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Cytosolic Ca^{2+} and phosphoinositide hydrolysis linked to constitutively active α_{1D}-adrenoceptors in vascular smooth muscle

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Running title: Ca^{2+} and constitutively active α_{1D} adrenoceptors

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ABSTRACT

In the present study we analyzed changes in intracellular Ca^{2+} levels and inositol phosphate accumulation related to a population of α_{1D} adrenoceptors in rat aorta resembling constitutively active receptors. Following intracellular Ca^{2+} store depletion by noradrenaline in Ca^{2+} free medium and removal of the agonist, restoration of extracellular Ca^{2+} induced four signals: a biphasic (transient and sustained) increase in $[Ca^{2+}]_i$, inositol phosphate accumulation and a contractile response in the aorta. The transient increase in Ca^{2+}, the inositol phosphate accumulation and the contractile response were not observed in aortae incubated with prazosin or BMY 7378 (a selective α_{1D} adrenoceptor ligand), relating the three signals to α_{1D} adrenoceptor activity. In presence of nimodipine, only the sustained increase in Ca^{2+} and the inositol phosphate accumulation were observed, relating both signals to calcium entry through L-channels. The four signals were abolished by Ni^{2+}. In the rat tail artery, where α_{1D} adrenoceptors are not functionally active, restoration of extracellular Ca^{2+} after store depletion induced only a sustained increase in $[Ca^{2+}]_i$ without IP accumulation nor contractile response. Taken together these results suggest that in the aorta, Ca^{2+} entry is required for the recovery of cytosolic calcium levels and the display of the membrane signals related to the constitutive activity of α_{1D} adrenoceptors, i.e. inositol phosphate formation and Ca^{2+} entry through L-type channels, which maintains a contractile response once the agonist has been removed.

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G protein-coupled receptors may exist in a spontaneously active form in the absence of an agonist, i.e. constitutively active (De Ligt et al, 2000). This phenomenon has been most readily observed in cell lines in which receptors are overexpressed or mutated. Two different groups have found constitutively active cloned α_{1D}-adrenoceptors in stably transfected Rat-1 fibroblasts (García-Sainz & Torres-Padilla, 1999; McCune et al., 2000) and human embryonic kidney 293 cells (Chalothorn et al., 2002) which are mainly located in a perinuclear location (McCune et al., 2000; Chalothorn et al., 2002). In addition, we found a population of α_{1D}-adrenoceptors in intact rat arterial vessels such as the aorta, the iliac or the proximal mesenteric artery which exhibit several features resembling those of constitutively active receptors (Noguera & D'Ocon, 1993; Noguera et al., 1996; Gisbert et al., 2000, 2002; Ziani et al., 2002) such as: a) its activity occurs in the absence of an agonist, b) it is inhibited by the α_1-adrenoceptor ligand prazosin and the selective α_{1D}-adrenoceptor ligand BMY 7378 which behave as inverse agonists, c) the irreversible α_1-adrenoceptor antagonist chloroethylclonidine, acting as a neutral antagonist, inhibited noradrenaline-induced contractions in this tissue and did not affect the constitutive response but prevented its inhibition by BMY 7378 and prazosin, and d) it is only observed in vessels (e.g. aorta, iliac or proximal mesenteric arteries) where α_{1D} adrenoceptors play a functional role. However, as opposed to constitutively active α_{1D} adrenoceptors in transfected cells, this type of response in native tissues requires a prior stimulation with an α_1 -adrenoceptor agonist. Once the stimulus is removed, the α_{1D} adrenoceptor-dependent response remains and can be inhibited by inverse agonists. A simple protocol, in which intracellular Ca^{2+} depletion by

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noradrenaline is followed by extracellular Ca^{2+} restoration, permits the differentiation between the agonist-induced and constitutive activation of the receptors (Noguera et al., 1996).

The aim of the present work was to analyze how the activity of these adrenoceptors is regulated by cytosolic Ca^{2+} and how it couples to membrane signals as phosphatidil inositol hydrolysis. Therefore, we examined the contractile response linked to the α_{1D}-adrenoceptor constitutive activity together with signals associated with stimulation of G-proteins in the cell membrane, the inositol phosphate accumulation and the changes in the cytosolic Ca^{2+} levels.

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METHODS

Tissue preparation. Thoracic aorta and tail artery from Wistar rats (200-250 g) were dissected in a Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2 and glucose 11) and cut into rings (approx 3-5 mm in length). Endothelium was removed by gentle rubbing of the intimal surface with a metal rod. The absence of a relaxant response after the addition of acetylcholine (10 µM) in preparations pre-contracted by noradrenaline (1 µM) indicated the absence of a functional endothelium.

Simultaneous measurements of $[Ca^{2+}]_i$ and tension. Aortic and tail artery rings were incubated for 4-6 and 2-3 h, respectively, at room temperature in Krebs solution containing the fluorescent dye fura-2 acetoximethylester (5 µM). The castor oil derivative Cremophor EL (final concentration in Krebs 0.05%) was used to solubilize and facilitate fura-2 AM penetration. The adventitial layer distorts the fluorescence so the ring has to be illuminated from the intimal side. Therefore, aortic rings were inverted so that the luminal face was exposed outwards. Arterial vessels were then suspended under 1 g of tension in a 5 ml organ bath containing Krebs solution, maintained at 37ºC and gassed with 95% O_2 and 5% CO_2. The bath was part of a fluorimeter (CAF 110, Jasco, Tokyo) which allows the estimation of changes in the fluorescence intensity of fura-2 simultaneously with force development (Perez-Vizcaino et al., 1999). Rings were alternatively illuminated (128 Hz) with two excitation wavelengths (340 and 380 nm) from a xenon lamp coupled with two monochromators. The emitted fluorescent light at the two excitation wavelengths (F340 and F380) was measured by a photomultiplier through a 510 nm filter and recorded by using data acquisition hardware (Mac Lab, model 8e, AD

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Instruments Pty Ltd., Castle Hill, Australia) and data recording software (Chart v3.2, AD Instruments Pty Ltd.). Force data were recorded simultaneously by an isometric force-displacement transducer coupled to the Mac Lab data acquisition system. The absolute values of $[Ca^{2+}]_i$ were estimated from the ratio of emitted fluorescence obtained at the two excitation wavelengths (F340/F380) using the Grynkiewicz equation as described (Kanaide, 1999). The maximal and minimal F340 and F380 values for this equation were obtained by treatment with ionomycin (1.4 µM) and then with EGTA (8 mM), respectively. Autoflorescence, determined by quenching fura-2 fluorescence with $MnCl_2$ (1 mM) at the end of the experiment, was subtracted.

After equilibration for 30-45 min, the experimental procedure shown in Fig. 1 designed to evidence the constitutive activity of the α_{1D}-adrenoceptors was performed (Noguera et al., 1993; Gisbert et al., 2000). Initially, a response to a maximal concentration of noradrenaline (1 µM in the aorta and 10 µM in the tail artery) was elicited. After drug washout, the preparations were placed in a Ca^{2+}-free Krebs solution (containing 0.1 mM EDTA) for 20 min, which led to a weak loss in tension (<10-15 %) and a reduction in the $[Ca^{2+}]_i$ levels below resting values, and then they were exposed to noradrenaline for 5 min. This procedure was repeated twice and then after another 20 min in Ca^{2+}-free solution the bath media was replaced by a normal Ca^{2+}-containing Krebs solution which induced an increase in $[Ca^{2+}]_i$ levels and a contractile response indicative of constitutive activity of α_{1D}-adrenoceptors. In some experiments, the Ca^{2+} channel blockers nimodipine and Ni^{2+} and the α_{1D}-adrenoceptor ligands prazosin and BMY7378 were added during the last 10 min in Ca^{2+}-free and during the exposure to the Ca^{2+}-containing Krebs solution. In the experiments where guanethidine was used it was present throughout the experiment.

