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Title page

Does phospholipase C mediate muscarinic receptor-induced rat urinary bladder contraction?

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Running title page

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Abstract

Muscarinic acetylcholine receptors, particularly M_3 receptors, are the physiologically most important mechanism to induced urinary bladder smooth muscle contraction. Their prototypical signaling response is a stimulation of phospholipase C (PLC), and this has also been shown in the urinary bladder. Nevertheless, it has remained controversial whether PLC signaling mediates bladder contraction induced by muscarinic receptor agonists. Studies in favor and against a role for PLC differed in their experimental protocol (single vs. repeated concentration-response curves within a single preparation) as well as in the PLC inhibitors which have been used. We have now tested whether previous differential conclusions regarding a role for PLC are related to inhibitors and/or experimental protocols. In a single curve protocol U 73,122 [1-(6-[(17β]-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino]hexyl)-1H-pyrrole-2,5-dione] did not attenuate carbachol responses. In a repeated curve protocol ET-18-OCH_3 (1-*O*-octadecyl-2-*O*-methyl-sn-glycero-3-phosphorylcholine) lacked significant inhibition relative to vehicle time controls. In contrast, D609 (*O*-tricyclo[5.2.1.02,6]dec-9-yl dithiocarbonate potassium salt) depressed maximum carbachol effects but also non-specifically inhibited contraction induced by KCl. Neomycin did not affect the carbachol-induced rat urinary bladder contraction. We conclude that previously reported differences relate to the use of inhibitors rather than experimental protocols and that the overall data do not support a role for PLC in M_3 muscarinic receptor-mediated rat bladder contraction.

Introduction

Muscarinic receptors are physiologically the most important mechanism mediating urinary bladder contraction (Andersson, 1993). The mammalian bladder (including human) mainly expresses M_2 and M_3 muscarinic receptors, which coexist in an approximate 3:1 ratio (Hegde, 2006;Abrams et al., 2006). While M_2 receptors can contribute to bladder smooth muscle tone under certain conditions, the primary mediator of normal mammalian bladder contraction is the minor population of M_3 receptors (Hegde2006;Abrams et al., 2006). Muscarinic receptor subtypes can couple to a range of signal transduction pathways, and the primary signaling of M_3 receptors is thought to occur by stimulation of a phospholipase C (PLC) to generate inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (Caulfield, 1993). Muscarinic receptor coupling to PLC activation and IP_3 formation has also been reported from the mammalian urinary bladder (An et al., 2002;Kajioka et al., 2005;Kories et al., 2003;Schneider et al., 2004b).

Nevertheless, the role of PLC, specifically of phosphatidylinositol-PLC (PI-PLC) in mediating muscarinic receptor-stimulated bladder contraction has remained controversial, particularly in rats. Some investigators have proposed a PLC involvement based upon the finding that the PI-PLC inhibitor ET-18-OCH_3 reduced carbachol potency and the phosphatidylcholine-PLC (PC-PLC) inhibitor D609 attenuated both the carbachol potency and maximal contractile response (Braverman et al., 2006a;Braverman et al., 2006b). Other investigators proposed a lack of PLC involvement based upon the finding that the PI-PLC inhibitor U 73,122 did not affect carbachol contractile responses in a concentration where it abolished carbachol-induced IP formation in the bladder (Schneider et al., 2004b). A comparison between those studies shows that they have applied not only different inhibitors

but also different experimental protocols; while the former investigators tested inhibitor and its vehicle in parallel bladder strips (i.e. performing only one carbachol curve per strip, “Philadelphia protocol”), the latter have tested increasing inhibitor concentrations in subsequent carbachol curves within the same bladder strip (“Amsterdam protocol”). To resolve this “PLC-controversy” we have now performed cross-over experiments. Thus, each inhibitor was tested by the other group using their protocol. Our joint efforts suggest that previously reported controversies relate to the use of inhibitor rather than protocol and do not support a role for PLC in muscarinic receptor-stimulated rat bladder contraction.

Methods

All experiments were in line with NIH guidelines on the care and use of experimental animals and had been approved by the respective animal care and use committees. Based upon our previous studies, experiments either followed the "Philadelphia protocol" as routinely used in the Ruggieri lab or the "Amsterdam protocol" as routinely used in the Michel lab.

