Bradykinin contracts rat urinary bladder largely independent of phospholipase C*

Carsten Sand, Martin C. Michel

Dept. of Pharmacology, University of Duisburg-Essen, Essen, Germany (CS)
Dept. of Pharmacology, Johannes Gutenberg University, Mainz, Germany (MCM)
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Address for correspondence:
Prof. Martin C. Michel
Dept. of Pharmacology
Johannes Gutenberg Universität
Obere Zahlbacher Str. 67
55101 Mainz, Germany
phone +49-6132-7799000
fax +49-6132-7299000
mail marmiche@uni-mainz.de

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List of non-standard abbreviations:
PL, phospholipase
Several receptor systems in the bladder causing detrusor smooth muscle contraction stimulate phospholipase C (PLC). PLC inhibition abolishes bladder contraction via P2Y₆ but not that via M₃ muscarinic receptors, indicating a receptor-dependent role of PLC. Therefore, we have explored the role of PLC in rat bladder contraction by bradykinin. The PLC inhibitor U 73,122 did not significantly affect the bradykinin response to a greater degree than its inactive analogue U 73,343 (10 µM each), whereas the phospholipase D inhibitor butan-1-ol relative to its inactive control butan-2-ol caused a weak but significant inhibition (0.3% each). The cytosolic phospholipase A₂ inhibitor arachidonyltrifluoromethyl ketone (300 µM) and the cyclooxygenase inhibitor indomethacin (10 µM) caused strong inhibition of the bradykinin response. The L-type Ca²⁺-channel blocker nifedipine (10-100 nM) concentration-dependently caused strong inhibition whereas only a small but significant inhibition was seen with SK&F 96,365 (10 µM), an inhibitor of receptor-operated Ca²⁺-channels. Several protein kinase C inhibitors yielded an equivocal picture (inhibition by 10 µM bisindolylmaleimide I and 1 µM calphostin but not by 10 µM chelerythrine). The rho kinase inhibitor Y 27,632 (1-10 µM) caused a strong and concentration-dependent inhibition of the bradykinin response. Our data support that not only M₃ but also bradykinin receptors cause bladder contraction by a largely PLC-independent mechanism. Both responses strongly involve L-type Ca²⁺-channels and rho kinase, whereas only the bradykinin response additionally involves the phospholipase A₂/cyclooxygenase pathway.
INTRODUCTION

The main physiological mechanism to induce urinary bladder contraction is the stimulation of muscarinic receptors (Abrams, et al., 2006; Hegde, 2006). Similar to the airways (Michel and Parra, 2008) and uterus (Kitazawa, et al., 2008), the bladder expresses more M2 than M3 receptors, but M3 receptors are the main if not exclusive mediator of physiological contraction (Abrams, et al., 2006; Hegde, 2006). The prototypical signaling pathway of M3 receptors is the activation of a phospholipase (PL) C followed by the mobilization of Ca\textsuperscript{2+} from intracellular stores (Caulfield and Birdsall, 1998). This has also been assumed to be the molecular mechanism underlying bladder contraction (Ouslander, 2004). Indeed, stimulation of M3 muscarinic receptors in the bladder activates PLC (Kories, et al., 2003; Schneider, et al., 2004b). Surprisingly, however, several studies in mice (Wegener, et al., 2004), rats (Schneider, et al., 2004b; Frazier, et al., 2007) and humans (Schneider, et al., 2004a) demonstrate that such activation contributes only little to bladder contraction. Rather L-type voltage-operated Ca\textsuperscript{2+} channels and rho kinase appear to be key mediators of muscarinic receptor-induced bladder contraction (Frazier, et al., 2008). On the other hand, PLC was recently proposed to mediate P2Y6 receptor-mediated contraction of rat detrusor (Yu, et al., 2013).

Bradykinin is also involved in the regulation of bladder function but only gains full relevance in pathophysiological states and hence has been proposed as a target for the treatment of bladder dysfunction (Yoshimura, et al., 2008). This concept has been validated by studies demonstrating that both B1 and B2 receptor antagonists can reduce
bladder overactivity in a rat spinal cord injury model (Forner, et al., 2012). Among the known bradykinin receptor subtypes, the B1 receptor apparently is only poorly expressed in the healthy urinary bladder but becomes more prominent under pathophysiological conditions (Butt, et al., 1995; Bellucci, et al., 2007; Forner, et al., 2012; Ribeiro, et al., 2014). In contrast, B2 receptor expression has been shown at the mRNA (Chopra, et al., 2005) and protein level as detected by radioligand binding (Figueroa, et al., 2001) and immunohistochemistry (Chopra, et al., 2005; Ribeiro, et al., 2014). While recent findings question antibody-based data on receptor expression (Michel, et al., 2009), the presence of B2 receptors in the bladder is also supported by considerable functional data. Thus, similar to muscarinic receptors, bladder bradykinin receptors couple to activation of a PLC (Nakahata and Nakanishi, 1988; Butt, et al., 1995; Bellucci, et al., 2007) and induce bladder contraction in various mammalian species (Nakahata and Nakanishi, 1988; Calixto, 1995; Meini, et al., 2000; Michel and Sand, 2009; Ribeiro, et al., 2014), perhaps partly via receptors located on the urothelium (Ochodnicky, et al., 2013; Ribeiro, et al., 2014). However, in contrast to muscarinic receptors (Frazier, et al., 2008), bradykinin receptors in the bladder strongly activate a PLA₂ and subsequently stimulate prostaglandin formation (Nakahata and Nakanishi, 1988; Nakahata, et al., 1987; Pinna, et al., 1992; Meini, et al., 1998; Bellucci, et al., 2007) as they do in other tissues (Meini, et al., 2012). Moreover, caveolae play different roles in bladder contraction elicited by muscarinic and bradykinin receptors (Cristofaro, et al., 2007). Therefore, the present study was primarily designed to explore whether bradykinin-induced detrusor contraction is PLC-dependent or, as shown for muscarinic receptors, largely PLC-independent; other
signaling pathways potentially involved in bradykinin-induced rat detrusor contraction were studied in comparison.
MATERIAL AND METHODS