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Inositol phosphate determination. The determination of total inositol phosphate (IP) accumulation was adapted from Berridge et al. (1982) as has been previously described (Gisbert et al., 2000). Briefly, rat thoracic aortae or tail arteries were exposed to Krebs solution containing 10 $\mu Ci \cdot mL^{-1}$ of myo-[^{3}H]inositol (specific activity 70.0 –100.0 $Ci \cdot mM^{-1}$) for 2 h at 37ºC and gassed with 95% O_2 plus a 5% CO_2 mixture. Afterwards, tissues were washed twice with Krebs solution. Vessels were cut into rings (1 mm for aorta, 2 mm for tail artery) and pooled. Two pieces of tail artery or four rings of aorta were placed in individual tubes which were incubated at 37ºC. Different experimental conditions were applied in each determination (carried out in triplicate), as detailed in Figure 2. LiCl (10 mM) was added to inhibit the metabolism of inositol monophosphates. Incubation was stopped by placing the samples in a cold water bath (4ºC) and adding 2 ml of a cold mixture of methanol/chloroform/HCl (40:20:1, v/v/v). Samples were sonicated for 35 min at 2º-3ºC in an ultrasonic water bath and, after the addition of 0.63 ml chloroform and 1.26 ml distilled water, centrifuged at 1500 g for 10 min to facilitate phase separation. The aqueous layer was removed from the tubes to assay the IP formation. Each sample was neutralized and run through an AG1-X8 column, formate form, 100-200 mesh (Bio-Rad, Hercules, Ca). The resin was washed successively with 6 ml of water and 6 ml of 60 mM ammonium formate-5 mM sodium tetraborate to eliminate free myo-[^{3}H]- inositol and glycerophosphoinositol, respectively. Total IPs were eluted with 3 ml of 1M ammonium formate-0.1 M formic acid. The eluent fractions were collected and counted in a scintillation counter. The lipid layer remaining after removal of the aqueous phase was used for measurement of [^{3}H]-phosphatidylinositols. Accumulation of [^{3}H]-IP was routinely calculated as a percentage (dpm %) of total [^{3}H]-inositol labeled lipids in each individual sample to correct interexperimental

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variations in label incorporation and sample sizes or was expressed as a percentage above the unstimulated [^{3}H]-IP accumulation (basal).

Chemicals. Acetylcholine, (-)-noradrenaline, prazosin, lithium chloride, $NiCl_2$, cremophor EL, nimodipine and guanethidine were purchased from Sigma (St. Louis MO, U.S.A.), BMY 7378 from RBI (Natick MA, U.S.A.), myo-[^{3}H]inositol from Amersham (Buckinhamshire, England) and fura-2 acetoximethylester (1 mM solution in dimethyl sulfoxide) from Calbiochem (La Jolla, CA, USA). Other reagents were of analytical grade. All compounds were dissolved in distilled water.

Statistical analysis. The results are presented as the mean ± S.E.M. for *n* determinations obtained from different animals. Where ANOVA showed significant differences ($P< 0.05$), the results were further analyzed using the Student Newman-Keuls test (Graph Pad Software; San Diego, California, U.S.A). Differences between phasic and tonic responses were analyzed by means of a paired Student t test.

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RESULTS

$[Ca^{2+}]_i$ signal and contractility linked to the constitutively active population of α_{1D}-adrenoceptors. Representative traces of the changes in contractile force and $[Ca^{2+}]_i$ obtained using the protocol designed to evidence the constitutive activity of the α_{1D}-adrenoceptors in fura-2 loaded arteries are shown in Figure 1. The contractile responses were similar to those previously obtained in tissues not loaded with fura-2 and mounted in conventional organ baths (Noguera et al., 1996). The basal $[Ca^{2+}]_i$ values were 120 ± 19 and 129 ± 15 nM, in the aorta and tail artery, respectively (n = 4). Noradrenaline (NA1 in Figures 1, 3 and 4) evoked a rapid and transient ("phasic response") increase in $[Ca^{2+}]_i$ (224 ± 33 and 251 ± 17 nM, respectively), followed by a decrease to a sustained level ("tonic response") (193 ± 45 and 187 ± 14 nM, respectively, after 5 min) Changes in intracellular calcium levels were accompanied by a slower contractile response (7.86 ± 1.25 and 9.87 ± 1.38 mN, respectively). The responses measured at 5 min were used as a control (100%) of the responses obtained thereafter in each preparation. After washing and recovery of basal contractile tone the bathing media was changed to a Ca^{2+}-free solution, and a decrease in the basal tone (-10 ± 5%, n = 8, and –5 ± 4%, n = 5, in the aorta and tail artery, respectively, $P > 0.05$) and in the $[Ca^{2+}]_i$ (-81 ± 12% and –87 ± 26% in the aorta and tail artery, respectively, $P < 0.05$ for both) was observed. After 20 min under these conditions, the addition of noradrenaline (NA2 in Figures 1, 3 and 4) induced a transient increase in $[Ca^{2+}]_i$, which represents an index of the content of agonist-sensitive intracellular Ca^{2+} stores, and a phasic contraction. Washing with Ca^{2+}-free medium induced a further decrease in $[Ca^{2+}]_i$. Upon a second application of the agonist in Ca^{2+}-free solution (NA3 in Figures 1, 3 and 4),

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only weak increases in contractile force and $[Ca^{2+}]_i$ were evoked, which indicated an almost complete depletion of internal Ca^{2+} stores sensitive to noradrenaline. In the aorta, after repeated washing with Ca^{2+}-free solution in the absence of noradrenaline for 20 min, restoring the Ca^{2+}-containing solution induced a transient increase (phasic) followed by a decrease to a sustained level (tonic) in $[Ca^{2+}]_i$, so that the $[Ca^{2+}]_i$ returned to the initial resting levels. This was associated with a contractile response (60.2 ± 11.2% of the control response to noradrenaline, n = 8) (Figures 1 and 3). In contrast, in the tail artery, upon restoring the Ca^{2+}-containing solution, $[Ca^{2+}]_i$ also returned to the initial resting values (Figures 1 and 4) but the response was not clearly biphasic as in the aorta and only a slight increase in force was observed (8.8 ± 2.1% of the control response to noradrenaline, n = 5).

IP accumulation linked to the constitutively active population of α_{1D}-adrenoceptors. The experiments of IP accumulation were performed following a protocol previously described by us (Gisbert et al., 2000) which attempts to reproduce the conditions of the contractility studies (Figure 2).

Basal IP accumulation (sample 1 in Figure 2) in rat aorta (9.55 ± 0.62 dpm %, n = 14) was significantly higher ($p<0.001$) than that obtained in rat tail artery (6.96 ± 0.44 dpm %, n = 14). In order to test if this increase in activity was due to the constitutive activity of α_{1D}-adrenoceptors in rat aorta, we analyzed the effects of two α_1 ligands on this basal activity. Prazosin (1 μM) and BMY 7378 (10 μM) which exhibit selective affinity for α_1- and α_{1D}-adrenoceptors, respectively, did not inhibit the basal accumulation of IP in rat aorta (Prazosin: 98.0 ± 7.7 % respect to control, n = 5; BMY7378 : 102.7 ± 7.7 % respect to control, n=4)

Noradrenaline increased IP accumulation in Ca^{2+}-containing solution in the aorta (19.9 ± 1.6 dpm %, n=16) and tail artery (79.8 ± 1.7 dpm %, n=5). Despite this

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the increase in $[Ca^{2+}]_i$ was progressively reduced upon successive applications of noradrenaline in Ca^{2+}-free medium and the IP accumulation in Ca^{2+}-free medium was similar to that in a Ca^{2+}-containing medium (NA1, NA2 and NA3 in Figures 3 and 4). These results confirm that the progressive reduction in $[Ca^{2+}]_i$ responses to successive applications of noradrenaline in Ca^{2+}-free medium was due to Ca^{2+}-store depletion but not to changes in IP accumulation. After the depletion of the intracellular Ca^{2+} stores sensitive to noradrenaline, restoration of extracellular Ca^{2+} (sample 6, S) induced a significant increase in the IP accumulation in the aorta (Figure 3C) but not in the tail artery (Figure 4C).

Effects of α_1-adrenoceptor ligands and guanethidine. The effects of prazosin (1 µM) and BMY 7378 (0.1 µM) were tested to analyze whether the responses observed upon restoration of extracellular Ca^{2+} were due to the constitutive activity of α_1-adrenoceptors. In the rat aorta, prazosin and BMY 7378 added 10 min before and during the exposure to Ca^{2+}-containing solution had no effect on basal $[Ca^{2+}]_i$ or resting tone but produced strong (> 80%) inhibitory effects on the contraction induced by restoring extracellular Ca^{2+} (Figure 5). In the presence of prazosin or BMY 7378, the initial component of the $[Ca^{2+}]_i$ signal (phasic in Figure 5) was similar to the sustained increase in $[Ca^{2+}]_i$ (tonic in Figure 5) induced by restoring extracellular Ca^{2+}. Both of them were similar to the sustained increase in $[Ca^{2+}]_i$ observed in the control aortae. The time courses of the $[Ca^{2+}]_i$ signals and contractile responses in control and in BMY 7378-treated arteries are compared in Figure 7.

