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Does cyclic AMP mediate rat urinary bladder relaxation by isoproterenol?*

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Running title: Role of cAMP in β-adrenergic rat bladder relaxation

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Abbreviations: L-NNA, Nω-nitro-L-Arginine; ODQ, 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine 2 HCl; H89, N-[2-(p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide 2 HCl; PKA, protein kinase A; NO, nitric oxide; Rp-cAMPS, Rp-adenosine 3',5'-cyclic monophosphorothioate; SQ 22,536, 9-(tetrahydro-2-furanyl)-9H-purin-6-amine

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Abstract

Cyclic AMP is the prototypical second messenger of β-adrenergic receptors, but recent findings have questioned its role in mediating smooth muscle relaxation upon β-adrenergic receptor stimulation. We have investigated the signaling mechanisms underlying β-adrenergic receptor-mediated relaxation of rat urinary bladder. Concentration-response curves for isoproterenol-induced bladder relaxation were generated in the presence or absence of inhibitors, with concomitant experiments using passive tension and KCl-induced pre-contraction. The adenylyl cyclase inhibitor 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22,536, 1 μM), the protein kinase A inhibitors 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7, 10 μM), N-[2-(p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89, 1 μM) and Rp-adenosine 3',5'-cyclic monophosphorothioate (Rp-cAMPS 30 μM) and the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ 3 μM) produced only minor if any inhibition of relaxation against passive tension or KCl-induced pre-contraction. Among various potassium channel inhibitors, $BaCl_2$ (10 μM), tetraethylammonium (3 μM), apamin (300 nM) and glibenclamide (10 μM) did not inhibit isoproterenol-induced relaxation. Some inhibition of the isoproterenol effects against KCl-induced tone but not against passive tension was seen with inhibitors of calcium-dependent potassium channels such as charybdotoxin and iberiotoxin (30 nM each). A combination of SQ 22,536 and ODQ significantly inhibited relaxation against passive tension by about half, but not that against KCl-induced tone. Moreover, the combination failed to enhance inhibition by charybdotoxin against KCl-induced tone. We

conclude that cAMP and cGMP each play a minor role in β-adrenergic receptor-mediated relaxation against passive tension and calcium-dependent potassium channels play a minor role against active tension.

Introduction

During the storage phase of the micturition cycle the urinary bladder must accommodate increasing amounts of urine without major elevation of intravesical pressure. This enhancement of bladder compliance requires relaxation of smooth muscle cells of the detrusor which is controlled by reflex pathways involving an efferent activity of the sympathetic nervous system, particularly the hypogastric nerve originating from spinal cord segments Th12-L2 (Michel and Peters, 2004). Norepinephrine released from the hypogastric nerves primarily acts upon β-adrenergic receptors in the urinary bladder to promote relaxation during the storage phase. Therefore, β-adrenergic receptor activation is considered to be the most important physiological mechanism mediating urinary bladder relaxation during the filling/storage phase of the micturition cycle (Yamaguchi, 2002;Andersson, 2004).

Several recent reports have investigated the β-adrenergic receptor subtypes mediating urinary bladder relaxation in several species. Atypical β-adrenergic receptors, i.e. β_3 and/or other non-β_1-non-β_2 subtypes, appear to be important for bladder relaxation in all species, but β_2- and possibly even β_1-adrenergic receptors can additionally contribute in some species (Yamaguchi, 2002). Much less attention has been devoted to identifying intracellular signaling pathways mediating the bladder relaxation upon β-adrenergic receptor stimulation. Stimulation of adenylyl cyclase and hence formation of cAMP is the prototypical signaling pathway of β-adrenergic receptors (Bylund, et al., 1994). Until recently, the smooth muscle relaxant effect of β-adrenergic stimulation was considered to

occur via cAMP, partly because the adenylyl cyclase stimulator forskolin, which also increases cellular cAMP content in the bladder (Kories, et al., 2003), frequently mimicked the effects of β-adrenergic stimulation. However, more recent data have implicated other signaling pathways including certain potassium channels (for review (Peters and Michel, 2003). Specifically, it has been shown in guinea pig urinary bladder that a β-adrenergic receptor agonist can activate potassium channels and that inhibition of such channels attenuates relaxation (Kobayashi, et al., 2000).

Therefore, we have investigated the role of cAMP-dependent and –independent pathways in the relaxation of rat urinary bladder by the β-adrenergic agonist isoproterenol. Since previous studies have failed to unequivocally identify the β-adrenergic receptor subtype mediating bladder relaxation in the rat, we have based our present study on isoproterenol, an agonist which similarly acts on all known β-adrenergic receptor subtypes (Hoffmann, et al., 2004). Previous studies on bladder relaxation by β-adrenergic agonists have employed two different approaches, i.e. relaxation against passive tension (Yamazaki, et al., 1998;Igawa, et al., 1999;Lecci, et al., 1998) or relaxation against active tone, induced e.g. by depolarizing concentrations of KCl (Oshita, et al., 1997;Longhurst and Levendusky, 1999;Yamanishi, et al., 2002;Kobayashi, et al., 2000). Since we expected this methodological difference to affect the relative role of potassium channels in bladder relaxation, all our experiments were performed under both conditions.

Material and Methods

Tissue preparation

Adult male Wistar rats (260-280 g) were purchased from Charles River. Animals were anesthetized using pentobarbital (75 mg/kg i.p.) and sacrificed by decapitation. The bladders were harvested, adipose and soft connective tissues were removed and the middle parts were cut longitudinally into four strips (1 mm diameter, 18 ± 1 mm length, 9.4 ± 0.7 mg, n = 70). All experimental procedures were in line with NIH guidelines for the use of laboratory animals and approved by the Animal Care Committee of Academisch Medisch Centrum.