Philadelphia protocol

Experiments were basically performed as described previously (Braverman et al., 2006a;Braverman et al., 2006b). Briefly, urinary bladders were removed from female Sprague-Dawley rats (Ace, Boyertown PA, body weight 200-250 g, bladder weight approx 100 mg) which had been euthanized by N_2 asphyxiation following anesthesia with 5% isoflurane in oxygen. The bladder was divided in the mid-sagittal plane and cut into longitudinal smooth muscle strips (approximately 3 X 8 mm). The muscle strips were then suspended with 9.8 mN of tension in tissue baths containing 15 ml of modified Tyrode's solution (125 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 23.8 mM $NaHCO_3$, and 5.6 mM glucose) and equilibrated with 95% O_2/5% CO_2 at 37ºC.

After equilibration to the bath solution for 30 min, a maximal contraction induced by a 3-min exposure to 120 mM KCl was recorded. The strips were ranked based on their contractile response to KCl and sorted so that the average response in each treatment group was equal. The strips were incubated for 30 min in the presence or absence of a PLC inhibitor. Concentration-response curves were derived from the peak tension developed after

cumulative addition of carbachol (10 nM to 300 µM final bath concentration) and normalized to the response to 120 mM KCl. Only one concentration of enzyme inhibitor was used for each muscle strip and each muscle strip was only exposed to a single carbachol concentration response curve.

Amsterdam protocol

Experiments were basically performed as described previously (Schneider et al., 2004b). Briefly, urinary bladders were removed from male Wistar rats (Charles River, Netherlands; body weight 298 ± 5 g; bladder weight 91 ± 3 mg) which had been euthanized by decapitation under pentobarbital anesthesia. Urinary bladder strips were cut longitudinally into four strips (1 mm in diameter, 16 ± 0.4 mm in length, 8.4 ± 0.3 mg in weight). Bladder strips were mounted under tension of 10 mN in 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^oC, 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.

After 75 min of equilibration, including washes with fresh buffer every 15 min, the bladder strips were challenged two times with 50 mM KCl with 60-min rest and washes between each challenge. After washout and an additional 45 min equilibration, cumulative concentration-response curves were constructed for carbachol in the absence of vehicle or inhibitor. Using 10 min washout and then 30 min equilibration periods in between, up to three additional curves were then generated in the presence of increasing concentrations of the indicated inhibitors or their vehicles.

Data Analysis

Carbachol concentration-response curves were analyzed by fitting sigmoidal curves to the experimental data. The force of contraction was expressed as the percentage of the effect of 120 mM KCl observed in the same bladder strip (Philadelphia protocol) or in the same bladder strip in the first concentration-response curve, i.e., before addition of any inhibitor or vehicle (Amsterdam protocol). Alterations in E_{max} or pEC_{50} in its presence relative to the vehicle or first curve were compared within curves in the same experiment using one way analysis of variance. If the effect of individual inhibitor concentration relative to the first curve was significant, Dunnett post test was performed. All curve fitting and statistical calculations were performed with the Prism program (GraphPad Software Inc., San Diego, CA) and a $p < 0.05$ was considered to be significant.

Chemicals

Carbachol HCl, U 73,122 [1-(6-[(17β]-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino]hexyl)-1H-pyrrole-2,5-dione] and neomycin were purchased from Sigma-Aldrich (St Louis, MO). ET-18-OCH_3 (1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine) and D609 (tricyclodecan-9-yl xanthogenate.K) were obtained from BIOMOL Research Laboratory (Plymouth Meeting, PA). Et-18-CHO_3 (at 10 mM) was dissolved in ethanol. D609 and neomycin (at 10 mM) were dissolved in distilled water. U 73,122 (at 1 mM) was dissolved in dimethylsulphoxide (DMSO).

Results

Philadelphia protocol

In the absence of any inhibitor, carbachol induced rat urinary bladder contraction with a pEC_{50} of 5.86 ± 0.10 and a maximum response of 16.7 ± 2.9 mN tension (n = 12). U 73,122 (10 μM) did not affect the potency or efficacy of carbachol to induce bladder contraction when expressed as % of each strip's maximum carbachol response (Figure 1), as absolute tension or as % of KCl-induced contraction (data not shown). When darifenacin (30 nM) was added concomitantly with U 73,122 it induced a right-shift of the carbachol concentration-response curve from which an apparent pA_2 value of 8.5 was calculated (Figure 1), indicating that the response in the presence of U 73,122 indeed occurs via a M_3 receptor.