To rule out the possibility that the release of noradrenaline from nerve terminals could play a role on the $[Ca^{2+}]_i$ signals and contractile responses upon restoring extracellular Ca^{2+}, the aortae were treated with guanethidine (5 µM)

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throughout the experimentn the presence of this drug, the transient and sustained changes in [Ca2+]i and the contractile responses induced by restoring extracellular Ca2+ were similar to those in parallel control experiments (85 ± 10%, 86 ± 6% and 102 ± 7%, respectively, n = 3).

In the tail artery, prazosin (1 μM) had no effect on either the increase in the $[Ca^{2+}]_i$ levels or the contractile response induced by restoration of extracellular Ca^{2+} (n = 3).

Sensitivity of the intracellular signals to Ca^{2+} channel blockers in the rat aorta. In order to analyze the role of extracellular Ca^{2+} entry on intracellular signals ($[Ca^{2+}]_i$ and IP accumulation), another set of experiments were performed in the presence of Ni^{2+} (1 mM) or the specific L-type voltage-dependent Ca^{2+} channel blocker nimodipine (0.1 nM). After depletion of internal Ca^{2+} stores by noradrenaline, the Ca^{2+} channel blockers were added 10 min before and during the exposure to Ca^{2+}-containing solution. In the experiments addressed to determine the IP accumulation, the Ca^{2+} channel blockers were added in sample 7 (Figure 2). Neither nimodipine nor Ni^{2+} modified resting $[Ca^{2+}]_i$ or basal tone. Figure 6 shows that nimodipine inhibited the contractile response (Figure 6A) but not the increase in the IP levels (Figure 6C) upon restoration of extracellular Ca^{2+}. In addition, nifedipine (0.1 μM) was also without effect on the IP accumulation in the aorta (41.2 ± 7.7%, n = 5 *vs* 55.5 ± 6.1% above basal values, n = 13, P > 0.05). In the presence of nimodipine, the initial increase in $[Ca^{2+}]_i$ (phasic in Figure 6B) was similar to the sustained response (tonic in Figure 6B) and both of them were similar to the sustained component of the control (Figure 6B). This can be more clearly observed

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in Figure 7 which also shows that the time course of the changes in $[Ca^{2+}]_i$ and tone in the presence of nimodipine was very similar to that observed in the presence of BMY 7378. Addition of Ni^{2+} produced a very strong inhibitory effect (> 90%) on all the signals associated with the restoration of extracellular Ca^{2+}, i.e. the increases in contractile tone, the phasic and tonic increases in $[Ca^{2+}]_i$ and the IP levels (Figure 6). The IP accumulation in Ni^{2+}-treated arteries was very similar to that observed when Ca^{2+} was not included in the medium (sample 8 in Figure 2 ; Figure 6C).

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DISCUSSION

We have previously shown that in vessels where the α_{1D}-adrenoceptors play a functional role, following an adrenergic stimulus, a population of α_{1D}-adrenoceptors temporarily remains in a constitutively active state when the stimulus disappears (Noguera et al., 1996; Gisbert et al., 2000). There is an experimental procedure which allows us to easily differentiate this constitutive activity from the response induced by an adrenergic stimulus. The assumption that the contractile response observed after removal of the agonist is due to the constitutive activity of α_{1D}-adrenoceptors is based on previous evidences as described above (refer to introduction). In addition, present results show that guanethidine, a noradrenergic neuron blocking drug, had no effect on these responses, ruling out a possible role for noradrenaline release from nerve terminals. However, despite noradrenaline not being present during the constitutive response, it is absolutely necessary as a previous α_1-adrenoceptor stimulus. In fact, a similar response was obtained by depletion of internal Ca^{2+} stores by other α_1-adrenoceptor agonists such as methoxamine and phenylephrine, whereas clonidine, serotonin, caffeine, ryanodine, thapsigargin and cyclopiazonic acid, which deplete Ca^{2+} stores in an α_1 – adrenoceptor-independent manner, did not elicit any contractile response when extracellular calcium was restored (Noguera & D'Ocon, 1993; Noguera et al., 1996; 1997; 1998).

α_1–Adrenoceptors signal through both pertussis toxin-sensitive G-proteins and $G_{q/11}$ proteins located in the cell membrane (Garcia-Sainz et al., 1999; Piascik & Perez, 2001). They mobilize intracellular Ca^{2+} as a consequence of the IP accumulation and activate Ca^{2+} influx via voltage-dependent and independent Ca^{2+}

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channels (Minneman, 1988; Zhong & Minneman, 1999; Inoue et al., 2001). The α_{1D}-adrenoceptor subtype is located mainly intracellularly in a perinuclear orientation(McCune et al., 2000; Chalothorn et al., 2002), then, the receptors must migrate to the cell membrane in order to interact to $G_{q/11}$-proteins located in it, therefore, the mechanisms involved in this migration would control the contractile response related to the constitutively active α_{1D}–adrenoceptors. This hypothesis prompted us to analyze, the increase in the contractile tone and the intracellular signalling ($[Ca^{2+}]_i$ and IP accumulation) associated with the constitutive activity of the α_{1D} subtype.

Ca^{2+} signal

In rat aorta, after depletion of noradrenaline sensitive Ca^{2+} stores and restoration of extracellular Ca^{2+} in the absence of the agonist, the increase in $[Ca^{2+}]_i$ represents a complex phenomenon in which transient and sustained components could be differentiated. In order to pharmacologically analyze the changes in $[Ca^{2+}]_i$, we tested the effects of two Ca^{2+} channel blockers, nimodipine and Ni^{2+}, an α_1 adrenoceptor ligand, prazosin, and a selective α_{1D} adrenoceptor ligand BMY7378.

In presence of nimodipine the transient component of $[Ca^{2+}]_i$ increase was not evident and the contractile response was strongly inhibited. Therefore, Ca^{2+} entry through L-type voltage operated channels is responsible for the transient component of $[Ca^{2+}]_i$ increase and it is involved in the contractile process. The transient and the sustained components of Ca^{2+} entry as well as the associated contractile response were blocked by Ni^{2+}, a non specific Ca^{2+} channel blocker. This cation is commonly used to block store operated Ca^{2+} channels (SOCs, Jung et al., 2000; Kukkonen & Akerman, 2001), non selective cationic channels which activate upon Ca^{2+} store depletion. Despite SOCs being the most reasonable targets for Ni^{2+} in our

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conditions, the present experiments do not exclude other Ca^{2+}-entry pathways such as the store depletion-independent Ca^{2+} entry pathway recently associated with α_1 adrenoceptors (Inoue et al., 2001).

In presence of prazosin or BMY 7378, the transient component has not been observed, but the sustained increase in $[Ca^{2+}]_i$ was not affected, which confirmed that both components of calcium entry could be pharmacologically distinguished. The α_1-adrenoceptor ligand-sensitive increase in $[Ca^{2+}]_i$ was remarkably similar to the nimodipine-sensitive one, suggesting that the constitutive activity of α_{1D}-adrenoceptors is required for the activation of L-type channels permitting calcium entry which contributes to contraction. The sustained increase in $[Ca^{2+}]_i$ restores cytosolic calcium levels but is not involved in the contractile response.

In tail artery, where a functional role of α_{1D}-adrenoceptors can be excluded (Lachnit et al., 1997; Gisbert et al., 2000) only a sustained increase in $[Ca^{2+}]_i$ was observed upon restoration of extracellular Ca^{2+}. This response was insensitive to α_1-adrenoceptor ligands and was not accompanied by contraction. The lack of functional α_{1D}-adrenoceptors in the tail artery is likely to be responsible for the absence of the transient $[Ca^{2+}]_i$ increase and the contractile response observed in this tissue.

IP signal

Previous (Gisbert et al., 2000) and present results indicate that, in the absence of the agonist, restoration of extracellular Ca^{2+} after depletion of noradrenaline-sensitive intracellular stores increased IP accumulation in rat aorta but not in tail artery. This IP accumulation was inhibited by prazosin and BMY 7378 (Gisbert et al., 2000), which confirmed the dependence of the signal on α_{1D}-

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adrenoceptor activity. Interestingly, as present results show, the IP accumulation observed in absence of the agonist is only obtained when extracellular calcium entry restores cytosolic calcium levels. Moreover, Ni^{2+}, which almost suppressed the increase in $[Ca^{2+}]_i$, also abolished the IP accumulation upon restoration of extracellular Ca^{2+}. The fact that nimodipine, which inhibits Ca^{2+} entry through L-channels strongly inhibited the associated contractile response, but did not affect the restoration of cytosolic Ca^{2+} levels nor abolish the IP accumulation, confirms that Ca^{2+} entry through L-type channels is the consequence of the constitutive activity of α_{1D}-adrenoceptors and is essential for contraction but not for IP accumulation due to constitutively active α_{1D} adrenoceptors. Therefore, and this is a crucial point of this study, these results suggest that the constitutively active α_{1D}-adrenoceptors, located intracellularly, require a physiological level of cytosolic Ca^{2+} to promote IP accumulation.

