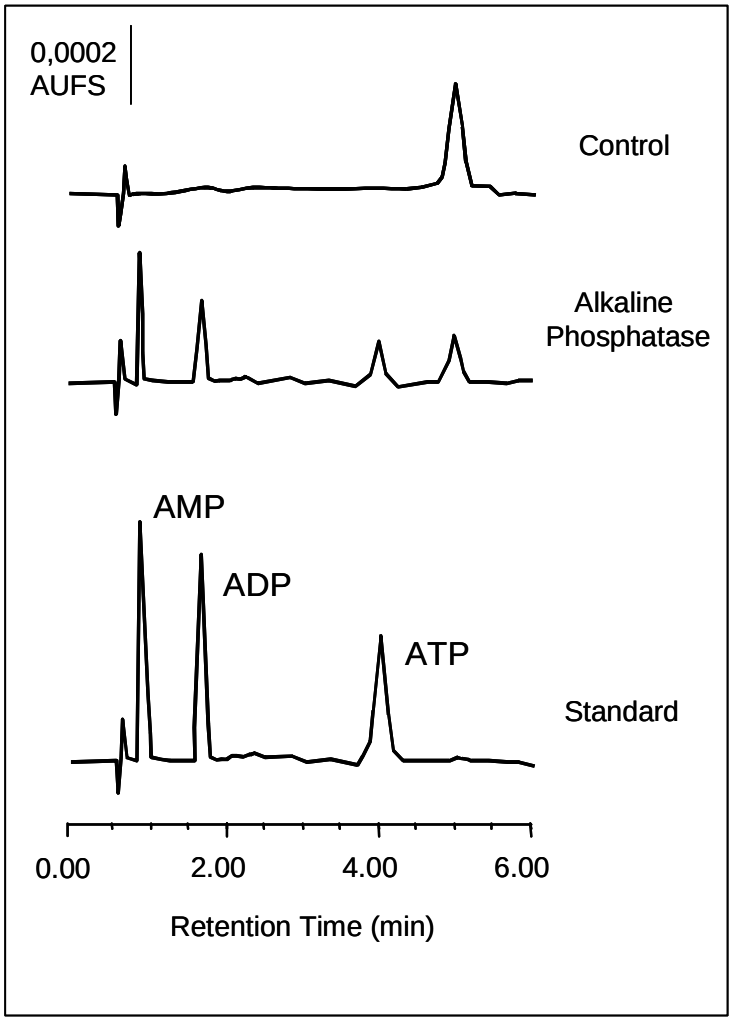

FIGURE 2

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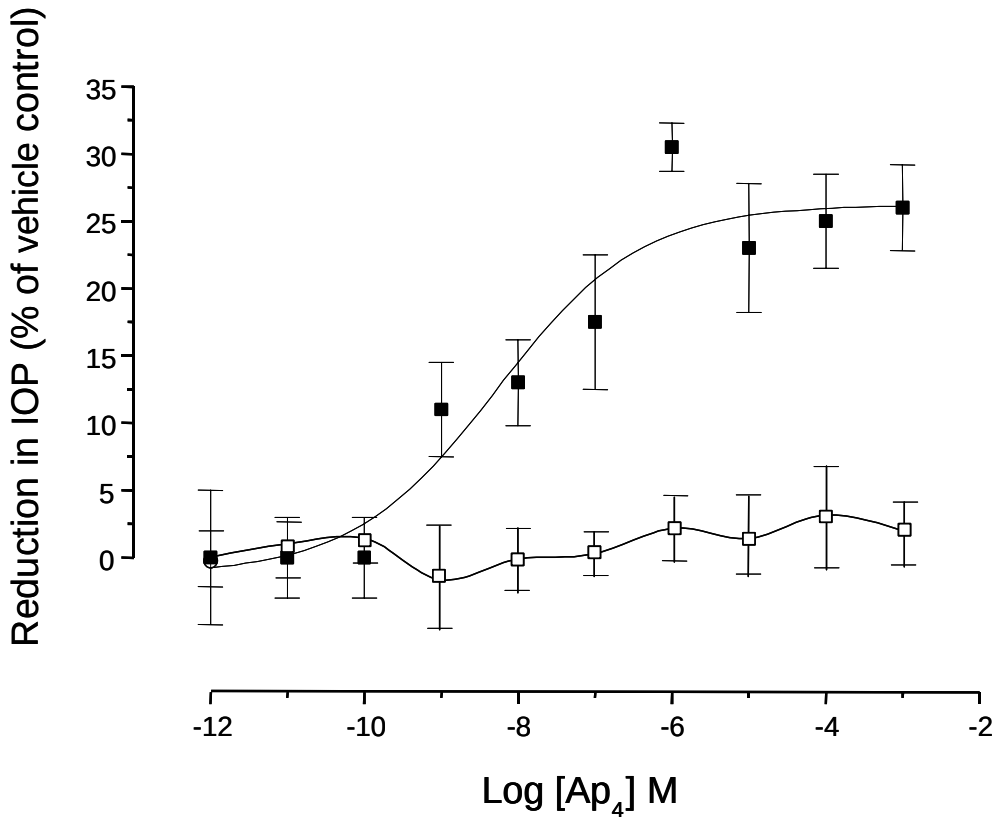

