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# Association of muscarinic M<sub>3</sub> receptors and Kir6.1 with caveolae in human detrusor muscle

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1 **ABSTRACT**

2 Caveolae are 50-100 nm large membrane invaginations that play a role in cellular signaling.  
3 The aim of the present study was to assess whether muscarinic M<sub>3</sub> receptors and the K<sub>ATP</sub>  
4 channel subunit Kir6.1 are associated with human detrusor caveolae, and to  
5 pharmacologically assess the relevance of this organization for contractility. Detrusor strips  
6 were dissected and used in ultrastructural, biochemical and mechanical studies. Caveolae  
7 were manipulated by cholesterol desorption using methyl-β-cyclodextrin (mβcd). Mβcd  
8 disrupted caveolae and caused a cholesterol-dependent ~3-fold rightward shift of the  
9 concentration-response curve for the muscarinic receptor agonist carbachol. The effect of  
10 mβcd was inhibited by the K<sub>ATP</sub> blockers glibenclamide, repaglinide and PNU-37883, and it  
11 was mimicked by the K<sub>ATP</sub> activator levcromakalim. Immunoelectron microscopy showed  
12 muscarinic M<sub>3</sub> receptors and Kir6.1 to be enriched in caveolae. In conclusion,  
13 pharmacological K<sub>ATP</sub> channel inhibition antagonizes the effect of caveolae disruption on  
14 muscarinic contractility in the human detrusor, and the K<sub>ATP</sub> channel subunit Kir6.1 co-  
15 localizes with M<sub>3</sub> receptors in caveolae.

16

17

18 Key words: carbachol, detrusor, glibenclamide, cyclodextrin, smooth muscle

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1

## 2 **1. Introduction**

3 Caveolae are 50-100 nm large plasma membrane invaginations (Cohen et al., 2004). The  
4 density of caveolae is high in smooth muscle cells and the organelles are often arranged in  
5 parallel longitudinally oriented strands (Gabella, 1976; Gabella and Blundell, 1978; North et  
6 al., 1993). Formation of caveolae depends on caveolin and cavin proteins (Cohen et al., 2004;  
7 Hill et al., 2008; Bastiani et al., 2009). Caveolins and cavin-1 are expressed in detrusor  
8 smooth muscle (Woodman et al., 2004; Shakirova et al., 2010a; Sadegh et al., 2011).  
9 Biochemical purification has revealed that signaling proteins such as mono- and  
10 heterotrimeric G-proteins and tyrosine kinases are enriched in caveolae (Chang et al., 1994).  
11 Some G protein-coupled receptors similarly reside there (Chun et al., 1994). Recently, a  
12 nanoscale labeling technique was used to demonstrate an enrichment of PtdIns 4,5-P<sub>2</sub> at the  
13 rims of caveolae in mouse detrusor (Fujita et al., 2009). Such examples of heterogeneous  
14 membrane distribution of signaling molecules are cornerstones of the “caveolae signaling  
15 hypothesis” which postulates that caveolae modulate the coupling of receptors to downstream  
16 signaling effectors (Lisanti et al., 1994; Shaul and Anderson, 1998).

17

18 Cholinergic activation of the urinary bladder is mediated by muscarinic M<sub>3</sub> receptors (Matsui  
19 et al., 2000; Fry et al., 2010; Andersson, 2011), and accumulating evidence implicates a role  
20 of caveolae in M<sub>3</sub> signaling. Using fluorescence microscopy and fractionation Gosens et al.  
21 (2007) showed that M<sub>3</sub> receptors are localized in caveolae domains in human airway smooth  
22 muscle cells, and that disruption of caveolae impairs contraction and Ca<sup>2+</sup> responses at  
23 submaximal concentrations of acetylcholine. In addition, disorganization of caveolae arrays  
24 by β-dystroglycan silencing displaced caveolin-1 with associated effects on metacholin-  
25 induced Ca<sup>2+</sup> signaling (Sharma et al., 2010). In accord with these findings, disruption of the

1 caveolin-1 gene leads to impairment of cholinergic contractility in the mouse bladder  
2 (Woodman et al., 2004; Lai et al., 2004; Sadegh et al., 2011). Desorption of cholesterol, which  
3 disrupts caveolae (Rothberg et al., 1992; Dreja et al., 2002), moreover inhibits nerve- and  
4 carbachol-induced contraction in the human bladder (Shakirova et al., 2010a). The exact  
5 mechanism by which caveolae modulate muscarinic signaling is however unclear.  
6  
7 Studies in rat arterial smooth muscle have shown that  $K_{ATP}$  channels, consisting of Kir6.1 and  
8 SUR2B, are localized in caveolae (Sampson et al., 2004; Sampson et al., 2007). Caveolin-1  
9 impairs  $K_{ATP}$  activation by MgADP in HEK293 cells (Davies et al., 2010).  $K_{ATP}$  channels are  
10 expressed in the human detrusor, and their pharmacological activation relaxes muscarinic  
11 contraction (Aishima et al., 2006). Electrophysiological experiments using the guinea pig  
12 detrusor have shown that muscarinic receptor stimulation leads to inhibition of  $K_{ATP}$  current  
13 via protein kinase C (PKC) (Bonev and Nelson, 1993). Because human detrusor  $K_{ATP}$   
14 channels appear to have a molecular composition similar to the corresponding channels in  
15 vascular smooth muscle (Kajioka et al., 2011), we hypothesized that they are associated with  
16 caveolae, and that they are involved in the effect of caveola disruption on muscarinic  
17 contractility. Our objective was to test this hypothesis using pharmacological experiments and  
18 immunoelectron microscopy.

19

## 1 **2. Material and methods**

### 2 *2.1. Detrusor preparation*

3 Transmural specimens were cut from the healthy part of the bladder after radical cystectomy  
4 for localized urothelial cancer and transported to the laboratory in  $\text{Ca}^{2+}$ -free HEPES buffered  
5 Krebs solution (composition in mM: NaCl 135.5, KCl 5.9,  $\text{MgCl}_2$  1.2, glucose 11.6, HEPES  
6 11.6, pH 7.4) on ice. All patients (45 males and 12 females, median age 72 years) gave  
7 informed consent and experiments were approved by the regional human ethics committee.  
8 1x1x10 mm large muscle bundles were prepared by dissection in  $\text{Ca}^{2+}$ -free HEPES buffered  
9 Krebs solution. For Western blotting muscle bundles were directly frozen in liquid nitrogen.

10

### 11 *2.2. Conventional electron microscopy*

12 6 bladder strips from one individual were used. Two strips were fixed after equilibration in  
13 HEPES-buffered Krebs solution at 37°C for 2h (controls); two strips were fixed after 1h in  
14 HEPES buffered Krebs followed by 1h in mβcd (1h depletion, 10 mM, 37°C); and two were  
15 fixed after 2h in mβcd (2h depletion, 10 mM). The fixative contained 2.5% glutaraldehyde in  
16 150 mM sodium cacodylate buffer (pH 7.4, room temperature). Specimens were fixed for 2  
17 hrs, followed by post-fixation in 1% osmium tetroxide, block-staining with uranyl acetate, and  
18 embedding in Araldite. Semi-thin cross sections were examined and areas were chosen and  
19 cut for transmission electron microscopy using a JEOL 1230 microscope (Jeol, Tokyo, Japan).  
20 Caveolar morphometry was determined on 60K digital photos using ImageJ (NIH, Bethesda,  
21 MD, USA).

22

### 23 *2.3. Force recording*

24 6-0 silk loops were tied at the ends of 1x1x3 mm preparations followed by mounting in four-  
25 channel myographs (610M, Danish Myo Technology, Aarhus, Denmark). Force data was

1 digitized and acquired using the WinDaq system. Preparations were allowed to equilibrate for  
2 30 min in aerated HEPES buffered Krebs (in mM: NaCl 135.5, KCl 5.9, MgCl<sub>2</sub> 1.2, glucose  
3 11.6, HEPES 11.6, CaCl<sub>2</sub> 2.5, pH 7.4) for 30 min at 37 °C. A basal tension of 8 mN was  
4 applied and preparations were contracted twice with 60 mM K<sup>+</sup> (obtained by exchange of  
5 NaCl and KCl). Following relaxation from the second contraction the preparations were either  
6 incubated for 1 h in fresh HEPES buffer or with 10 mM methyl-β-cyclodextrin (mβcd)  
7 dissolved directly in the physiological buffer in a pair-wise fashion. Responses were  
8 normalized to the second reference (high K<sup>+</sup>, HK in the figures) contraction. Carbachol was  
9 prepared as a stock solution (10<sup>-2</sup> M) followed by serial dilutions in physiological buffer.  
10 After mβcd treatment and three washes, concentration-response curves were generated by  
11 cumulative addition of carbachol (10<sup>-8</sup> → 10<sup>-5</sup> M). Each concentration was maintained for 7  
12 min and analyses were made by force integration over this period.

13

#### 14 *2.4. Experimental design*

15 In the mechanical experiments 12 preparations from one patient were mounted and  
16 cholesterol was depleted from every second preparation. Half of the control preparations and  
17 half of the depleted preparations were then drug-treated. The remaining preparations were  
18 treated with vehicle. Data from the identically treated strips in each experiment was averaged  
19 and entered into the spreadsheets as n=1. Except when indicated, n therefore refers to the  
20 number of patients. This design was chosen to allow for pairwise comparisons of the  
21 rightward shift in the presence and absence of drug in the same individual.

22

#### 23 *2.5. Sucrose gradient fractionation*

24 Detrusor strips were frozen in liquid nitrogen and crushed to a powder that was further  
25 homogenized in lysis buffer (40 mM HEPES, 1% Triton-X, 10 mM NaF, 1 mM PMSF, 0.25

1 mM vanadate and protease inhibitors, pH 7.4). The homogenate was left on ice for 1h with  
2 homogenization every 15 min. After centrifugation at 5000g (7 min) the supernatant was  
3 mixed with 0.5 M Na<sub>2</sub>CO<sub>3</sub> (pH 11) and sonicated on ice. An equal volume of 90% sucrose (25  
4 mM MES and 0.15 M NaCl) was added and a discontinuous sucrose gradient was layered on  
5 top (4 ml 35% sucrose, 4 ml 5% sucrose) followed by centrifugation. Equal volumes of each  
6 fraction were loaded on SDS-PAGE gels followed by Western blotting (Sadegh et al., 2011)  
7 using antibodies against caveolin-1 (BD610407, BD Biosciences, 1:20000), human M<sub>3</sub> (H-  
8 210, Santa Cruz Biotechnology Inc. 1:500), Kir6.1 (R-14, sc-11224, Santa Cruz  
9 Biotechnology Inc., Sampson et al., 2007 1:200), and SM22 $\alpha$  (transgelin, ab14106, Abcam,  
10 1:5000).

11

## 12 *2.6. Quantitative reverse transcription PCR (RT-qPCR)*

13 Biopsies transported to the lab in RNAlater (Qiagen, Valencia, CA, USA) were frozen in  
14 liquid nitrogen after further dissection of bundles in RNA later. Tissue was homogenized in  
15 TRIzol® (Invitrogen) with an Omni homogenizer (Omni International, Kennesaw, USA).  
16 Homogenates were centrifuged and RNA isolated using RNeasy Mini Kit (Qiagen, Valencia,  
17 CA, USA). The purity and concentration of RNA was determined using a ND-1000  
18 spectrophotometer (Nanodrop Technologies Inc. Wilmington, USA). 1 $\mu$ g RNA was  
19 transcribed to cDNA with Precision qScript™ Reverse transcription kit (Primer Design,  
20 Southampton,UK). Expression levels for Kir 6.1 and Kir 6.2 were determined using SYBR  
21 green quantitative Real-Time PCR (M3000P Stratagene, La Jolla, CA, USA). Amplification  
22 was initiated by incubation at 95 °C for 10 min, followed by 45 cycles between 95 °C for 20 s,  
23 and 60 °C for 1 min. The protocol was ended by incubation for 1 min at 95 °C, 1 min at 55 °C,  
24 and 30 s at 95 °C and the reaction volume was 20  $\mu$ l. Kir6.1 expression was related to Kir6.2  
25 expression in the same sample using the  $2^{-\Delta\Delta C_t}$  formula. Primer dimers and other unspecific



1 artifacts were evaluated by melting curve analysis. Primers for Kir6.1, Kir6.2, SUR1 and pan-  
2 SUR2 were from Qiagen. Additional primers for Kir6.x were from Primer Design and had the  
3 following sequences: CATTGTGACTGAGGAAGAAGGA (Kir6.1, sense),  
4 GGTCTGAATAAGGATGGAAGGTT (antisense), GATGGTGGGAGGGACTGAG (Kir6.2,  
5 sense), and CAGAGTTCTAGGCAAGTTCACA (antisense). The efficiencies for the Kir6.x  
6 primers were not different (Kir6.1, Qiagen:  $2.1 \pm 0.02$ ; Kir6.2, Qiagen:  $2.1 \pm 0.02$ ; Kir6.1,  
7 Primer Design:  $2.1 \pm 0.04$ ; Kir6.2, Primer Design:  $2.2 \pm 0.10$ ). The primer sequences for  
8 SUR2A and SUR2B were as specified by Kajioka et al. (2011).

9

## 10 2.7. Immunoelectron microscopy

11 Strips were fixed (1.5% paraformaldehyde, 0.5% glutaraldehyde, in 0.1 M phosphate buffer,  
12 pH 7.4) for 1 h at room temperature, followed by washing with 0.1 M phosphate buffer, pH  
13 7.4. The samples were subsequently dehydrated in ethanol and further processed for Lowicryl  
14 embedding (Carlemalm et al., 1985). Sections were cut and mounted on gold grids. For  
15 immunostaining, the grids were floated on top of drops of immune reagents dispensed on a  
16 sheet of parafilm. Free aldehyde groups were blocked with 50 mM glycine, and the grids were  
17 then incubated with 5% (v/v) goat serum in incubation buffer (0.2% acetylated bovine serum  
18 albumin in PBS (pH 7.6) for 15 min. This blocking procedure was followed by overnight  
19 incubation at 4 °C with M<sub>3</sub> receptor antibody (GTX13063, rabbit, 1:10, from GeneTex®, Inc.,  
20 Shakirova et al., 2010a) and Kir6.1 antibody (sc-11224, goat, 1:10, Santa Cruz Biotechnology  
21 Inc., Sampson et al., 2007). Controls without primary antibodies were included. The grids  
22 were washed in a large volume (200 µl) of incubation buffer, floating on drops containing the  
23 gold conjugate reagents. 1 µg/ml anti-rabbit IgG 5 nm gold (BBI) and 1 µg/ml anti-goat IgG  
24 20 nm gold were added, and the incubation was allowed to proceed for 2 h at 4 °C. After  
25 further washes, the sections were postfixated in 2% glutaraldehyde. Finally, sections were

1 washed with distilled water and post-stained with 2% uranyl acetate and lead citrate. Samples  
2 were examined with a JEOL JEM 1230 electron microscope operated at 80 kV accelerating  
3 voltage. Images were recorded with a Gatan Multiscan 791 charge-coupled device camera.