Amsterdam protocol

In the first concentration-response curve within a bladder strip, i.e. in the absence of any inhibitor, carbachol increased force of contraction with a pEC_{50} of 5.94 ± 0.03 and maximum effects of 6.0 ± 0.3 mN/mg (n = 41 muscle strips). In confirmation of previous studies (Kories et al., 2003;Schneider et al., 2004b), three subsequent curves in the absence of any inhibitor yielded rather similar values. The PLC inhibitor vehicles (distilled water, ethanol or DMSO) caused minor if any right-shifts of the carbachol curves (up to 0.2 log units) but those failed to reach statistical significance (data not shown).

In confirmation of our previous findings (Schneider et al., 2004b), addition of 1, 3 and 10 μM U 73,122 to a second, third and fourth carbachol curve, respectively, did not have significant

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effects on the potency or efficacy of carbachol as compared to vehicle time controls (Figure 2). ET-18-OCH_3 (10-100 μM) only at the highest concentration caused a minor right-shift of the carbachol curve (about 0.4 log units; Figure 3), which was not statistically significant as compared to vehicle time controls. D609 had little effect against carbachol at 10-30 μM, but at a concentration of 100 μM almost abolished the carbachol response (Figure 4). At 100 μM but not at 30 μM, D609 significantly attenuated contractile responses to 25, 50, 75, 100 and 120 mM KCl ($n = 5\text{-}6$, $p < 0.01$) when compared to vehicle control using the Philadelphia protocol (Figure 5), and 100 μM D609 similarly inhibited the response to 50 mM KCl in the Amsterdam protocol (data not shown). Neomycin (10-100 μM) did not affect the potency or efficacy of carbachol (Figure 6).

Discussion

Although M_2 receptors can contribute to mammalian bladder contraction, most studies including data from M_2 and M_3 knock-out mice suggest a predominant role for the M_3 subtype under normal physiological conditions in most species including humans (Hegde2006;Abrams et al., 2006). Activation of M_3-receptors prototypically leads to the hydrolysis of phosphatidylinositol 4, 5-biphosphaste to result in generation of the second messengers IP_3 and diacylglycerol which is then followed by elevation of intracellular calcium concentration and by activation of protein kinase C. It can also couple to the hydrolysis of phophatidylcholine by a PC-PLC which may contribute to sustaining smooth muscle contraction (Caulfield1993). However, the role of PLC, specifically of PI-PLC in mediating muscarinic receptor-stimulated bladder contraction has remained controversial and the two labs contributing to the present study have taken somewhat different views on this. As we had previously used both different inhibitors and different experimental protocols to explore a role of PLC, we have now collaborated in a cross-over study in which each inhibitor was tested in the opposite protocol.

Both protocols showed a very similar potency of carbachol to elicit rat bladder contraction under baseline conditions, thus providing cross-validation of our two techniques. The two labs also have used rats of different gender (females in Philadelphia and males in Amsterdam), but previous work shows that gender does not affect carbachol-induced contraction or IP formation in rat bladder (Kories et al., 2003). The key difference between the two protocols had been the use of single vs. repeated concentration-response curves within a single muscle strip. The reproducibility of pEC_{50} and E_{max} across multiple curves (Kories et al., 2003;Schneider et al., 2004b) argues that no major desensitization occurred

under these conditions in the Amsterdam protocol. However, it is possible that during repeated curves a switch from one underlying signaling mechanism to another could occur, which may not manifest in alterations of pEC_{50} or E_{max}, and may have led to missing a role for PLC in the Amsterdam protocol.

In total four different PLC inhibitors have been tested in the present study. In this regard U 73,122 and ET-18-OCH_3 are considered PI-PLC inhibitors, D609 is considered a PC-PLC inhibitor, and neomycin is considered a non-selective PLC inhibitor. U 73,122 had previously lacked inhibitory effects against carbachol-induced contraction in rat bladder in a concentration where if fully abolished carbachol-induced IP formation (Schneider et al., 2004b). This PI-PLC inhibitor had also failed to block carbachol-induced contraction of mouse (Wegener et al., 2004) or human bladder (Schneider et al., 2004a), all of which had used the Amsterdam or a similar protocol. In the present study U 73,122 also did not affect the carbachol response in the Philadelphia protocol. It could be argued that PI-PLC is involved in this response but its role can be concealed by a switch from M_3 to M_2 receptors in the presence of U 73,122. However, the high potency of darifenacin to antagonize contraction in the presence of U 73,122 indicates that this contraction is indeed M_3-mediated. Thus, the lack of U 73,122 to inhibit bladder contraction in a concentration where it inhibited IP formation does not support a role for PI-PLC in carbachol-induced bladder contraction.

