Adreno-muscarinic synergy in the bladder trigone: calcium-dependent and independent mechanisms.

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Conflicts of interest:

None.

Abstract

We have recently demonstrated a strong synergy between muscarinic and adrenergic excitatory pathways in the trigone which, as part of the bladder base, plays a key role in outflow control. This study aimed to characterise the role of intracellular Ca^{2+}, $[Ca^{2+}]_i$, in this synergistic effect. Muscle strips from the superficial trigone of male guinea-pigs were used for tension experiments. In isolated muscle cells, $[Ca^{2+}]_i$ was measured by epifluorescence microscopy using the fluorescent indicator Fura-2. Phenylephrine (PE, 10 μM) augmented contractions induced by carbachol (1 μM) to 4.0±0.8-fold of control, while corresponding $[Ca^{2+}]_i$ levels did not exceed 1.3±0.2-fold of control. Furthermore, PE generated significantly greater contractions for a given rise of $[Ca^{2+}]_i$, compared to depolarising KCl solutions. The protein kinase-C inhibitor GF 109203X (5 μM) and the Rho-kinase inhibitor Y-27632 (5 μM) reduced the PE contracture to 37.3±9.4 and 60.1±12.4 % of control, respectively, without significantly altering the $[Ca^{2+}]_i$ transients. GF 109203X reduced the augmentation of 1 μM carbachol by PE to 1.5±0.1-fold of control. Muscarinic and adrenergic receptor activation exert a powerful synergistic effect in the bladder trigone without equivalent changes to the $[Ca^{2+}]_i$ transient. Ca^{2+}-sensitisation of contractile proteins is likely to play a key role in this synergism, particularly for adrenergic activation.

Key words:

Trigone, synergy, alpha-adrenoceptor, muscarinic receptor, protein kinase c, rho kinase.

1. Introduction

We recently described a strong mutual interaction between adrenergic and muscarinic contractile activation in the bladder trigone [1]. In this study, 10 µM of the alpha-1-agonist phenylephrine (PE) was effective in enhancing contractions elicited by electrical-field stimulation, as well as by exposure to other agonists. The superficial trigone develops, with the ureter, from an outgrowth of the mesonephric duct and provides, as a transverse-orientated interureteric muscle, competent vesico-ureteric anchoring. Situated between the ureteric orifices and bladder outlet, it is considered to play a crucial role in uretero-vesical function, continence and micturition [2], [3]. Within the lower urinary tract, it represents an area of dual parasympathetic-muscarinic and sympathetic-adrenergic innervation. Reports about the proportion of adrenergic, muscarinic, and other transmitter systems in the trigone are variable [4], [5]. We found the muscarinic component of the electrically-evoked contraction to be about twice as significant as the adrenergic component (with a small purinergic component) [1]. However, this study demonstrated the physiological relevance of a dual receptor expression by showing a synergistic interaction. Synergy between both neurotransmitters in generating tension is of real interest because combined treatment with anti-adrenergics and anti-muscarinics for patients with both bladder outlet obstruction and overactive bladder symptoms has been shown to be highly effective not only in alleviating symptoms of urgency, but also in improving urinary outflow [6], [7], [8], [9]. The adreno-muscarinic interaction might be a result of Ca^{2+}-sensitisation of the contractile proteins. Ca^{2+}-sensitisation has been subject to intense research during the last few years: G-protein linked Rho-kinase (ROK) and protein kinase C (PKC) are thought to inhibit myosin phosphatase activity and therefore enhance myosin light-chain phosphorylation and cause an increase of force

76 [10]. Both pathways have been detected in detrusor and urethral muscle activation in
77 different species such as human, rat, guinea-pig, and pig [11], [12]. These pathways
78 were also found to exist in adrenoceptor-mediated contractions in trigonal smooth
79 muscle in our preceding study [1].
80 The aim of this study was to reveal the role $[Ca^{2+}]_i$ within the strong interaction
81 between adrenergic and muscarinic pathways in the trigone and to examine the
82 relevance of PKC and ROK in this effect.

2. Materials and Methods

2.1 Tissue preparation

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986. Male guinea-pigs (400 – 500 g, Dunkin-Hartley) were killed by cervical dislocation (schedule – 1 procedure), and the bladder with adjacent urethra removed. The bladder dome was resected cranially to the ureteral entrance into the dorsal bladder base. The bladder base was then longitudinally opened on the ventral site and the trigonal area exposed. After removing the mucosa by blunt dissection, a thin strip of muscle (4-5 mm in length, average weight 6.4 mg) between the ureteral orifices was cut from the superficial part of the trigone. The superficial layer could easily be distinguished from the underlying detrusor by its paler, whitish appearance.