4  
5 Three human bladder specimens were used for quantitative analysis of M<sub>3</sub> and Kir6.1  
6 labeling. Ultrathin sections from different areas of the specimens were examined following  
7 the rules of systematic uniform random sampling. For each caveolae-containing plasma  
8 membrane stretch, the numbers of gold particles were counted and compared to non-specific  
9 background staining. A total of 50 cellular profiles were analyzed in each case.

10

#### 11 *2.8. Drugs and chemicals*

12 Carbachol, glibenclamide, levcromacalim, chelerythrine and methyl- $\beta$ -cyclodextrin were  
13 from Sigma. PNU-37883 (U37883A; 4-morpholinecarboximidine-N-1-adamantyl-N'-  
14 cyclohexyl-hydrochloride) and repaglinide were from Tocris. Vehicle (0.1% DMSO or 0.1%  
15 ethanol) was included in all control experiments ( $\pm$ m $\beta$ cd).

16

#### 17 *2.9. Statistics*

18 With the exception for m $\beta$ cd + PNU-37883-treated preparations, a four parametric Hill  
19 equation was fitted to concentration-response data using the least squares method in order to  
20 retrieve the EC<sub>50</sub> value. Student's t-test was used to test for significant differences. Bonferroni  
21 correction was used for multiple comparisons. P<0.05 was considered significant. In the  
22 figures \*, \*\* and \*\*\* denote P<0.05, P<0.01, and P<0.001, respectively. For clarity,  
23 significant differences are not indicated in the concentration-response curves.

24

25

26

1

## 2 **3. Results**

### 3 *3.1. Effect of mβcd on the ultrastructure of caveolae in human detrusor*

4 Caveolae, clustered between dense bands (membrane-associated dense plaques), were  
5 numerous along the sarcolemma in control detrusor myocytes (**1A** and **B**). Schwann cells  
6 around detrusor nerves also contained caveolae (**1B**), but no caveolae were seen in neurons.  
7 After 1 and 2h of treatment with methyl-β-cyclodextrin (mβcd) caveolae in detrusor myocytes  
8 were flattened and dilated (**C**, **D**). The depths of 24 caveolae with visible necks were 90±3 nm  
9 (control), 69±4 nm (1 h mβcd), and 60±4 nm (2 h mβcd), while their opening diameters were  
10 30±1 nm in controls, 59±3 nm at 1 h and 61±4 nm at 2 h (compare **1A-D**). Their maximal  
11 widths changed only marginally (69±2 nm, control; 67±2 nm, 1 h; 62±4 nm, 2 h).

12

### 13 *3.2. Mechanical effects of mβcd*

14 Few (87 out of 690 =13%) detrusor strips exhibited spontaneous contractile activity during  
15 equilibration. On treatment with mβcd following the reference contraction, a time-dependent  
16 shift to the right of the concentration-response curve for the muscarinic agonist carbachol was  
17 observed (**2A**, 3 strips from the same individual at each time). Consequently, the negative  
18 logarithm of the EC<sub>50</sub> value for carbachol decreased (**2B**). Mβcd (1h, n=5) did not reduce K<sup>+</sup>-  
19 induced contraction at any K<sup>+</sup> concentration between 10 and 50 mM (**2C**). When cholesterol  
20 was reloaded after 1h treatment with mβcd, the concentration response relationship for  
21 carbachol was shifted back left (**2D**, n=6). While reloading of cholesterol led to a slight  
22 reduction of the E<sub>max</sub> value, contractility was fully restored at carbachol concentrations below  
23 1 μM (**2E**), and the EC<sub>50</sub> value recovered completely (**2F**). Thus, the mechanical effects of

1 mβcd are time-dependent, correlate with ultrastructural effects on caveolae, are specific for  
2 receptor-induced contractions, and depend largely on removal of cholesterol.

3

### 4 *3.3. Effects of drugs that target K<sub>ATP</sub> channels*

5 An increased extracellular K<sup>+</sup> concentration should reduce the driving force for an outward K<sup>+</sup>  
6 current. We contracted detrusor preparations partially using 25 mM K<sup>+</sup> and then added  
7 carbachol in a cumulative manner. Under these conditions cholesterol desorption did not  
8 inhibit muscarinic contractility (**3A**, n=6). In control strips, from the same patients and run in  
9 parallel, a clear-cut inhibition was seen (effect at 0.1 μM carbachol is shown in **3B**).

10

11 To test the involvement of K<sub>ATP</sub> channels, the rightward shift of the concentration response  
12 curve in the absence (**3C**) and presence (**3D**) of the K<sub>ATP</sub> current inhibitor glibenclamide (10  
13 μM) was compared. Mβcd increased the EC<sub>50</sub> value 2.6±0.4-fold in control conditions and  
14 1.5±0.1-fold in the presence of glibenclamide (10 μM, P<0.05, n=6, **3E**). Similarly, no  
15 significant right shift was induced by mβcd in the presence of the K<sub>ATP</sub> inhibitor repaglinide  
16 (10 μM, n=6, P>0.05 for the effect of mβcd). Incubation with the K<sub>ATP</sub> activator  
17 levcromakalim (1 μM, **3F**) mimicked the effect of mβcd and caused a 3.9±0.4-fold shift to the  
18 right of the concentration-response curve (n=6, **3G**). Thus, the effect of mβcd on cholinergic  
19 detrusor activation is inhibited by K<sub>ATP</sub> channel blockers and mimicked by K<sub>ATP</sub> channel  
20 activation.

21

22

1

2 *3.4. PNU-37883 reverses the effect of cholesterol desorption in human bladder*

3 Kir6.1 is a candidate pore component of the human bladder  $K_{ATP}$  channel. To test its role in  
4 the effect of cholesterol desorption we used the Kir6.1 blocker PNU37883. This substance (at  
5 20  $\mu$ M) had a pronounced effect after desorption of cholesterol (**4B** vs. **4A**); at submaximal  
6 concentrations of carbachol, force was increased ( $P < 0.05$  at 0.1  $\mu$ M carbachol) rather than  
7 decreased, and the concentration-response curve exhibited two apparent phases (**4B**). No  
8 rightward shift was seen in the presence of PNU37883 whereas a  $2.7 \pm 0.4$ -fold rightward shift  
9 was induced by  $m\beta cd$  in the matched control preparations from the same individuals (**4C**,  
10  $n=6$ ). We also tested the reverse experiment, i.e. adding PNU37883 to control and  $m\beta cd$ -  
11 treated preparations after having increased the carbachol concentration cumulatively to 0.1  
12  $\mu$ M. As expected, control strips contracted transiently to about 40% of the reference  
13 contraction at this concentration whereas the  $m\beta cd$  treated strips contracted little (**4D**). On  
14 addition of PNU37883, control strips continued to relax after the transient response to  
15 carbachol (**4D**, four out of four strips) whereas a sustained contraction was elicited in four out  
16 of four  $m\beta cd$ -treated strips.

17

18 *3.5. Probing the composition of  $K_{ATP}$  channel in human urinary bladder using RT-qPCR*

19 Because our mechanical experiments suggested involvement of  $K_{ATP}$  channels and Kir6.1 in  
20 the effect of cholesterol desorption in human bladder, we examined expression of  $K_{ATP}$   
21 channel subunits using RT-qPCR. Kir6.x primer efficiencies were comparable, and using two  
22 different primer pairs for each target, it was clear that Kir6.1 expression dominated over  
23 Kir6.2 (**5A**). We were unable to achieve a quantitative comparison of sulphonylurea receptor

1 expression, but products of expected sizes were seen using SUR1, pan-SUR2, and SUR2B  
2 primers (**5B**).

3

### 4 *3.6. Detection of Kir6.1 by Western blotting and co-fractionation with M<sub>3</sub> and caveolin-1*

5 In keeping with Kir6.1 mRNA expression in human bladder, the protein product was detected  
6 by Western blotting (**6A**). Mouse aorta was included as positive control. To probe the  
7 subcellular distribution of Kir6.1 and muscarinic M<sub>3</sub> receptors we used sucrose density  
8 fractionation (n=4). Caveolin-1 was primarily distributed in fractions 5-8 with a peak in  
9 fractions 5-6 (**6B**). M<sub>3</sub> and Kir6.1 similarly showed a broad distribution with a peak in  
10 fraction 6 (**6B**). The cytoskeletal protein SM22 $\alpha$  was present in the high density fractions, as  
11 was the majority of cellular protein (not shown). These findings are consistent with the  
12 possibility that M<sub>3</sub> and Kir6.1 reside in caveolae.

13

### 14 *3.7. Immunoelectron microscopy shows co-localization of M<sub>3</sub> and Kir6.1 in caveolae*

15 Kir6.1 and M<sub>3</sub> were next labeled with immunogold. Figure **7A** shows an overview acquired by  
16 conventional electron microscopy using the same detector as in panels **B** and **C**. Panels **B** and  
17 **C** show immunogold labeling of M<sub>3</sub> (5 nm gold) and Kir6.1 (20 nm gold). Both proteins were  
18 enriched in caveolae with less labeling of planar membrane (**7B** and **C**). Clusters of  
19 submembranous caveolae were also labeled, and vesicles with gold particles were  
20 occasionally observed deeper in the cytoplasm. Panels **D** and **E** show M<sub>3</sub> and Kir6.1  
21 distribution in two additional individuals.

22

23 The total number of small gold particles (M<sub>3</sub>) counted was 1549, 1898 and 1721, and the  
24 number of large gold particles (Kir6.1) was 581, 629, and 601, respectively. Of these, 1177  
25 (76%), 1480 (78%) and 1256 (73%) small gold particles, and 604 (71%), 434 (69%) and 421

1 (70%) large gold particles were localized to caveolae. Thus, ~70% of both Kir6.1 and M<sub>3</sub> is  
2 confined to caveolae, which constitute ~28% of the membrane surface length. M<sub>3</sub> and Kir6.1  
3 are therefore enriched 6-fold in caveolae.

4

5 *3.8. Chelerythrine inhibits the rightward shift of the concentration-response curve for*  
6 *carbachol*

7 Co-localization of M<sub>3</sub> and Kir6.1 in caveolae may be a prerequisite for M<sub>3</sub>-mediated  
8 inhibition of Kir6.1. Indeed, the PKC translocation inhibitor chelerythrine (3 μM) effectively  
9 inhibited the rightward shift induced by mβcd (1.6±0.2-fold with chelerythrine vs. 3.9±0.6-  
10 fold in the controls, n=6, Figure **8A-C**). However, chelerythrine had no effect in control  
11 conditions (**8D**).

12

#### 1 4. Discussion

2

3 We present ultrastructural evidence favoring a caveolar localization of  $M_3$  receptors and  
4 Kir6.1-containing  $K_{ATP}$  channels in the human detrusor. We moreover show that three  
5 different  $K_{ATP}$  channel blockers, representing different structural classes and with non-  
6 overlapping off-target effects, antagonize the effects of caveolae disruption on muscarinic  
7 detrusor activation. Findings with chelerythrine moreover suggest that protein kinase C is  
8 involved in the effect of cholesterol desorption.

9

10 Muscarinic receptor-mediated and protein kinase C-dependent inhibition of  $K_{ATP}$  current has  
11 been demonstrated in guinea pig detrusor (Bonev and Nelson, 1993). One possibility therefore  
12 is that colocalization of  $M_3$  receptors and  $K_{ATP}$  channels in caveolae facilitate inhibition of the  
13 channels as a component of the excitatory influence of muscarinic stimulation. A restricted  
14 inhibition of  $K_{ATP}$  channels within caveolae implies the presence of a caveolar “signalosome”.  
15 Indeed,  $G_{\alpha q}$  (Oh and Schnitzer, 2001), phospholipase  $C_{\beta 1}$  (Sharma et al., 2010), and protein  
16 kinase C (Mineo et al., 1998, Sampson et al., 2007), have been demonstrated to associate with  
17 caveolae in a static or dynamic manner in other cells and tissues. Our findings with the protein  
18 kinase C inhibitor chelerythrine, which is known to mitigate receptor-induced  $K_{ATP}$  current  
19 inhibition (Thorneloe et al., 2002), are however not entirely consistent with this possibility.  
20 While chelerythrine inhibited the effect of  $m\beta cd$  on muscarinic contraction, it had little effect  
21 on the muscarinic contraction as such.

22

23 Our present and previous (Shakirova et al., 2010a) data on the effects of cholesterol  
24 desorption in the human bladder do not support loss of  $M_3$  receptors, reduced ligand binding  
25 to  $M_3$  receptors, or a generalized impairment of  $M_3$  receptor coupling. Treatment with either  
26 25 mM  $K^+$  or PNU-37883, a Kir6.1 channel blocker (Surah-Narwal et al., 1999), completely



1 antagonized the effect of cholesterol desorption. This would not occur if desorption of  
2 cholesterol had a major effect on ligand binding or on the contractile machinery. Similar  
3 conclusions were reached by Gosens et al. (2007) on the basis of ligand binding assays using  
4 airway smooth muscle cells.

5

6 We find that Kir6.1 is expressed in human detrusor. This confirms work on Kir6.x mRNA  
7 expression in commercial human bladder RNA preparations (Kajioka et al., 2011). Similarly,  
8 SUR2B was readily detectable. It is therefore reasonable to infer that Kir6.1/SUR2B  
9 containing  $K_{ATP}$  channels are present, if not dominating, in human detrusor myocytes, which  
10 implies regulation by caveolin-1/caveolae similar to that seen in HEK293 cells (Davies et al.,  
11 2010).

12

13 Disruption of caveolae has little effect on muscarinic contraction in some species (Shakirova  
14 et al., 2006, 2010a, 2010b). A difference in regulation depending on the molecular make-up of  
15 the  $K_{ATP}$  channel is one putative reason for this. Indeed, substantial differences in the effect of  
16 drugs that target  $K_{ATP}$  channels exist between man and rodent bladders (Wammack et al.,  
17 1994). Interestingly, caveolin-1 levels drop in human detrusor hypertrophy associated with  
18 benign prostatic hyperplasia (Boopathi et al., 2011). This could result in altered excitability  
19 via  $K_{ATP}$  channels.

20

21 In conclusion, our pharmacological findings suggest that protein kinase C and  $K_{ATP}$  channels  
22 are involved in the effect of cholesterol desorption, which disrupts caveolae, on muscarinic  
23 contraction in the human detrusor.  $M_3$  receptors and Kir6.1 moreover localize to detrusor  
24 caveolae. To the best of our knowledge, no previous description has been provided of the  
25 ultrastructural localization of these important signaling proteins in the human bladder.