An important issue which arises from the present results is whether the α_{1D} adrenoceptors are “truly” constitutively active in native tissues if they need calcium to evidence their activity. We can suppose that calcium acts directly on the receptor changing its conformation from an inactive to an active state. However, our results do not sustain this hypothesis because neither, prazosin nor BMY7378 affected the basal accumulation of IP in aorta indicating that, in presence of physiological levels of cytosolic calcium, we can not observe constitutive activity in vessels not previously stimulated by an α_1-adrenoceptor agonist. This evidence suggests that calcium plays a role facilitating the coupling of constitutively active α_{1D} adrenoceptors to G proteins but does not act directly on changing the conformation of the receptor.

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It is well known that α_{1D} adrenoceptors are intracellularly located in a perinuclear orientation (McCune et al., 2000; Chalothorn et al.,2002), but $G_{q/11}$ proteins which mediate IP accumulation are in the cell membrane then, if α_{1D} adrenoceptors are in an active conformation but intracellularly located, they need to migrate to the cell membrane to couple to $G_{q/11}$ proteins and induce IP accumulation. We propose that calcium permits this migration and, when cytosolic calcium levels are very low, the constitutively active receptors can not migrate therefore, membrane signals such as IP accumulation can not be observed.

Another essential question is the exact role that the previous adrenergic stimulus played in the population of α_{1D} adrenoceptors. We can exclude that residual noradrenaline could be activating the receptors giving a "persistent activation" instead of a "constitutive activity" for the following evidences: i) it is clear from previous works (Noguera et al., 1996; 1997; Gisbert et al., 2000) that noradrenaline-induced contractile responses and constitutively active α_{1D}–adrenoceptor induced responses are pharmacologically distinguishable: the first is chloroethylclonidine sensitive and nimodipine insensitive whereas the second is chloroethylclonidine insensitive and nimodipine sensitive; ii) noradrenaline-induced IP accumulation was not dependent on $[Ca^{2+}]_i$ because noradrenaline induced similar increases in IP accumulation in Ca^{2+}-containing (NA1 in Figure 3) and Ca^{2+}-free medium (NA2 and NA3 in Figure 3) despite $[Ca^{2+}]_i$ levels being much lower in the latter conditions; however, as has been previously discussed, IP accumulation related to constitutively active α_{1D} adrenoceptors depends on calcium then, if NA persistently activates α_{1D} adrenoceptors, Ca^{2+} would not be needed to observe the IP accumulation.

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Then, if noradrenaline is not present, two different possibilities must be considered. One of them is the possibility that intracellularly located α_{1D} adrenoceptors were activated by adrenergic agonists and remained temporarily in an active conformation when the agonist disappeared. In this case, the constitutive activity of the α_{1D} adrenoceptors is temporal, not an intrinsic property of the receptor, and depends directly on a previous stimulus. The other possibility is the consideration that the α_{1D} adrenoceptors would always be in an active conformation but intracellulary located and the adrenergic stimulus was needed as a signal for its recruitment to the external membrane permitting the coupling to G proteins located in it. This agonist-induced recruitment of cytosolic adrenoceptors has been previously described with regard to the α_{1A} subtype (Holtbäck et al., 1999) Our present results do not permit to differentiate if the constitutive activity of α_{1D} adrenoceptors is a temporal or an essential property of these receptors but previous studies (García-Sainz & Torres-Padilla, 1999; McCune et al., 2000) using cloned α_{1D} adrenoceptors expressed in different cell lines have shown constitutive activity for this subtype, independent on previous adrenergic stimulus, then, we can suggest that this is an essential property of the receptor also, in native tissues.

In conclusion, our experiments demonstrated that, after an adrenergic stimulus, a population of α_{1D} adrenoceptors remains in an active conformation. In Ca^{2+} free medium, the low level of cytosolic Ca^{2+} reached following the depletion of noradrenaline-sensitive intracellular Ca^{2+} stores does not permit the display of the membrane signals associated to the constitutive activity of α_{1D}-adrenoceptors. When extracellular Ca^{2+} is restored, Ca^{2+} entries into aortic smooth muscle cells by means of two processes with different time courses and pharmacology. A Ni^{2+}-sensitive

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increase in $[Ca^{2+}]_i$ is required to evidence the constitutive activity of α_{1D}-adrenoceptors in native tissues as indicated by increased IP accumulation. An additional $[Ca^{2+}]_i$ increase through L-type channels is triggered by constitutively active α_{1D}-adrenoceptors and is responsible of the contractile response.

Under physiological conditions the cytosolic Ca^{2+} levels are high enough to permit the coupling of the constitutively active α_{1D}-adrenoceptors to the membrane signals, and its participation in the contractile response. These processes permit that the contraction, triggered by an adrenergic stimulus, would be temporarily sustained, even when the stimulus was removed (Ziani et al., 2002).

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References

Berridge MT, Dawson RMC, Downes ES, Heslop JP, Irvine RF (1982) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem J* **212**:473-482.

Chalothorn, D., McCune DF, Edelmann SE, García-Cazarin ML, Tsujimoto G, Piascik MT 2002. Differences in the cellular localization and agonist-mediated internalization properties of the α_1-adrenoceptor subtypes. *Mol Pharmacol* **61**: 1008-1016.

De Ligt RA, Kourounakis AP, IJzerman AP. (2000) Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. *Br J Pharmacol*;**130**:1-12.

Garcia-Sainz J, Torres-Padilla ME (1999) Modulation of basal intracellular calcium by inverse agonists and phorbol myristate acetate in rat-1 fibroblats stably expressing α_{1D}-adrenoceptors. *FEBS Lett* **443**:277-281.

Garcia-Sainz J, Vazquez-Prado J, Del Carmen Medina L (1999) α_1-adrenoceptors: function and phosphorylation. *Eur J Pharmacol* **389**:1-12.

Gisbert R, Noguera MA, Ivorra MD, D'Ocon P (2000) Functional evidence of a constitutively active population of α_{1D}-adrenoceptors in rat aorta. *J Pharmacol Exp Ther* **295**:810-817.

Gisbert R, Ziani K, Miquel R, Noguera MA, Ivorra MD, Anselmi E, D'Ocon, P (2002) Pathological role of a constitutively active population of α_{1D}-adrenoceptors in arteries of spontaneously hypertensive rats. *Br J Pharmacol* **135**:206-216.

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JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.046169
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Holtbäck U, Brismar H, DiBona GF, Fu M, Greengard P, Aperia A (1999) Receptor recruitment: a mechanism for interactions between G protein-coupled receptors *Proc Natl Acad Sci* **96**: 7271-7275

Inoue R, Okada T, Onoue H, Hara Y, Shimizu S, Naitoh S, Ito Y, Mori Y (2001) The transient receptor potential protein homologue TRP6 is the essential component of vascular α_1-adrenoceptor-activated Ca^{2+} -permeable cation channel. *Circ Res* **88**: 325-332.

Jung S, Pfeiffer F, Deitmer JVV (2000) Histamine-iduced calcium entry in rat cerebellar astrocytes: evidence for capacitative and non-capacitative mechanisms. *J Physiol* **527**:549-561.

Kanaide H (1999) Measurement of $[Ca^{2+}]_i$ in smooth muscle strips using front surface fluorimetry. *Methods Mol Biol* **114**: 269-277.

Kukkonen JP, Akerman KE (2001) Orexin receptors couple to Ca^{2+} channels different from store-operated Ca^{2+} channels. *Neuroreport* **12**:2017-2020.

Lachnit WG, Tran AM, Clarke DE, Ford APD (1997) Pharmacological characterization of an α_{1A}-adrenoceptor mediating contractile responses to noradrenaline in isolated caudal artery of rat. *Br J Pharmacol* **120**:819-826.

McCune DF, Edelmann SE, Olges JR, Post GR, Waldrop BA, Waugh DJJ, Perez DM, Piascik MT (2000) Regulation of the cellular localization and signalling properties of the α_{1B} and α_{1D} -adrenoceptors by agonists and inverse agonists. *Mol Pharmacol* **57**:659-666.