FIGURE 3

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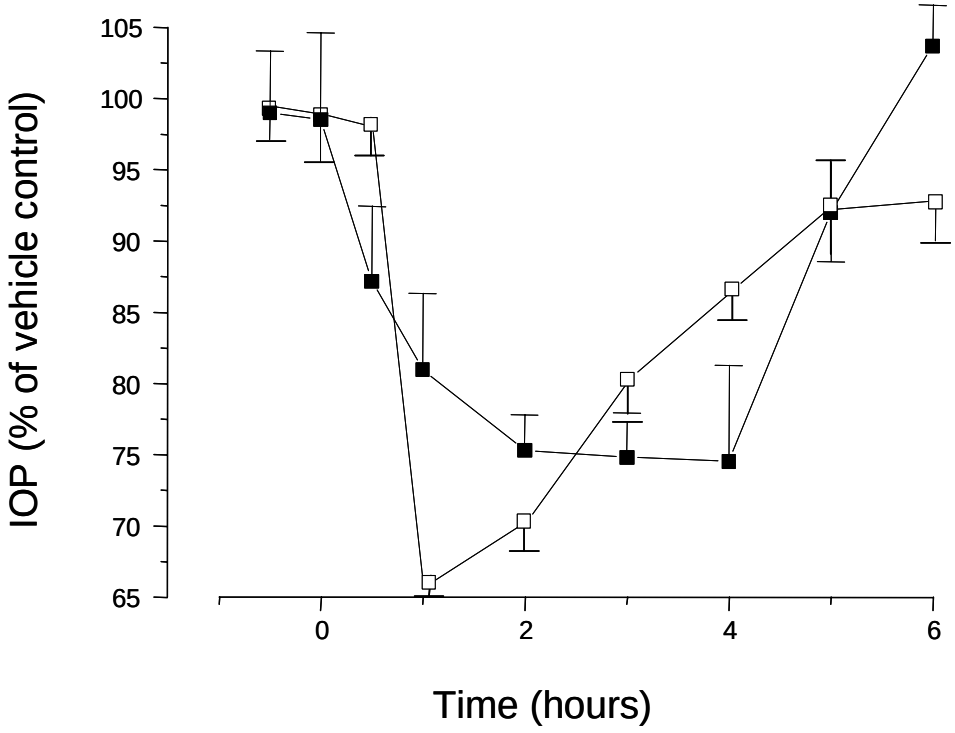

FIGURE 4

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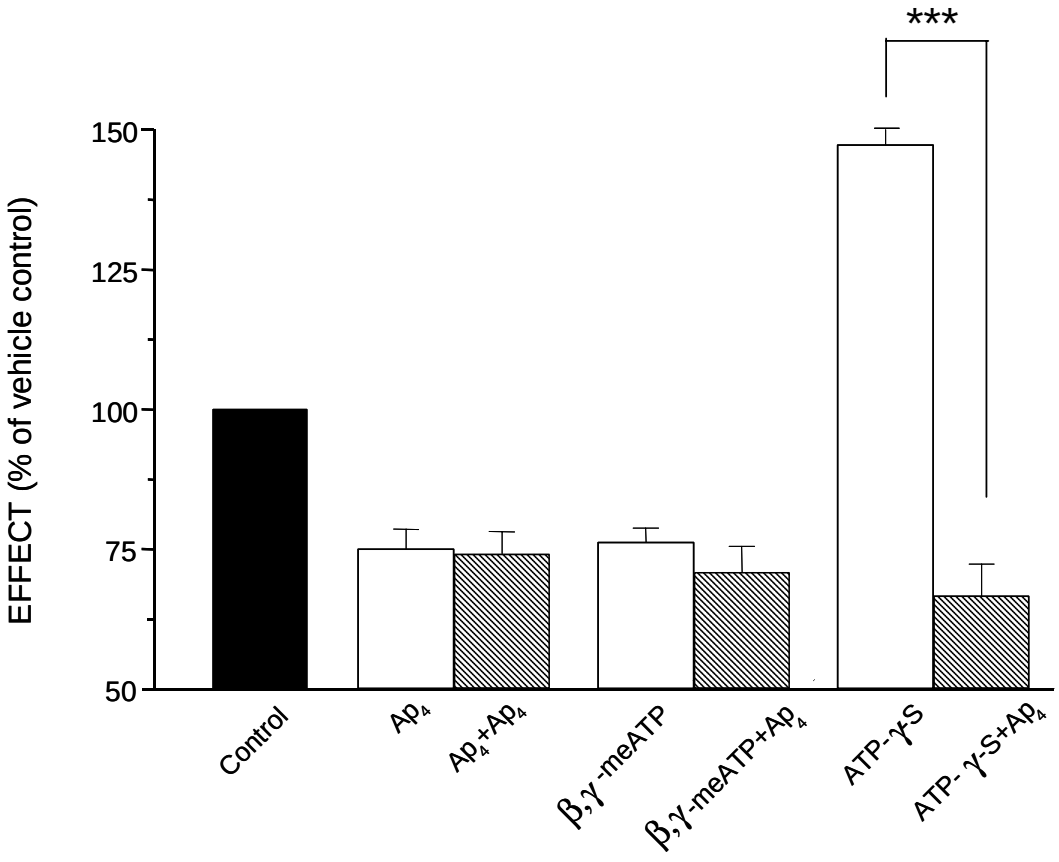