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## Legends

**Figure 1.** Desorption of cholesterol using methyl- $\beta$ -cyclodextrin (m $\beta$ cd) causes a time-dependent deterioration of human detrusor caveolae. Human bladder preparations were incubated in HEPES-buffered Krebs buffer alone (**A** and **B**) or in 10 mM m $\beta$ cd dissolved in the same buffer for one (**C**) and two (**D**) hours, respectively. In control conditions, caveolae (highlighted with arrowheads) were numerous (**A**). Caveolae were also seen in Schwann cells wrapped around detrusor nerves (**B**, center structure), but not in neurons. After desorption of cholesterol, caveolae were shallower with an increased neck-to-width ratio (**C**, **D**). After 1h normal appearing caveolae were still present in some regions (inset in **C**). At 2h a more complete change was apparent (arrowheads in **D**). All panels were acquired at 60K magnification and the scale bars represent 0.5  $\mu$ m.

**Figure 2.** Desorption of cholesterol induces a rightward shift of the concentration-response curve for carbachol (Cch) in human detrusor. Cumulative concentration-response curves were generated using the muscarinic agonist carbachol at time 0 and after 1, 2, and 3 h of cholesterol desorption (**A**, means of three strips from one individual are shown, i.e. n=1). Force was normalized to the reference, K<sup>+</sup>-high (60 mM; HK), contraction at the start of the experiment. Desorption of cholesterol time-dependently reduced the negative logarithm of the EC<sub>50</sub> value for carbachol (**B**). Contraction induced by cumulative addition of K<sup>+</sup> was unaffected by m $\beta$ cd (1h, n=5, **C**). Reloading of cholesterol reversed the effect of m $\beta$ cd (1h) on carbachol contraction at low to intermediate carbachol concentrations (n=6, **D**). **E** shows force at 0.1  $\mu$ M carbachol from the experiment in **D**. **F** shows potency (-log (EC<sub>50</sub>)) from experiment in **D**.



1 **Figure 3.** Effect of KCl and  $K_{ATP}$  channel inhibition on the effect of cholesterol desorption in  
2 the human detrusor. Panel **A** shows force in control and  $m\beta cd$ -treated human detrusor strips  
3 when 25 mM KCl was added just prior to the cumulative addition of carbachol. **B** shows force  
4 at 0.1  $\mu M$  carbachol from experiment in **A**. **C** and **D** show concentration response curves for  
5 carbachol from the same individuals ( $n=6$ ) in the absence (**C**) and presence of glibenclamide.  
6 Cholesterol desorption for 1h caused a 2.6-fold right shift of the concentration-response curve  
7 for carbachol and  $K_{ATP}$  current blocker glibenclamide (10  $\mu M$ ) reduced this effect (**E**). The  
8 dotted line in **E** represents the fold shift of the concentration response curve with 25 mM KCl  
9 in the solution. Levromakalim (1  $\mu M$ ), a  $K_{ATP}$  channel activator, mimicked the effect of  
10 cholesterol desorption (**F** and **G**,  $n=6$ ).

11

12 **Figure 4.** The Kir6.1 blocker PNU37883 (U37883A) reverses the effect of cholesterol  
13 desorption in the human detrusor. Vehicle-treated ( $\pm m\beta cd$ , **A**) and PNU37883-treated (20  
14  $\mu M$ ,  $\pm m\beta cd$ , **B**) preparations from the same patients were run in parallel. Open circles  
15 represent  $m\beta cd$  treated strips. **C** shows the fold shift induced by  $m\beta cd$  in the absence and  
16 presence of PNU37883 ( $n=6$ ). **D** shows an original record of force where PNU37883 was  
17 added to a control (thick trace) and an  $m\beta cd$ -treated (thin trace) preparation after increasing  
18 the carbachol concentration in a step-wise fashion to 0.1  $\mu M$  (4 preparations from one  
19 individual). The dotted line represents basal force. Responses elicited by low and intermediate  
20 concentrations were typically transient.

21

22 **Figure 5.** RT-qPCR showing that Kir6.1 mRNA level exceeds Kir6.2 mRNA level in human  
23 detrusor (**A**,  $n=10-12$ ). Two different primer pairs for each target were used and each primer  
24 pair is represented by a bar. Sulphonylurea receptor expression is shown in **B** ( $n=6$ ).  
25 Examples from two individuals are shown.

1 **Figure 6.** Detection of Kir6.1 by Western blotting and co-fractionation of muscarinic M<sub>3</sub>  
2 receptors and Kir6.1 with caveolin-1. Human detrusor and mouse aortic lysates were loaded  
3 on SDS-PAGE gels and Kir6.1 was detected by Western blotting (**A**). The amount of protein  
4 loaded is indicated below each lane. Bands are from the same blot, but four lanes were  
5 excised and omitted (indicated by the central vertical line). Blotting of sucrose gradient  
6 fractions (1-12) showing distribution of caveolin-1, M<sub>3</sub> receptors, Kir6.1, and the actin  
7 binding protein SM22 $\alpha$  (**B**, n=4).

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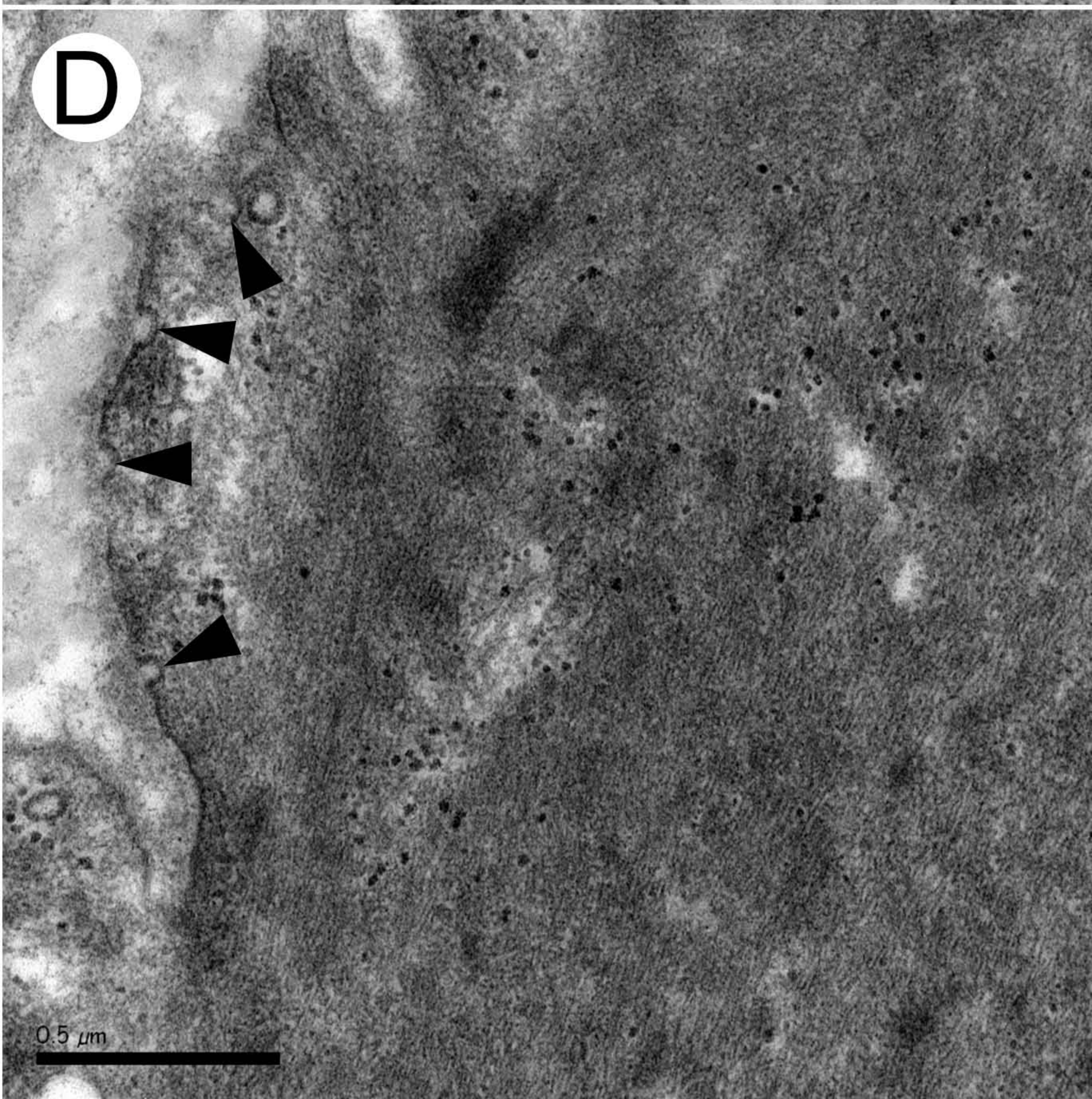
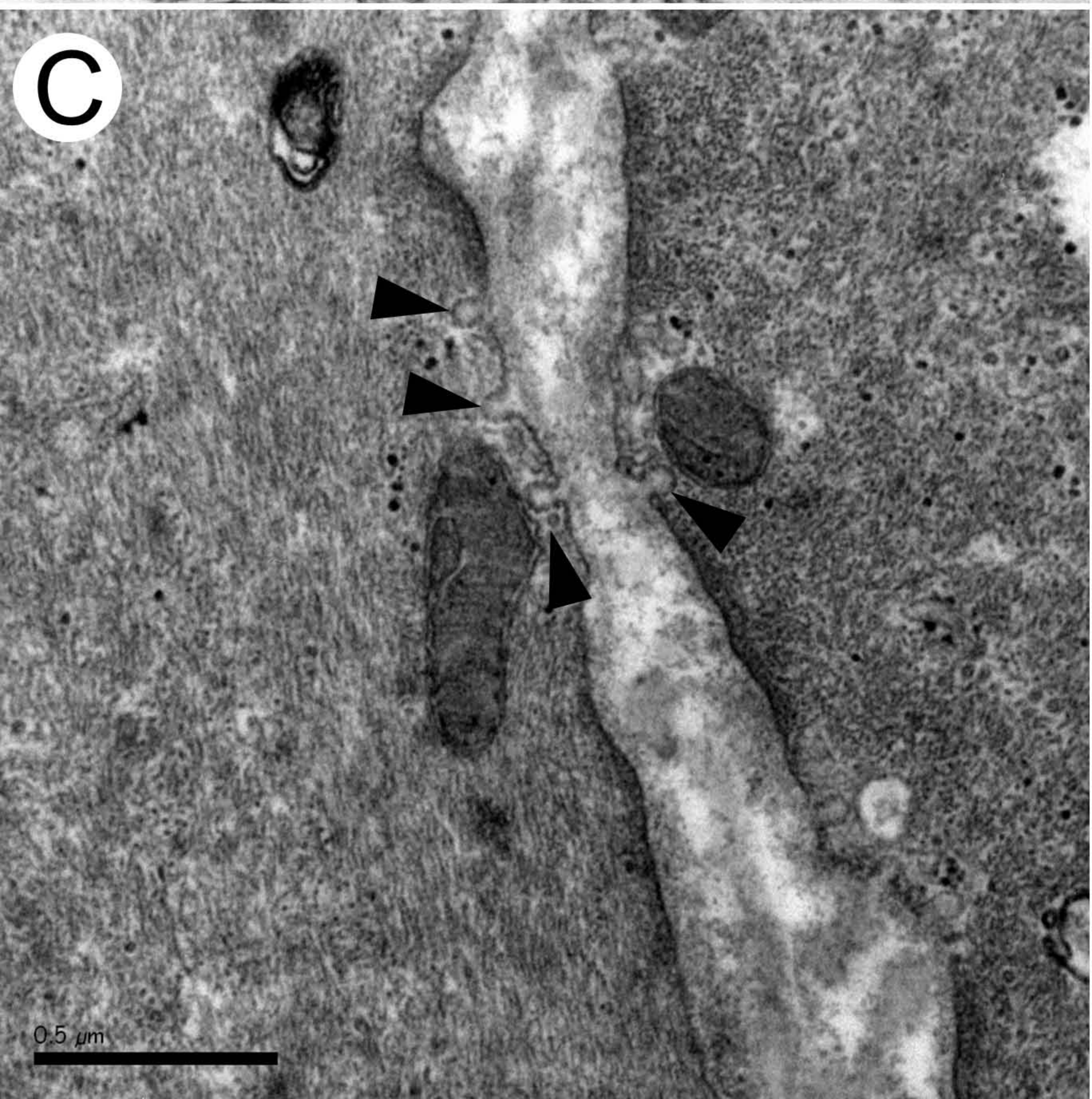
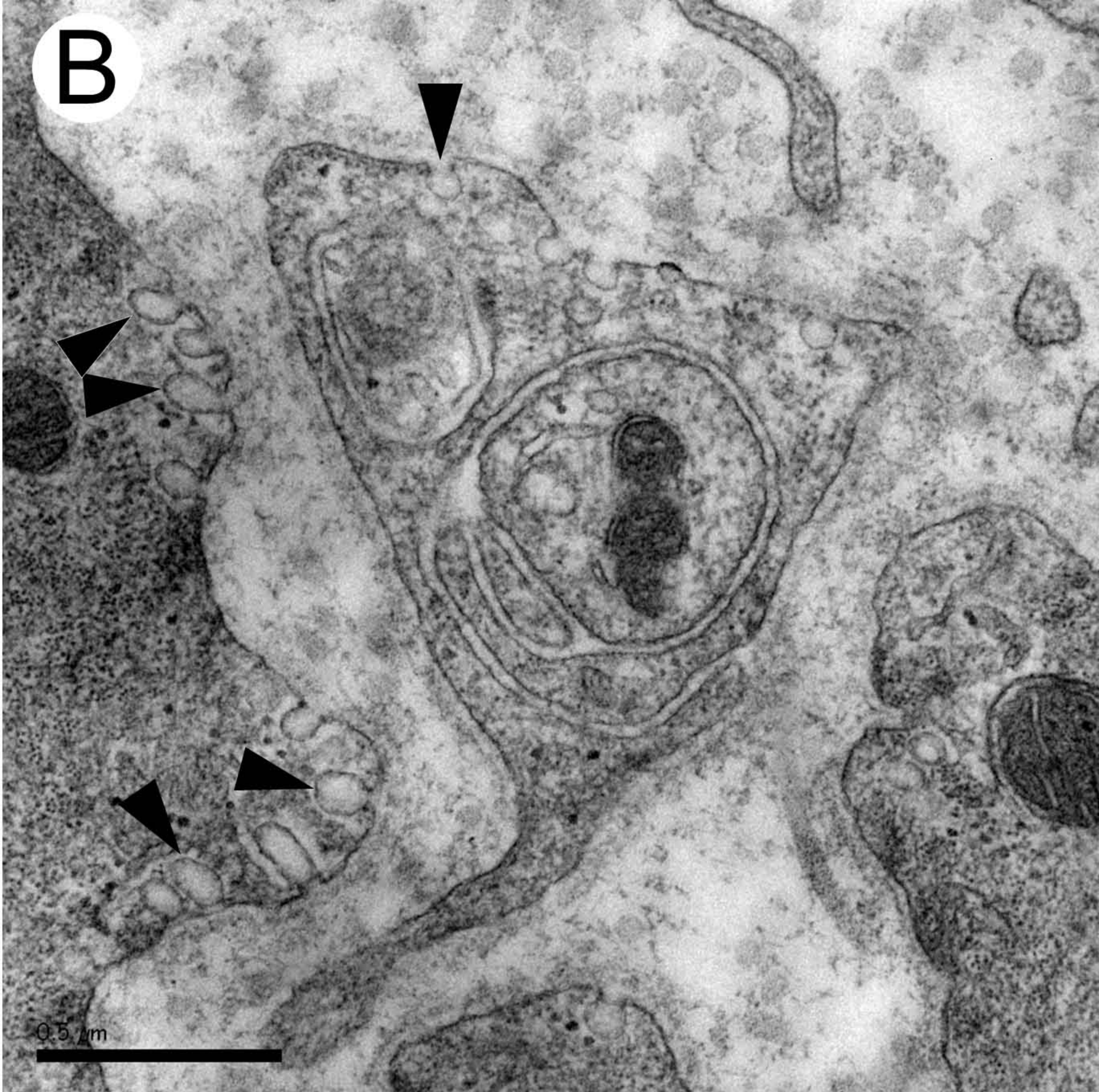
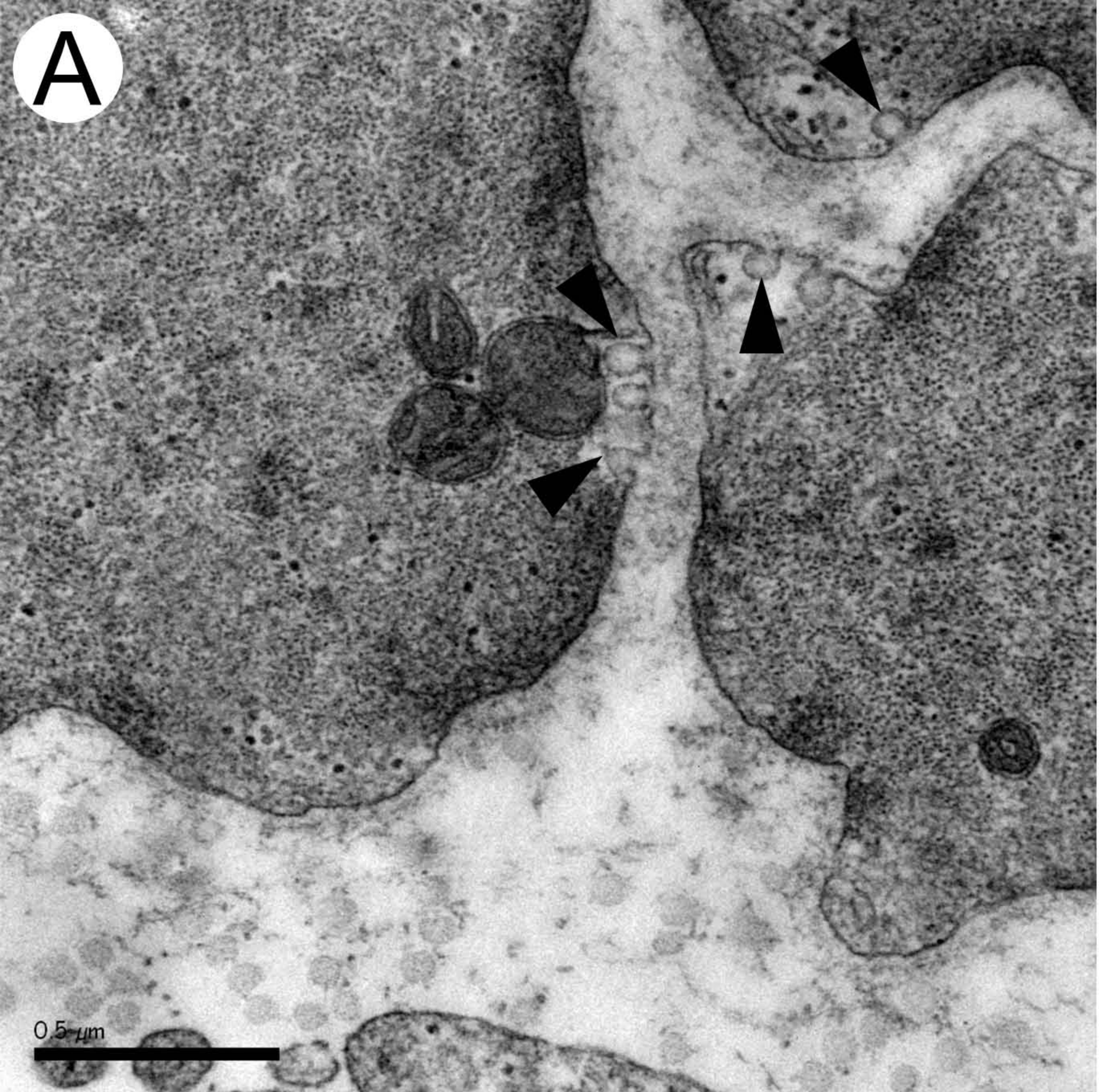
9 **Figure 7.** Immunoelectron microscopy demonstrates co-localization of muscarinic M<sub>3</sub> (5 nm  
10 gold) receptors and Kir6.1 (20 nm gold) in caveolae of human detrusor smooth muscle cells.  
11 Panel **A** shows an overview by conventional electron microscopy of the same bladder that  
12 was used for labeling in **B** and **C**. Caveolae are highlighted with arrow heads. Panel **B** is  
13 focused on flat membrane and panel **C** on a cluster of caveolae in the lower cell. Note that the  
14 caveolae-free membrane segment in the upper cell essentially is devoid of labeling. Panels **D**  
15 and **E** show localization of M<sub>3</sub> and Kir6.1 in two additional bladders. The scale bars in **A** and  
16 **E** represent 0.5 and 0.1  $\mu\text{m}$ , respectively. The latter scale bar applies for panels **B** through **E**.