Relaxation experiments

The bladder strips were mounted under a resting tension of 10 mN in organ baths containing 7 ml Krebs-Henseleit buffer of the following composition (mM): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, Na_4EDTA 0.025, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.5 at 37^{o}C, yielding a total potassium concentration of 5.9 mM. The organ baths were continually gassed with 95% O_2/5% CO_2 to maintain a pH of 7.4. The bladder strips were equilibrated for 75 minutes during which the buffer solution was refreshed every 15 minutes. Following the equilibration, the tissues were challenged with 50 mM KCl for 6 minutes. Thereafter, they were again equilibrated with normal buffer and readjustment of passive tension to 10 mN every 10 minutes until stabilization had occurred, usually

within 45 minutes. Thereafter, inhibitors of signal transduction or their vehicles were added. The further experimental design is depicted in figure 1: After 10 minutes some strips were pre-contracted with 50 mM KCl. After 30 minutes (20 minutes after KCl), when the KCl-exposed strips had reached a stable tension, cumulative isoproterenol concentration-response curves (0.3 nM – 30 μM with concentration increases every 3 minutes) were started (figure 1). At the end of each experiment, 10 μM forskolin was added to each preparation to assess receptor-independent relaxation. To avoid desensitization, only a single relaxation curve was generated in each bladder strip. To avoid false negative findings, we have generally used high inhibitor concentrations corresponding to those used in previous studies (Czyborra, et al., 2002).

Preliminary experiments had shown that a passive tension of 10 mN and a KCl concentration of 50 mM yielded more stable contractions than various other combinations of passive tension and KCl concentration (data not shown). α-Adrenergic receptor antagonists (e.g. phentolamine or phenoxybenzamine) or uptake blockers (e.g. desipramine, hydrocortisone or phenoxybenzamine) were not used because isoproterenol is unlikely to stimulate α-adrenergic receptors and because uptake blockers did not even affect relaxation by norepinephrine, a much better uptake substrate than isoproterenol (data not shown). Moreover, a high concentration of desipramine (10 μM) markedly reduced contraction responses to both carbachol and KCl, phenoxybenzamine (50 μM) markedly reduced the contraction to carbachol, and phentolamine increased myogenic activity of the preparation (data not shown).

Chemicals

Pentobarbital was obtained from OPG^{FARMA} Groothandel B.V. (Utrecht, Netherlands). Hydrocortisone was acquired from Bufa B.V. (Uitgeest, Netherlands). All other chemicals were purchased from Sigma (Deisenhofen, Germany). Stock solutions of the various experimental compounds were made as follows: 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) at 30 mM in dimethylsulphoxide; glibenclamide and N-[2-(p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide 2 HCl (H89) at 1 and 10 mM, respectively, in ethanol; apamin at 3 mM in 5% acetic acid; 1-(5-isoquinolinesulfonyl)-2-methylpiperazine 2 HCl (H7), Rp-cAMPS, tetraethylammonium chloride, Nω-nitro-L-arginine (L-NNA), iberiotoxin, charybdotoxin and 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22,536) at 10 mM, 30 mM, 300 μM, 10 mM, 3 μM and 3 μM, 1 mM respectively, in deionized water.

Data analysis

Non-linear regression was used to fit sigmoidal curves to the isoproterenol concentration-response curves to determine agonist potency (pEC_{50}) and maximum effects (E_{max}). The force of contraction immediately prior to addition of the first isoproterenol concentration within a given experiment was defined as 0% relaxation, and a force of contraction of 0 mN was defined as 100% relaxation.

All data are expressed as mean ± SEM of *n* experiments. Statistical significance of inhibitor effects on the E_{max} or pEC_{50} of isoproterenol was assessed by two-tailed t-tests if a single inhibitor was compared to a given vehicle or by one-way ANOVA if multiple inhibitors were compared to a given vehicle. If the latter indicated that variance between groups was significantly greater than within groups, individual inhibitors were compared to vehicle with Dunnet post-hoc tests. Similar calculations were made for the forskolin effects. All statistical analysis was calculated using the Prism program (GraphPad, San Diego, California, U.S.A), and a $P < 0.05$ was considered statistically significant.

Results

Signal transduction underlying relaxation against passive tension

In the absence of inhibitors, resting tension immediately prior to addition of isoproterenol was approximately 10 mN. None of the vehicles or inhibitors substantially altered resting tension (data not shown). Moreover, resting tension declined by less than 5% over the course of an experiment if no isoproterenol was added (figure 2). Therefore, no correction for such spontaneous decline was made in the analysis of the isoproterenol concentration-response curves.

Neither ethanol, nor acetic acid nor dimethylsulphoxide significantly affected the relaxing response to isoproterenol relative to that in the presence of water (table 1), and even triple vehicle had no effect (data not shown). When data for all vehicles were pooled, isoproterenol relaxed bladder tone with a pEC_{50} of 8.16 ± 0.04 and maximum reduction of 33 ± 2% (n = 35, figure 2).

The role of cAMP- and cGMP-dependent pathways on bladder relaxation was assessed using the adenylyl cyclase inhibitor SQ 22,536 (1 μM), the protein kinase A (PKA) inhibitors H7 (10 μM), H89 (1 μM) and Rp-cAMPS (30 μM) and the guanylyl cyclase inhibitor ODQ (3 μM). All five inhibitors caused only minor attenuation of the isoproterenol effects, and such inhibition reached statistical significance only for Rp-

cAMPS (figures 3-4, table 1). L-NNA (100 µM), an inhibitor of NO synthase, also failed to affect the E_{max} of isoproterenol but significantly enhanced its potency (figure 4, table 1); however, the meaning of this alteration is difficult to judge due to a change in the shape of the concentration-response curve in the presence of L-NNA.

Six different inhibitors were used to study the role of various types of potassium channels in the isoproterenol-induced bladder relaxation, i.e. $BaCl_2$ (10 µM), tetraethylammonium chloride (3 µM), glibenclamide (10 µM), apamin (300 nM), charybdotoxin (30 nM) and iberiotoxin (30 nM). None of the potassium channel blockers significantly altered the E_{max} or pEC_{50} of isoproterenol (figure 5, table 1).

Finally, we tested the effects of the combined inhibition of the signaling pathways. A combination of SQ 22,536 and ODQ significantly inhibited maximum isoproterenol responses without altering its potency (figure 6, table 1). Addition of charybdotoxin to SQ 22,536, ODQ or its combination, however, had no additional inhibitory effect (figure 6, table 1).

Forskolin (10 µM, added after the highest isoproterenol concentration) yielded only little additional relaxation (figure 1), i.e. caused a total relaxation of 38 ± 2% (pooled data for all vehicles). All of the above inhibitors had similar effects against forskolin (table 2) as against the maximum effect of isoproterenol, i.e. only a combination of SQ 22,536 and ODQ significantly attenuated the forskolin responses (table 1).

