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Signal transduction underlying carbachol-induced contraction of rat urinary bladder.

II. Protein kinases*

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Running title: Protein kinases in muscarinic bladder contraction

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Non-standard abbreviations:

DMSO; dimethylsulfoxide

ERK; extracellular signal-regulated kinase

MAPK, mitogen-activated protein kinases

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PI-3-kinase, phosphatidylinositol-3-kinase

PKC, protein kinase C

Recommended section assignment: other

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ABSTRACT

We have investigated the role of several protein kinases in carbachol-stimulated, M_3 muscarinic receptor-mediated contraction of rat urinary bladder. Concentration-response curves for the muscarinic receptor agonist carbachol were generated in the presence of multiple concentrations of inhibitors of various protein kinases, their inactive controls or their vehicles. Bladder contraction was not significantly inhibited by three protein kinase C inhibitors (chelerythrine 1-10 μM, calphostin C 0.1-1 μM and Gö 6850 1-10 μM), by the tyrosine kinase inhibitor genistein or its inactive control daidzein (3-30 μM each) or by two inhibitors of activation of mitogen-activated protein kinase (PD 98,059 10-100 μM and U 126 3-30 μM) or their negative control U 124 (3-30 μM). While high concentrations of wortmannin (3-30 μM) inhibited bladder contraction, this was not mimicked by another inhibitor of phosphatidylinositol-3-kinase, LY 294,002 (3-30 μM) and hence more likely due to direct inhibition of myosin light chain kinase by wortmannin than to an involvement of phosphatidylinositol-3-kinase. In contrast, Y 27,632 (1-10 μM), an inhibitor of rho-associated kinases, concentration-dependently and effectively attenuated the carbachol responses. We conclude that carbachol-induced contraction of rat urinary bladder does not involve protein kinase C, phosphatidylinositol-3-kinase, tyrosine kinases or extracellular signal-regulated kinases; in contrast rho-associated kinases appear to play an important role in the regulation of bladder contraction.

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Muscarinic acetylcholine receptors are the physiologically most important mechanism to mediate contraction of the urinary bladder (Andersson, 1993). Although rat bladder expresses more M_2 than M_3 receptors, contractile responses to the exogenous muscarinic agonist carbachol and to endogenous agonist released by field stimulation occur predominantly if not exclusively via M_3 receptors in rats (Longhurst et al., 1995;Hegde et al., 1997;Choppin et al., 1998;Tong et al., 1997;Braverman et al., 1998;Longhurst and Levendusky, 2000;Kories et al., 2003). In the accompanying paper we have confirmed the involvement of M_3 but not M_2 receptors in carbachol-induced contraction and have demonstrated that this involves nifedipine-sensitive Ca^{2+} channels and, to a smaller degree, phosphoplipases D and A_2 and store-operated Ca^{2+} channels, but not cyclooxygenase and surprisingly also not phospholipase C (Schneider et al., 2003). Overall, this pattern of proximal signaling mediating urinary bladder smooth muscle contraction shares many properties with that of other types of smooth muscle such as vascular smooth muscle. Such proximal signaling pathways are connected to smooth muscle contraction by a network of protein kinases. Kinases which have been implied mediating the contraction of vascular smooth muscle include protein kinase C (PKC) (Aburto et al., 1995;Dessy et al., 1998), phosphatidylinositol-3-kinase (PI-3-kinase) (Ibitayo et al., 1998), tyrosine kinases (Jinsi et al., 1996;Di Salvo et al., 1997) including those of the src family (Roberts, 2001), mitogen-activated protein kinases (MAPK) particularly those of the extracellular signal-regulated kinase (ERK) family (Dessy et al., 1998;Fetscher et al., 2001), and rho-associated kinase (Mukai et al., 2001;Fukata et al., 2001;Altmann et al., 2003). Therefore, we have investigated possible roles of these kinases in the carbachol-induced M_3 receptor-mediated contraction of rat bladder.

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METHODS

Force of contraction: Urinary bladder strips were prepared from female Wistar rats (265 ± 4 g bladder weight, 77 ± 1 mg bladder weight, n = 40) obtained from the central animal breeding facility at the University of Essen. Experiments were performed as previously described (Kories et al., 2003). Briefly, longitudinal bladder strips (approximately 1 mm diameter, 17 ± 1 mm length, 10.1 ± 0.3 mg weight, n = 143) were mounted under a tension of 10 mN in 10 ml organ baths containing Krebs-Henseleit solution (119 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.027 mM EDTA, 5.5 mM glucose and 10 mM HEPES) which was aerated with 95% O_2 and 5% CO_2 to yield a pH of 7.4 at 37°C. After 60 min of equilibration including washes with fresh buffer every 15 min, the bladder strips were challenged three times with a combination of 50 mM KCl and 0.1 mM carbachol with 5 min rest and washes between each challenge. Following washout and an additional 30 min of equilibration, cumulative concentration-response curves were constructed for carbachol in the absence of any inhibitor or vehicle. Using washout and 15 min equilibration periods in between, up to 3 additional curves were then generated in the presence of increasing concentrations of the indicated inhibitors, their negative controls or their vehicles.