Minneman KP (1988) α_1-Adrenergic receptor subtypes, inositol phosphates and sources of cell Ca^{2+} *Pharmacol Rev* **40**:87-119.

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JPET Fast Forward. Published on March 6, 2003 as DOI: 10.1124/jpet.102.046169
This article has not been copyedited and formatted. The final version may differ from this version.

Noguera MA, D'Ocon MP (1993) Evidence that depletion of internal calcium stores sensitive to noradrenaline elicits a contractile response dependent on extracellular calcium in rat aorta. *Br J Pharmacol* **110**: 861-867.

Noguera MA, Ivorra MD, D'Ocon MP (1996) Functional evidence of inverse agonism in vascular smooth muscle. *Br J Pharmacol* **119**:158-154.

Noguera MA, Ivorra MD, Chuliá S, D'Ocon P (1997) Capacitative Ca^{2+} entry associated with α_1 adrenoceptors in rat aorta. *Naunyn-Schmi Arch Pharmacol* **356**: 83-89.

Noguera MA, Madrero Y, Ivorra MD, D'Ocon P (1998) Characterization of two different Ca^{2+} entry pathways dependent on depletion of internal Ca^{2+} pools in rat aorta. *Naunyn-Schmi. Arch Pharmacol* **357**: 92-99.

Perez-Vizcaino F, Cogolludo AL, Tamargo J (1999) Modulation of arterial Na^+/K^+-induced $[Ca^{2+}]i$ reduction and relaxation by noradrenaline, endothelin and PMA. *Am J Physiol* **276**:H651-H657.

Piascik MT, Perez DM (2001) α_1-Adrenergic receptors: new insights and directions. *J Pharmacol Exp Ther* **298**:403-410.

Zhong H, Minneman KP (1999) α_1-adrenoceptor subtypes. *Eur J Pharmacol* **375**:261-276.

Ziani K, Gisbert R, Noguera MA, Ivorra MD, D'Ocon P (2002) Modulatory role of a constitutively active population of α_{1D} -adrenoceptors in conductance vessels. *Am J Physiol* **282**:H475-481.

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Footnotes

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Figure 1. Representative tracings of simultaneous recordings of $[Ca^{2+}]_I$ and contractile force observed during the experimental procedure used to study the constitutively active population of α_{1D}-adrenoceptors in fura-2 preloaded aorta (A) and tail artery (B). Noradrenaline (1 μM in aorta and 10 μM in the tail artery) (NA1) was added and the sustained responses were taken as a control. After washing and recovery of the basal tone, tissues were incubated for 20 min in Ca^{2+}-free solution and then noradrenaline was added (NA2). Afterwards the tissues were washed in Ca^{2+}-free solution and exposed to noradrenaline (NA3). Arteries were then incubated for 20 min in Ca^{2+}-free solution and then extracellular $CaCl_2$ was restored which raised $[Ca^{2+}]_I$ and induced a contractile response only in the aorta (denoted by S). The dotted line represents the baseline and the small solid and open circles indicate washouts in Ca^{2+}-containing and Ca^{2+}-free solution, respectively.

Figure 2. Description of the experimental protocol designed to determine IP accumulation. Samples 1 and 2 were loaded in Ca^{2+}-containing solution (grey bars) and samples 3 to 8 were loaded in Ca^{2+}-free medium (white bars). In some samples, noradrenaline (NA 1 μM in aorta and 10 μM in the tail artery) was added once (sample 5) or twice (samples 6 to 8) in Ca^{2+}-free medium, followed by washings (W) to promote depletion of Ca^{2+} from internal stores. The exclusion of LiCl from the incubating medium permits noradrenaline-induced IP formation but prevents its accumulation (Gisbert et al., 2000). LiCl 10 mM was then added to all samples (30 min after the beginning of the experiment) and an antagonist (A) was added to sample 7. Finally, $CaCl_2$ 1.8 mM was added to samples 6 and 7 (grey bar), and

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noradrenaline (NA) was included in samples 2, 4 and 5 for 30 min. Following this protocol samples 2, 4, 5 and 6 are equivalent to the responses NA1, NA2, NA3 and S, respectively, in the $[Ca^{2+}]_i$ and contraction studies shown in Figure 1. In sample 7 the antagonists (A) BMY 7378, prazosin, nimodipine or Ni^{2+} were added. In sample 8, the experimental procedure was identical to sample 6 but addition of $CaCl_2$ was omitted.

Figure 3. Changes in contractile force (A), $[Ca^{2+}]_i$ (B) and IP accumulation (C) in rat aorta following the protocols shown in figures 1 and 2. NA1 indicates the effects of noradrenaline in Ca^{2+}-containing solution, NA2 and NA3 the effects of successive applications of noradrenaline in Ca^{2+}-free solution to deplete intracellular Ca^{2+}-stores and S the effects of restoring extracellular Ca^{2+}. The transient (phasic peak) and sustained (tonic, at 5 min) increases in $[Ca^{2+}]_i$ in panel B are denoted by P and T, respectively. The results are means ± s.e. means of 8 experiments (panels A and B) and 5-16 (panel C).

Figure 4. Changes in contractile force (A), $[Ca^{2+}]_i$ (B) and IP accumulation (C) in rat tail artery following the protocols shown in figures 1 and 2. NA1 indicates the effects of noradrenaline in Ca^{2+}-containing solution, NA2 and NA3 the effects of successive applications of noradrenaline in Ca^{2+}-free solution to deplete intracellular Ca^{2+}-stores and S the effects of restoring extracellular Ca^{2+}. The transient (phasic peak) and sustained (tonic, at 5 min) increases in $[Ca^{2+}]_i$ in panel B are denoted by P and T, respectively. The results are means ± s.e. means of 4-5 experiments.

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Figure 5. Inhibitory effects of BMY 7378 (0.1 μM) and prazosin (1 μM) on the changes in contractile force (A), and $[Ca^{2+}]_i$ (B) induced by restoring extracellular Ca^{2+} (indicated by S in previous figures) after intracellular Ca^{2+} store depletion in rat aorta. The transient (phasic) and sustained (tonic) increase in $[Ca^{2+}]_i$ in panel B are denoted by P and T, respectively. The results are means ± s.e. means of 5-8 experiments. * indicates $P < 0.01$ vs control (Newman Keuls´ test) and ‡ $P < 0.01$ P vs T (paired t test).

Figure 6. Inhibitory effects of nimodipine (0.1 nM), Ni^{2+} (1 mM) and Ca^{2+}-free solution, on the changes in contractile force (A), $[Ca^{2+}]_i$ (B) and IP accumulation (C) induced by restoring extracellular Ca^{2+} (indicated by S in previous figures) after intracellular Ca^{2+} store depletion in rat aorta. The transient (phasic) and sustained (tonic) increase in $[Ca^{2+}]_i$ in panel B are denoted by P and T, respectively. The results are means ± s.e. means of 4-8 experiments in panels. * indicates $P < 0.01$ vs control (Newman Keuls´ test) and ‡ $P < 0.01$ P vs T (paired t test).

Figure 7. Time-course of the changes in $[Ca^{2+}]_i$ (A) and contractile force (B) induced by restoring extracellular Ca^{2+} (indicated by S in previous figures) after intracellular Ca^{2+} store depletion in the absence (control) and in presence of nimodipine (0.1 nM) or BMY 7378 (0.1 μM) in rat aorta. The traces are the averaged recordings of 5-8 experiments. The solid circles are plotted to indicate the mean ± s.e. means at selected time points.