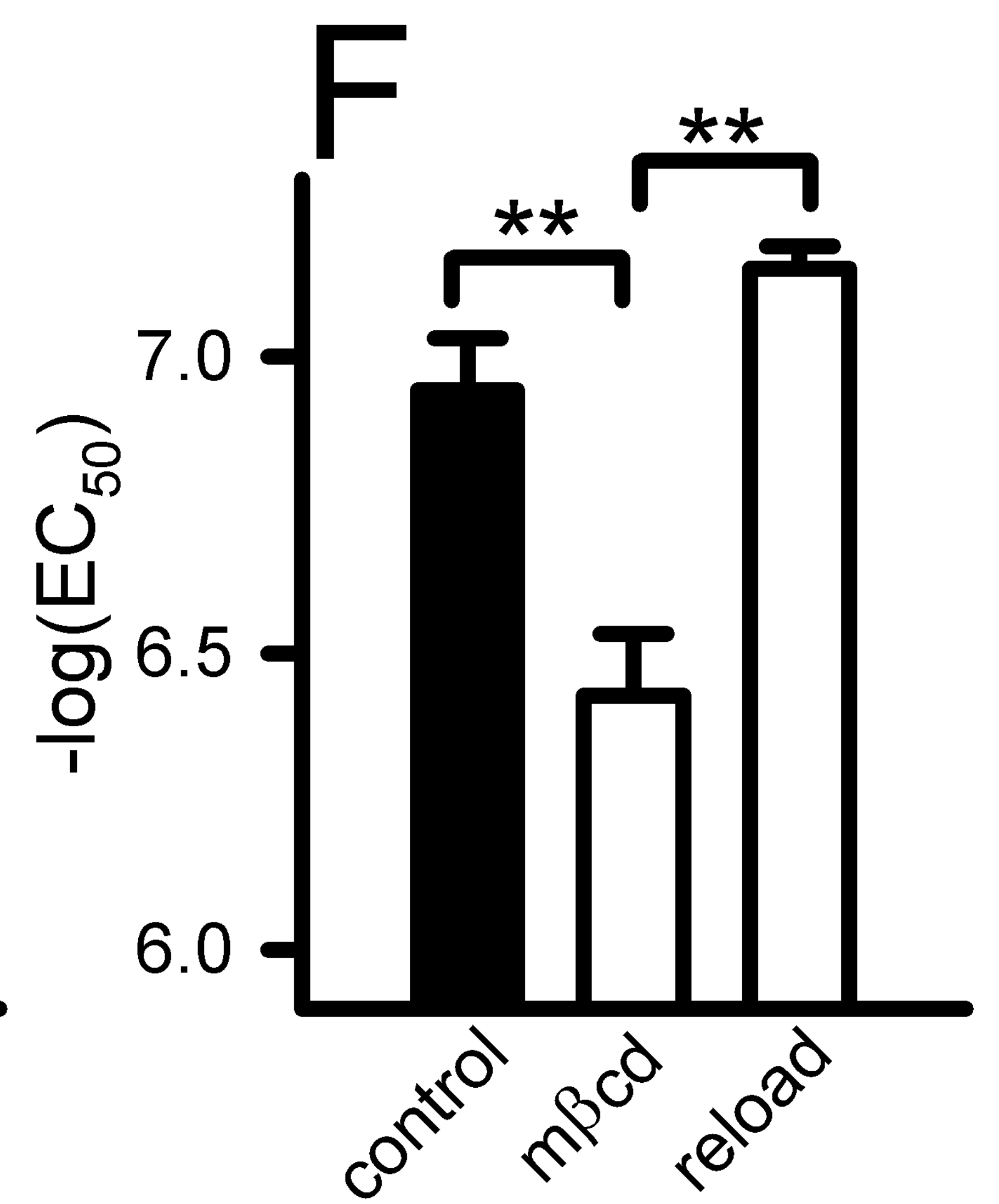
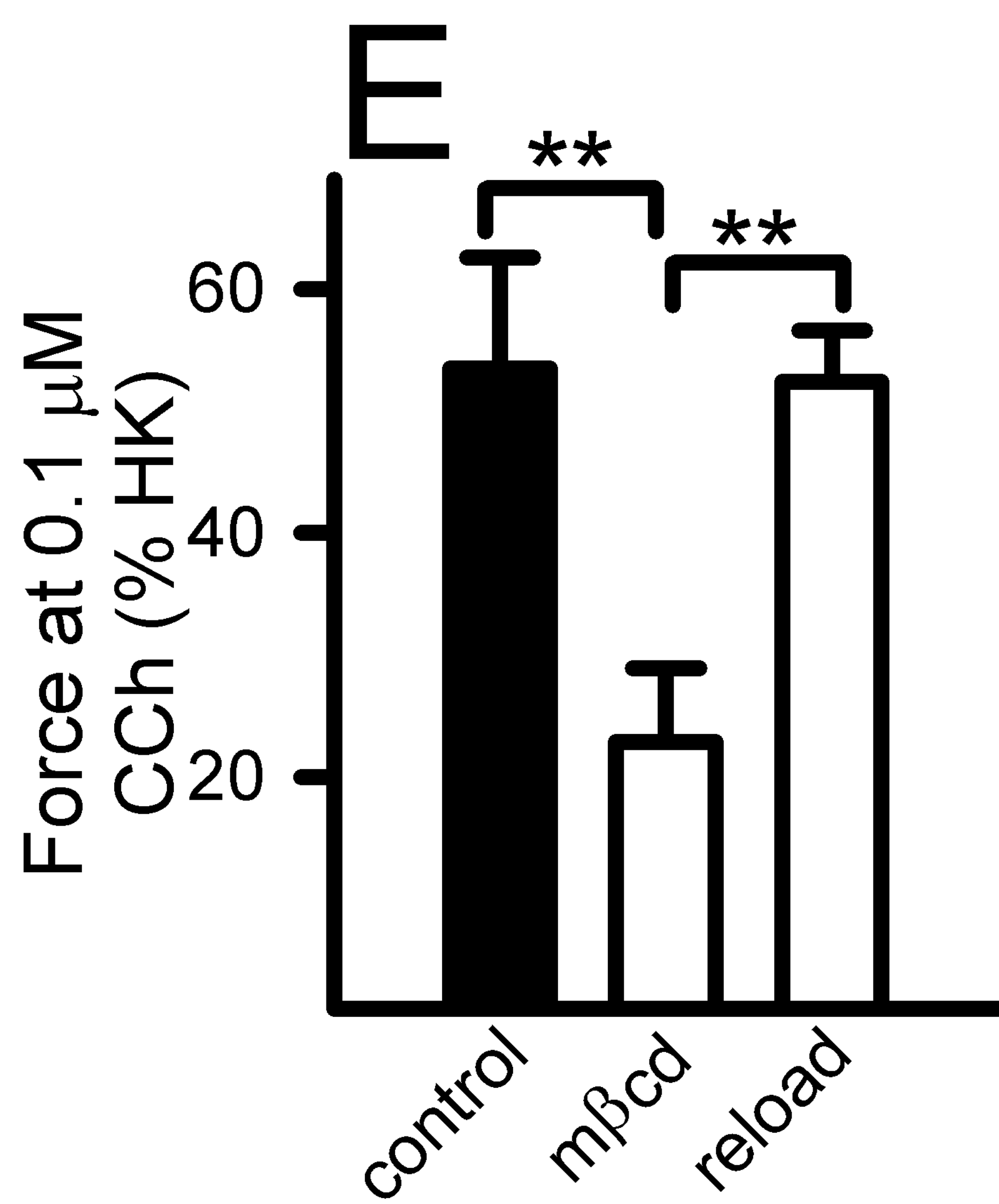
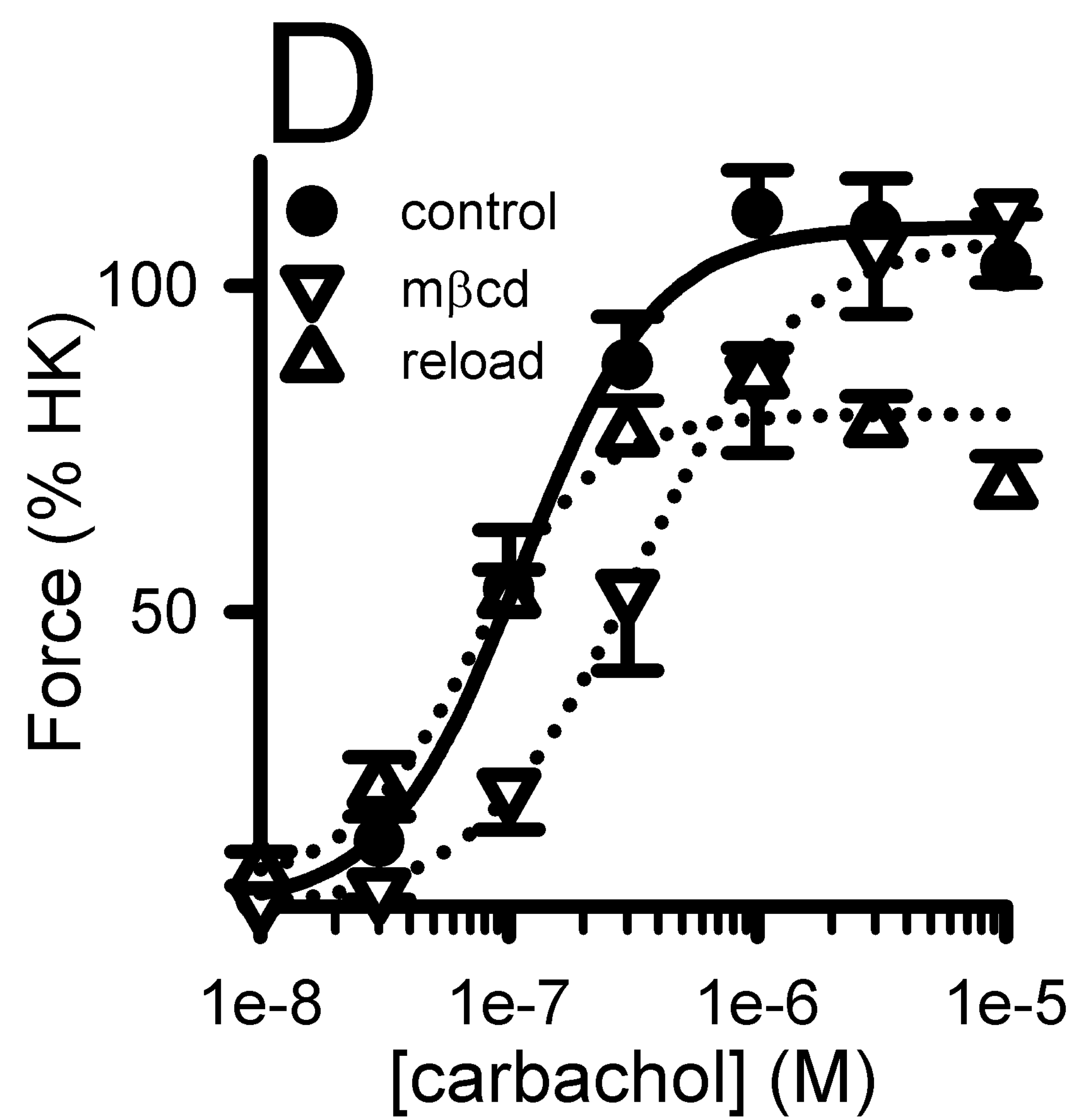
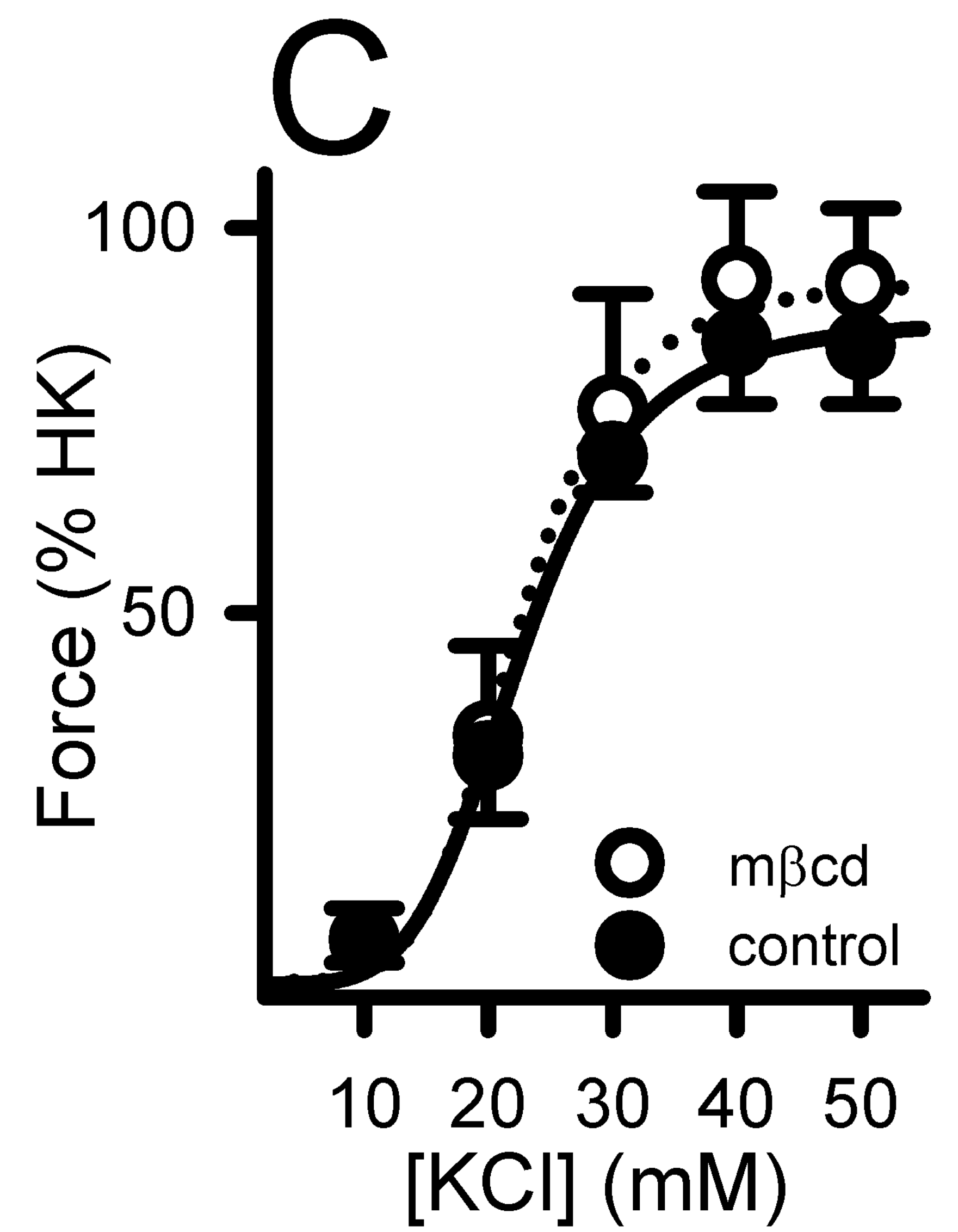
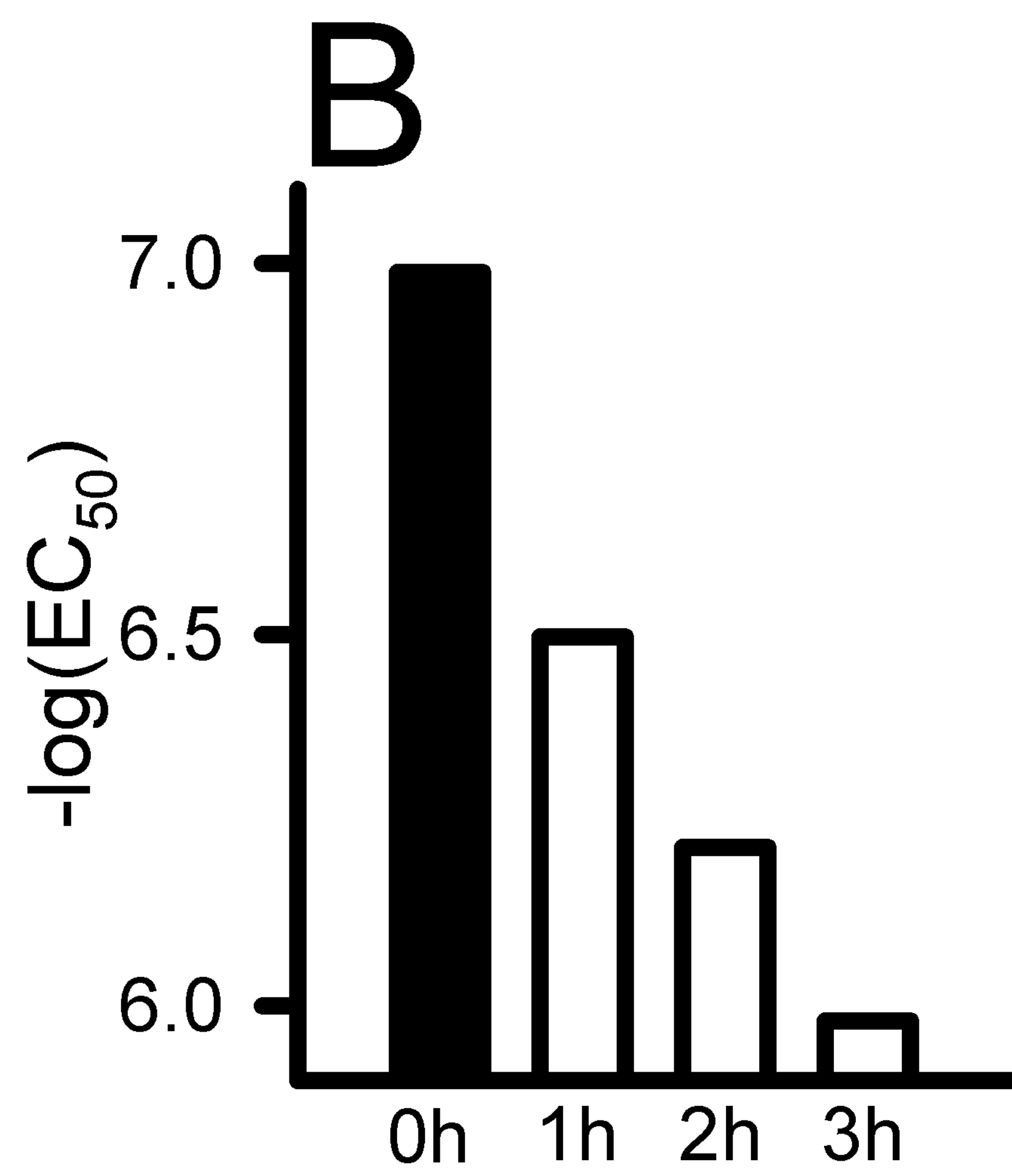
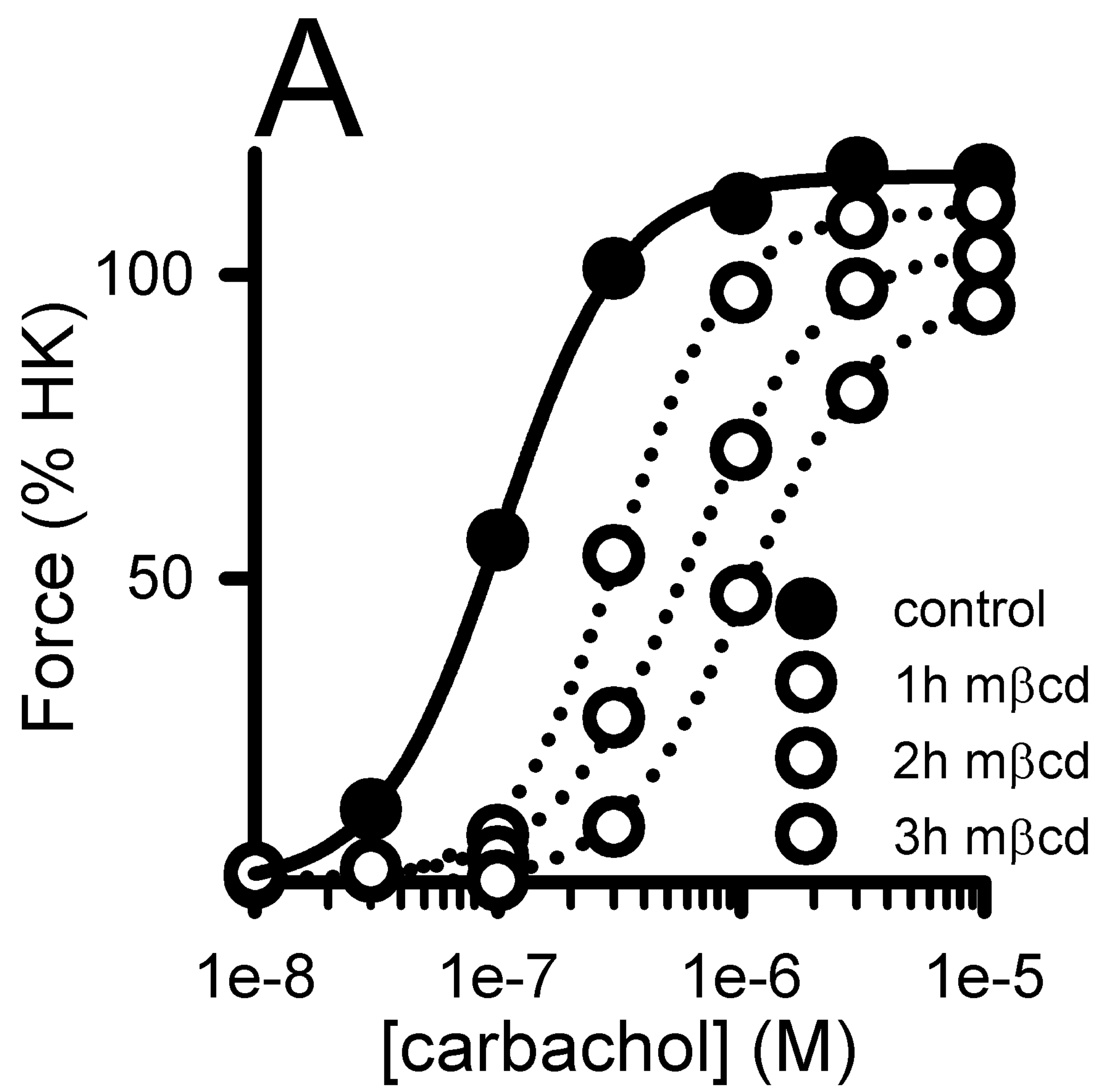
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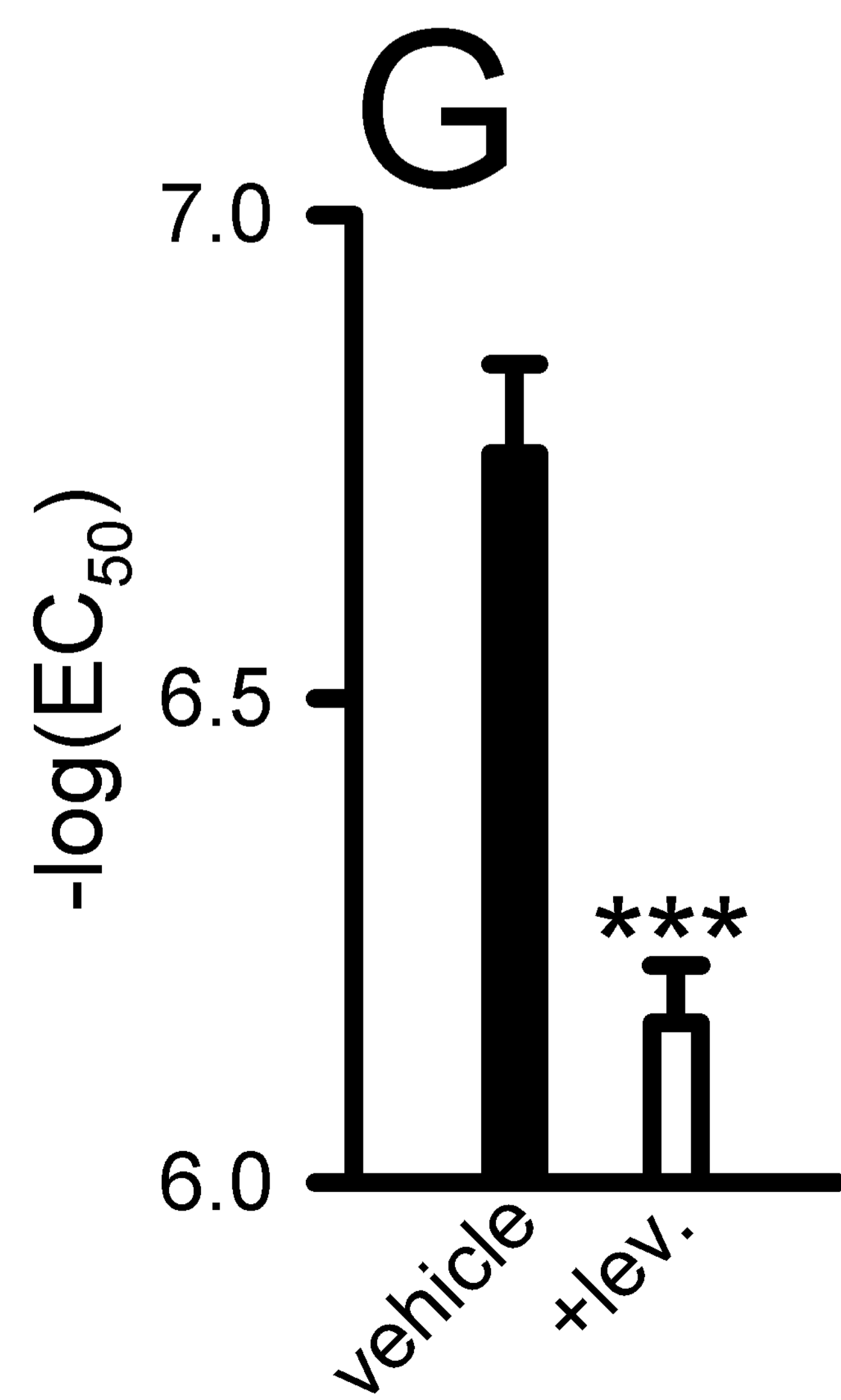