Carbachol concentration-response curves were analyzed by fitting sigmoidal curves to the experimental data, in which the bottom of the curve was fixed at 0. The force of contraction in the absence and presence of inhibitors were expressed as % of maximum carbachol effects observed within the same bladder strip in the first concentration-response curve, i.e. prior to addition of any inhibitor or vehicle. To assess inhibitor effects, alterations in E_{max} or pEC_{50} in its presence relative to the first curve were compared to those in the presence of a matching vehicle time

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control using two-way analysis of variance testing for main treatment effect and concentration-dependency; if this indicated statistical significance, the effect of individual inhibitor concentrations relative to time-matched controls was assessed by Bonferroni post-tests. A $p < 0.05$ was considered to be significant in all statistical analyses. All curve fitting and statistical calculations were performed with the Prism program (version 4.0, Graphpad Software, San Diego, CA).

Chemicals Carbachol HCl, PD 98,059 (2'-amino-3'-methoxyflavone) and wortmannin were obtained from Sigma-Aldrich (Taufkirchen, Germany). Calphostin C (from *Cladosporium cladosprioides*), chelerythrine HCl, LY 294,002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), LY 303,511 (2-piperazinyl-8-phenyl-4H-1-benzopyran-4-one), U 124 (1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene) and U 126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene) were obtained from Calbiochem (Bad Soden, Germany). Y 27,632 (trans-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide) was from Tocris (Bristol, UK).

Calphostin C (at 1 mM), chelerythrine (at 1 mM), daidzein (at 10 mM), genistein (at 10 mM) Gö 6850 (at 1 mM), LY 294,002 (at 10 mM), LY 303,511 (at 10 mM), PD 98,059 (at 100 mM), U 124 (at 10 mM), U 126 (at 10 mM) and wortmannin (at 1 mM) were dissolved in dimethylsulfoxide (DMSO). Y 27,632 (at 10 mM) was dissolved in water.

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RESULTS

Prior to addition of any inhibitor, carbachol concentration-dependently increased force of contraction with a pEC_{50} of 5.75 ± 0.02 and maximum effects of 38.1 ± 1.0 mN (n = 143 strips). All further data are expressed as % of the maximum carbachol effect in the same preparation prior to addition of any inhibitor, negative control or vehicle.

A potential role of PKC was assessed using the inhibitors chelerythrine (1-10 µM, figure 1), calphostin C (0.1-1 µM, supplementary figure 1) and Gö 6850 (1-10 µM, supplementary figure 2), with the latter being a selective inhibitor of classical PKC isoforms. None of the three inhibitors significantly affected the potency or maximum effects of carbachol-induced bladder contraction relative to paired time-control experiments in the presence of vehicle.

To test a possible role of PI-3-kinase in carbachol-induced bladder contraction, the inhibitors wortmannin (3-30 µM, figure 2) and LY 294,002 (3-30 µM, figure 3) as well as LY 303,511 (3-30 µM, supplementary figure 3), a negative control for LY 294,002 were tested. Wortmannin caused statistically significant inhibition of carbachol-induced contraction at concentrations starting at a concentration of 3 µM and almost completely abolished the carbachol effects at 30 µM; the inhibition by wortmannin involved reduction of both, the potency and maximal effects of carbachol (figure 2). In contrast, LY 294,002 did not cause statistically significant inhibition of carbachol-induced bladder contraction (figure 3). LY 303,511, despite not being an inhibitor of PI-3-kinase, caused statistically significant reductions of carbachol potency (but not maximal responses) at all concentrations which had been tested (supplementary figure 3).

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A potential role of tyrosine kinases was tested using the inhibitor genistein (3-30 μM, n = 6, figure 4) and its negative control daidzein (3-30 μM, n = 6, supplementary figure 4). Neither genistein nor daidzein significantly affected the potency or maximal effects of carbachol-induced bladder contraction.

To test a possible role MAPK of the ERK type, we have used the MAPK kinase inhibitors PD 98,059 (10-100 μM, n = 6, figure 5) and U 126 (3-30 μM, n = 6, supplementary figure 5) and U 124 (3-30 μM, n = 7, supplementary figure 6), a negative control for the latter. None of the three inhibitors significantly affected the potency or maximal effects of carbachol in rat urinary bladder.

To test a role of rho-associated kinase, its inhibitor Y 27,632 (1-10 μM, n = 6, figure 6) was tested. Y 27,632 concentration-dependently inhibited carbachol-induced bladder contraction, and this inhibition consisted mainly of a reduction of maximum responses (by 13 ± 1%, 22 ± 3% and 61 ± 4%) with only moderate reduction in pEC_{50} (by 0.028 ± 0.05, 0.45 ± 0.05 and 0.57 ± 0.06 log units) with 1, 3 and 10 μM Y 27,632, respectively.

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DISCUSSION

The muscarinic acetylcholine receptor agonist carbachol mediates contraction of rat bladder via M_3 receptors (Longhurst et al., 1995;Hegde et al., 1997;Choppin et al., 1998;Tong et al., 1997;Braverman et al., 1998;Longhurst et al., 2000;Kories et al., 2003). The accompanying manuscript has described the relative roles of proximal signalling pathways in mediating this response (Schneider et al., 2003). In other types of smooth muscle these proximal pathways are linked to smooth muscle contraction by a network of protein kinases, but the specific wiring depends on the receptor and type of smooth muscle under investigation. The present study has investigated the possible involvement of a number of protein kinases in carbachol-induced M_3 receptor-mediated contraction of rat urinary bladder which have previously been implied in mediating the contraction of vascular smooth muscle (see introduction). Recent studies have highlighted the problem that a number of prototypical kinase inhibitors can also have effects unrelated to inhibition of their cognate kinase (Davies et al., 2000;El-Kholy et al., 2003;Altmann et al., 2003). Therefore, the present study, whenever possible, has used multiple, chemically unrelated kinase inhibitors as well as negative controls, i.e. chemically related compounds which lack kinase inhibition, in order to minimize problems related to non-specific inhibitor effects.