2.2 Tension experiments

Strips were tied in a horizontal superfusion trough, to a fixed hook at one end and an isometric force transducer at the other. The preparation was superfused with Tyrode´s solution at 5-10 ml.min^{-1} containing (mM): NaCl, 118; $NaHCO_3$, 24.0; KCl, 4.0; $MgCl_2$, 1.0; NaH_2PO_4, 0.4; $CaCl_2$, 1.8; glucose, 6.1; Na pyruvate, 5.0; pH 7.4, gassed with 95 % O_2 and 5 % CO_2 at 37°C; pH 7.4. Contractile responses were generated by exposure to exogenous agonists. Agonists used were carbachol (1 and 10 μM) and phenylephrine (PE, 10 μM). To evaluate the effect of ROK inhibitors, tissues were incubated with (R)-(+)-*trans*-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide.2HCl (Y-27632) [13] and then challenged with different

agonists. Similarly, to examine the effect of PKC inhibitors, tissues were incubated with 2-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)maleimide (GF 109203X) [14] before agonist exposure. Control experiments, using only the vehicle (DMSO) for the test agents, were always carried out and exerted no significant effects.

Cell isolation. The bladder was quickly removed and placed in Ca^{2+}-free solution containing (mM): NaCl, 105.4; $NaHCO_3$, 20.0; KCl, 3.6; $MgCl_2$, 0.9; NaH_2PO_4, 0.4; glucose, 5.5; Na pyruvate, 4.5; Hepes, 4.9; pH 7.1. The trigonal area was then dissected as described above and cells were dissociated using a collagenase-based enzyme mixture dissolved in Ca^{2+}-free solution. Cells from the detrusor and urethral smooth muscle were similarly prepared.

2.3 Measurement of intracellular calcium ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured by epifluorescence microscopy using the fluorescent Ca^{2+} indicator Fura-2. Cells were loaded with 5 μM Fura-2 acetoxymethyl (AM) ester at 37° C for 9 min and then stored at 4°C for later use. An aliquot of cell suspension was placed in a chamber mounted on the stage of an inverted microscope and superfused at 37°C with Tyrode´s solution. 0-Ca^{2+} superfusate contained no added $CaCl_2$ and 0.1 mM EGTA, which buffered the ionised Ca^{2+} concentration to approximately pCa 8. Single cells were challenged with agonists at the same concentration as muscle strips, and to evaluate the effect of ROK- and PKC-inhibitors incubated according to the same protocol as for the intact preparation. The cells were illuminated alternately (32 Hz) at 340 and 380 nm. The emitted light was split by a dichroic mirror centred at 410 nm, and collected by a photomultiplier between 410 and 510 nm. The Fura-2 ratio signal was converted to $[Ca^{2+}]_i$ values using an in vitro

calibration method. The relationship between $[Ca^{2+}]_i$ and the ratio R (fluorescence ratio at 340 nm/380 nm excitation) is given by the equation [15]:

$$[Ca^{2+}]_i = K_d ß((R - R_{min})/(R_{max} - R)),$$

where R is the fluorescence ratio under study, R_{min} and R_{max} refer to ratio values at 0 $[Ca^{2+}]$ and saturating $[Ca^{2+}]$, respectively, ß is the ratio at 0 and saturating $[Ca^{2+}]$ at 380 nm alone, and K_d (224 nM) is the dissociation constant of Fura-2 for Ca^{2+} . The magnitude of the $[Ca^{2+}]_i$ rise was quantified as i) the peak deflection from the basal $[Ca^{2+}]_i$ before an intervention; ii) the duration of the transient; or iii) the integral of the rise of the ratio tracing above the baseline.

2.4 Statistics

Values are given as the mean ± SD with the number of animals (n). Differences between the mean values of different sets were examined by paired or unpaired Student´s *t*-tests and the null hypothesis rejected at $p<0.05$.

3. Results

3.1 Trigonal cells respond exclusively to both carbachol and phenylephrine

Figure 1A shows representative original traces of $[Ca^{2+}]_i$ in three cells, isolated from the bladder dome (A), the trigone (B), and the proximal urethra (C). All three cells were challenged with 10 µM carbachol as well as 10 µM PE. While detrusor and urethral cells would always respond respectively to either carbachol or PE alone, trigonal cells showed a clear response to both stimuli; to our knowledge, this observation has not yet been described for the trigone. Only those trigonal cells would be selected for experimentation that showed a clear response to both agonists.

3.2. Ca^{2+}- release from intracellular stores

To find out whether carbachol or PE mobilise Ca^{2+} from intracellular stores, we superfused trigonal single cells with 0-Ca^{2+} solution for three minutes and then challenged the cells with 10 µM PE or 10 µM carbachol (Fig. 1 B). Control experiments with 40 mM KCl as well as 80 mM KCl, were always carried out prior to the agonist intervention and never showed a response in 0-Ca^{2+} solution. Although agonist responses were clearly reduced – probably due to loss of cytosolic Ca^{2+} and consequent reduction in stored Ca^{2+} - we still observed a $[Ca^{2+}]_i$ rise of 25.4 ± 19.1 % (n = 12) for PE and of 21.8 ± 22 % (n = 11) for carbachol, compared to responses in Tyrode's solution.

