European Journal of Pharmacology 610 (2009) 106–109

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, Gastrointestinal and Urogenital Pharmacology

Phospholipase C mediated Ca^{2+} signals in murine urinary bladder smooth muscle

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ARTICLE INFO

Article history:
Received 5 January 2009
Received in revised form 27 February 2009
Accepted 10 March 2009
Available online 19 March 2009

Keywords:
Detrusor
Contraction
U73122

ABSTRACT

Muscarinic stimulation of urinary bladder induces contraction via an increase in intracellular Ca^{2+} concentration that results from Ca^{2+} influx through Ca^{2+} channels and/or IP_3-mediated Ca^{2+} release controlled by phospholipase C (PLC) signalling. The significance of PLC/IP_3 signalling in this cascade has recently been questioned because PLC inhibitors were without effect on carbachol-induced contractions in detrusor muscle strips. However, PLC/IP_3-mediated Ca^{2+} release was clearly observed in recordings of Ca^{2+} signals in isolated myocytes. Therefore, we investigated the presence of PLC/IP_3-dependent Ca^{2+} release by directly monitoring Ca^{2+} signals in intact detrusor muscle strips. Concomitant Ca^{2+} signals from Ca^{2+} channel activity were eliminated by the Ca^{2+} channel antagonist isradipine (3 μM) or by the use of muscles from $Ca_v1.2$ channel-deficient (SMACKO) mice. In absence of Ca^{2+} channel activity, carbachol elicited contractions and Ca^{2+} signals in muscles from wild type and SMACKO mice that were inhibited by the PLC inhibitor U73122 (10 μM). The results show that PLC/IP_3-dependent Ca^{2+} release is activated by stimulation with carbachol in urinary bladder smooth muscle but has a minor contribution to overall carbachol-induced Ca^{2+} signals.

1. Introduction

Micturition is predominantly controlled by muscarinic signalling (Frazier et al., 2008). The common cascade of muscarinic signalling in urinary bladder smooth muscle involves M3 receptors coupled to $G_{q/11}$ proteins that activate phospholipase C (PLC). PLC activity leads to generation of diacylglycerol and inositol-1,4,5-triphosphate (IP_3), the latter being responsible for IP_3-induced Ca^{2+} release from intracellular stores and probably followed by Ca^{2+}-induced Ca^{2+} release (Andersson and Arner, 2004). The released Ca^{2+} binds to calmodulin; Ca^{2+}/calmodulin, in turn, activates myosin light chain kinase that induces contraction of smooth muscle by phosphorylation of myosin light chain (Somlyo and Somlyo, 2003).

A number of studies on single myocytes support the concept of signalling via intracellular Ca^{2+} release (Sanders, 2001). For example, IP_3 concentrations were increased after muscarinic stimulation (Iacovou et al., 1990) and IP_3-induced Ca^{2+} signals have been shown in isolated urinary bladder smooth muscle cells (Chambers et al., 1996; Ganitkevich and Isenberg, 1992; Ji et al., 2006).

However, evidence from studies on intact urinary bladder smooth muscle challenged the concept that carbachol-induced Ca^{2+} release is solely responsible for contraction after muscarinic stimulation. For example, inhibitors of PLC did not influence muscarinic contractions (Frazier et al., 2007). In addition, L-type Ca^{2+} channels have been shown to control the major part of the contractile response to muscarinic stimulation (Bo and Burnstock, 1990; Fovaeus et al., 1987; Wegener et al., 2004; Wuest et al., 2007).

In the present study, we investigated the presence of PLC/IP_3 mediated Ca^{2+} signals in intact muscle preparations from urinary bladder by simultaneous recordings of tension and Ca^{2+} signals. To suppress Ca^{2+} signals carried by L-type Ca^{2+} channel activity, we used the Ca^{2+} channel blocker isradipine or muscles from $Ca_v1.2$ deficient mice. In the absence of Ca^{2+} channel activity, we were able to directly monitor PLC/IP_3-mediated Ca^{2+} signals activated by muscarinic stimulation in intact muscles.

2. Materials and methods

All experiments complied with the European guidelines for the use of experimental animals and were approved by the local animal ethics committee. Experiments were performed on wild type mice and mice with a defective L-type $Ca_v1.2$ Ca^{2+} channel gene in smooth muscle after treatment with tamoxifen (SMACKO, *s*mooth *m*uscle α_{1c}-subunit calcium channel *k*nock *o*ut) (Wegener et al., 2004). All mice used were killed by decapitation; the bladder was quickly transferred to buffer solution (in mM: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, $NaHCO_3$ 12, NaH_2PO_4 0.42, glucose 5.6) bubbled with carbogen (95% O_2, 5% CO_2). The urothelium was gently removed and detrusor muscle was cut into longitudinal translucent strips of about 1 to 2 mg. The muscle strips were loaded with 10 μM Fura-2 AM in the presence of 0.01% pluronic acid for 4 h at room temperature and 1 h at 37 °C and then mounted into the organ bath (Fibermic, SI, Heidelberg, Germany). Tension was recorded isometrically at 37° ± 1 °C by the Fibermic

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doi:10.1016/j.ejphar.2009.03.036

device (SI, Heidelberg, Germany). Extracellular dye and pluronic acid were washed out by superfusion with buffer solution at 1 ml/min for 30 min.