A role for PKC in mediating agonist-stimulated contraction of vascular smooth muscle has been identified in some vessels for some receptors (Aburto et al., 1995;Dessy et al., 1998) whereas the same or other receptors in different vessels elicited vasoconstriction without intermediate PKC activation (Fetscher et al., 2001;Shirao et al., 2002). In the present study, three chemically distinct PKC inhibitors failed to significantly affect carbachol-induced contraction of rat urinary bladder despite being tested in high concentrations. Since PKC activation, particularly of the

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classical PKC isoforms which are sensitive to inhibition by Gö 6850, frequently occurs secondary to phospholipase C activation, and since experiments shown in the accompanying paper did not detect a role for phospholipase C in urinary bladder contraction (Schneider et al., 2003), these data demonstrate that the phospholipase C/PKC cascade may be activated by muscarinic receptors in rat bladder (Livak and Schmittgen, 2001;Schneider et al., 2003) but is not crucial for induction of contraction.

Activation of a PI-3-kinase has also been implicated in contraction of colonic (Ibitayo et al., 1998) but not vascular smooth muscle (Altmann et al., 2003). In the present study, a high concentration of one inhibitor (wortmannin) inhibited carbachol-induced bladder contraction, whereas another one (LY 294,002) did not. In this context, it should be noted that high concentrations of wortmannin (but not of LY 294,002) were also shown to inhibit skeletal muscle contraction in a manner independent from PI-3-kinase inhibition (Hong and Chang, 1998), possibly by directly inhibiting myosin light chain kinase (Takayama et al., 1996). The unexpected inhibitory effect of LY 303,511, a negative control for LY 294,002 with regard to PI-3-kinase inhibition, was not further investigated in the present study but may relate to other actions of this compound such as inhibition of certain K^+ channels (El-Kholy et al., 2003) or direct blockade of certain receptors (Altmann et al., 2003). The overall data, however, indicate that PI-3-kinase is not involved in mediating carbachol-induced contraction of rat urinary bladder.

Tyrosine kinases (Jinsi et al., 1996;Di Salvo et al., 1997;Janssen et al., 2001) including those of the src family (Roberts, 2001) can also mediate smooth muscle contraction in some preparations but not in others (Fetscher et al., 2001;Altmann et al., 2003). In the present study neither the

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tyrosine kinase inhibitor genistein nor its negative control daidzein inhibited bladder contraction, indicating that tyrosine kinases do not play a major role in this regard.

Recently it was demonstrated that inhibitors of MAPK activation, particularly of MAPK of the ERK family, can also inhibit smooth muscle contraction in some cases (Dessy et al., 1998;Fetscher et al., 2001;Roberts, 2001) but not in others (Watts et al., 1998;Janssen et al., 2001;Altmann et al., 2003). In the present study neither PD 98,059 nor U 126 nor its negative control U 124 significantly inhibited carbachol-induced contraction of rat urinary bladder. Hence, ERK activation does not appear to be required to contract this type of smooth muscle.

Rho-associated kinase is gaining attention as a universal regulator of smooth muscle tone. It is expressed at high levels in rat urinary bladder, and its inhibitor Y 27,632 attenuates bladder contraction induced by stimulation of muscarinic, purinergic or neurokinin A receptors (Wibberley et al., 2003). A similar inhibition was also seen in the present study. Studies in other tissues indicate that this can involve several different mechanisms including a direct effect on myosin light chains, effects on CPI-17, a phosphorylation-dependent inhibitory protein of myosin phosphatase, and, perhaps most importantly, a direct inhibition of myosin phosphatase (Fukata et al., 2001). Which of these mediates the effects on bladder contraction, remains to be investigated.

Taken together it appears that a range of protein kinases, which has been implicated in mediating contraction of other types of smooth muscle such as PKC, PI-3-kinase, tyrosine kinases and MAPK of the ERK family, do not play a major role in mediating carbachol-induced M_3 muscarinic receptor-mediated contraction of rat urinary bladder. In contrast, this and previous

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data (Wibberley et al., 2003) suggest that rho-associated kinase is an important mediator of bladder contraction. Further studies at the subcellular level appear necessary to elucidate the molecular pathways linking proximal signal transduction of the muscarinic receptor to the activation of rho-associated kinase and ultimately urinary bladder smooth muscle contraction.

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Footnote page

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LEGENDS TO THE FIGURES

Figure 1: Effects of the protein kinase C inhibitor chelerythrine and its vehicle DMSO (final concentration ≤ 1%) on carbachol-induced contraction (n = 6).