FIGURE 5

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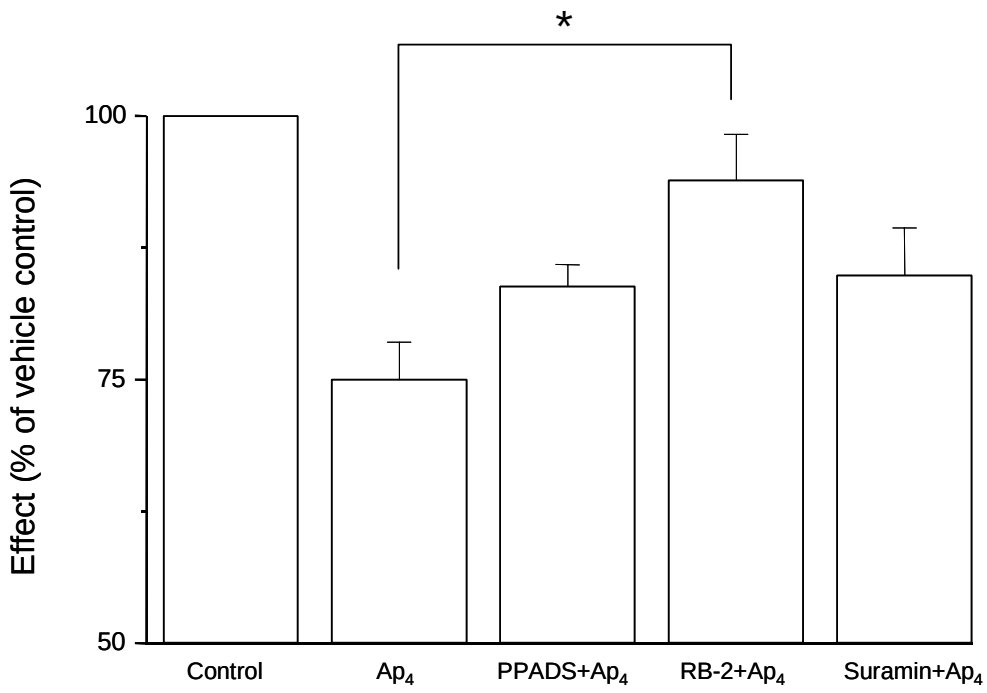

FIGURE 6

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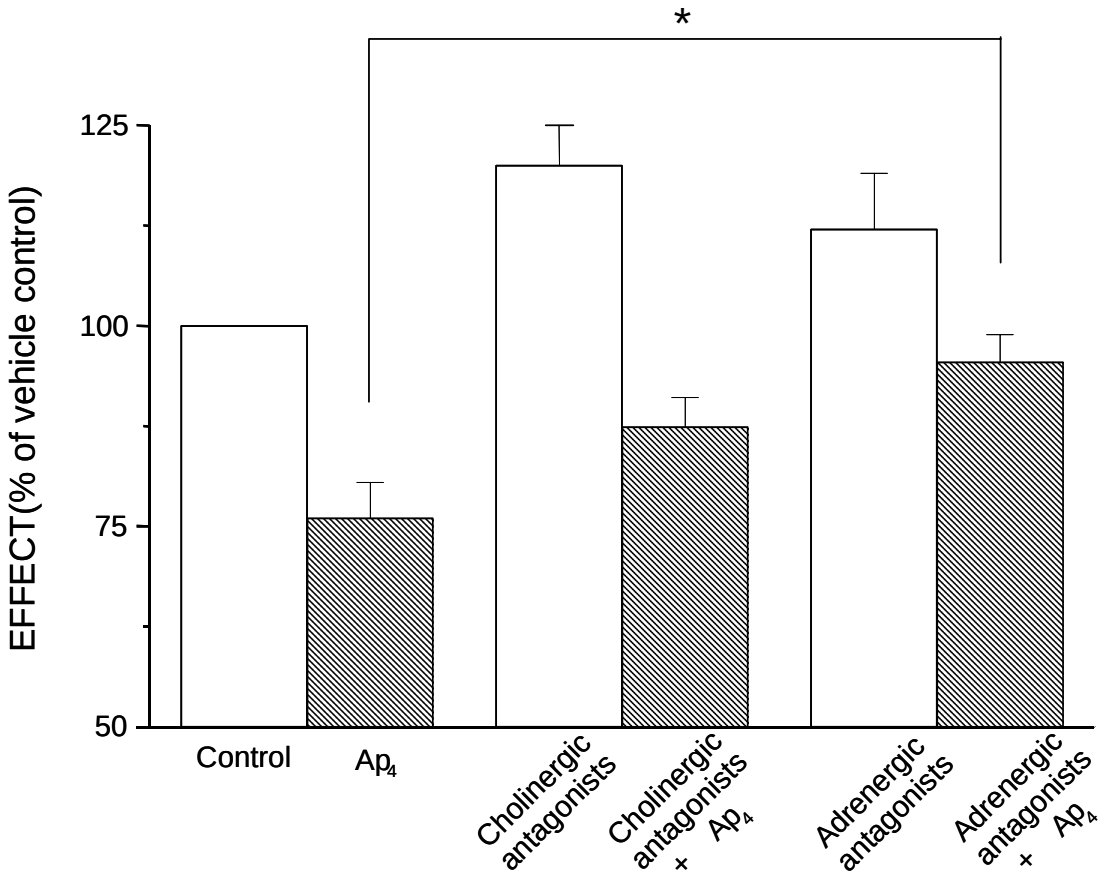