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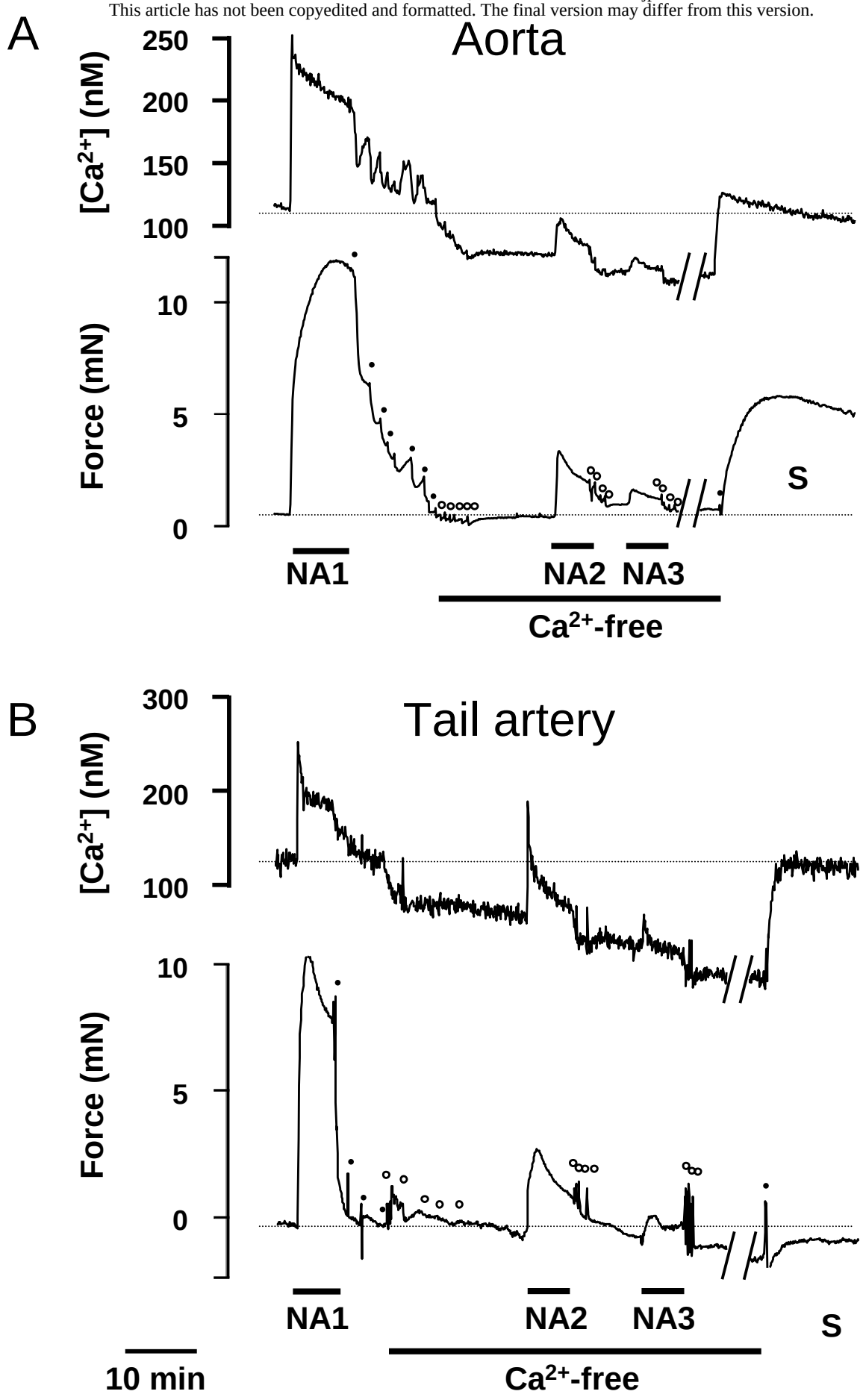

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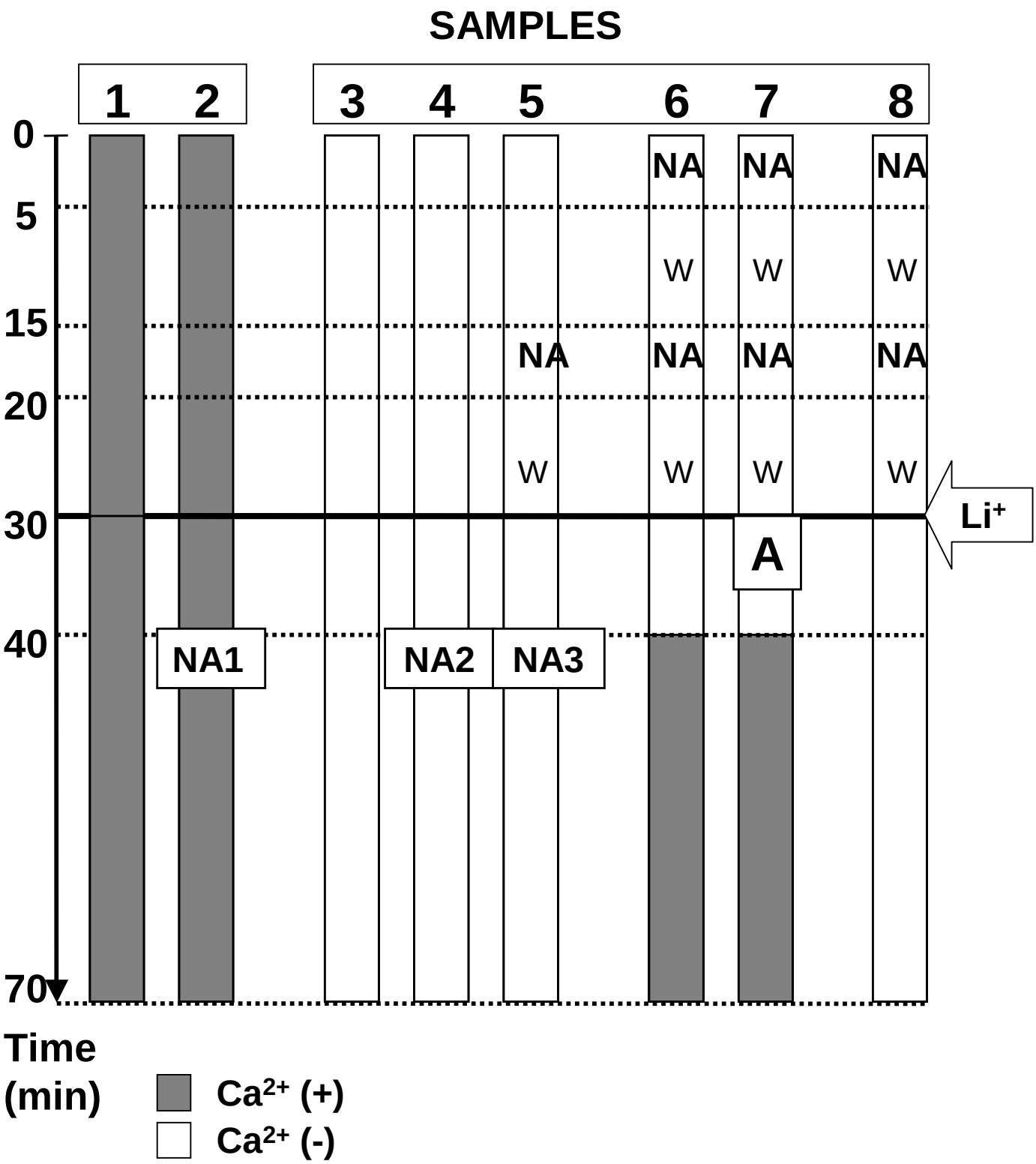