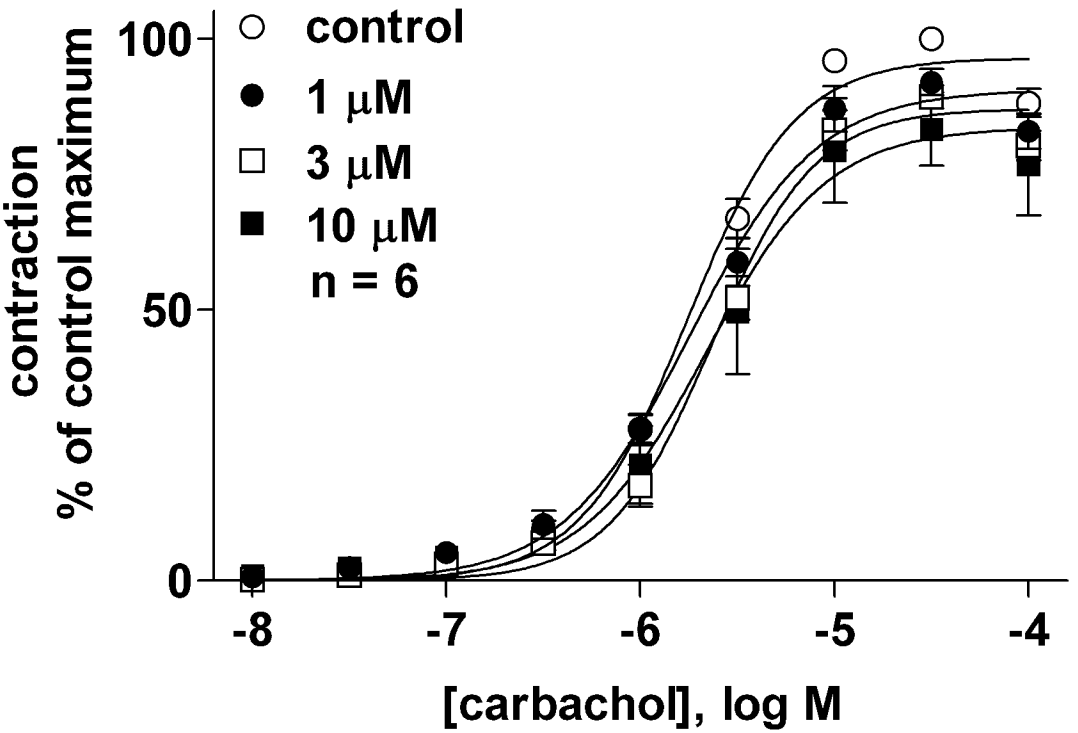
Figure 2: Effects of the phosphatidylinositol-3-kinase inhibitor wortmannin and its vehicle DMSO (final concentration ≤ 3%) on carbachol-induced contraction (n = 8). ** and ***: $p < 0.01$ and < 0.001, respectively, vs. matching time controls in the presence of vehicle in a two-way analysis of variance followed by Bonferroni post-tests.

Figure 3: Effects of the phosphatidylinositol-3-kinase inhibitor LY 294,002 and its vehicle DMSO (final concentration ≤ 0.3%) on carbachol-induced contraction (n = 8).

Figure 4: Effects of the tyrosine kinase inhibitor genistein and its vehicle DMSO (final concentration ≤ 0.3%) on carbachol-induced contraction (n = 6).

Figure 5: Effects of PD 98,059, an inhibitor of the activation of mitogen-activated protein kinases, and its vehicle DMSO (final concentration ≤ 0.1%) on carbachol-induced contraction (n = 6).