FIGURE 7

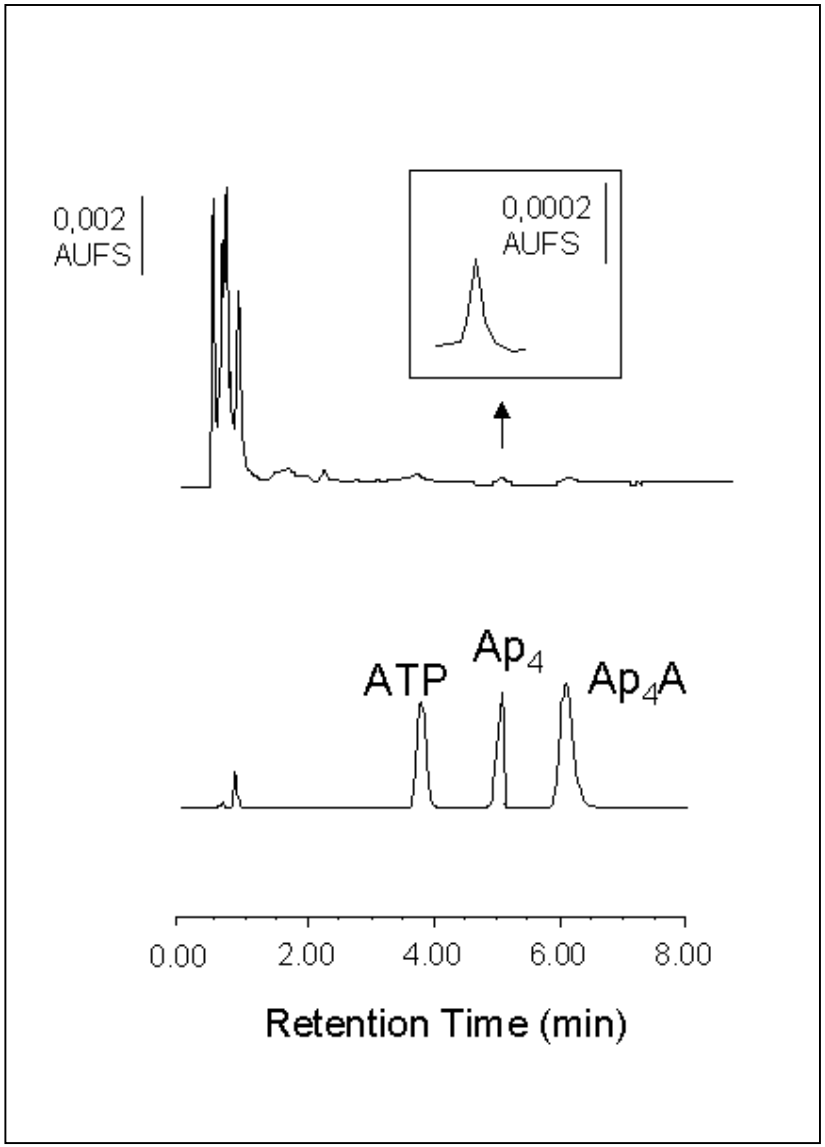

Figure 1

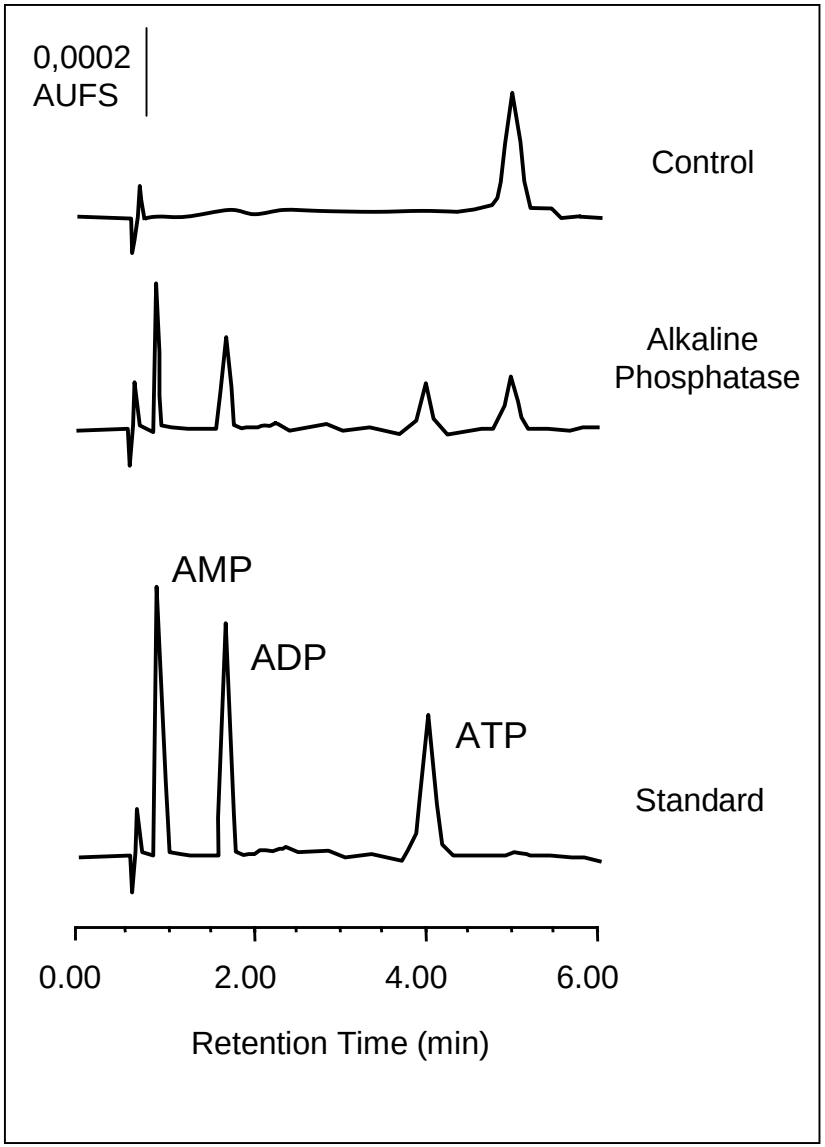