3.3 $[Ca^{2+}]_i$ elevation is insufficient to explain synergistic contractions

To evaluate the potentiating effect of α-adrenoreceptor (α-AR) stimulation on responses to the muscarinic agonist carbachol, intact trigonal preparations were first challenged with 1 µM carbachol for 1 min as a control response, then superfused by 10 µM PE for 4 min., immediately followed by a combined intervention of 10 µM PE and 1 µM carbachol for 1 min (Fig. 2 A). The contraction evoked by carbachol in the presence of PE was measured from the new baseline of the preceding PE stimulation, representing the additional increase of force on top of the PE contraction, and compared to the response to 1 µM carbachol alone. The potentiation by PE was more than 4-fold (404.8 ± 84.6 %, n = 13, Fig. 2 C). To a similar degree, 10 µM PE increased contractions evoked by 20 mM KCl (428.1 ± 42.0 %, n = 5, Fig. 2 A and D, data from [1]). This suggests that the potentiating effect of PE takes place immediately prior to activation of the contractile machinery.

To address the question whether force potentiation in the muscle preparation is directly correlated to augmented $[Ca^{2+}]_i$ levels, we exposed isolated trigonal cells to the same protocol while recording $[Ca^{2+}]_i$ (Fig. 2, B). Cells displayed variable responses of $[Ca^{2+}]_i$ to prolonged PE exposures: constant rises for the duration of the stimulation as well as transient increases were obtained. Combined interventions were compared to the respective single control stimuli (1 µM carbachol or 20mM KCl) with respect to i) the maximum recorded $[Ca^{2+}]_i$ rise; ii) the duration of $[Ca^{2+}]_i$ transients; or iii) the area under the rise of $[Ca^{2+}]_i$ traces (3) were used as variables similar results were obtained. The peak of the combined response was always measured from the new baseline when $[Ca^{2+}]_i$ levels were still elevated at the end of PE pre-stimulation. For both KCl and carbachol experiments, a significant increase of about 30 % was recorded for the combined intervention, using the various indices of

203 $[Ca^{2+}]_i$ responses as indicated above (carbachol: i) 130.3 ± 18.6 %, ii) 123.4 ± 26.0
204 %, iii) 146.5 ± 58 %, n = 19; KCl: i) 127.2 ± 20.6, n = 15). However, there was a great
205 discrepancy between force augmentation in muscle strips and the $[Ca^{2+}]_i$ rise in
206 single cells (Fig. 2 C and D).
207
208 *3.4 Phenylephrine and carbachol in relation to the Ca^{2+}/tension curve*
209
210 To test the hypothesis that contractile activation via the adrenergic pathway in
211 trigonal smooth muscle employs mechanisms additional to internal Ca^{2+} release, we
212 determined the position of 10 µM PE responses in relation to a Ca^{2+}/tension curve
213 (Fig. 3). We plotted the rise of $[Ca^{2+}]_i$ in single cell experiments against the amplitude
214 of contractions in strips evoked by 10, 20, and 40 mM KCl. The point representing
215 the 10 µM PE intervention which served as an internal reference was clearly above
216 the plot through the KCl intervention points. This indicates that for a given rise of
217 $[Ca^{2+}]_i$ evoked by adrenergic stimulation greater force is generated. 20 mM KCl
218 elicited about the same rise in $[Ca^{2+}]_i$ as 10 µM PE (94.3 ± 30.1 %, n = 12), but only
219 25.6 ± 11.1 % (n = 10) of the contractile force. In turn, 40 mM KCl evoked a
220 comparable contraction as 10 PE (98.3 ± 36.0 %, n = 11), but a larger rise of $[Ca^{2+}]_i$
221 (169.3 ± 49.3 %, n = 22). The 1 µM carbachol intervention point lies close to the KCl
222 Ca^{2+}/tension curve, showing that in trigonal muscle muscarinic force activation is
223 predominantly determined by Ca^{2+} rise.
224
225 *3.5 The effect of PKC and ROK inhibition on the response to phenylephrine*

226 Ca^{2+} sensitisation by ROK and PKC has recently been shown to play a role in
227 detrusor activation [13]. We investigated the effect of the ROK inhibitor Y-27632

(5 μM) and PKC inhibitor GF 109203X (5 μM) on contractions and $[Ca^{2+}]_i$ levels in the trigone when evoked by PE (Fig. 4 A, B). Pre-incubation with GF 109203X (30 min) caused a reduction of the PE contracture to 37.3 ± 9.4 % of control (n = 14). Contractile force completely recovered after 15 – 30 min. A longer incubation did not reduce the contracture any further and did not prevent full recovery. With single cells, levels of $[Ca^{2+}]_i$ were not affected by GF 109203 X (103.7 ± 5.6 %, n = 6). Y-27632 reduced the PE contraction to a lesser extent (60.1 ± 12.4 % of control, n = 8) without significant alteration of $[Ca^{2+}]_i$ (101.2 ± 29.4 %, n = 6).