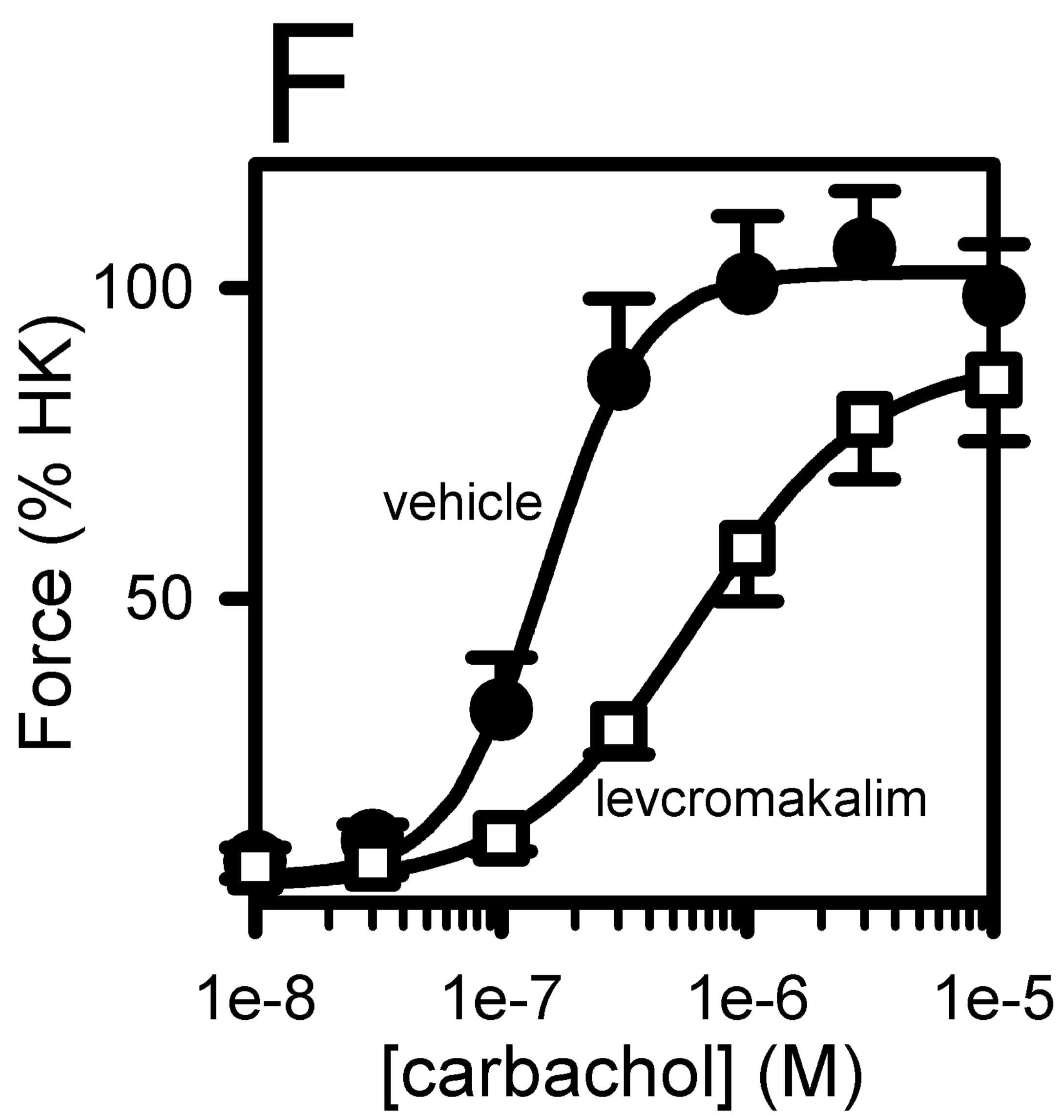
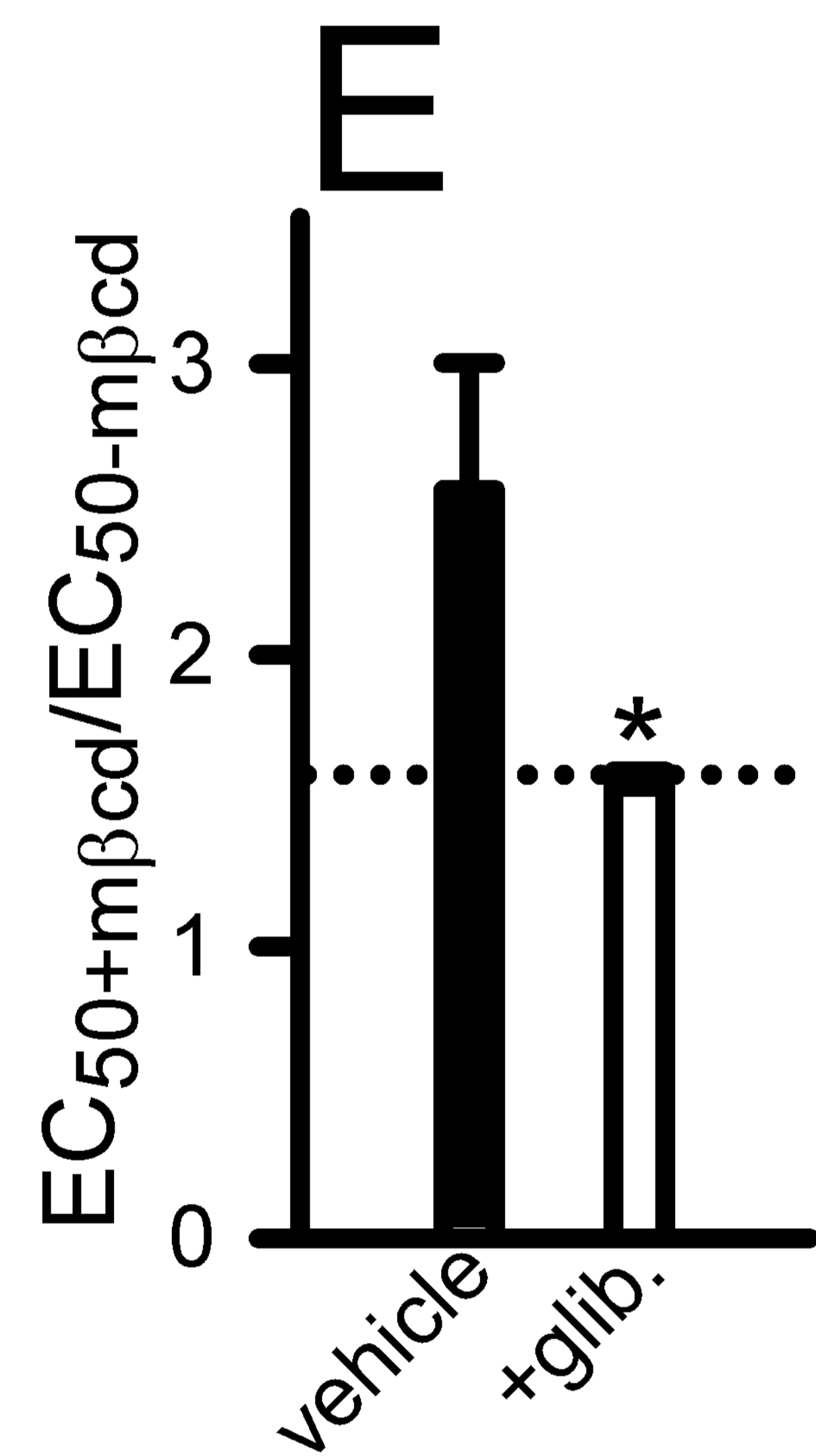
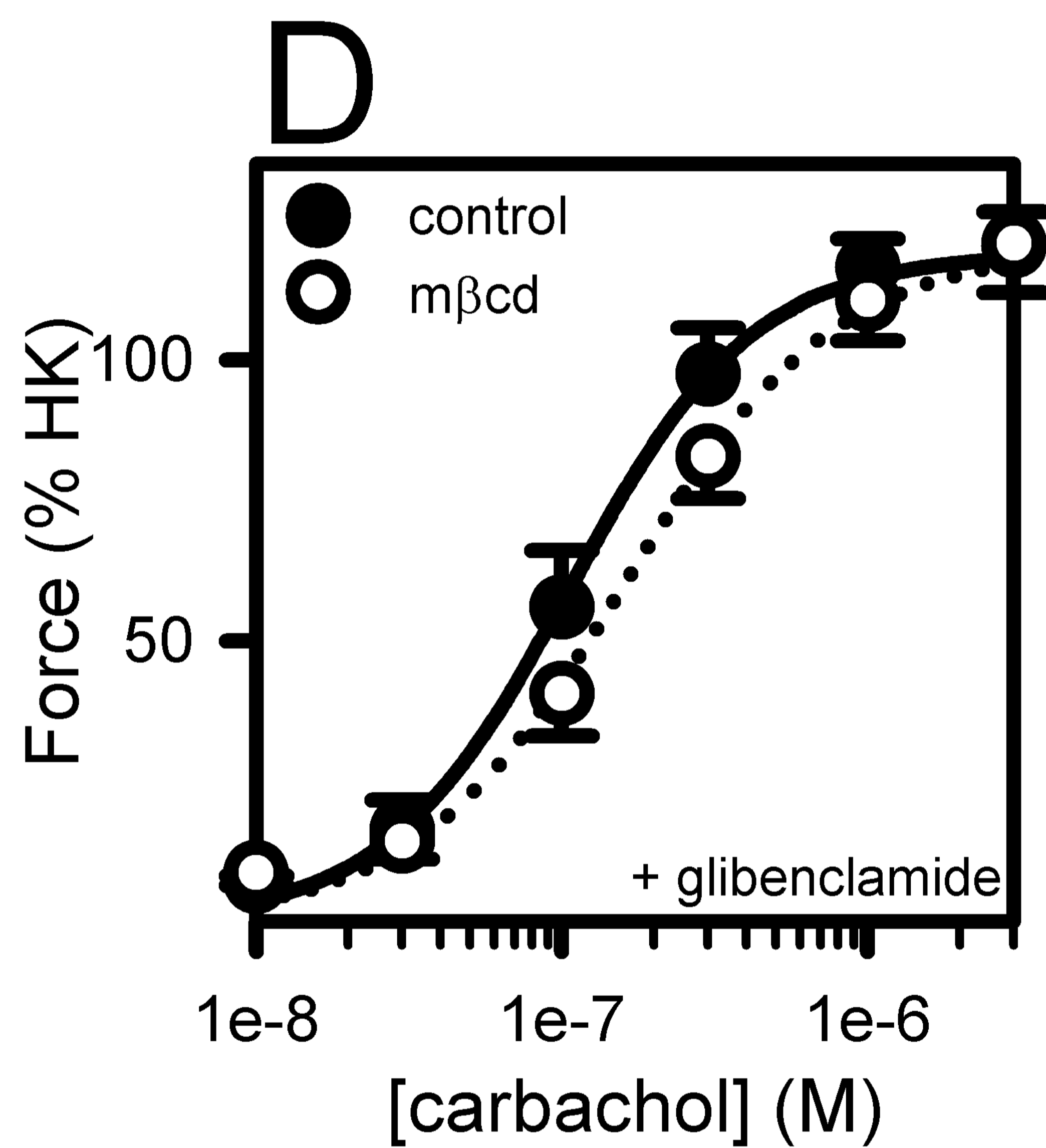
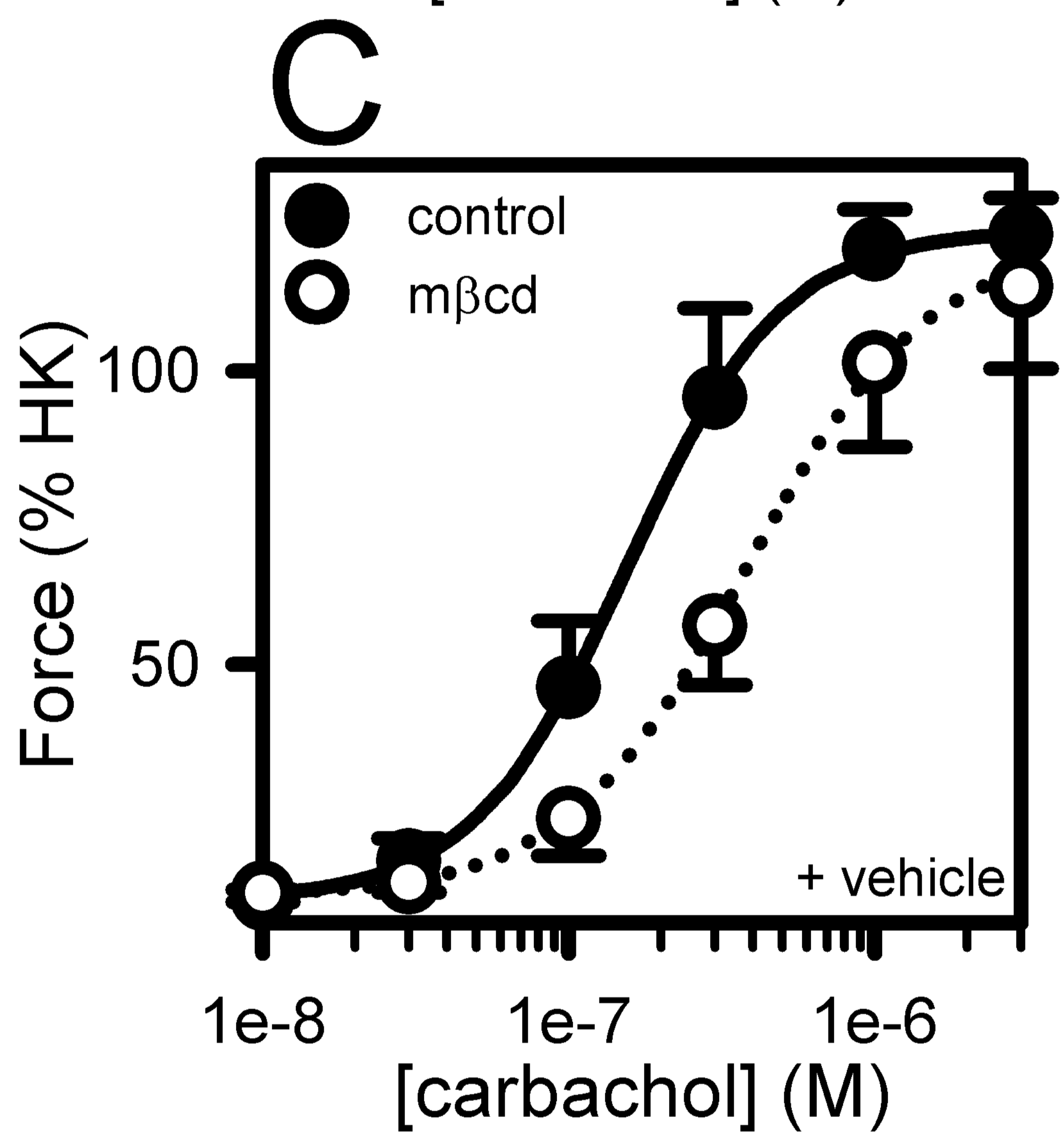
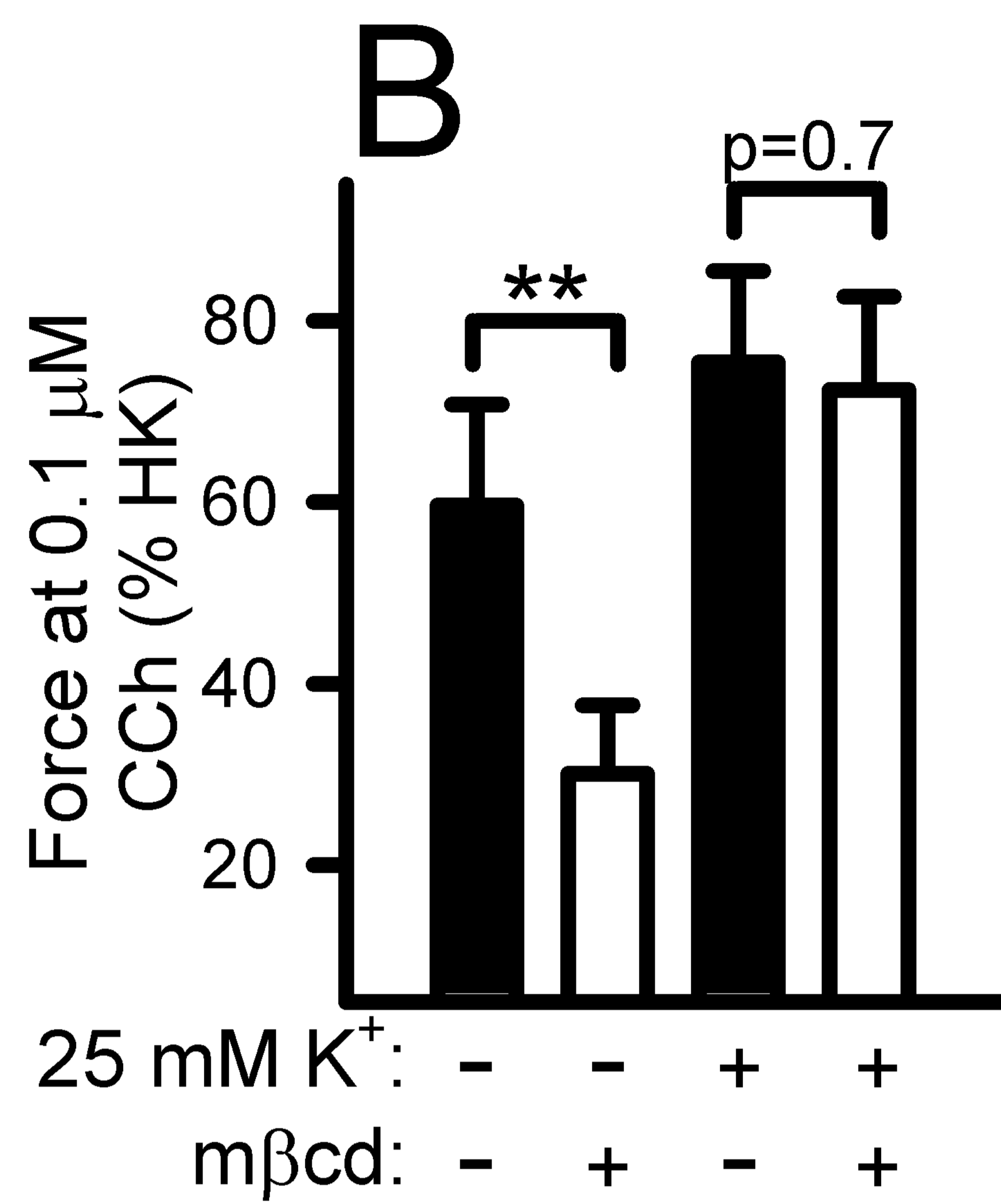
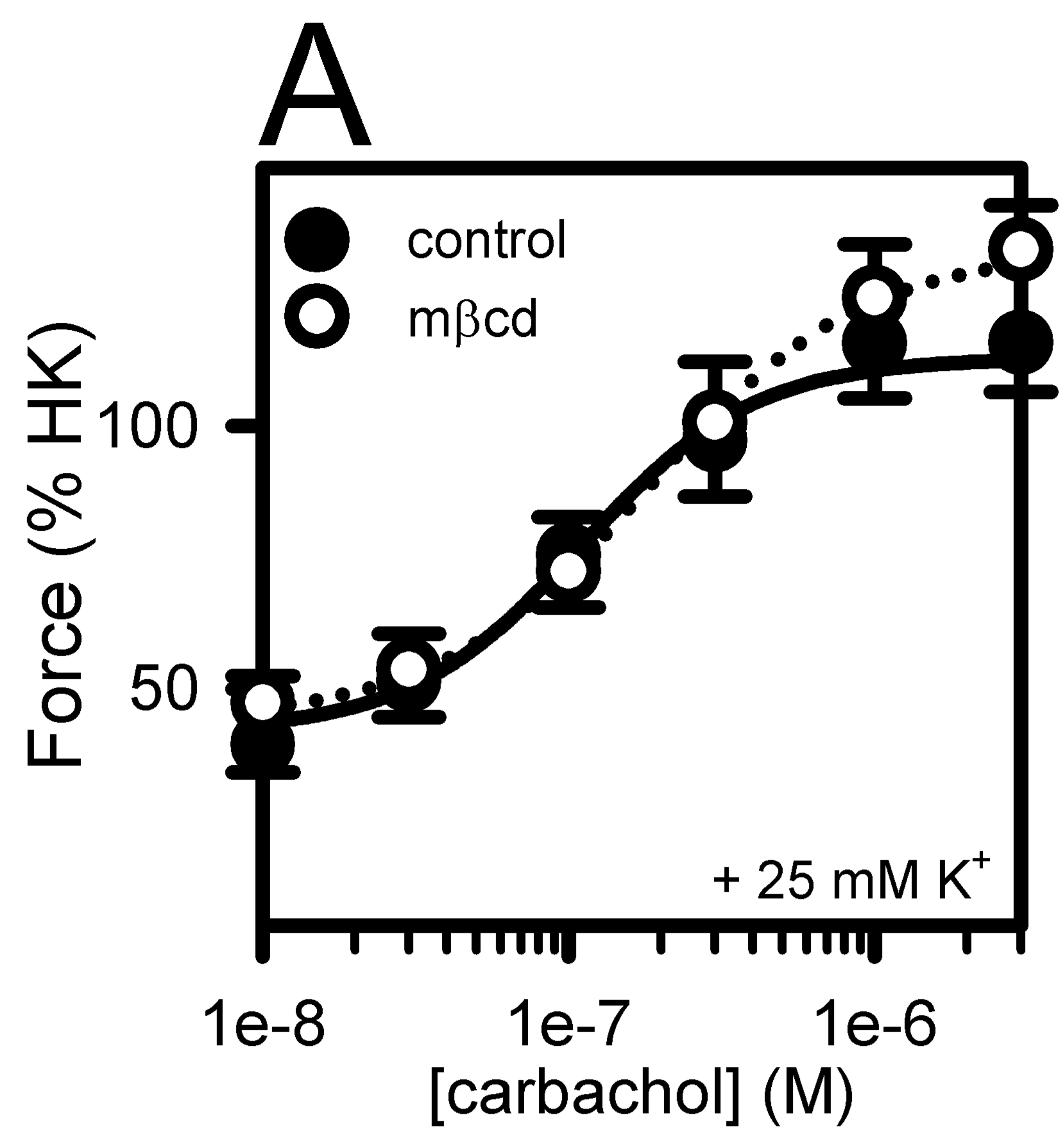
18 **Figure 8.** Chelerythrine, a protein kinase C translocation inhibitor, largely eliminates the  
19 rightward shift of the concentration-response relationship that is induced by cholesterol  
20 desorption in human detrusor. **A** and **B** show the effect of m $\beta$ cd in control preparations and in  
21 the presence of chelerythrine, respectively. **C** shows the fold shift induced by m $\beta$ cd in the  
22 absence and presence of chelerythrine (3  $\mu\text{M}$ , n=6 for both).