Figure 2

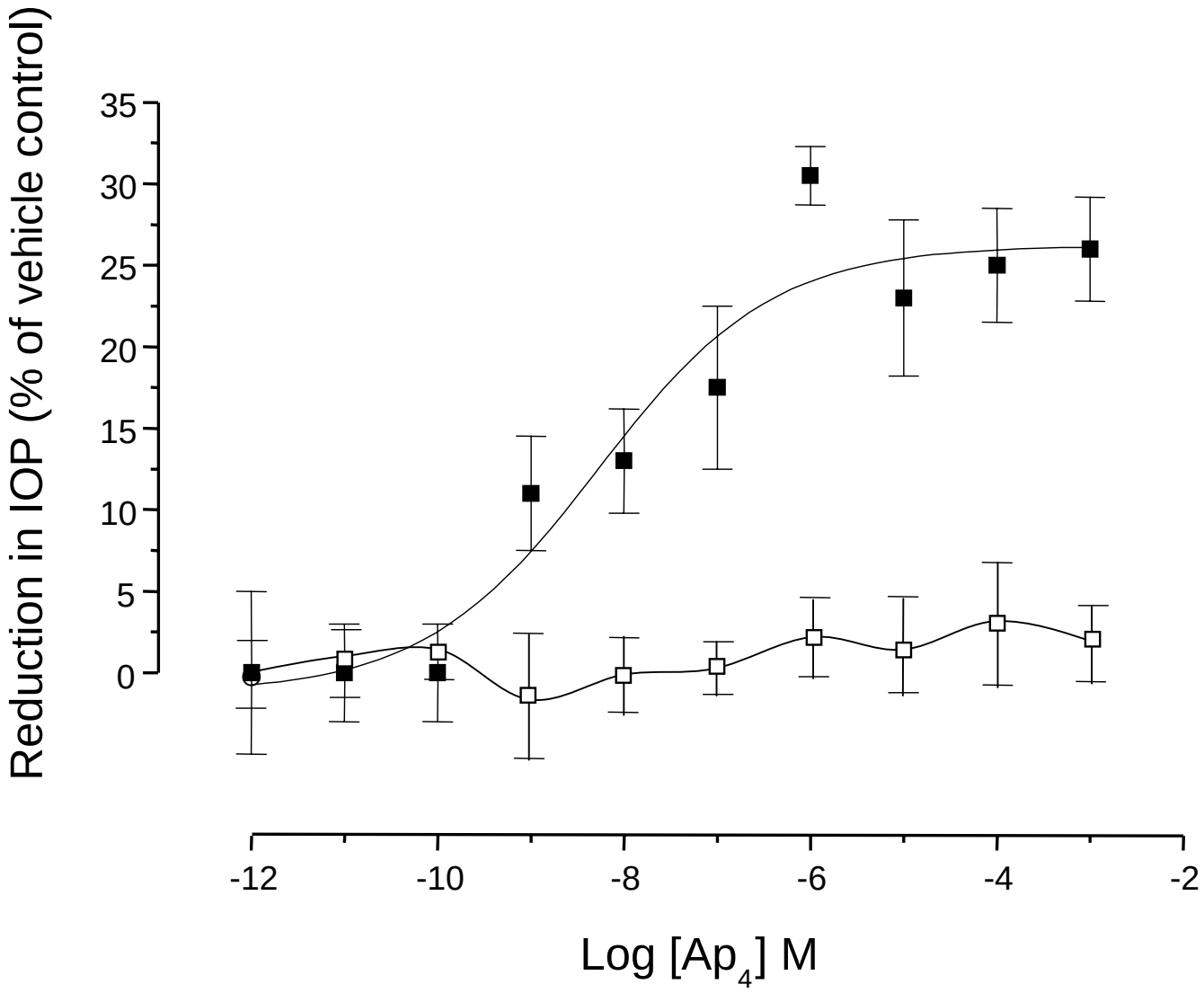

Figure 3