Figure 6: Effects of the rho kinase inhibitor Y 27,632 and its vehicle water (final concentration ≤ 0.1%) on carbachol-induced contraction (n = 6). ** and ***: $p < 0.01$ and < 0.001, respectively, vs. matching time controls in the presence of vehicle in a two-way analysis of variance followed by Bonferroni post-tests.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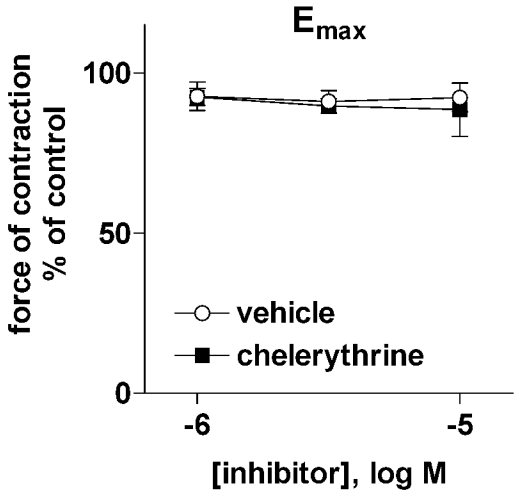
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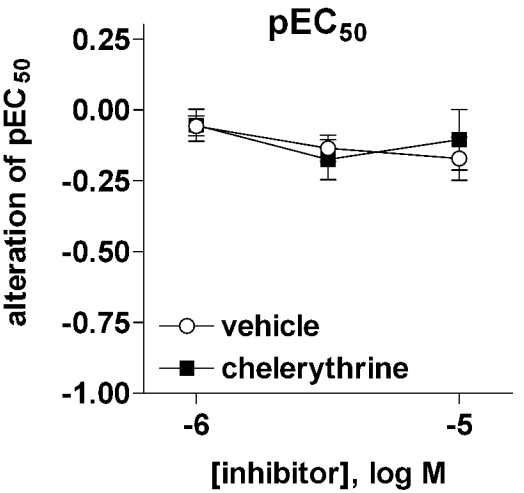