Signal transduction underlying relaxation of KCl-induced tension

From a passive tension of 10 mN, addition of 50 mM KCl initially increased tension to approximately 35 mN; thereafter tension slowly declined to a relatively stable plateau of approximately 25 mN (figure 1). This plateau was not markedly affected by any of the vehicles of inhibitors (data not shown) and declined by less than 5% over the course of an experiment if no isoproterenol was added (figure 2).

Neither ethanol, nor acetic acid nor dimethylsulphoxide significantly affected the relaxing response to isoproterenol relative to that in the presence of water (table 1). When data for all vehicles was pooled, isoproterenol relaxed bladder tone with a pEC_{50} of 7.43 ± 0.04 and an E_{max} of 53 ± 1% (n = 34, figure 2).

None of the inhibitors of cyclic nucleotide- or nitric oxide-dependent pathways caused statistically significant alterations of isoproterenol-induced relaxation of pre-contracted bladder strips (figures 3-4, table 1).

The BK_{Ca} channel inhibitors charybdotoxin and iberiotoxin significantly reduced the relaxing potency of isoproterenol, and charybdotoxin additionally significantly reduced its E_{max} in pre-contracted bladder (figure 5, table 1). In contrast, $BaCl_2$, tetraethylammonium chloride, glibenclamide or apamin did not significantly affect the isoproterenol responses (table 1).

A combination of SQ 22,536 and ODQ did not inhibit isoproterenol-induced relaxation of rat bladder (table 1). Moreover, SQ 22,536 and ODQ alone or in combination did not enhance the inhibitory effect of charybdotoxin (table 1).

Relaxation responses to 10 μM forskolin in the absence of signaling inhibitors but in the presence of pre-contraction were 69 ± 2% (n = 34, pooled data from all vehicles). The forskolin response was significantly inhibited by charybdotoxin or iberiotoxin (table 2). A significant inhibition of forskolin responses was also seen with a combination of SQ 22,536 and ODQ, but neither of them alone or their combination enhanced the inhibitory effect of charybdotoxin (table 2).

Discussion

Smooth muscle tone in the urinary bladder is largely regulated by a balance between contraction elicited via muscarinic acetylcholine receptors and relaxation elicited via β-adrenergic receptors. Recently, we have intensively characterized the signaling pathways underlying muscarinic receptor-mediated contraction of rat and human urinary bladder (Fleichman, et al., 2004;Schneider, et al., 2004a;Schneider, et al., 2004b). In the present study we have investigated the signal transduction underlying rat urinary bladder relaxation by the β-adrenergic agonist isoproterenol. Mechanisms involved in the relaxant effects of forskolin, added after the highest isoproterenol concentration, were preliminarily studied in comparison.

Methodological considerations

Previous studies have employed two different approaches to measure urinary bladder relaxation by β-adrenergic receptor agonists, i.e. relaxation against passive tension (Yamazaki, et al., 1998;Igawa, et al., 1999;Lecci, et al., 1998) or relaxation against active tone, induced e.g. by depolarizing concentrations of KCl (Oshita, et al., 1997;Longhurst and Levendusky, 1999;Yamanishi, et al., 2002;Yamanishi, et al., 2003;Kobayashi, et al., 2000). Since we hypothesized that the extracellular potassium concentration may affect the relative role of potassium channels, we have used both approaches in parallel. Our data show that the presence of KCl-induced pre-contraction has indeed important implications for the quantification of the isoproterenol-induced bladder relaxation. Thus,

measurements in the presence of KCl yielded approximately one log unit lower isoproterenol potency but somewhat greater maximum effects. This is in line with indirect comparisons in the published literature, where an pEC_{50} for isoproterenol of 8.3 (Yamazaki, et al., 1998) vs. 7.2 (Longhurst and Levendusky, 1999) and of 9.1 (Yamazaki, et al., 1998) vs. 7.3 (Oshita, et al., 1997) were reported in rats and rabbits, respectively, for passive tension vs. pre-contraction. Physiological urinary potassium concentrations range between 1 and 50 mM, but due to the presence of the urothelium it is not fully clear whether and/or how much of this variation is sensed by bladder smooth muscle cells. Moreover, increasing bladder filling itself alters bladder distension. Therefore, we cannot definitively determine which of the two experimental conditions approximate the physiological situation most closely. Irrespective of such considerations, these differences will impact estimates of relative selectivity of an agonist for bladder vs. e.g. cardiac β-adrenergic receptors and hence may be important in defining uroselectivity of such agents.

Signaling underlying bladder relaxation

Cyclic AMP is the classical second messenger of β-adrenergic receptors, and the adenylyl cyclase activator forskolin mimics the effect of isoproterenol on bladder tone in the present and many previous studies. Therefore, we have evaluated the role of the cAMP/PKA pathway in rat bladder relaxation using the adenylyl cyclase inhibitor SQ 22,536 and the three PKA inhibitors H7, H89 and Rp-cAMPS. All four inhibitors caused only weak inhibition against either isoproterenol or forskolin, and only inhibition by Rp-

cAMPS of isoproterenol or forskolin responses against passive tension reached statistical significance. While we cannot rule out that some of these negative results were affected by an insufficient tissue penetration of the inhibitors, it should be noticed that the chosen inhibitor concentrations were rather high and similar to those used by many other investigators in studies on vascular or bronchial smooth muscle. Therefore, our data do not support a major role for cAMP and/or PKA in isoproterenol or forskolin-induced bladder relaxation. They are in line with those of several other tissues where smooth muscle relaxation by β-adrenergic agonists was insensitive or only partially sensitive to inhibitors of the cAMP/PKA pathway (for review see (Peters and Michel, 2003). A rather minor role for the cAMP/PKA pathway may particularly be relevant for atypical β-adrenergic receptors, since e.g. in rabbit corpus cavernosum relaxation via β_2-adrenergic receptors involved adenylyl cyclase activation whereas that via atypical β-adrenergic receptors did not (Teixeira, et al., 2004). If isoproterenol acts cAMP-independently, this could be a direct effect of the receptor or its linked G-protein to a different effector such as a potassium channel. Direct coupling of β-adrenergic receptors to at least some types of potassium channels has indeed been demonstrated (Kathöfer, et al., 2003). On the other hand, it remained surprising that the cAMP/PKA pathway also explained only a minor part of the relaxation by the adenylyl cyclase activator forskolin. While forskolin is a direct adenylyl cyclase activator, but nevertheless its effect upon intracellular cAMP content may be partly G_s-dependent (Laurenza, et al., 1989). A future study with full concentration-response curves for forskolin in the absence of isoproterenol may be necessary to fully define the signaling underlying forskolin-induced bladder relaxation.