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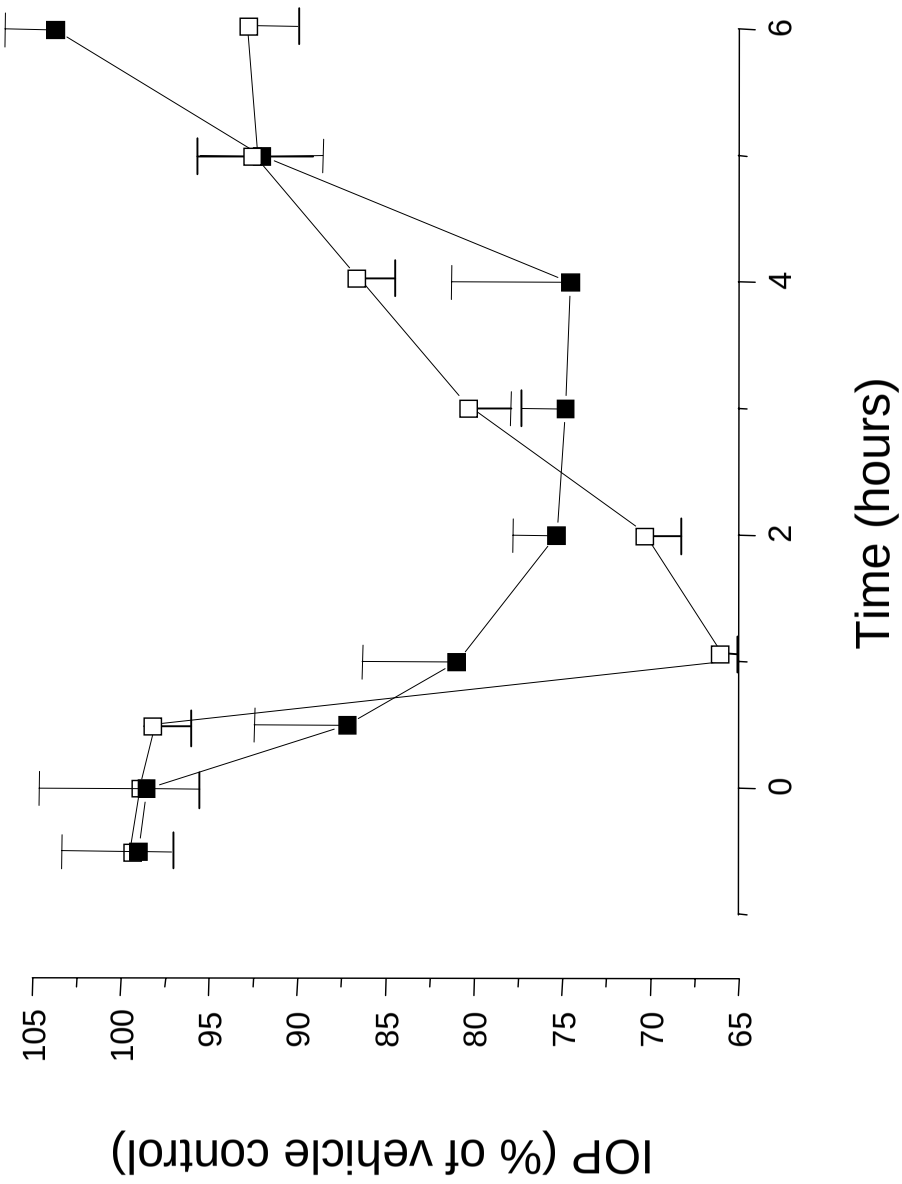
Figure 4