3.6 The effect of PKC and ROK inhibition on the potentiated muscarinic component

To explore further the hypothesis that the synergy described above between adrenergic and muscarinic activation is due to kinase activity, we incubated muscle strips with the PKC or ROK inhibitor for 30 min. The preparation was then superfused with 10 μM PE alone, followed by the combined intervention (10 μM PE + 1 μM carbachol), and the enhanced muscarinic component compared to that from 1 μM carbachol alone (Fig. 4 C). Similar results were obtained when the sequence of the interventions was changed. There was still a significant, but greatly reduced increase of force (145.7 ± 14.5 %, n = 5, vs. 404.8 ± 84.6 % of control) after PKC inhibition. Incubation with the ROK inhibitor, in turn, reduced the potentiating effect by a smaller, but still significantly different, degree (295.8 ± 34.24 %, n = 4, of control).

4. **Discussion**

4.1 Cellular synergism is mediated through calcium sensitisation

In contrast to previously reported facilitation of acetylcholine release by prejunctional α-ARs in the detrusor [16], the force-potentiating interaction between adrenergic and muscarinic neurotransmission in the trigone is a true synergistic effect on the muscle cells themselves. In this study, we measured changes to $[Ca^{2+}]_i$ in parallel experiments to tension recording. It is important to note that the same cell responded to both muscarinic and adrenergic stimulation, discounting the possibility that the dual innervation of the trigone might be a mere result of two distinct cell populations. The strong synergistic effect seen when recording tension with intact muscle preparations was not mirrored by changes to $[Ca^{2+}]_i$, although a significant $[Ca^{2+}]_i$ increase to carbachol of about 30 % was recorded with 10 μM PE. Even assuming that preactivation by PE caused a shift to the steep part of the Ca^{2+}/tension curve, where a similar rise of $[Ca^{2+}]_i$ would result in a larger contraction, an increase of not more than 30 % in $[Ca^{2+}]_i$ could not explain the more than 4-fold potentiation of tension.

The hypothesis that the adrenergic pathway must involve mechanisms additional to IP_3-mediated Ca^{2+}-release from intracellular stores, was supported by the finding that PE required lower $[Ca^{2+}]_i$ levels than high-KCl solutions to generate the same contractile force. When $[Ca^{2+}]_i$ is constant, agonists activating G-protein-coupled receptors might cause a leftward shift of the Ca^{2+}/tension curve, i.e. Ca^{2+}-sensitisation. The two major G-protein-coupled pathways operate through PKC and ROK. It was therefore of interest to determine if the adrenergic pathway in trigonal

muscle activation involves Ca^{2+}-sensitisation, and by which route. We found the contraction to phenylephrine to be dramatically reduced by the PKC-inhibitor GF 109203X and, to a smaller extent, by the ROK-inhibitor Y-27632, while levels of intracellular Ca^{2+} changes were similar. The effect of PKC- and ROK-inhibition on the augmented muscarinic response in the combined intervention clearly shows that both PKC and ROK mediate synergism in contraction. In previous experiments [1], PKC as well as ROK inhibition were without significant effect on the contracture evoked by carbachol alone, whereas in the combined intervention, the enhanced muscarinic component was reduced by 73 % and 36 %, respectively.

4.2 Source of $[Ca^{2+}]_i$

The observation that under α-AR stimulation, cell depolarisation by KCl elicits a 30% higher $[Ca^{2+}]_i$ rise, suggests also a modulation of voltage-dependent Ca^{2+} channels - an effect which has been established in cardiac myocytes and employs PKC to phosphorylate L-type Ca^{2+}-channels [21]. To the same degree, α-AR activation was shown to facilitate carbachol-induced Ca^{2+}-release from intracellular stores. However, this interaction may not be mediated by PKC, as PKC inhibition had no effect on $[Ca^{2+}]_i$ transients elicited by PE itself, which – like carbachol – activates IP_3 and mobilises Ca^{2+} from intracellular stores. This is in accordance with previous reports where inhibition of PKC or ROK had no effect on carbachol-evoked $[Ca^{2+}]_i$ transients in the detrusor [12]. We have not characterised in detail the nature of the intracellular Ca^{2+}-stores and messengers in this study – this is subject of current investigations.

However, $[Ca^{2+}]_i$ contributes to some extent to the synergistic interaction between adrenergic and muscarinic pathways in the bladder trigone, and might (together with

still active ROK) be responsible for the 40 % force augmentation of the combined intervention still seen under PKC inhibition.