Previously, ET-18-OCH_3 (30-100 μM), i.e. at 6-20 times its reported K_i value for PI-PLC (Braverman et al., 2006b) was reported to decrease the apparent potency of carbachol without effect on E_{max} in the Philadelphia protocol (by about 1 log unit at 100 μM) (Braverman et al., 2006b). In the present studies using the Amsterdam protocol such antagonism was less pronounced and did not reach statistical significance as compared to vehicle time controls.

The reason for this discrepancy remains unclear but it should be noted that the final ethanol concentration in the assay in the original Philadelphia experiments had been only 0.35% whereas it was 1.0% in the present experiments using the Amsterdam protocol. Moreover, in light of the unequivocal U 73,122 data, this minor discrepancy between reported data has little effect on the overall conclusion regarding a relative lack of a PI-PLC component in the contractile carbachol response.

It has been proposed that a PC-PLC may be involved in the sustained phase of smooth muscle contraction (Somlyo and Somlyo, 1994;Makhlouf and Murthy, 1997). Using the Philadelphia protocol it had previously been reported that the PC-PLC inhibitor D609 at 100 μM, but not at lower concentrations, can reduce the E_{max} of carbachol in rat bladder (Braverman et al., 2006b). The present study using the Amsterdam protocol confirms this observation. However, this may not reflect a role for PC-PLC in carbachol-induced bladder contraction as the same concentration of D609 also suppressed the receptor-independent contraction induced by KCl in both the Philadelphia and the Amsterdam protocol. Moreover, D609 may have additional effects unrelated to PC-PLC (van Dijk et al., 1997;Kiss and Tomono, 1995). Which if any of those D609 effects is involved in the inhibition of the carbachol response in the bladder remains to be investigated. However, regardless of this consideration these data do not support a specific role of PC-PLC in the carbachol response.

Finally, neomycin is a rather non-specific inhibitor of PLC, which had been used to support a claim for an involvement of PLC in feline bladder smooth muscle contraction (An et al., 2002). However, neither the previous data using the Philadelphia protocol (Braverman et al., 2006b) nor the present data using the Amsterdam protocol support such effects, at least in rats.

Taken together the available data clearly show that muscarinic receptors in the bladder can couple to PLC activation (An et al., 2002;Kajioka et al., 2005;Kories et al., 2003;Schneider et al., 2004b) but do not support a major role for such activation in mediating bladder contraction induced by muscarinic receptor agonists. In contrast, undisputed roles exist for an influx of extracellular calcium through voltage-dependent channels (Schneider et al., 2004a;Schneider et al., 2004b;Ikeda et al., 1999) and for activation of a rho-kinase (Peters et al., 2006) in mediating bladder contraction. Moreover, calcium release from intracellular stores mediated by a ryanodine receptor may also play a role (Kajioka et al., 2005).

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Legends for figures

Figure 1: Effect of U 73,122 (10 μM) alone and in combination with darifenacin (30 nM) on carbachol-induced rat bladder contraction using the Philadelphia protocol (one curve per muscle strip). Data are means ± SEM of 6-12 experiments. The maximum contractile response (mean ± SEM) in mN of tension are 17 ± 3 for control vehicle, 23 ± 5 for U 73,122 and 17 ± 2 for U73,122 + darifenacin.

Figure 2: Effect of U-73,122 (1-10 μM) on carbachol-induced rat bladder contraction using the Amsterdam protocol (multiple curves per muscle strip). Data are means ± SEM of 6 experiments.

Figure 3: Effect of ET-18-OCH_3 (10-100 μM) on carbachol-induced rat bladder contraction using the Amsterdam protocol (multiple curves per muscle strip). Data are means ± SEM of 7 experiments.

Figure 4: Effect of D609 (10-100 μM) on carbachol-induced rat bladder contraction using the Amsterdam protocol (multiple curves per muscle strip). Data are means ± SEM of 6 experiments.