Ca^{2+} signals were recorded using a ratio master system (www.pti.com) as described previously (Himpens et al., 1990). Data from the photomultiplier and the force transducer were recorded at 1 Hz using the A/D digitizer of the ratio master system.

All chemicals were used as pure as commercially available and purchased from Sigma (www.sigmaaldrich.com). U73122 (1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione), Xestospongin C, *m*-3M3FBS (2,4,6-Trimethyl-N-(*m*-3-trifluoromethylphenyl)benzenesulfonamide) and Fura-2AM were purchased from Calbiochem (www.merckbiosciences.co.uk); NCDC (2-nitro-4-carboxyphenyl-*N*,*N*-diphenyl-carbamate) was obtained from Sigma. Substances were added to the bath solution as single dose or cumulatively to achieve the concentrations as indicated.

Results are presented as original recordings or expressed as means ± S.E.M. Effects of substances were analysed in steady-state conditions. Changes in tension were determined with respect to the maximum of contraction and the baseline. Baseline values were 27.9 ± 1.7 mN ($n = 25$) from wild type mice and 27.1 ± 1.7 mN ($n = 14$) from SMACKO mice. Ca^{2+} signals were calculated as difference between maximum and baseline of the emission ratio at 510 nm during alternate excitation at 340 and 380 nm. Baseline values were 1.03 ± 0.05 ($n = 25$) and 1.08 ± 0.08 ($n = 14$) for wild type and SMACKO mice, respectively. Statistical comparisons of data sets were performed by a paired Student's *t*-test using Prism 4 software (www.graphpad.com). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Carbachol-induced Ca^{2+} signals and contractions in wild type mice

Simultaneous measurements of intracellular Ca^{2+} signals and tension were performed in translucent detrusor muscle strips from mice. The muscarinic agonist carbachol (10 μM) elicited Ca^{2+} signals (Δratio with 0.14 ± 0.02; $n = 25$) and simultaneously induced contractions (Δtension with 111 ± 9 mN; $n = 25$) in intact muscle strips (Fig. 1A, left). The contractions elicited by carbachol (10 μM) were not significantly influenced by the PLC inhibitors U73122 at 10 μM (Wegener et al., 2004) and NCDC (100 μM; 88 ± 5% of control; $n = 4$) or by the proposed IP_3 receptor antagonist Xestospongin C (10 μM; 95 ± 5% of control; $n = 4$). The Ca^{2+} channel blocker isradipine (3 μM) reduced the effects of carbachol on Ca^{2+} signals and tension (Fig. 1) confirming that Ca^{2+} channels contribute to carbachol-induced contractions and Ca^{2+} signals in urinary bladder of wild type mice.

In the presence of isradipine, carbachol still elicited Ca^{2+} signals and contractions (Fig. 1). The PLC inhibitor U73122 was used to further characterize these effects. In the presence of both isradipine (3 μM) and U73122 (10 μM), the effects of carbachol on Ca^{2+} signals and on contractions were inhibited (Fig. 1). These results suggest that, in the presence of isradipine, carbachol-induced Ca^{2+} signals and contractions are mediated by PLC/IP_3 signalling.

3.2. Carbachol-induced Ca^{2+} signals and contractions in SMACKO mice

Next, we investigated the effects of U73122 on carbachol-induced contractions and Ca^{2+} signals using SMACKO mice that exhibit a smooth muscle selective inactivation of the $Ca_v1.2$ channel gene (Wegener et al., 2004). Carbachol induced Ca^{2+} signals (Δratio with 0.06 ± 0.01; $n = 14$) and increased simultaneously contractions (Δtension with 9 ± 2 mN; $n = 14$) in detrusor muscle strips from SMACKO mice (Fig. 2A, left). In the presence of U73122 (10 μM), carbachol-induced Ca^{2+} signals and carbachol-induced contractions were inhibited (Fig. 2). The results strengthen the view that, in absence of L-type Ca^{2+} channel activity, the carbachol-induced Ca^{2+} signals are mediated by PLC/IP_3 signalling.