Interpretation of the reduced carbachol-response following L-type Ca^{2+} channel knock-out in a recent study initiated a debate as to whether agonist stimulation of bladder smooth muscle opens membrane Ca^{2+}-channels or release Ca^{2+} from intracellular stores [18]. Our data suggest that agonist stimulation of trigonal smooth muscle involves Ca^{2+} release from intracellular stores, evidenced by the persistence of PE and carbachol-induced [Ca^{2+}] rise in Ca^{2+}-free solution when KCl-induced Ca^{2+} entry was abolished. A Ca^{2+}-free environment inevitably causes continuous loss of cytosolic Ca^{2+} and depletion of intracellular stores, which explains the reduction of resting $[Ca^{2+}]_i$ and the decrease in $[Ca^{2+}]_i$ response to agonists; however, significant responses to PE and carbachol were measured, in contrast to KCl interventions.

4.3 Clinical implications

Our data suggest a strong interaction between muscarinic and adrenergic pathways in the bladder trigone: the muscarinic pathway predominantly operates through modulation of $[Ca^{2+}]_i$; the adrenergic pathway significantly alters the Ca^{2+}-sensitivity of the contractile machinery. The latter provides an effective mechanism to allow the bladder neck to switch between a closed to open state, especially in combination with an NO-based relaxation process [3].

Recent reports suggest the advantages of a combined anti-muscarinic and anti-adrenergic therapy in patients suffering from both bladder outflow obstruction and overactive bladder symptoms; only the combined use of the muscarinic antagonist

tolterodine and the alpha-adrenergic antagonist tamsulosin has been shown to be effective with respect to both parameters quality of life and maximum urine flow rate [6], [7], [8], [9]. This is the first experimental study providing an explanation for the beneficial effect of a simultaneous muscarinic and adrenergic blockade in the trigone as the dorsal part or the bladder base. It might well be that in conditions of simultaneous mechanical obstruction and increased muscarinic tone, the inhibition of both synergistic components – adrenergic-driven Ca^{2+}-sensitisation and muscarinic-driven $[Ca^{2+}]_i$ increase – achieves the highest possible reduction of outflow resistance in the bladder base and neck.

5. Conclusion

This study demonstrates that the adrenergic pathway in the bladder trigone mainly operates through Ca^{2+}-sensitisation and is thereby capable of a more than 4-fold potentiation of muscarinic force activation. It supports recent reports about the high effectiveness of a combined anti-muscarinic and anti-adrenergic therapy in patients with both OAB and outflow obstruction.

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Figure legends

Figure 1

A: $[Ca^{2+}]_i$ recordings of myocytes isolated from the bladder dome (D, top), the trigone (T, middle), and the proximal urethra (U, bottom), challenged with 10 μM carbachol and the α_1-adrenergic agonist phenylephrine (PE, 10 μM). The diagram (left) shows the approximate sites from where the cells were obtained. **B:** Effect of 0-Ca^{2+} solution on $[Ca^{2+}]_i$ transients elicited by 10 μM PE (top), 10 μM carbachol (middle) and 40 mM KCl (bottom). Traces in part A (middle) and B were all from one cell preparation.

Figure 2

Representative traces showing contractures of intact trigone strips **(A)** and $[Ca^{2+}]_i$ transients **(B)** in isolated trigonal cells. Responses were elicited by 1 μM carbachol or 20 mM KCl alone or in the presence of 10 μM PE. Summary of the effects of 1 μM carbachol **(C)** or 20 mM KCl **(D)** on tension and $[Ca^{2+}]_i$ in the absence and presence of 10 μM PE, mean ± sd; * $p<0.05$; † data from [1].