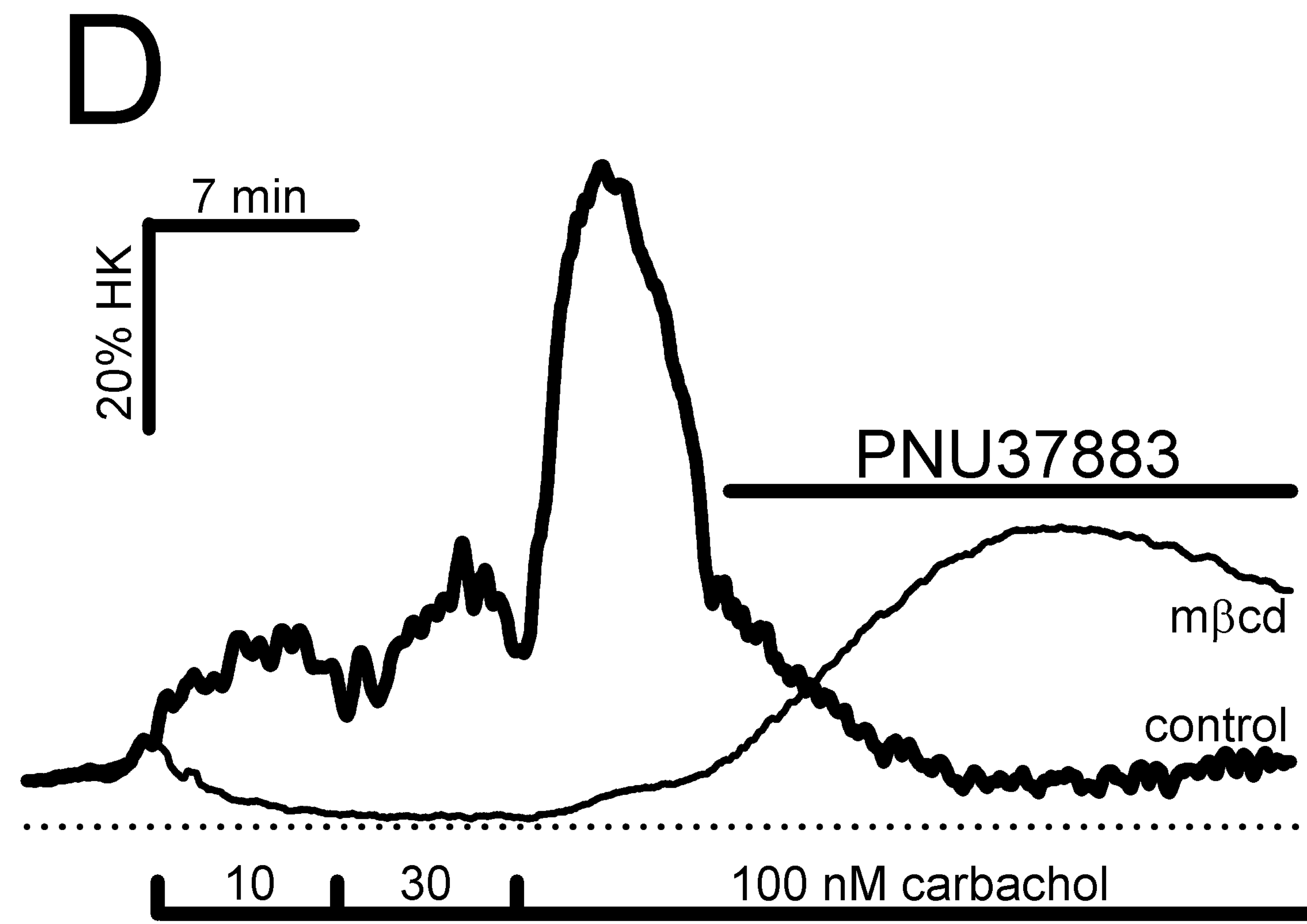
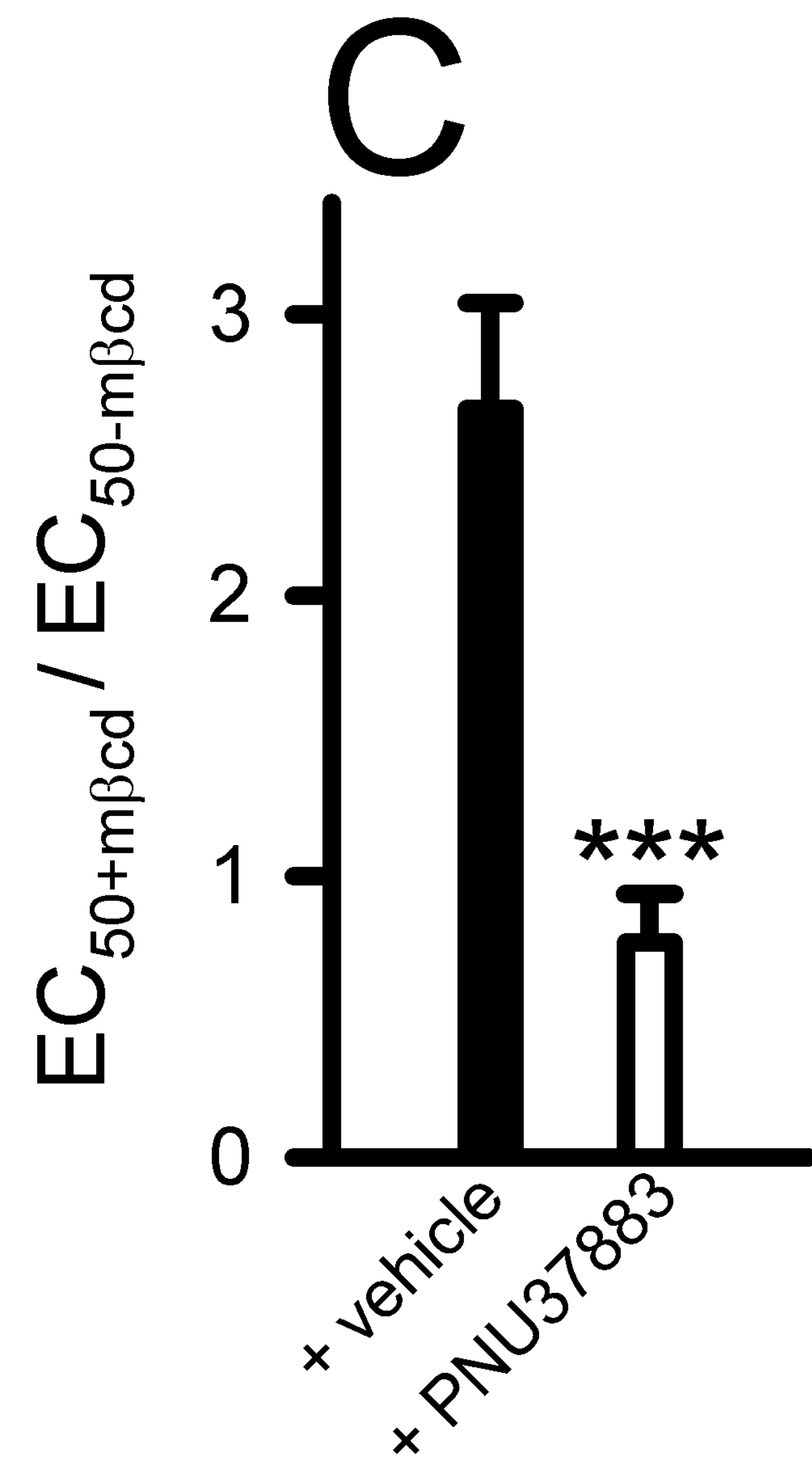
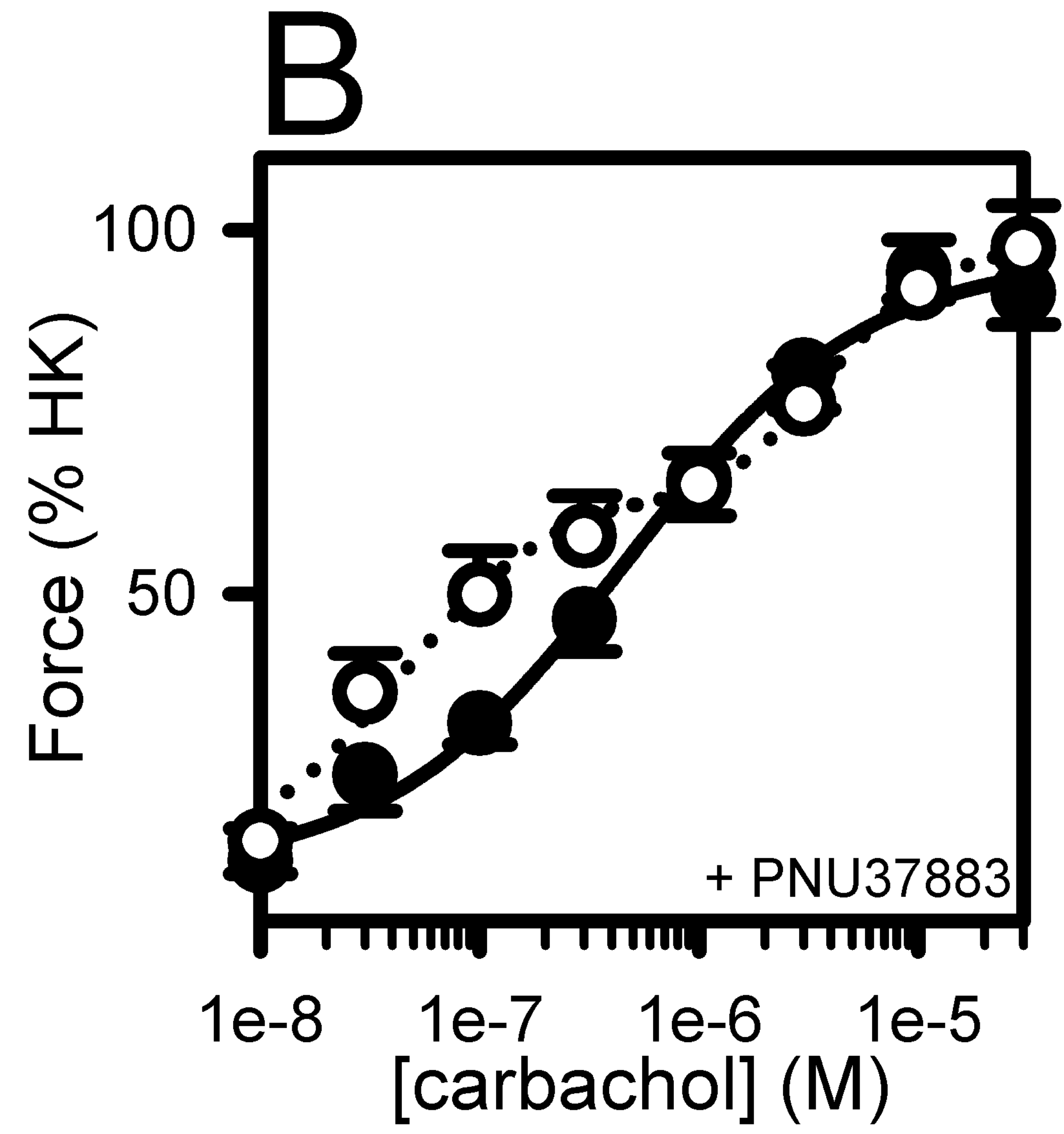
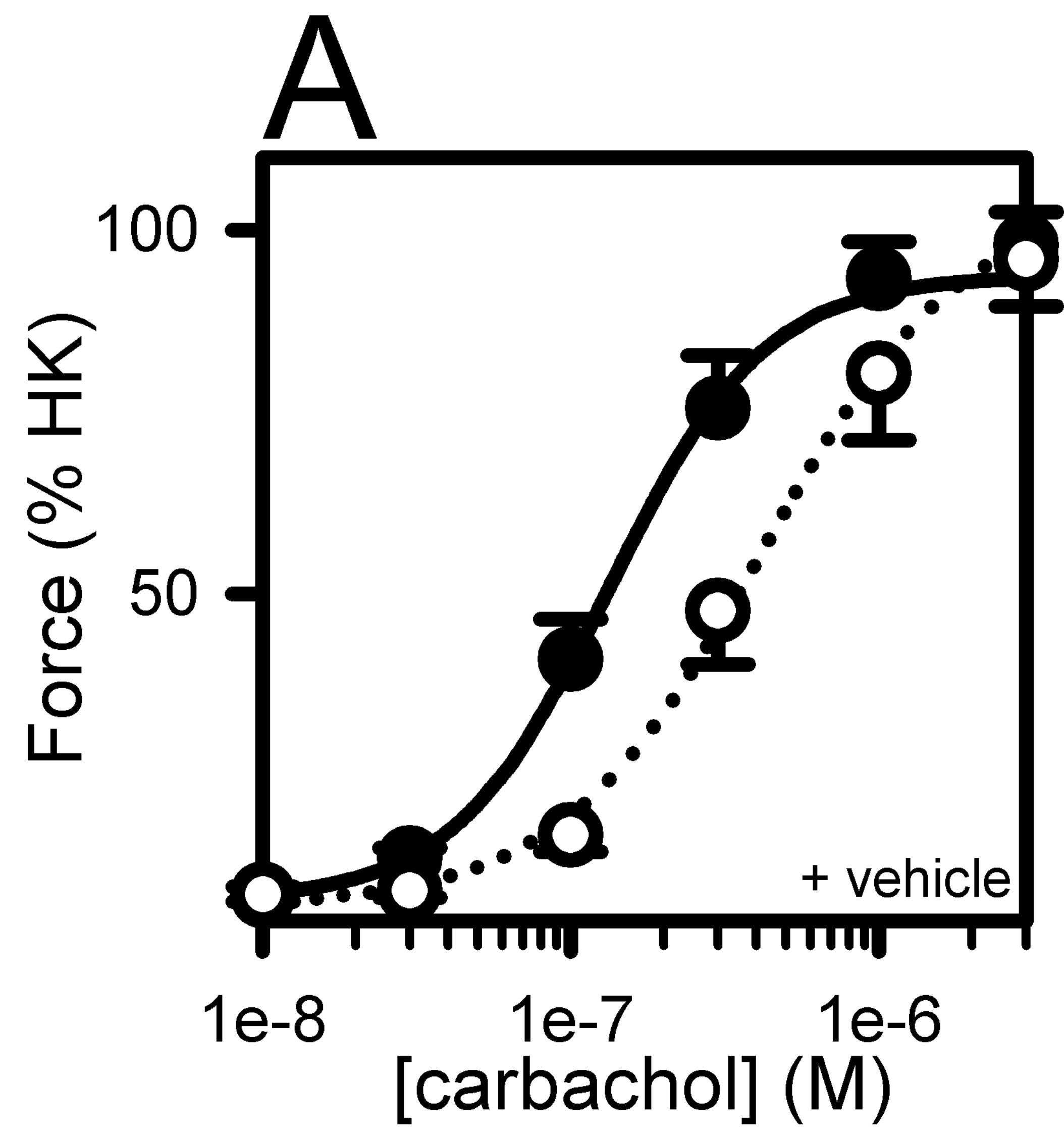
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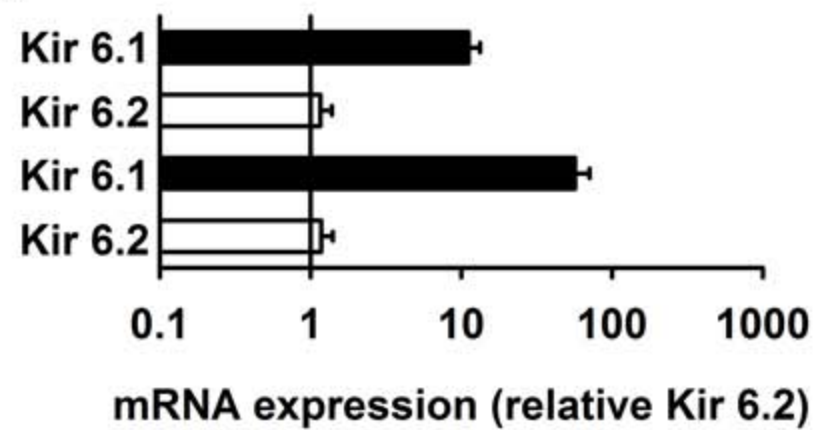