NO, guanylyl cyclase and cGMP are not prototypical signaling molecules of β-adrenergic receptors, but recent studies in vascular preparations have shown that inhibition of these pathways can attenuate β-adrenergic relaxation of smooth muscle in vascular (Cardillo, et al., 1997;Hutri-Kähönen, et al., 1999) and non-vascular preparations (Li, et al., 2004;Teixeira, et al., 2004). Therefore, we have assessed their potential role in isoproterenol-induced bladder relaxation using the NO synthesis inhibitor L-NNA and the guanylyl cyclase inhibitor ODQ. While ODQ did not significantly attenuate the isoproterenol or forskolin effect, L-NNA lacked inhibition of maximum isoproterenol effects but may have enhanced its potency These findings are difficult to interpret since the overall role of NO for detrusor function remains controversial (Liu and Lin-Shiau, 1997;Garcia-Pascual, et al., 1996).

Interestingly, concomitant inhibition of both adenylyl and guanylyl cyclase inhibited the isoproterenol response in rat urinary bladder against passive tension by about two thirds. Therefore, we propose that adenylyl cyclase and guanylyl cyclase each contribute to isoproterenol-induced bladder relaxation in a minor way only because the two pathways may compensate for each other. Only combined inhibition reveals their relevance. Nevertheless it must be stated that even combined inhibition accounted for only two thirds of the overall response against passive tension and did not at all explain relaxation against active tension. A similar synergistic effect was recently demonstrated for vascular smooth muscle relaxation by ceramide, which was only weakly sensitive to inhibition of guanylyl cyclase or BK_{Ca} channels, whereas their combined inhibition abolished it (Czyborra, et al., 2002).

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Potassium channels are present in many tissues including smooth muscle of the lower urinary tract (Gopalakrishnan, et al., 1999;Chen, et al., 2004). Potassium channel opening, particularly as induced by BK_{Ca} channel openers, mediates relaxation (Malysz, et al., 2004). Some previous studies have linked β-adrenergic receptor stimulation to potassium channel opening (Czyborra, et al., 2002;Kathöfer, et al., 2003;Horinouchi, et al., 2003). Specifically it has been shown that β-adrenergic receptor stimulation in guinea pig urinary bladder activates BK_{Ca} channels (Kobayashi, et al., 2000). Therefore, we have studied the role of various potassium channels in isoproterenol-induced rat urinary bladder relaxation using a variety of blockers including the broad-spectrum inhibitors $BaCl_2$ and tetraethylammonium, the K_{ATP} channel inhibitor glibenclamide, the SK_{Ca} small conductance channel inhibitor apamin, and the BK_{Ca} high conductance calcium-dependent potassium-channel inhibitors charybdotoxin and iberiotoxin. Previous studies in guinea pigs had already demonstrated that iberiotoxin can attenuate isoproterenol-induced bladder relaxation (Kobayashi, et al., 2000). In the present study only inhibitors of calcium-dependent potassium-channels, particularly charybdotoxin, inhibited the isoproterenol effects whereas inhibitors of other potassium channels lacked significant effects. Similar to the attenuation by adenylyl or guanylyl cyclase inhibitors and in agreement with the previously reported guinea pig data (Kobayashi, et al., 2000), this was only a partial inhibition. Opposite to the effects of the adenylyl or guanylyl cyclase inhibitors, the inhibition was seen only in pre-contracted bladder strips and not against passive tension.

Since adenylyl cyclase, guanylyl cyclase and calcium-dependent potassium-channels each contributed to isoproterenol-induced bladder relaxation in a minor way only, we tested combinations of the various inhibitors. While the combination of the adenylyl and the guanylyl cyclase inhibitor was quite effective against passive tension (see above), combination of either alone or both with charybdotoxin did not produce any additional effects either against passive tension or in pre-contracted bladder strips.

In summary, we propose that adenylyl and guanylyl cyclase but not potassium channels contribute to isoproterenol effects against passive tension, whereas calcium-dependent potassium channels but not adenylyl or guanylyl cyclase contribute to effects against KCl-induced pre-contraction. Since physiological potassium concentrations in urine can vary over a wide range, it remains to be studied *in vivo* which of these mechanisms is operative under physiological conditions.

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Footnotes:

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LEGENDS TO THE FIGURES

Figure 1. Experimental design in the absence (passive tension, upper panel) and presence of pre-contraction with 50 mM KCl (lower panel). Data are from a representative experiment performed in the absence of inhibitors. The distance between two ticks on x axis denotes a ten minutes period. Unless otherwise noted, arrows indicate increasing isoproterenol concentrations.

Figure 2. Development of rat urinary bladder tone in the absence (time control, TC) and presence of isoproterenol (ISO) under conditions of passive tension and pre-contraction induced by 50 mM KCl. Data are means ± SEM of 7-13 experiments performed in the absence of inhibitor or vehicles.

Figure 3. Effect of the adenylyl cyclase inhibitor SQ 22,536 (1 μM) and the protein kinase A inhibitor H7 (10 μM) on isoproterenol-induced relaxation of rat urinary bladder. Data are means ± SEM of 5-13 experiments. A quantitative analysis of these data is shown in table 1.

Figure 4. Effect of the guanylyl cyclase inhibitor ODQ (3 μM) and NO synthase inhibitor L-NNA (100 μM) on isoproterenol-induced relaxation of rat urinary bladder. Data are means ± SEM of 5-13 experiments. A quantitative analysis of these data is shown in table 1.

Figure 5. Effects of the BK_{Ca} channel inhibitors iberiotoxin (30 nM) and charybdotoxin (30 nM) on isoproterenol-induced relaxation of rat urinary bladder. Data are means ± SEM of 6-13 experiments. A quantitative analysis of these data is shown in table 1.