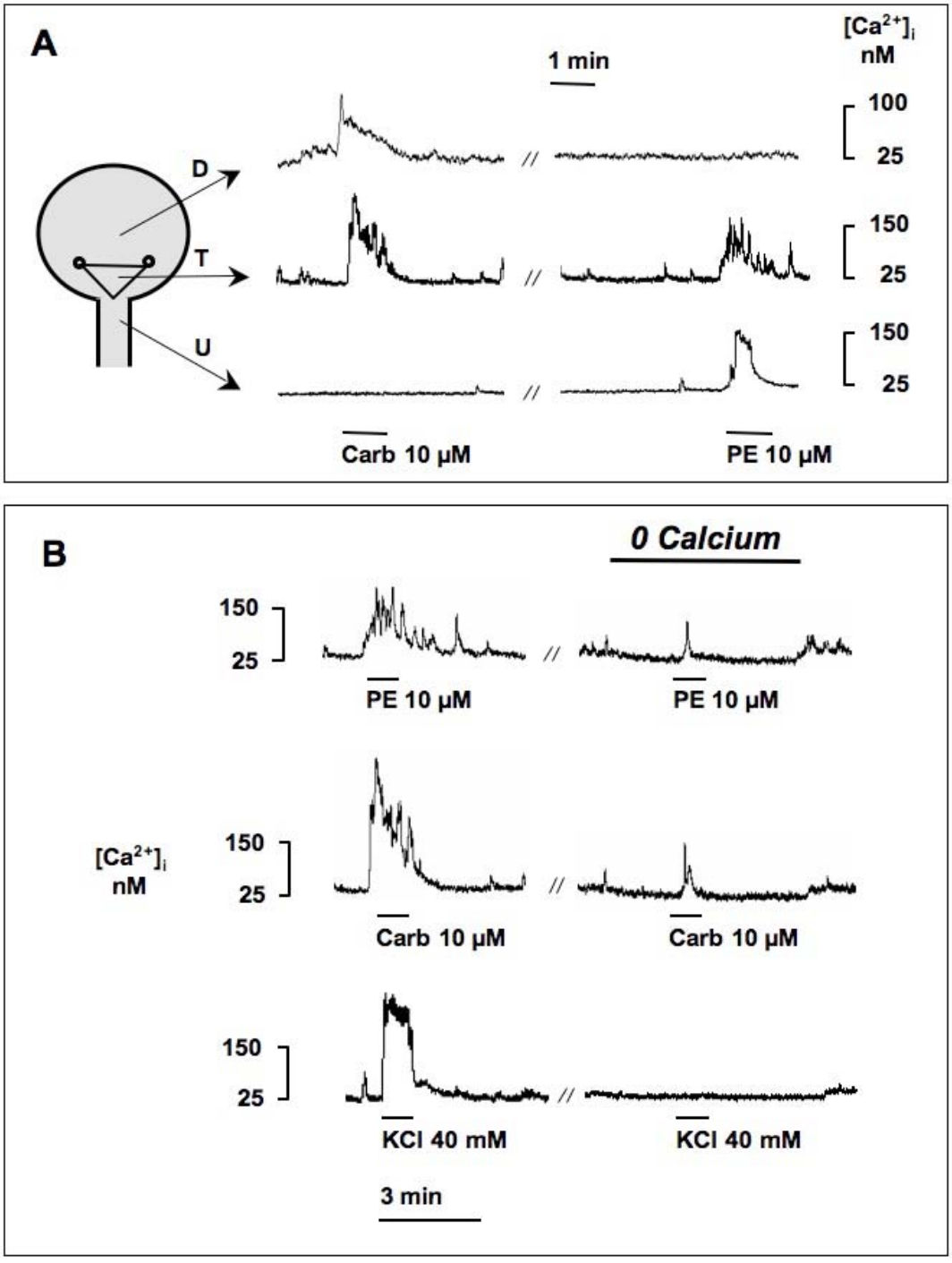
Figure 3

The relationship between isometric contracture tension and the increase of $[Ca^{2+}]_i$ by 10, 20, and 40 mM KCl solutions (closed circles), and 1 μM carbachol (open square). Data are expressed as a proportion of these variables elicited by 10 μM PE (large closed square). The curve was fitted by a least-squares method (KaleidaGraph™, Synergy Software).

Figure 4

A: The effect of the protein kinase-C inhibitor GF 109203 X (5 μM) on 10 μM PE contractures (top) and $[Ca^{2+}]_i$ (bottom). Tension and $[Ca^{2+}]_i$ records were obtained from different preparations. **B:** Force and $[Ca^{2+}]_i$ elicited by 10 μM PE after incubation with GF 109203 X and Rho-kinase inhibitor Y-27632 (5 μM). Data are shown as a percentage of the values in the absence of these inhibitors. **C:** The effect of GF 109203 X and Y-27632 on the augmentation of the 1 μM carbachol contracture by 10 μM PE; format as in part B; * $p<0.05$ compared to control; † $p<0.05$ compared to carbachol + PE without pre-incubation.