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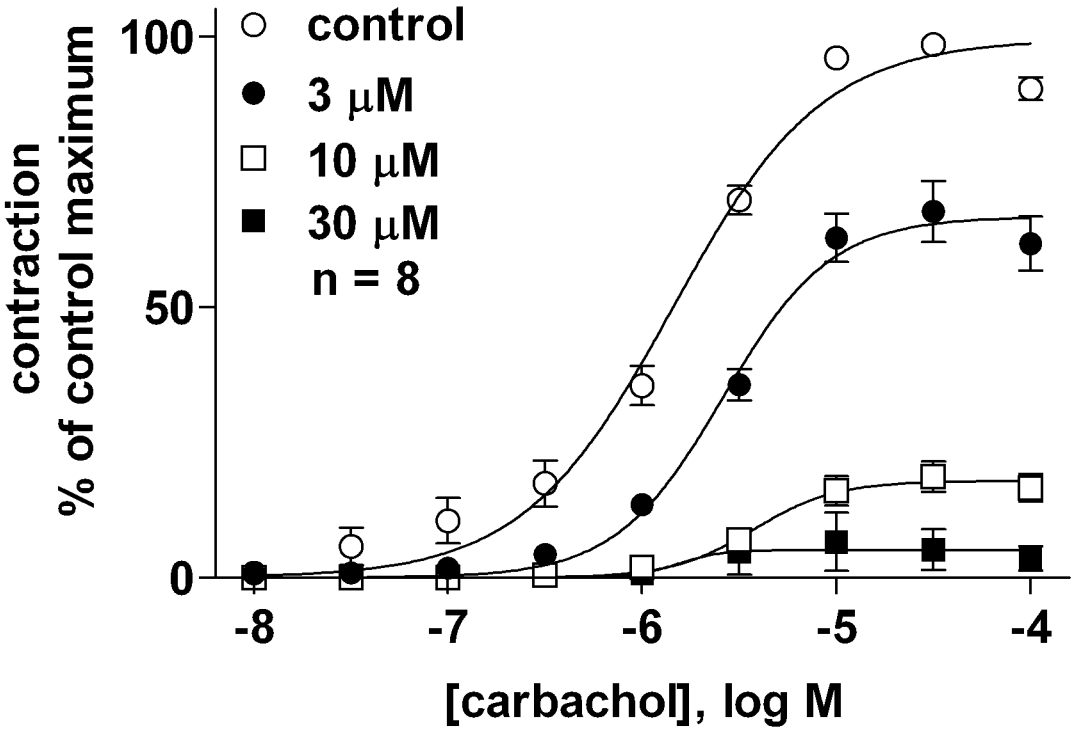
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% of control maximum
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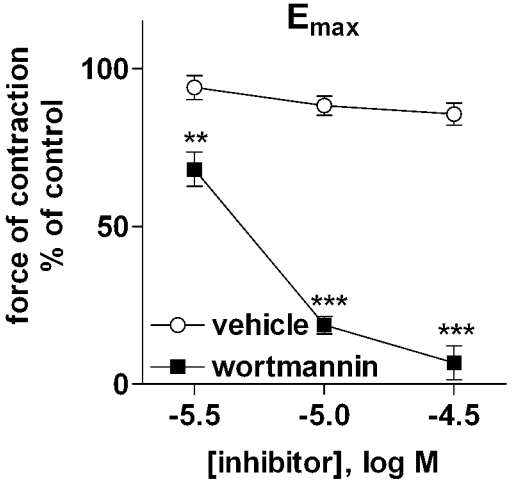
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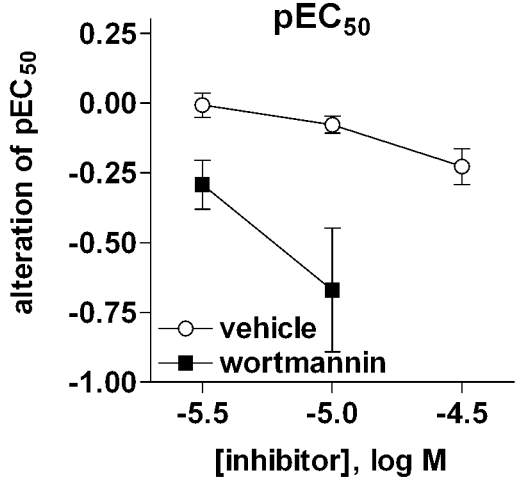
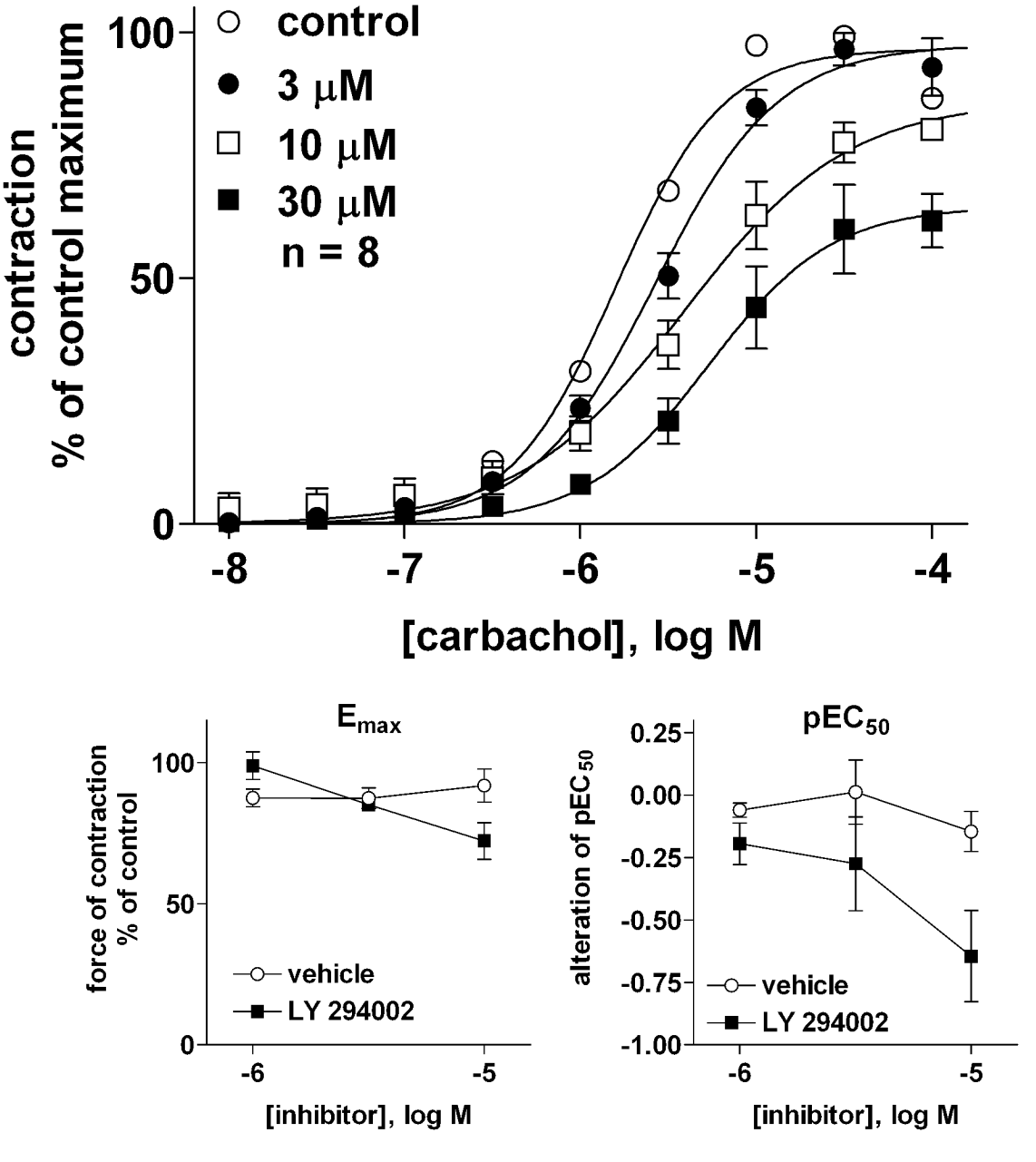
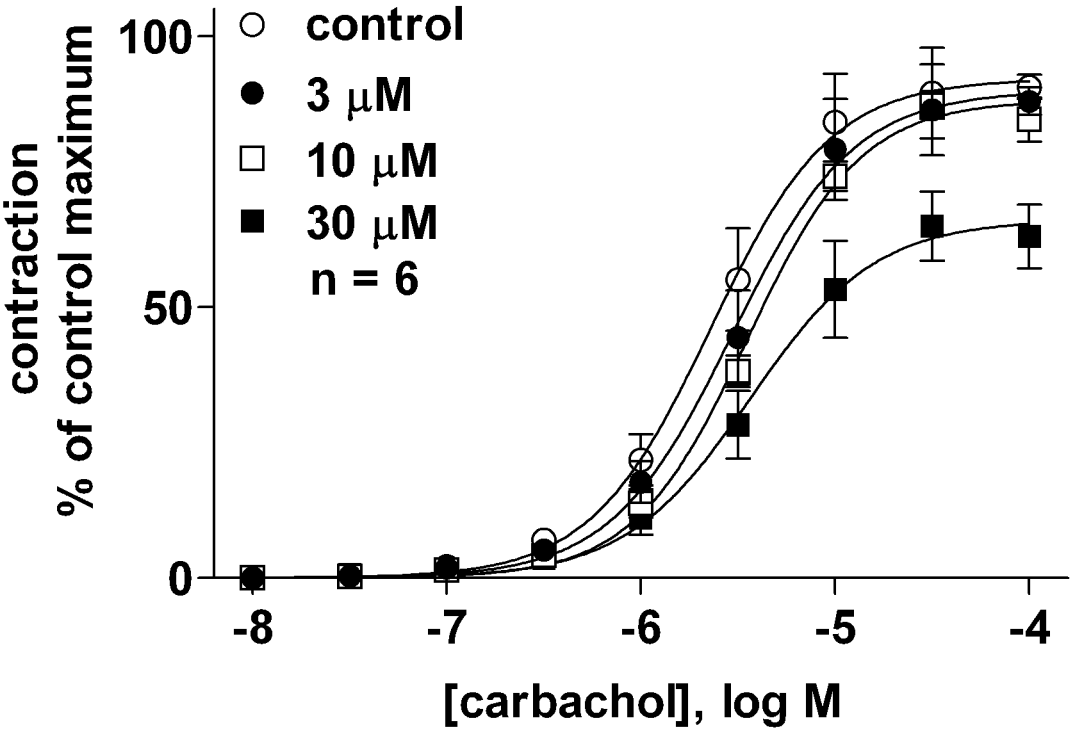
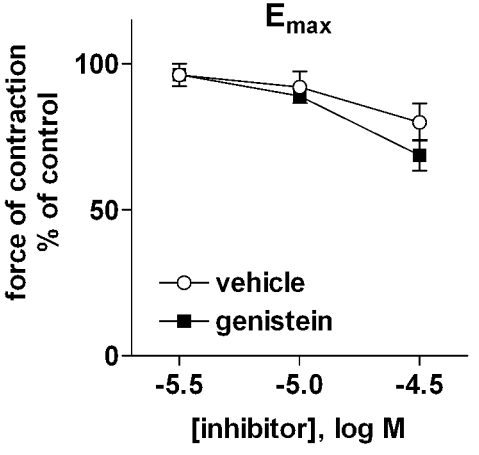
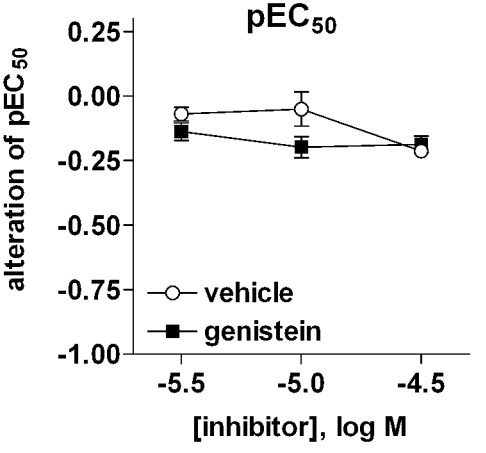
wortmannin
-5.5
-5.0
-4.5
[inhibitor], log M