Figure 5: Effect of D609 (30 and 100 μM) on KCl-induced rat bladder contraction using the Philadelphia protocol (one curve per muscle strip). Data are means ± SEM of 5-6 experiments. The initial 120 mM KCl contractile response (mean ± SEM) in mN of tension are 34 ± 4 for control, 34 ± 4 for 30 μM D609 and 38 ± 4 for 100 μM D609.

Figure 6: Effect of neomycin (10-100 μM) on carbachol-induced rat bladder contraction using the Amsterdam protocol (multiple curves per muscle strip). Data are means ± SEM of 4 experiments.

Figure 1

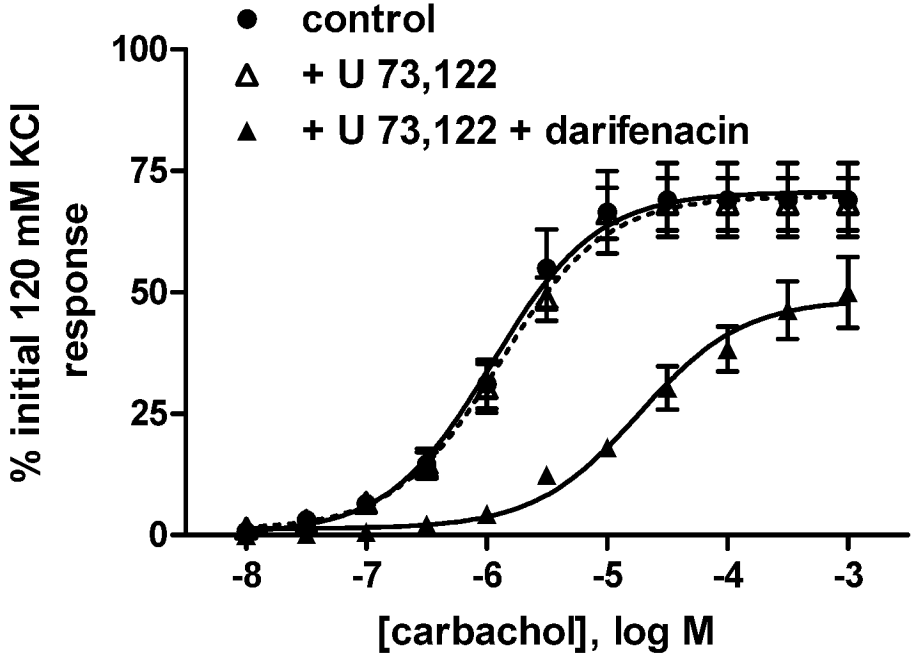

control
+ U 73,122
+ U 73,122 + darifenacin
% initial 120 mM KCl response
100
75
50
25
0
-8
-7
-6
-5
-4
-3
[carbachol], log M

Figure 2

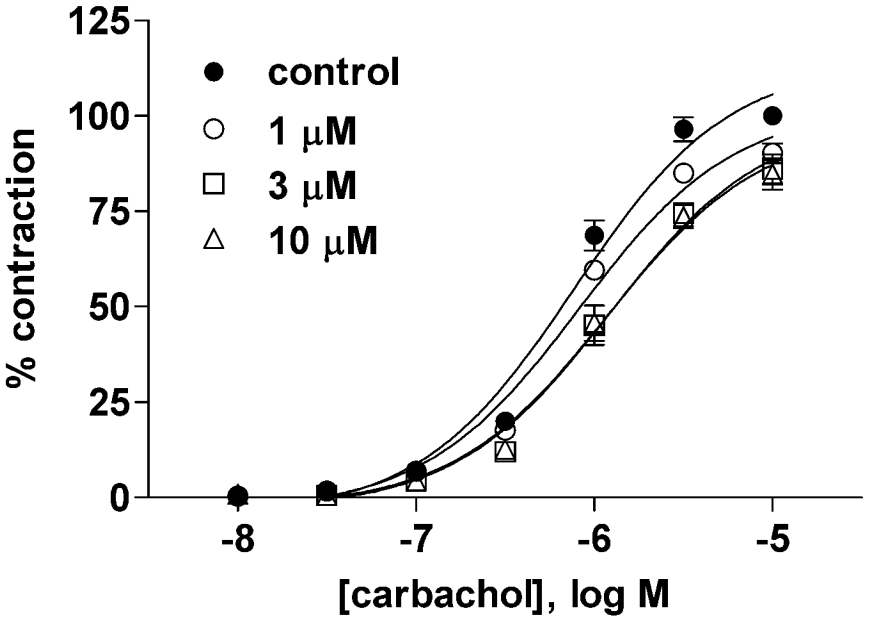

125
100
75
50
25
0
% contraction
control
1 μM
3 μM
10 μM
-8
-7
-6
-5
[carbachol], log M