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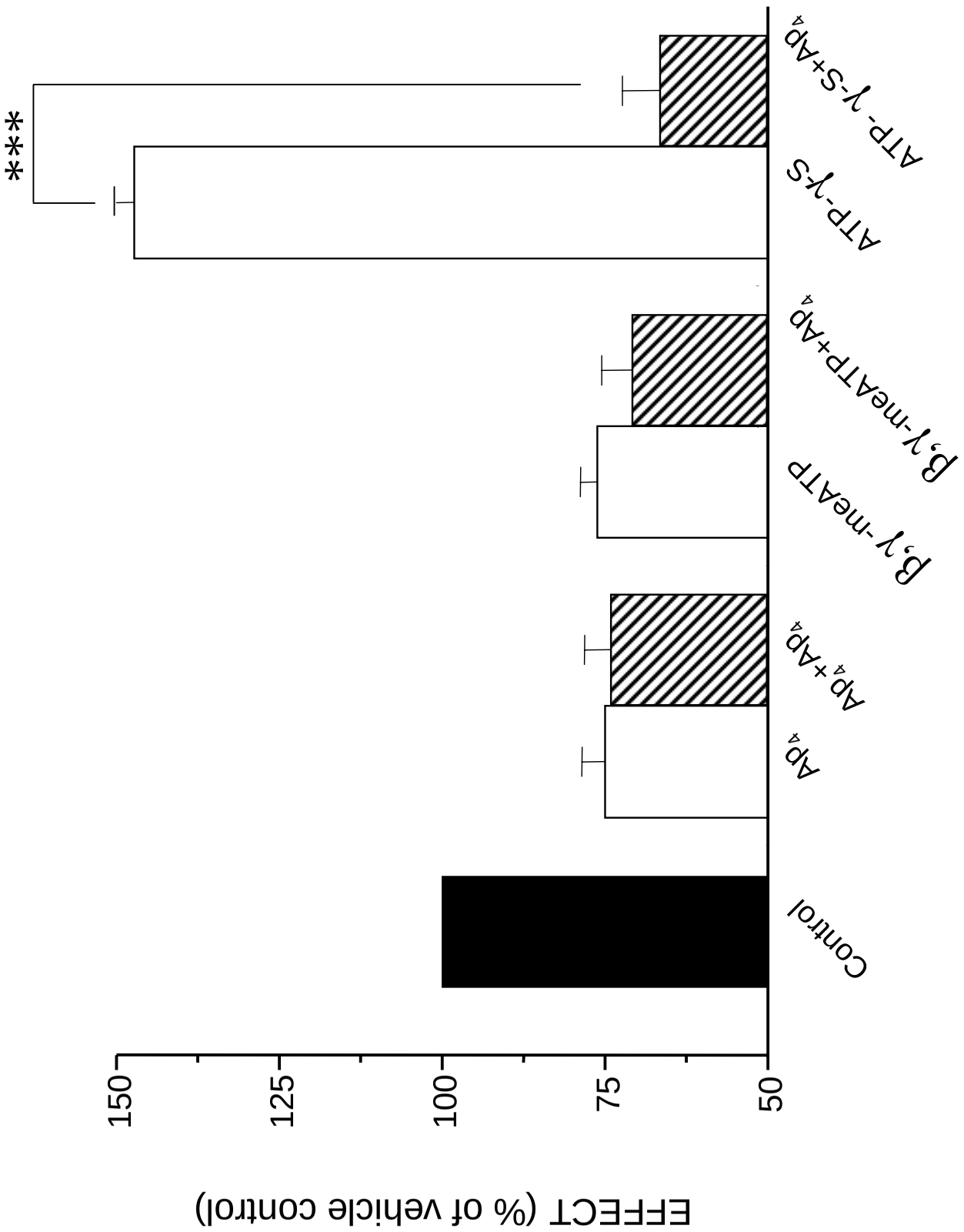