436 Fig 1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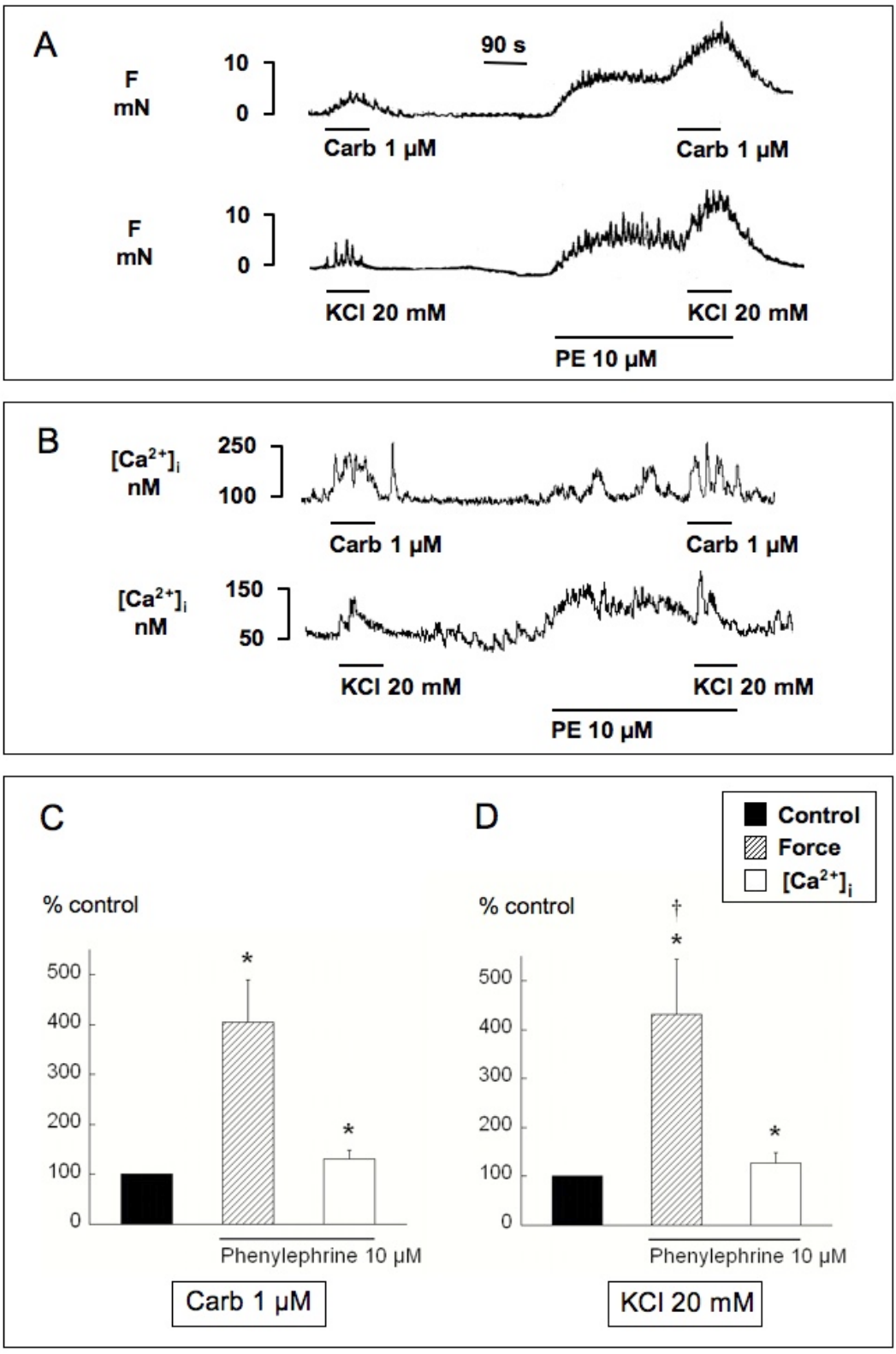
438 Fig 2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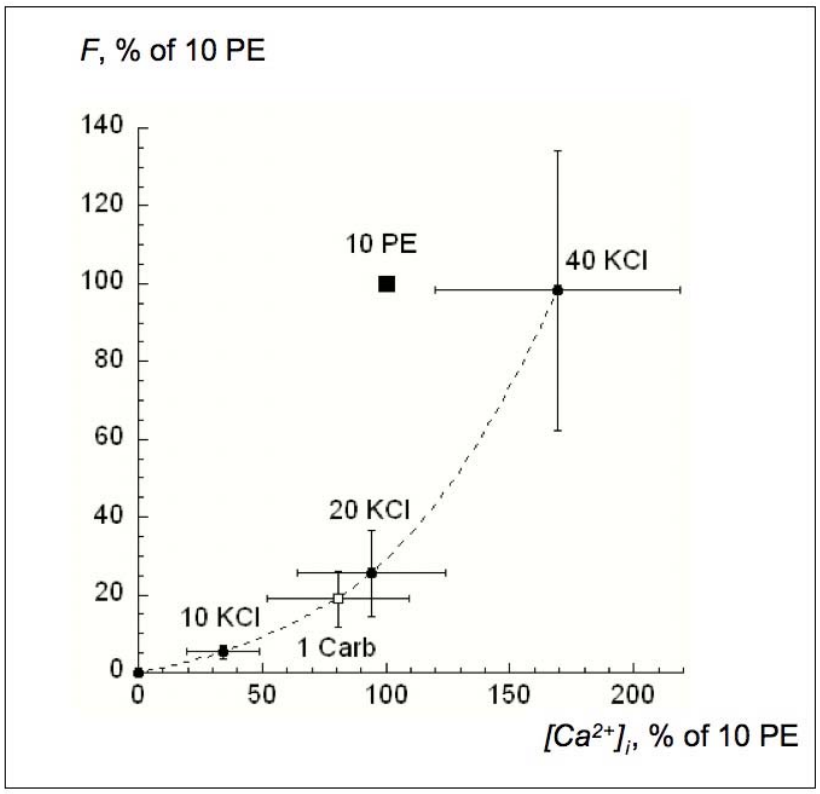
440 Fig 3