Figure 3

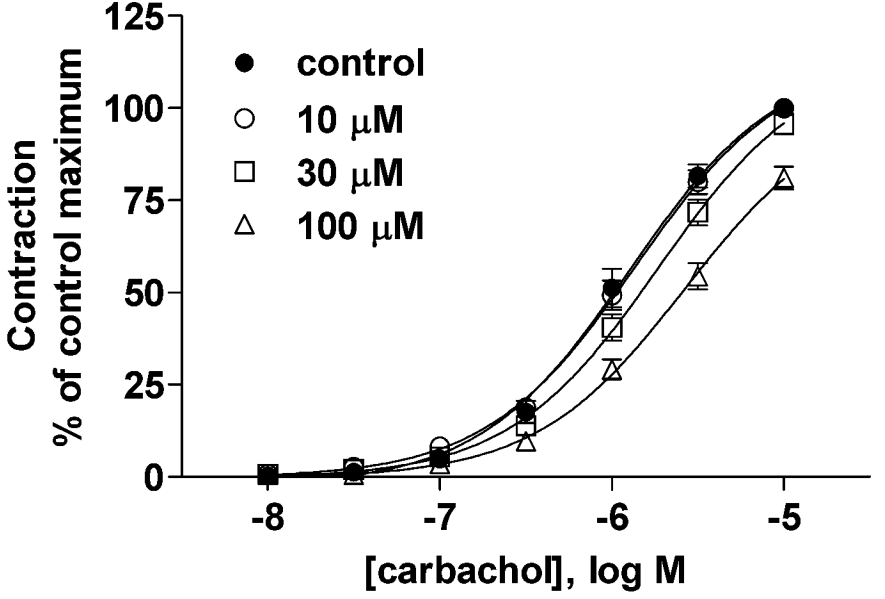

125
100
75
50
25
0
Contraction
% of control maximum
control
10 μM
30 μM
100 μM
-8
-7
-6
-5
[carbachol], log M

Figure 4

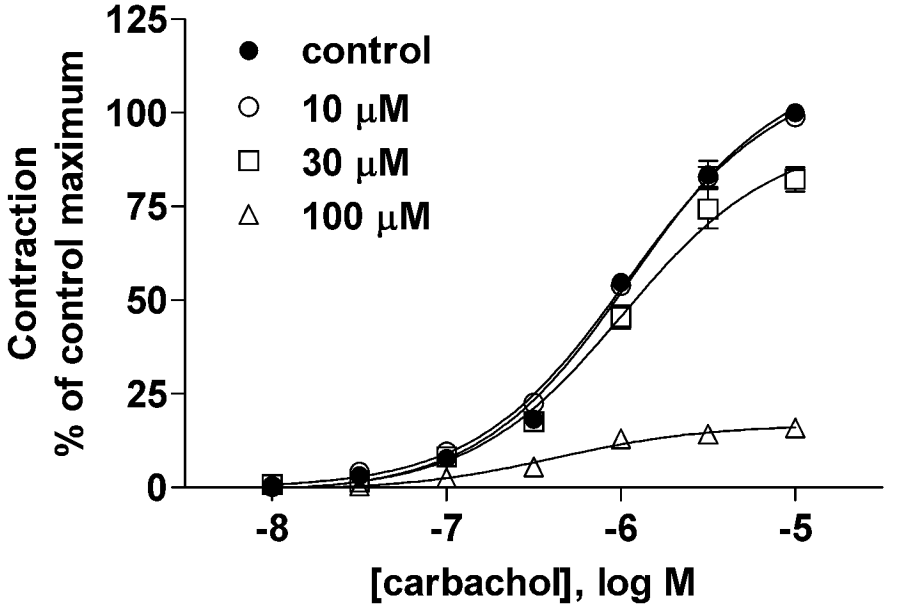

125
100
75
50
25
0
Contraction
% of control maximum
control
10 μM
30 μM
100 μM
-8
-7
-6
-5
[carbachol], log M