Figure 6. Effects of combinations of the adenylyl cyclase inhibitor SQ 22,536 (10 μM), the guanlylyl cyclase inhibitor ODQ (3 μM) and the BK_{Ca} channel inhibitor charybdotoxin (30 nM) on isoproterenol-induced relaxation of rat urinary bladder. Data are means ± SEM of 5-6 experiments. A quantitative analysis of these data is shown in table 1.

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Table 1: Effect of signaling inhibitors on isoproterenol-induced relaxation of rat bladder strips.

	E_{max}		pEC_{50}	
	Against passive tension	Against KCl-induced pre-contraction	Against passive tension	Against KCl-induced pre-contraction
Vehicles				
H_2O	32 ±3	53 ± 2	8.17 ± 0.11	7.40 ± 0.09
Ethanol	34 ±3	55 ± 2	8.23 ± 0.07	7.48 ± 0.06
DMSO	34 ±5	54 ± 3	8.05 ± 0.07	7.33 ± 0.11
Acetic Acid	33 ±2	51 ± 4	8.14 ± 0.04	7.49 ± 0.06
Cyclic nucleotide-dependent pathways				
SQ 22,536	24 ± 3	56 ± 3	8.14 ± 0.07	7.53 ± 0.11
H7	24 ± 2	63 ± 2	8.43 ± 0.03	7.72 ± 0.09
H89	35 ± 4	57 ± 4	8.13 ± 0.06	7.49 ± 0.09
Rp-cAMPS	22 ± 3*	57 ± 3	8.87 ± 0.43	7.86 ± 0.31
ODQ	28 ± 3	56 ± 3	8.24 ± 0.07	7.41 ± 0.05
L-NNA	26 ± 3	52 ± 4	8.98 ± 0.22*	7.43 ± 0.14
K-channels				

$BaCl_2$	40 ± 2	57 ± 2	8.20 ± 0.17	7.26 ± 0.04
Tetraethylammonium	31 ± 4	50 ± 4	8.17 ± 0.23	7.56 ± 0.20
Glibenclamide	32 ± 6	55 ± 3	8.25 ± 0.15	7.56 ± 0.06
Apamin	40 ± 3	43 ± 2	8.33 ± 0.10	7.59 ± 0.06
Charybdotoxin	42 ± 4	38 ± 2*	8.07 ± 0.10	6.87 ± 0.27*
Iberiotoxin	35 ± 6	43 ± 4	8.06 ± 0.16	6.87 ± 0.30*
Combinations				
SQ + ODQ	16 ± 3*	45 ± 3	8.04 ± 0.04	7.43 ± 0.11
SQ + charybdotoxin	31 ± 2	36 ± 4*	8.03 ± 0.12	7.55 ± 0.17
ODQ + charybdotoxin	36 ± 2	37 ± 4*	8.16 ± 0.10	7.53 ± 0.13
SQ + ODQ + charybdotoxin	14 ± 2*	37 ± 5*	8.10 ± 0.10	7.18 ± 0.07

Data are means ± SEM of 5-13 experiments and expressed as % relaxation. *: $p < 0.05$ vs. corresponding vehicle control in a two-tailed t-test or one-way ANOVA followed by Dunnet tests. ODQ was compared to dimethylsulphoxide, glibenclamide and H89 to ethanol, apamin to acetic acid, and all other inhibitors to deionized water. Note that the Rp-cAMPS experiments were done at a later point in time and hence were compared to a different control group (E_{max} against passive 36 ± 5%, E_{max} against KCl 52 ± 3%, pEC_{50} against passive 9.01 ± 0.18, pEC_{50} against KCl 7.82 ± 0.27; n = 6 each).

Table 2. Effects of signaling inhibitors on 10 μM forskolin-induced relaxation of rat bladder strips.

	Against passive tension	Against KCl-induced pre-contraction
Absence of inhibitors		
H_2O	37 ± 3	70 ± 2
Ethanol	38 ± 3	73 ± 1
DMSO	42 ± 4	70 ± 2
Acetic acid	37 ± 3	61 ± 6
Cyclic nucleotide-dependent pathways		
SQ 22,536	26 ± 3	74 ± 3
H7	27 ± 3	73 ± 1
H89	38 ± 5	66 ± 5
Rp-cAMPS	23 ± 3*	75 ± 2
ODQ	30 ± 3	72 ± 3
L-NNA	29 ± 4	67 ± 3
K-channels		
$BaCl_2$	44 ± 4	73 ± 2
Tetraethylammonium	35 ± 6	69 ± 4
Glibenclamide	35 ± 7	64 ± 2
Apamin	43 ± 4	63 ± 2
Charybdotoxin	45 ± 4	49 ± 2*
Iberiotoxin	41 ± 6	56 ± 1*

Combinations		
SQ + ODQ	17 ± 3*	59 ± 3*
SQ + charybdotoxin	32 ± 3	46 ± 1*
ODQ + charybdotoxin	41 ± 3	51 ± 1*
SQ + ODQ + charybdotoxin	17 ± 3*	49 ± 2*

Data are means ± SEM of 5-13 experiments and expressed as % relaxation. *: $p < 0.05$ vs. corresponding vehicle control in a two-tailed t-test or one-way ANOVA followed by Dunnet tests. ODQ was compared to dimethylsulphoxide, glibenclamide and H89 to ethanol, apamin to acetic acid, and all other inhibitors to deionized water. Note that the Rp-cAMPS experiments were done at a later point in time and hence were compared to a different control group (against passive 39 ± 5%, against KCl 74 ± 3%, n = 6 each).