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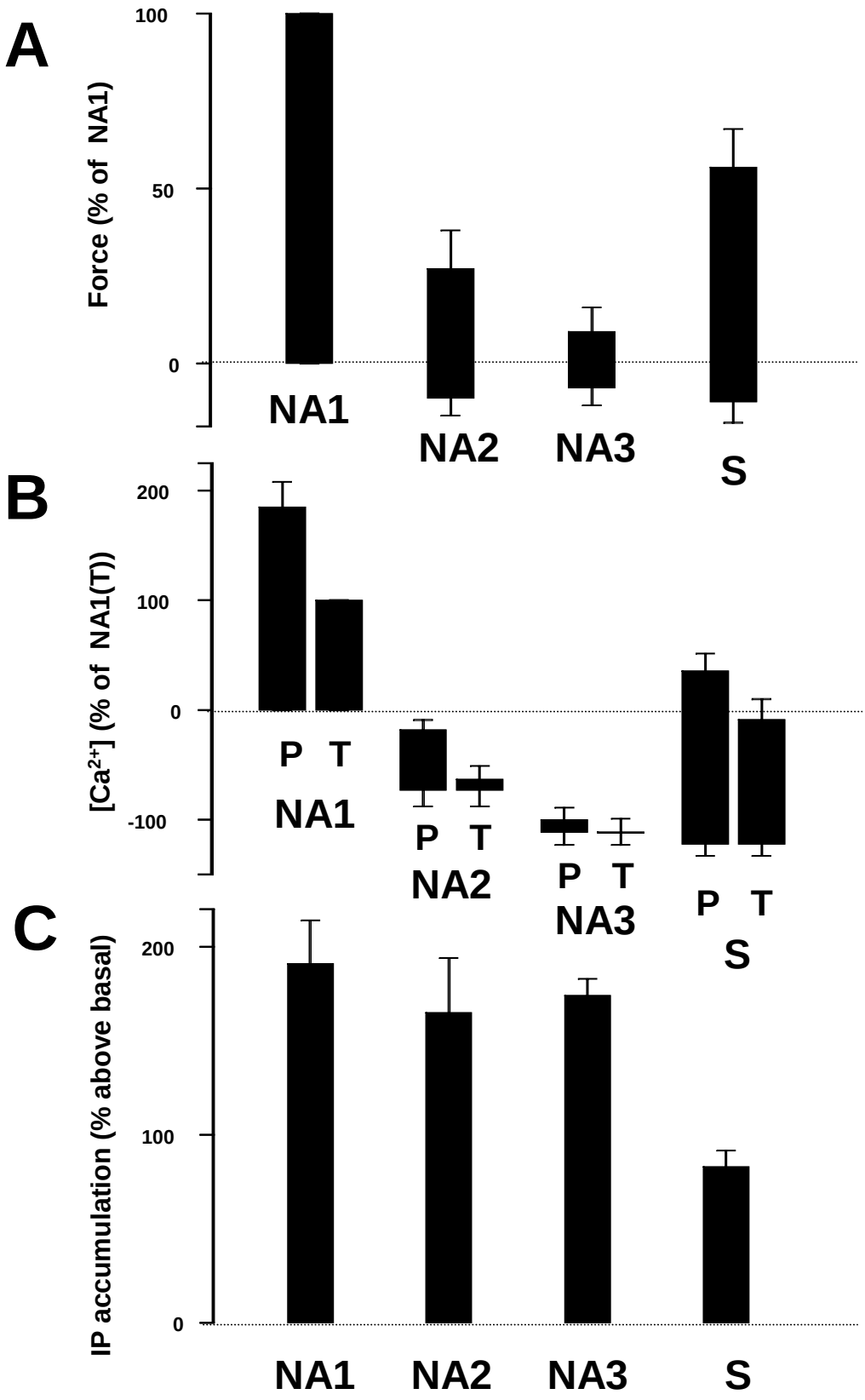

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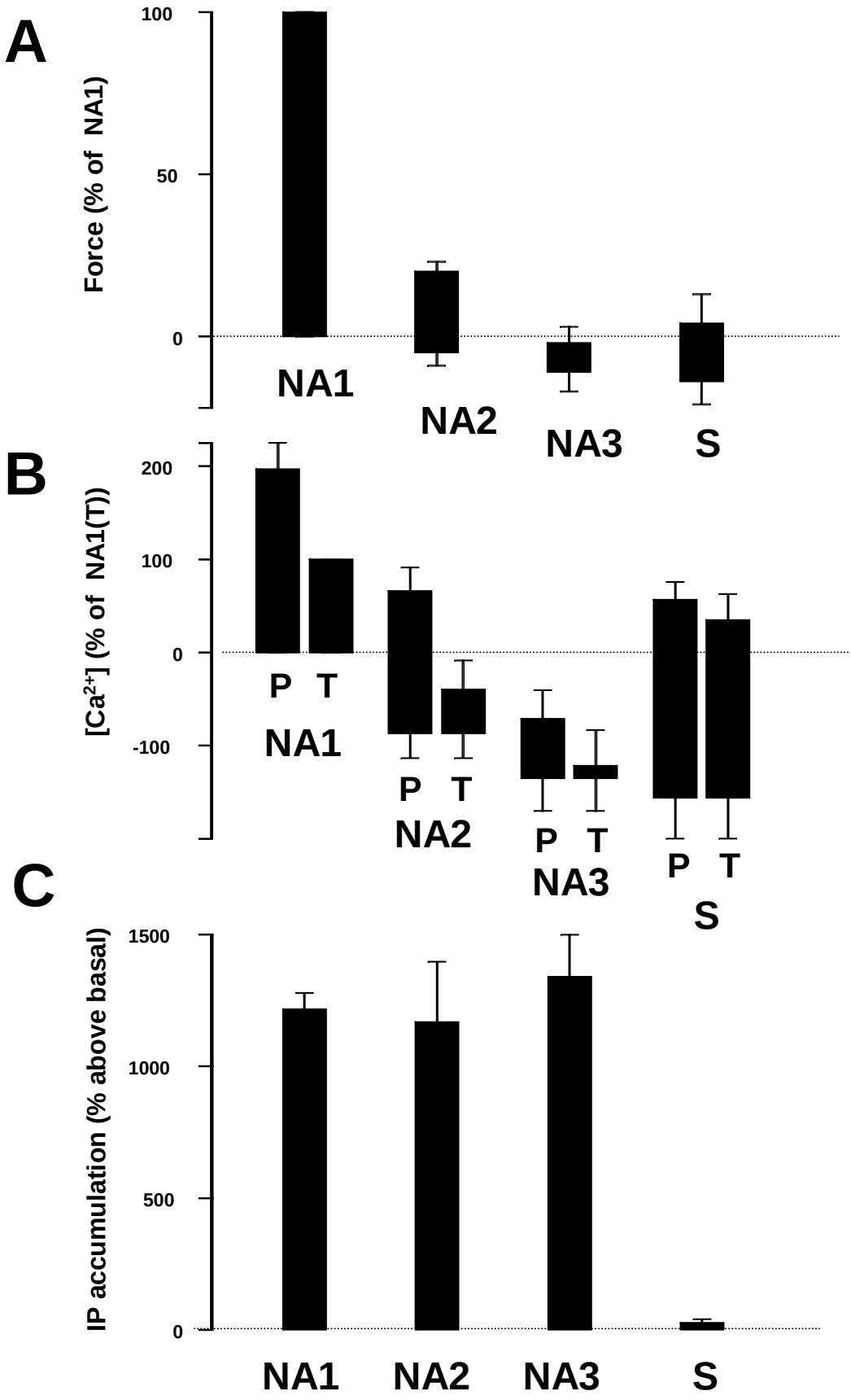

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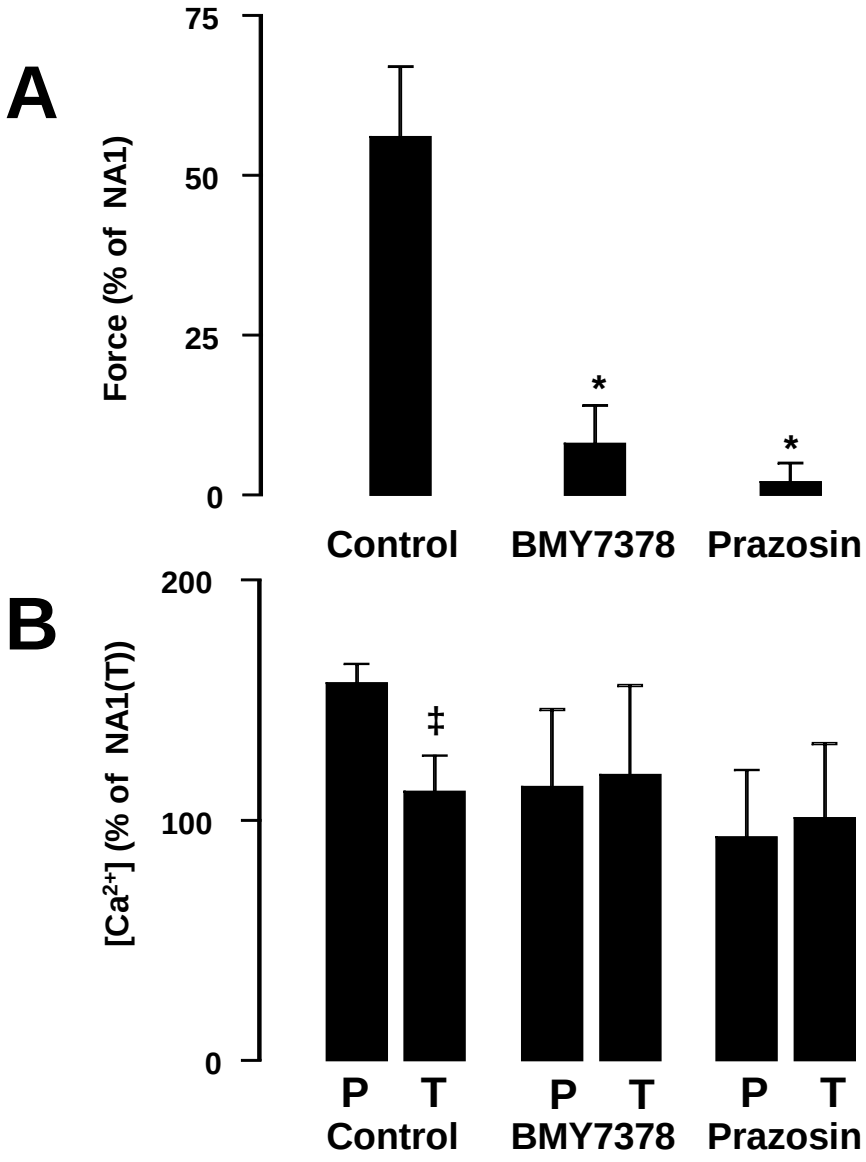