F, % of 10 PE
140
120
100
80
60
40
20
0
10 PE
40 KCl
20 KCl
10 KCl
1 Carb
0
50
100
150
200
[Ca2+]i, % of 10 PE

441

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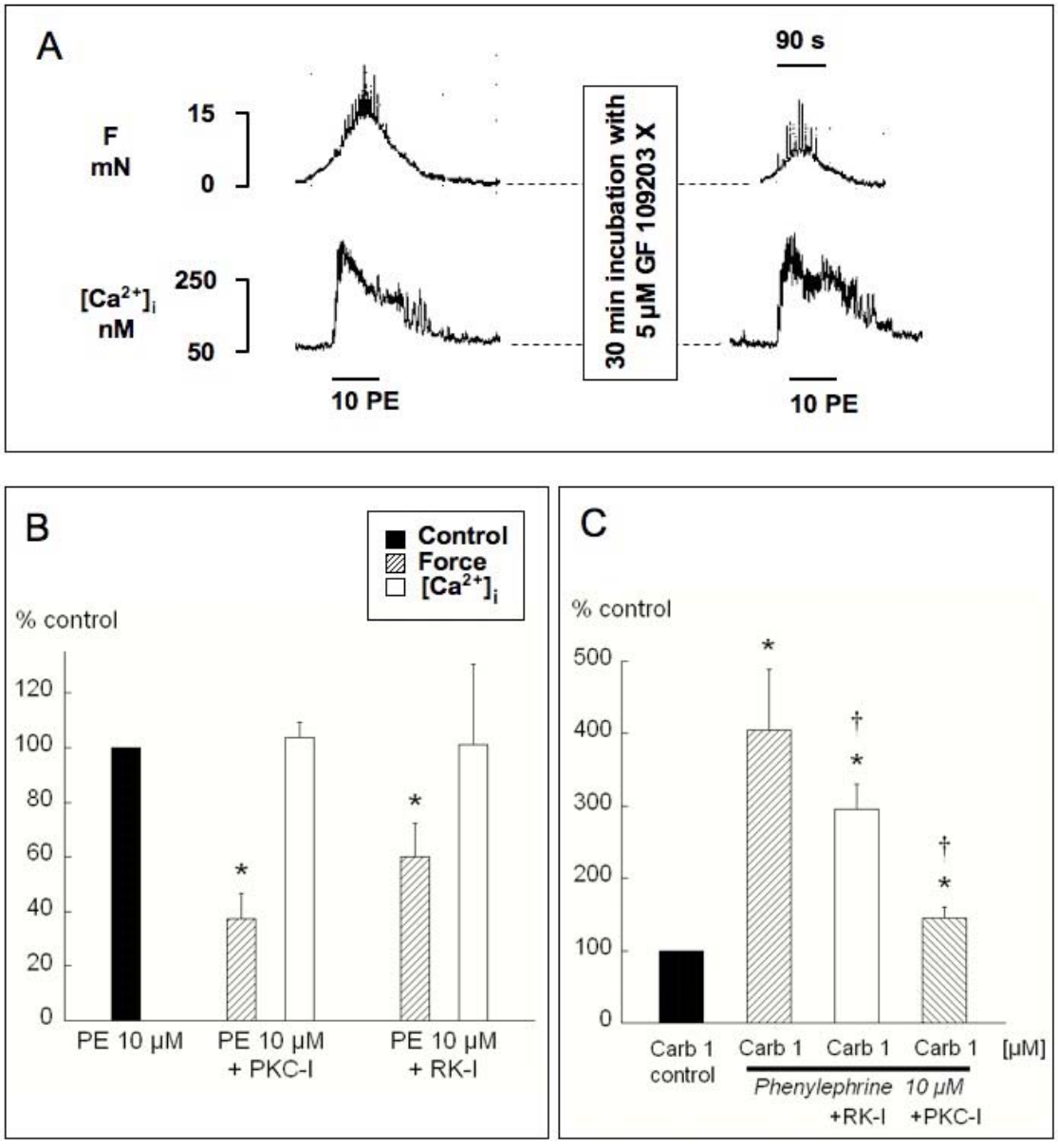
A
90 s
F
mN
15
0
[Ca2+]i
nM
250
50
30 min incubation with
5 µM GF 109203 X
10 PE
10 PE
B
Control
Force
[Ca2+]i
% control
120
100
80
60
40
20
0
PE 10 µM
PE 10 µM
+ PKC-I
PE 10 µM
+ RK-I
C
% control
500
400
300
200
100
0
Carb 1
control
Carb 1
Carb 1
Carb 1
[µM]
Phenylephrine 10 µM
+RK-I
+PKC-I

443