4. Discussion

It is well accepted that hormone-induced stimulation of smooth muscle induces contraction by PLC/IP_3-dependent release of Ca^{2+} from intracellular stores (Somlyo and Himpens, 1989). In urinary bladder muscle, Ca^{2+} signals corresponding to IP_3-dependent Ca^{2+} release from intracellular stores have been observed mainly in isolated myocytes. For example, Ca^{2+} release was induced by IP_3 in permeabilzed myocytes (Chambers et al., 1996; Zhao and Muallem, 1990). In addition, Ca^{2+} signals corresponding to IP_3 receptor-mediated Ca^{2+} release were observed after flash-photolysis of caged Ca^{2+} in single myocytes (Ji et al., 2006). However, studies on intact detrusor muscle preparations report no effects of PLC inhibitors on contractions in response to muscarinic stimulation (Frazier et al., 2007). In addition, most studies reporting IP_3-dependent Ca^{2+} signals did not report a concomitant contraction in their single cell preparations (Chambers et al., 1996; Ganitkevich and Isenberg, 1992; Ji et al., 2006), except for Imaizumi et al. (1998). The present study shows that carbachol elicited

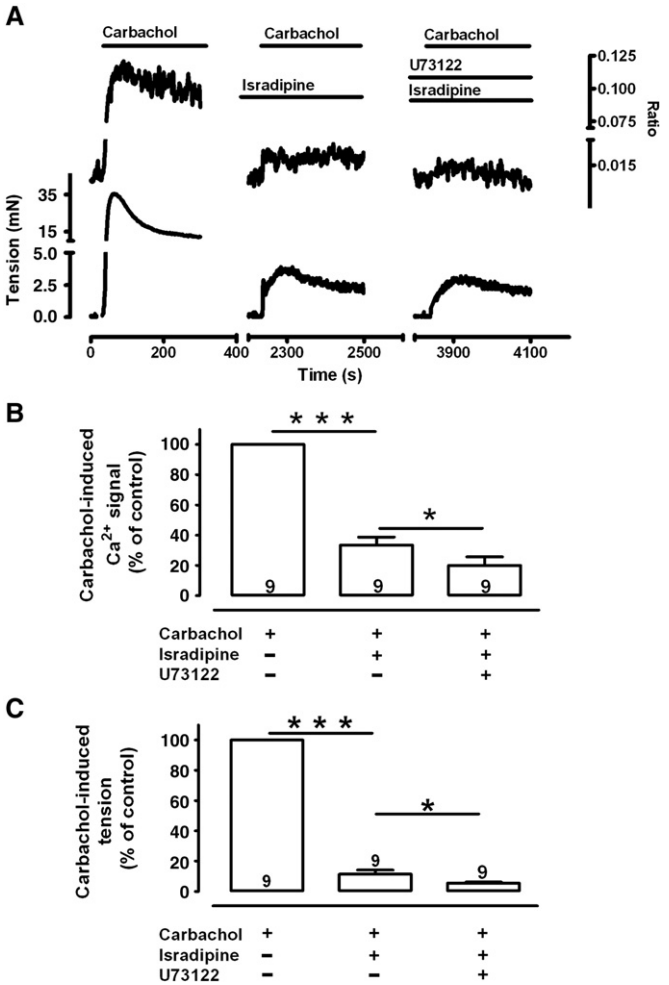

Fig. 1. Effect of isradipine and U73122 on carbachol-induced contractions and Ca^{2+} signals in bladder smooth muscle strips from wild type mice. (A) Simultaneous recordings of tension (lower traces, left axis) and Ca^{2+} signals (upper traces, right axis) in strips from wild type mice. Lines indicate the presence of carbachol (10 μM), isradipine (3 μM) or U73122 (10 μM). Gaps in the recordings were used to wash out the previously added carbachol. (B, C) Carbachol-induced Ca^{2+} signals (B) and contractions (C) in strips from wild type mice. Data were normalized to the magnitude of the Ca^{2+} signal and contraction induced by the first stimulation with carbachol. Bars represent means ± SEM. Numbers in bars represent the number of experiments. Differences were calculated using a paired Student's *t*-test. ***, $P < 0.001$; *, $P < 0.05$.