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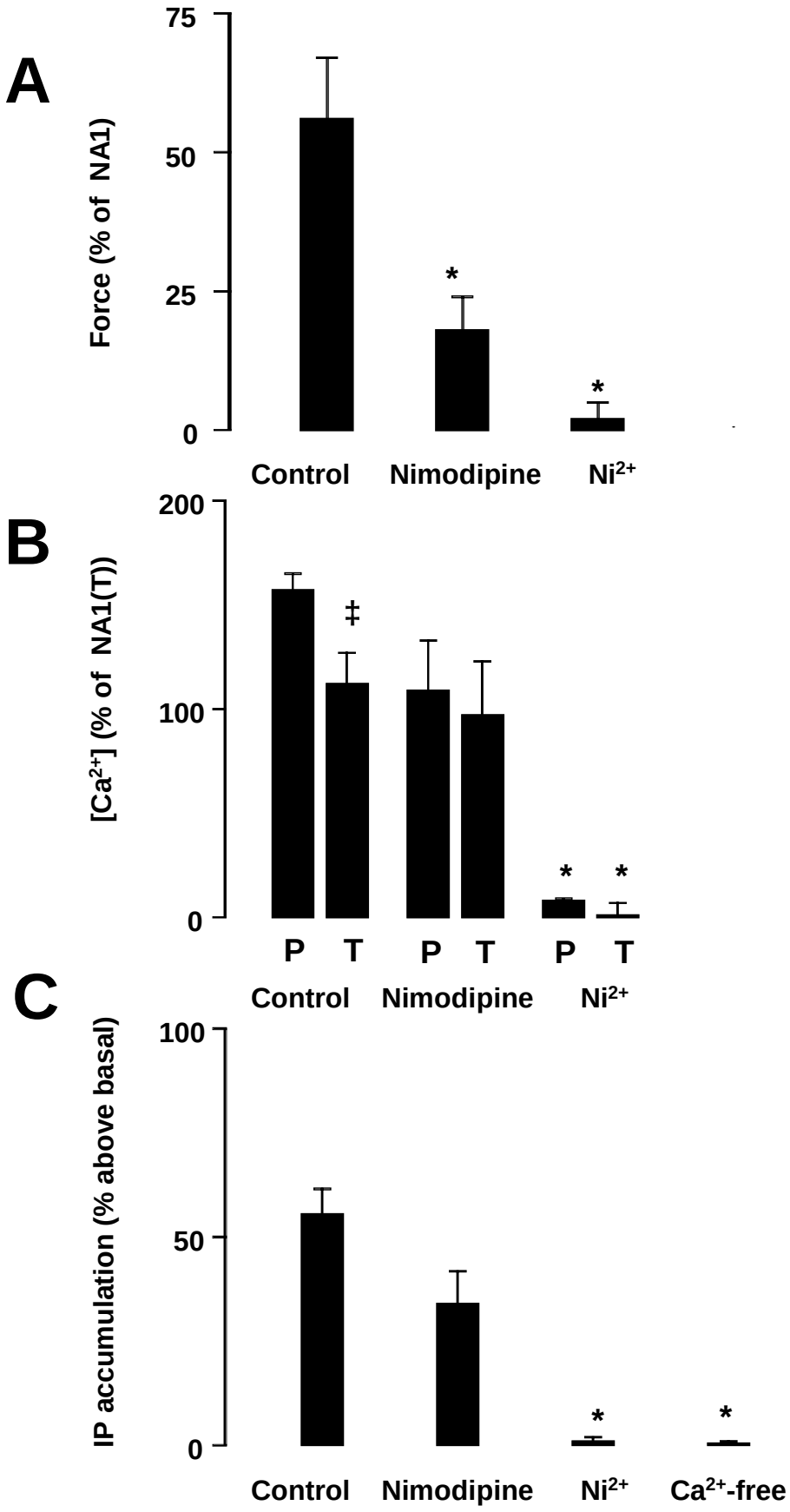

Figure 6

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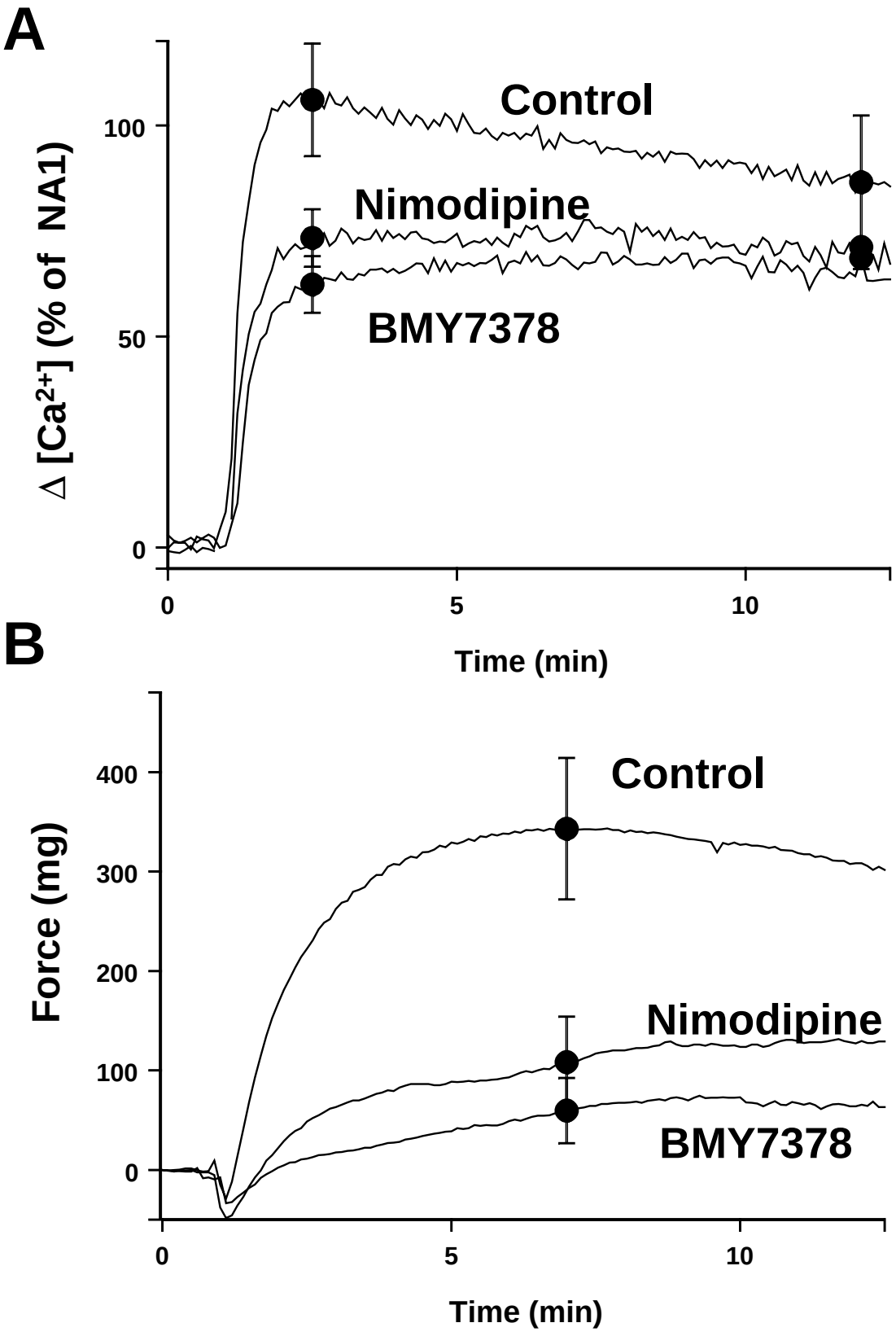

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Figure 7