Figure 1

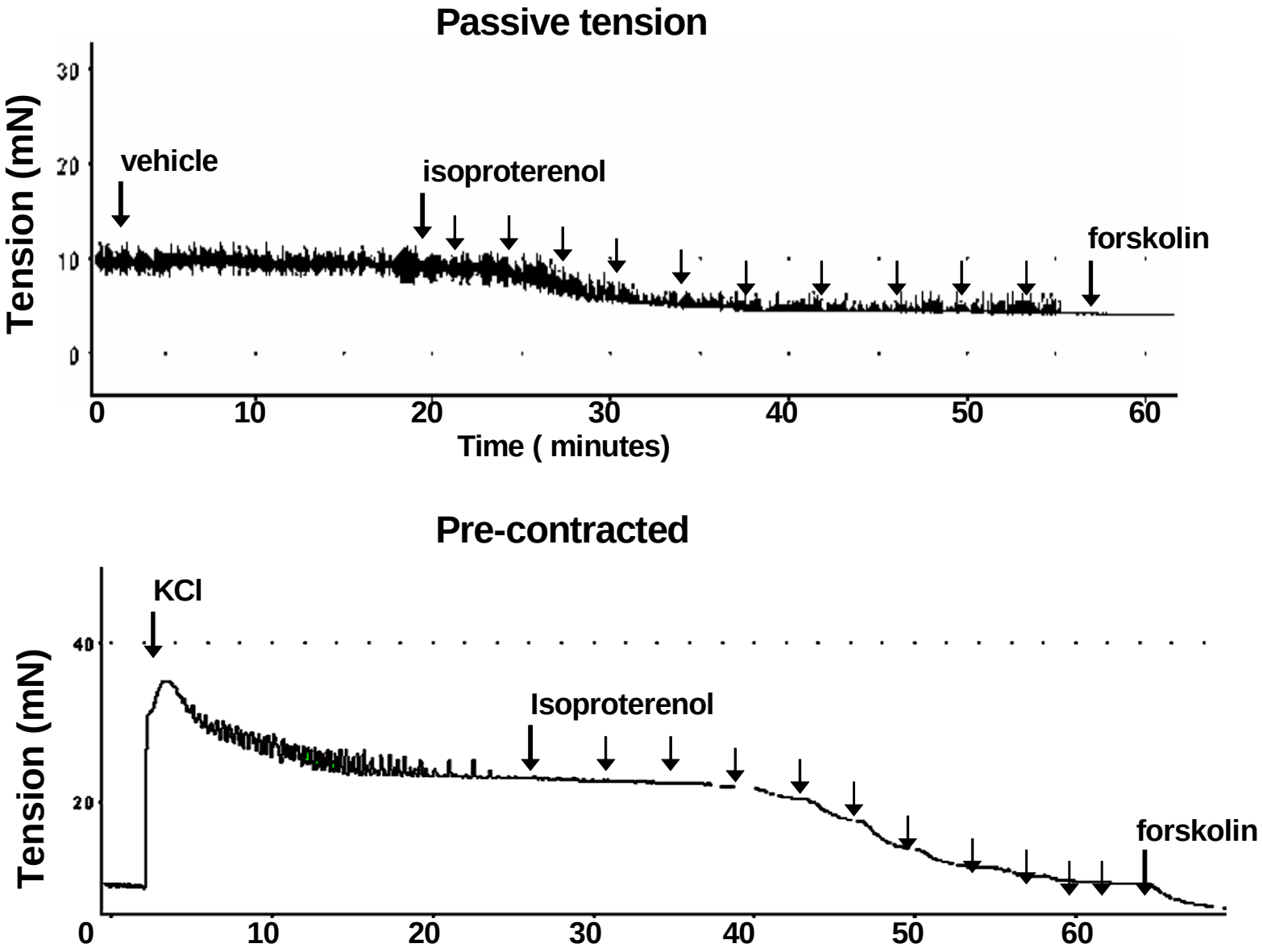

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Figure 2

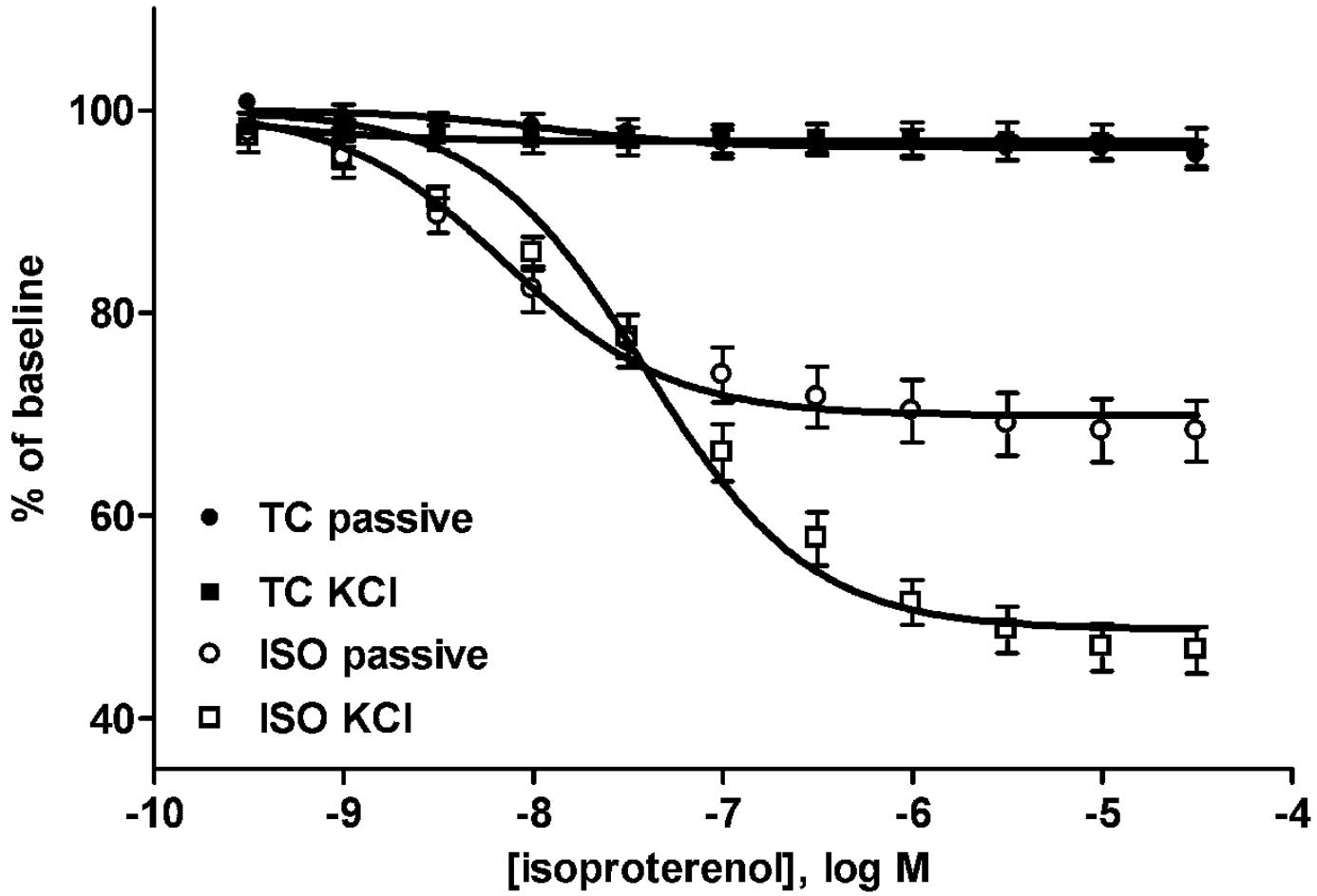