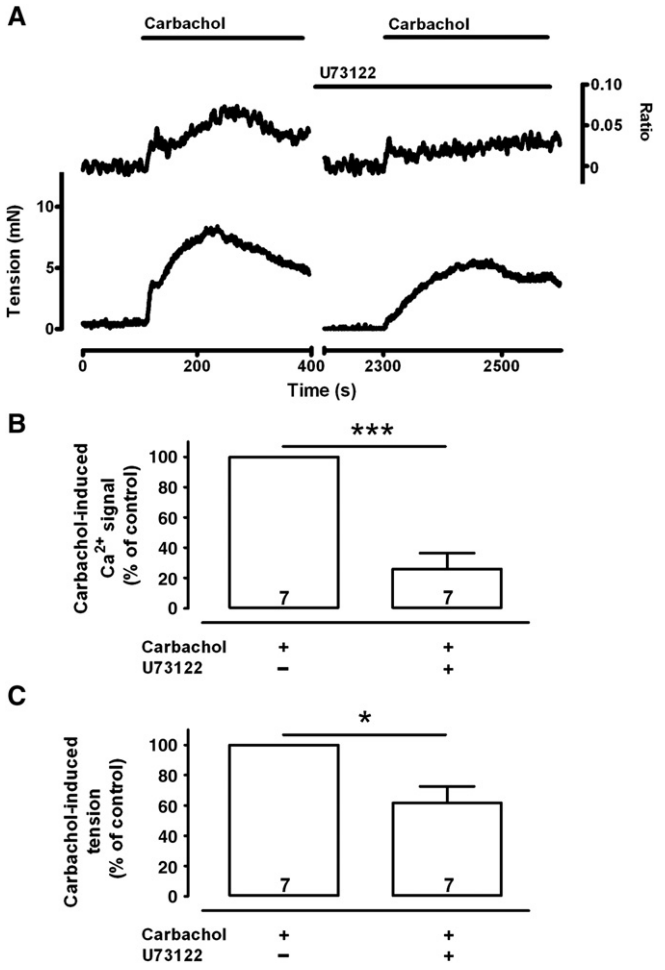

Fig. 2. Effect of U73122 on carbachol-induced contractions and Ca^{2+} signals in bladder smooth muscle strips from SMACKO mice. (A) Simultaneous recordings of tension (lower traces, left axis) and Ca^{2+} signals (upper traces, right axis) in strips from SMACKO mice. Lines indicate the presence of carbachol (10 μM) or U73122 (10 μM). Gaps in the recordings were used to wash out the previously added carbachol. (B, C) Carbachol-induced Ca^{2+} signals (B) and contractions (C) in strips from SMACKO mice. Data were normalized to the magnitude of the Ca^{2+} signal and contraction induced by the first stimulation with carbachol. Bars represent means ± SEM. Numbers in bars represent the number of experiments. Differences were calculated using a paired Student's *t*-test. ***, $P<0.01$; *, $P<0.05$.

PLC/IP_3-dependent Ca^{2+} signals in intact muscle preparations and confirms the results observed in single myocytes. However, these signals were much smaller as compared to the overall Ca^{2+}-signals without inhibiting L-type Ca^{2+} channel activity. Further, the PLC/IP_3-dependent Ca^{2+} signals were associated with tiny contractions as compared to contractions under control conditions. In addition, the proposed activator of PLC, *m*-3M3FBS at 100 μM, did only induce about 1% of the carbachol-mediated contraction (data not shown). Thus, the results indicate that carbachol-induced PLC/IP_3-mediated Ca^{2+} release is sufficient to be detected but seems to account for less than 15% of the muscarinic-mediated Ca^{2+} signal in urinary bladder. It is suggested that there exists a functional dissociation of Ca^{2+} being released and probably controlling STOC activity and Ca^{2+} triggering contraction (Ganitkevich and Isenberg, 1996). The observed muscarinic-induced contraction that was not accompanied by a Ca^{2+} signal, may be due to Ca^{2+} independent contraction mediated by sensitization of the contractile elements (Durlu-Kandilci and Brading, 2006; Somlyo and Somlyo, 2003).

The results presented cannot exclude a participation of Ca^{2+}-induced Ca^{2+} release (CICR) on the muscarinic-induced Ca^{2+} signals. However, evidence against this participation comes from findings showing that emptying intracellular Ca^{2+} stores with SERCA inhibitors did not influence phasic muscarinic contraction in detrusor muscle (Munro and Wendt, 1994; Wegener et al., 2004). In addition, ryanodine did only slightly reduce carbachol-induced contraction in detrusor muscle from mice (data not shown, (Wuest et al., 2007)). Furthermore, deficiency of ryanodine receptor type 2 which is the major form mediating CICR in urinary bladder smooth muscle (Ji et al., 2004), did not influence the contractile response to acetylcholine (Hotta et al., 2007).

In summary, the results show the presence of muscarinic-induced Ca^{2+} signals elicited by both Ca^{2+} channel-mediated Ca^{2+} entry and by PLC/IP_3-mediated Ca^{2+} release in intact urinary bladder muscles from mice. However, PLC/IP_3-mediated Ca^{2+} release after muscarinic receptor stimulation does only account for less than 15% of the overall induced Ca^{2+} signal and Ca^{2+}-dependent contraction in murine urinary bladder smooth muscle.

Acknowledgement

The present study was supported by the Deutsche Forschungsgemeinschaft.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2009.03.036.

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