Figure 5

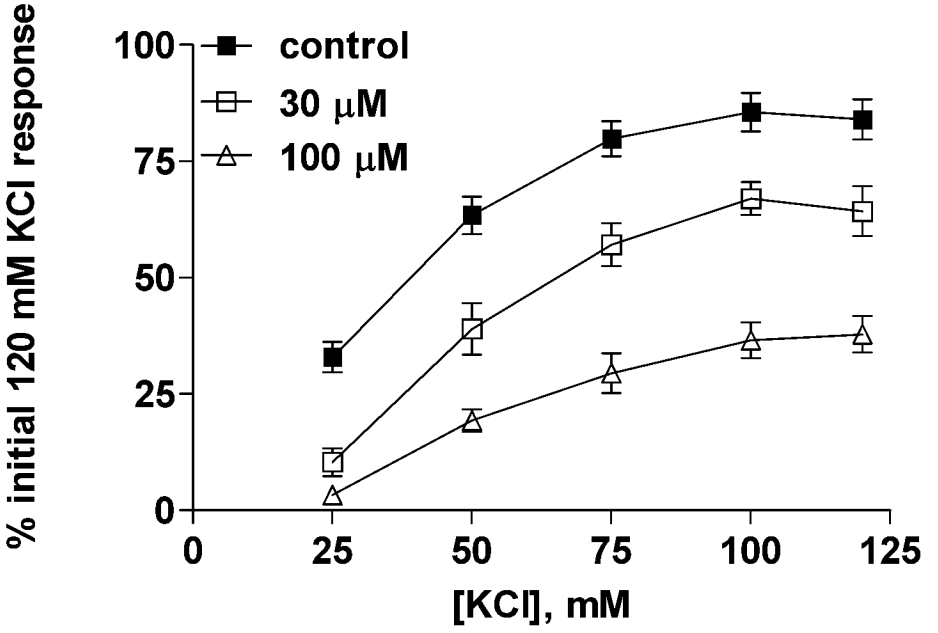
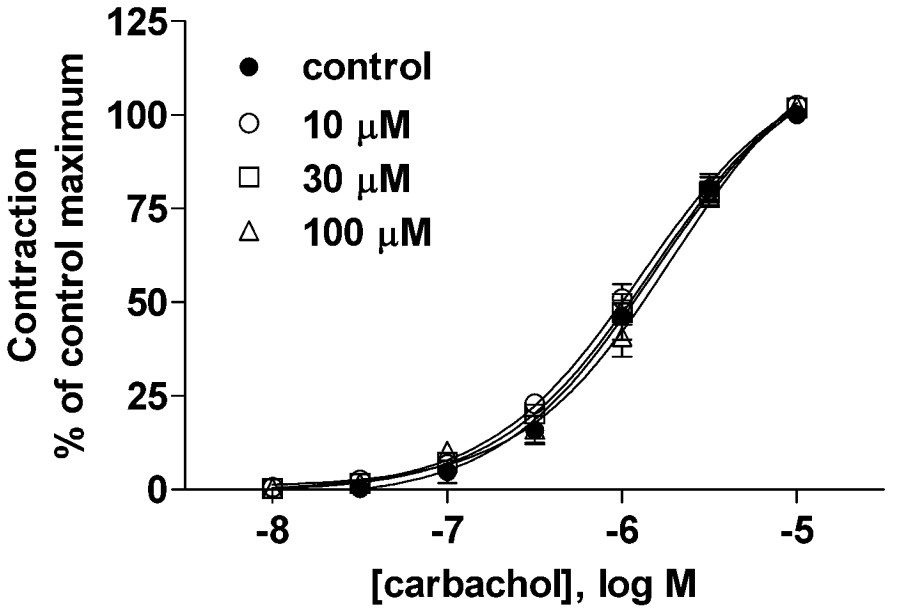

% initial 120 mM KCl response
100
75
50
25
0
control
30 μM
100 μM
0
25
50
75
100
125
[KCl], mM

Figure 6


125
100
75
50
25
0
Contraction
% of control maximum
control
10 μM
30 μM
100 μM
-8
-7
-6
-5
[carbachol], log M