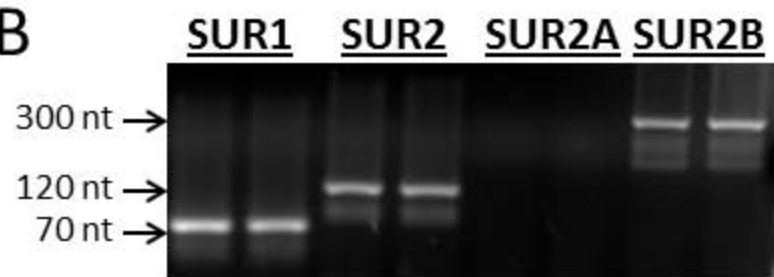


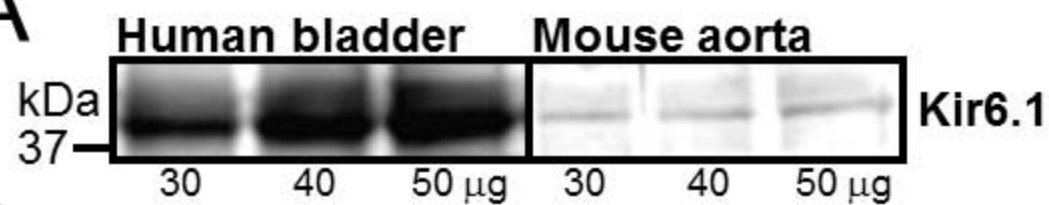


**A**



**B**



**A****B**