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Figure 3

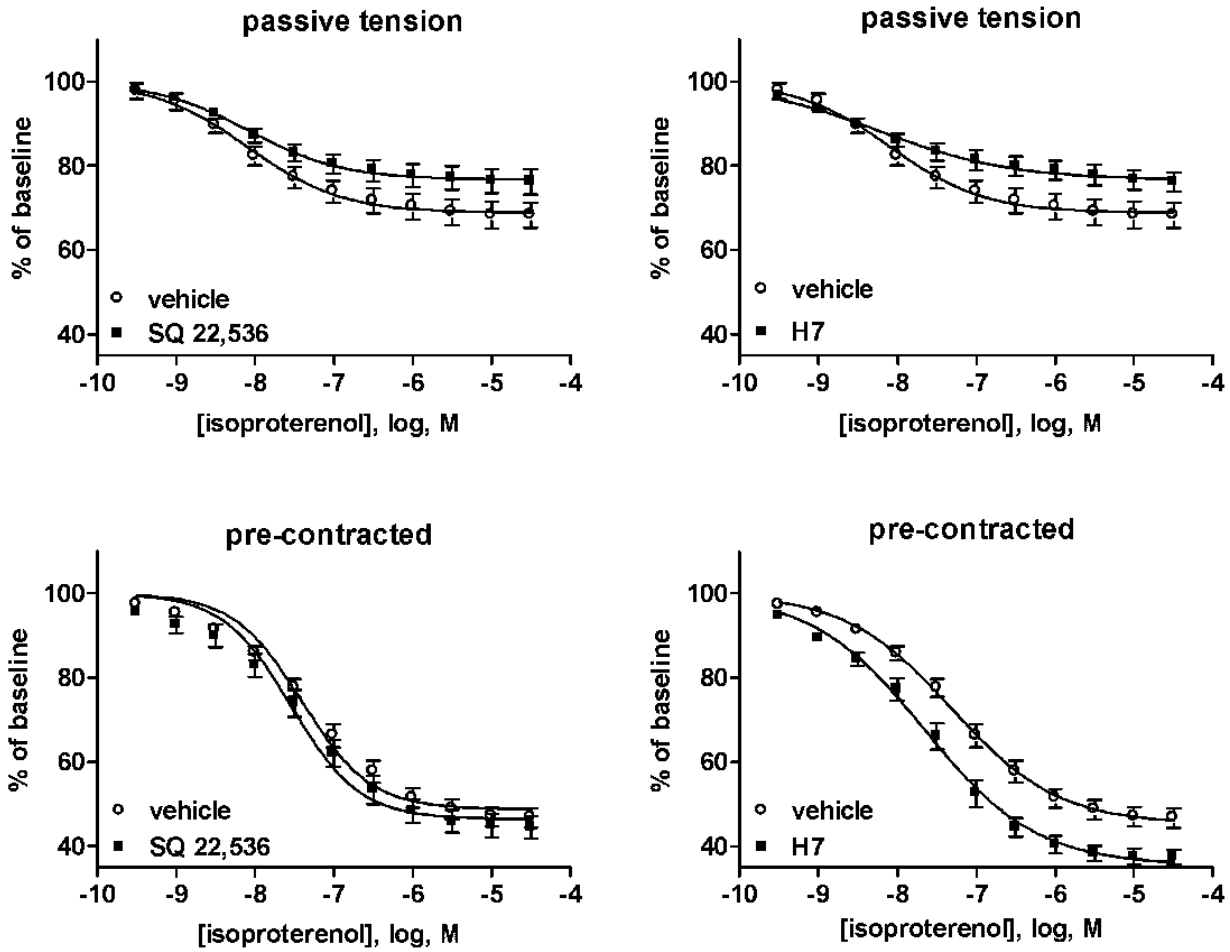

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Figure 4

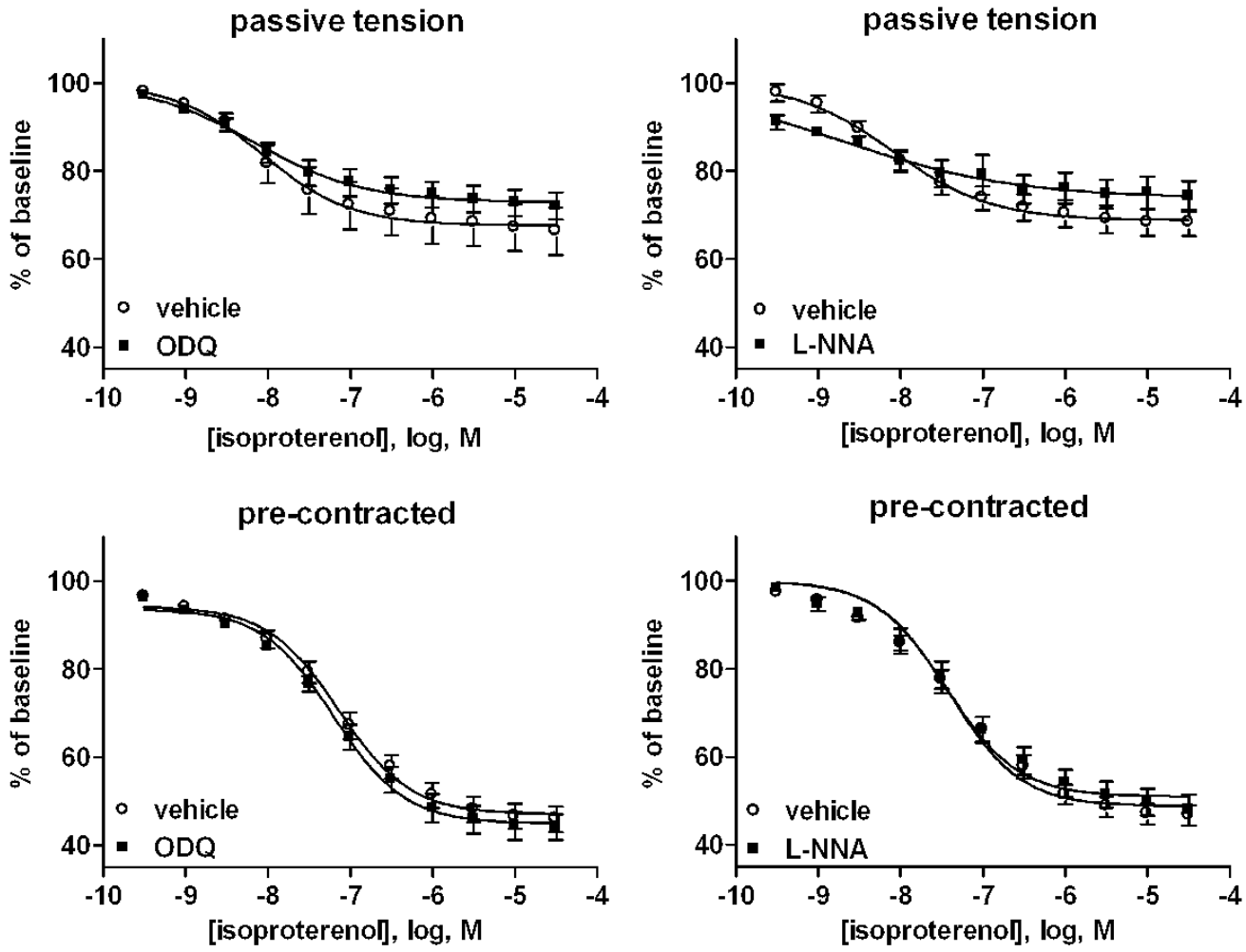