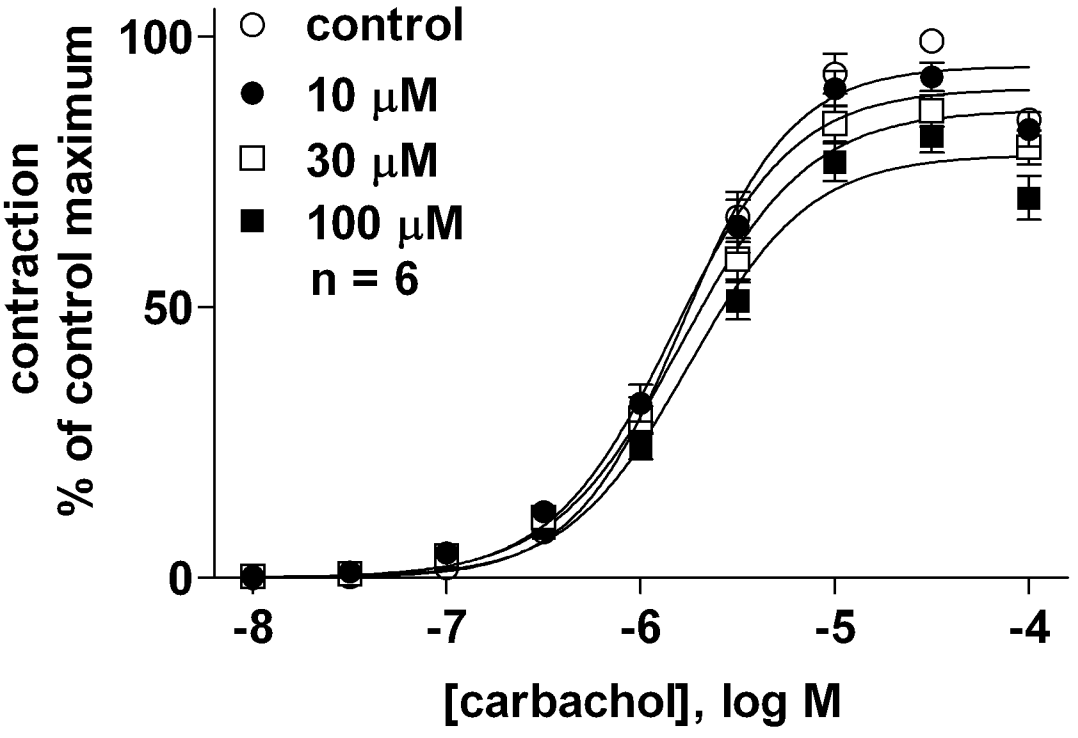

pEC50
alteration of pEC50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
vehicle
wortmannin
-5.5
-5.0
-4.5
[inhibitor], log M

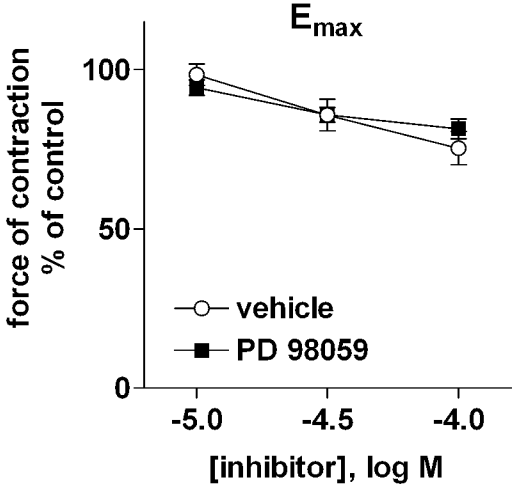

control
3 μM
10 μM
30 μM
n = 8
contraction
% of control maximum
100
50
0
-8
-7
-6
-5
-4
[carbachol], log M
Emax
force of contraction
% of control
100
50
0
vehicle
LY 294002
-6
-5
[inhibitor], log M
pEC50
alteration of pEC50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
vehicle
LY 294002
-6
-5
[inhibitor], log M

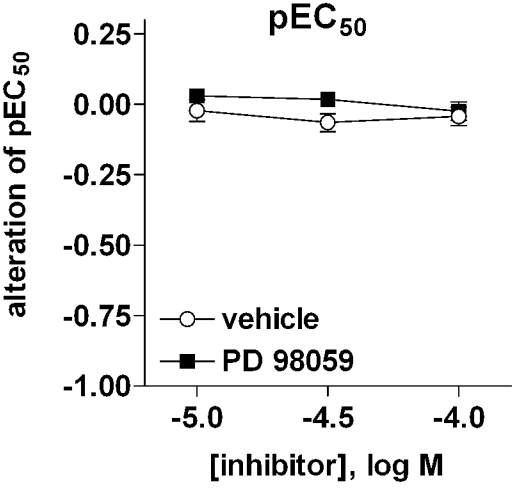

control
3 μM
10 μM
30 μM
n = 6
100
50
0
contraction
% of control maximum
-8
-7
-6
-5
-4
[carbachol], log M