Figure 5

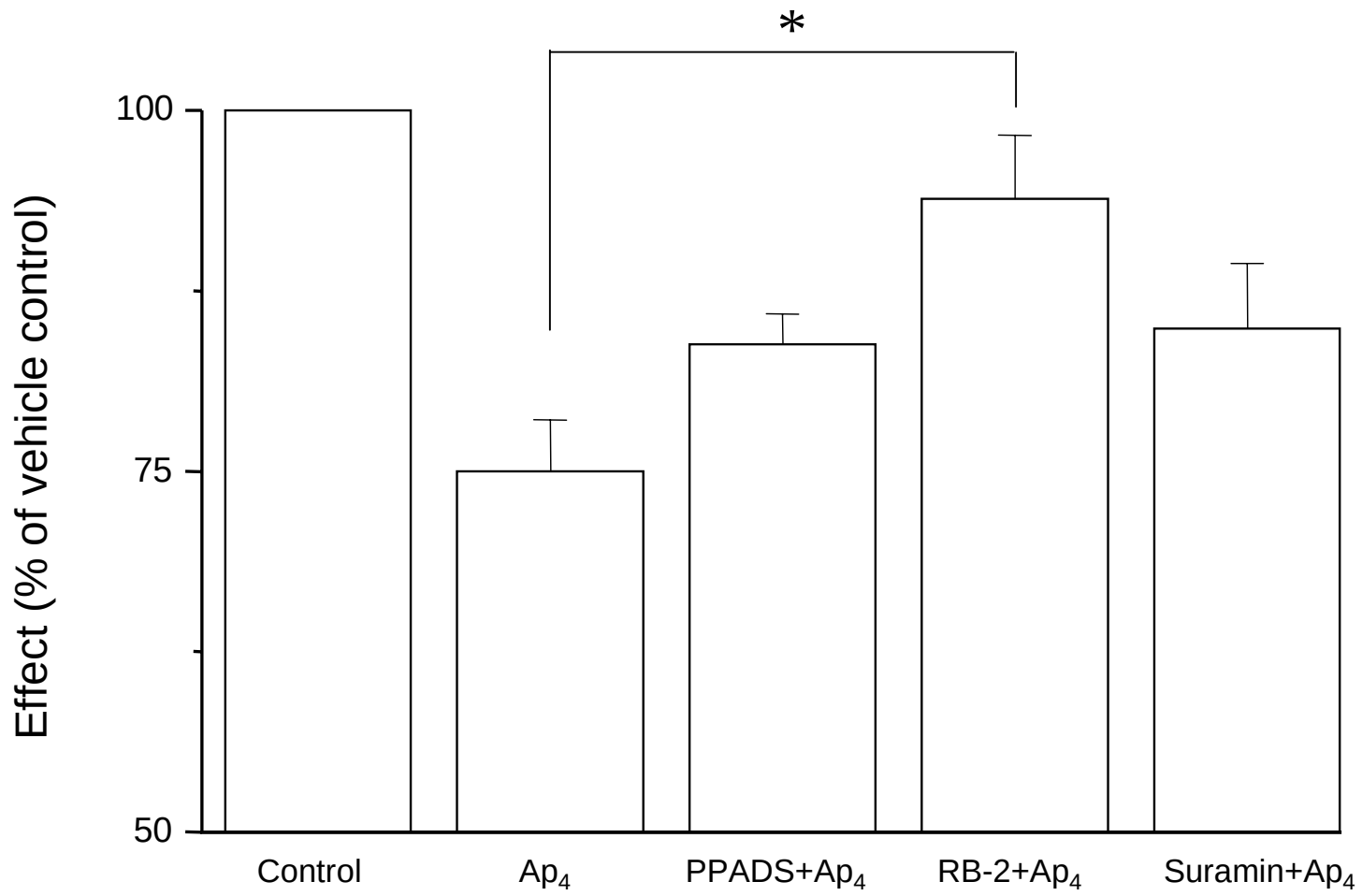

Figure 6

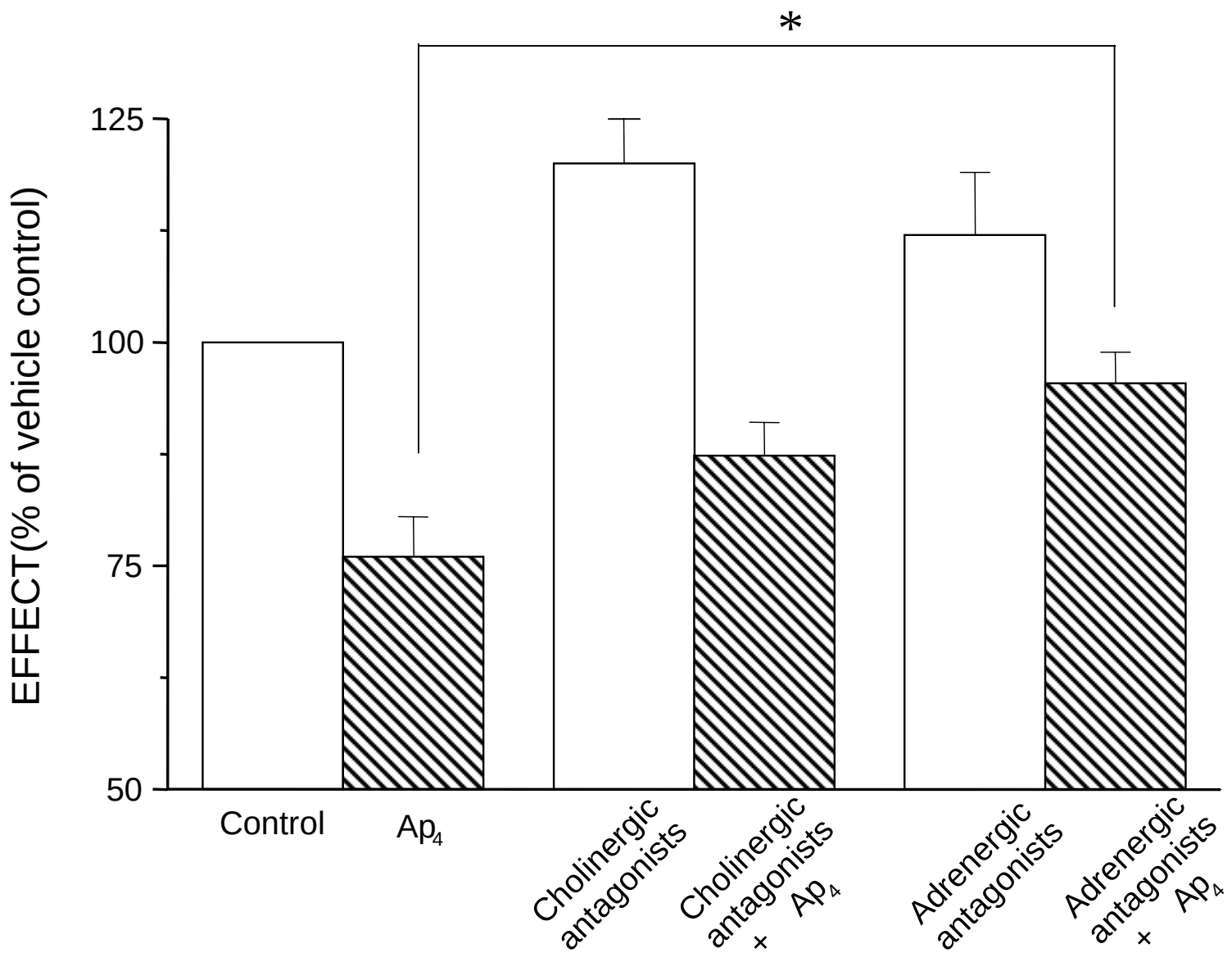

Figure 7