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Figure 5

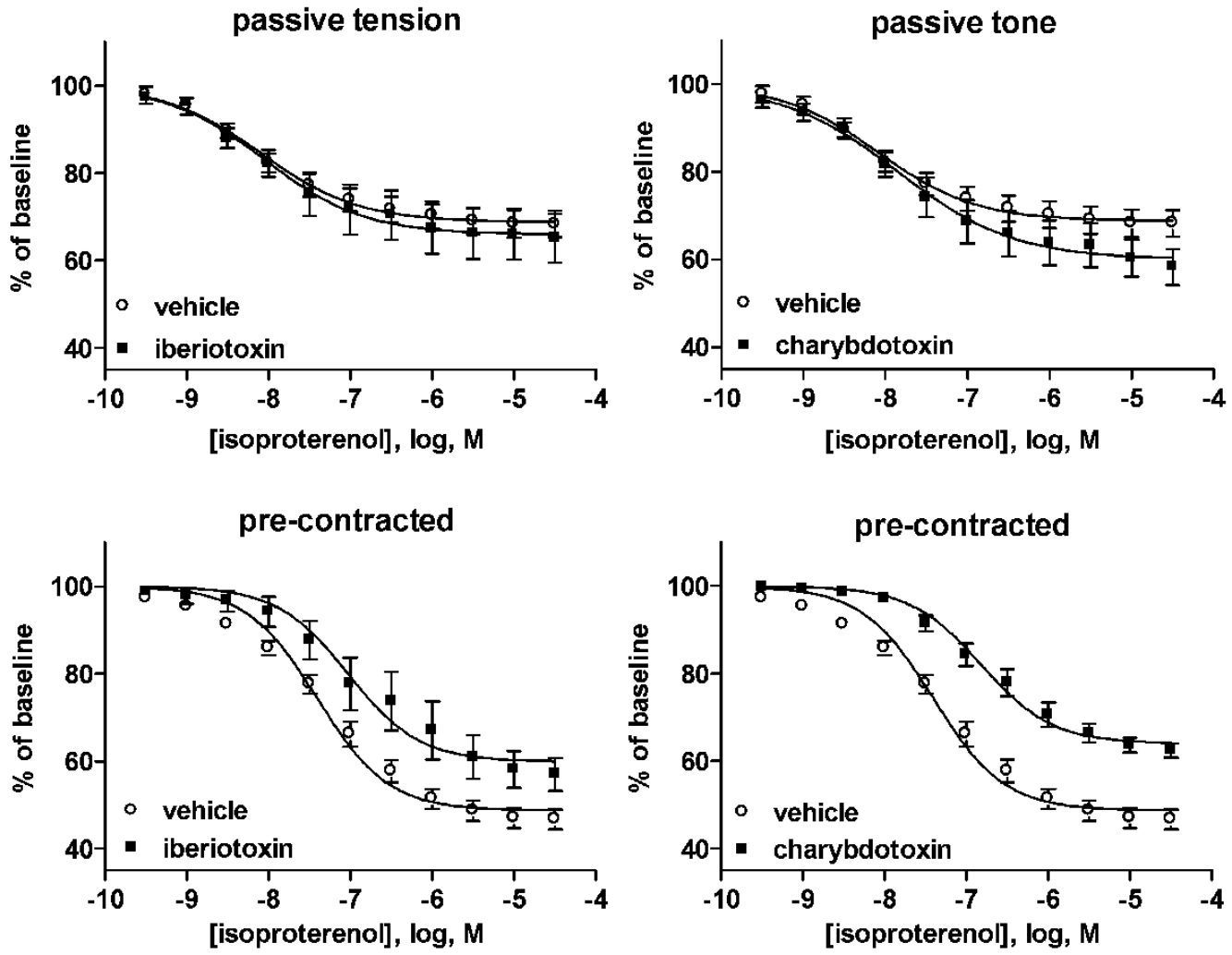

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Figure 6