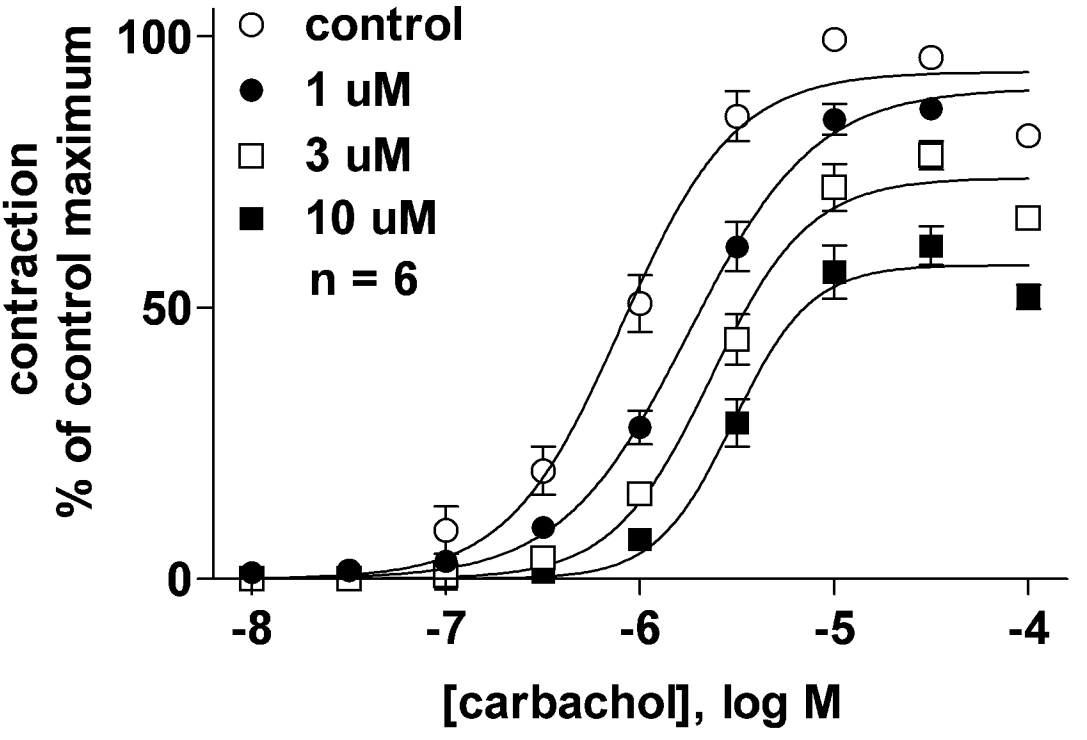

Emax
100
50
0
force of contraction
% of control
vehicle
genistein
-5.5
-5.0
-4.5
[inhibitor], log M

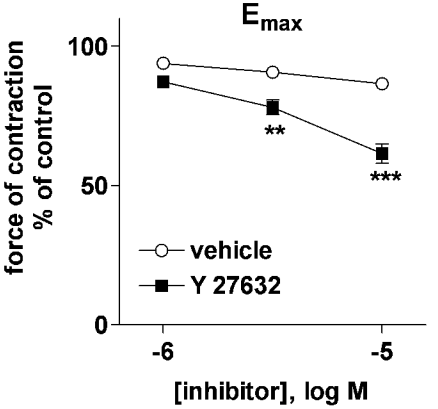

pEC50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
alteration of pEC50
vehicle
genistein
-5.5
-5.0
-4.5
[inhibitor], log M

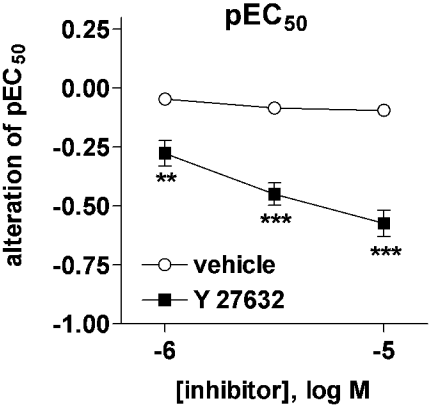

contraction
% of control maximum
100
50
0
control
10 μM
30 μM
100 μM
n = 6
-8
-7
-6
-5
-4
[carbachol], log M


E_{max}
force of contraction
% of control
100
50
0
vehicle
PD 98059
-5.0
-4.5
-4.0
[inhibitor], log M


pEC_{50}
alteration of pEC_{50}
0.25
0.00
-0.25
-0.50
-0.75
-1.00
vehicle
PD 98059
-5.0
-4.5
-4.0
[inhibitor], log M


control
1 uM
3 uM
10 uM
n = 6
contraction
% of control maximum
100
50
0
-8
-7
-6
-5
-4
[carbachol], log M


E max
force of contraction
% of control
100
50
0
**
***
vehicle
Y 27632
-6
-5
[inhibitor], log M


pEC50
alteration of pEC50
0.25
0.00
-0.25
-0.50
-0.75
-1.00
**
***
***
vehicle
Y 27632
-6
-5
[inhibitor], log M