Contraction studies

The present study was performed in accordance with the German law on animal protection which is in line with Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Urinary bladder strips were prepared from male Wistar rats (body weight 349 ± 3 g, bladder weight 83.6 ± 1.4 mg, n = 88) obtained from the central animal breeding facility at the University of Essen; urothelium was not removed. Experiments were performed as previously described (Kories, et al., 2003) with minor modifications. Briefly, longitudinal bladder strips (approximately 1 mm diameter, 17 ± 0 mm length, 11.6 ± 0.2 mg weight, n = 321) were mounted under a tension of 10 mN in 10 ml organ baths containing Krebs-Henseleit solution (119 mM NaCl, 25 mM NaHCO3, 4.7 mM KCl, 1.18 mM KH2PO4, 1.17 mM MgSO4, 2.5 mM CaCl2, 0.027 mM EDTA, 5.5 mM glucose and 10 mM HEPES) which were aerated with 95% O2 and 5% CO2 to yield a pH of 7.4 at 37°C. This was supplemented with 3 μM captopril to prevent bradykinin breakdown unless otherwise indicated. After 60 min of equilibration including washes with fresh buffer every 15 min, the bladder strips were challenged three times with 50 mM KCl/100 μM carbachol with 5 min rest and washes between challenges. Following washout and an additional 30 min of equilibration, a cumulative concentration-response curve was constructed for bradykinin in the absence or presence of the indicated inhibitor or its vehicle; only a single bradykinin curve was generated in each preparation and increasing bradykinin
concentrations were added in 10 min intervals. Therefore, bladder strips in the absence and presence of inhibitor were tested in parallel strips from the same rat within a given experiment. Because initial experiments had shown that bradykinin elicited much smaller responses than the muscarinic agonist carbachol, only muscle strips yielding a KCl/carbachol-induced force of contraction of at least 40 mN were included in analysis in order to allow robust quantification of bradykinin effects in the absence and presence of the inhibitors. Of note, some of the rats in the present study had bladder stones but this did not have an obvious effect on bladder strip contractile responses.

Data analysis

Contraction data were analyzed based on peak force amplitude. To reduce inter-experimental data variability, the force amplitude of contraction in response to bradykinin was expressed as % of peak response to the last addition of 50 mM KCl/100 μM carbachol within the same bladder strip, i.e. prior to addition of any inhibitor or vehicle, which was 61 ± 2 mN. Nevertheless, bradykinin responses remained highly variable and, therefore, about twice the number of experiments was performed per condition as compared to our previous studies with muscarinic agonists to obtain robust data. Since bradykinin concentration-response curves were quite shallow and did not reach obvious maximum values in many cases (Figure 1), no curve fitting was performed. Rather, the effect of a given inhibitor was determined by two-way ANOVA testing for overall effect of the presence of inhibitor. In additional post-hoc analyses, inhibitor effects on the response to 10 μM bradykinin were analyzed by one-way ANOVA followed by
Dunnett’s multiple comparison test or by t-tests. A p < 0.05 was considered to be significant. All statistical calculations were performed with the Prism program (version 6.0, Graphpad Software, San Diego, CA).

Chemicals

Bradykinin, its antagonists icatibant (also known as Hoe 140) and [Leu^8, des-Arg^9]-bradykinin acetate, carbachol HCl, nifedipine, SK&F 96,365 (1-[8-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole HCl), U 73,122 (1-(6-[(17B)-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino]hexyl)-1H-pyrrole-2,5-dione) and U 73,343 (1-(6-[(17B)-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino]hexyl)-2,5-pyrrolidinedione) were obtained from Sigma-Aldrich (Taufkirchen, Germany).

AACOCF₃ (arachidonyltrifluoromethyl ketone), bisindolylmaleimide I (also known as GF 109203X or Gö 6850), calphostin C (from Cladosporium cladosporioides), chelerythrine HCl and indomethacin were from Calbiochem (Bad Soden, Germany). Y 27,632 (trans-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide) was from Tocris (Bristol, UK).

Bradykinin, icatibant and [Leu^8, des-Arg^9]-bradykinin were dissolved in water at concentrations of 10, 0.1 and 1 mM, respectively. AACOCF₃ (at 10 mM), bisindolylmaleimide I (at 1 mM), calphostin C (at 1 mM), chelerythrine (at 1 mM) and U 73,122 and U 73,343 (at 3 mM) were dissolved in dimethylsulfoxide. Indomethacin (at 10 mM) and nifedipine (at 1 mM) were dissolved in ethanol. SK&F 96,365 and Y 27,632
(at 10 mM) were dissolved at 1 mM in distilled water. The experiments involving nifedipine were performed in light-shielded organ baths.
RESULTS

In the absence of captopril, bradykinin (1 pM – 10 μM) concentration-dependently contracted rat bladder strips, but the increases in force amplitude of contraction were small compared to those of KCl/carbachol in the same strips and the concentration-response curves were shallow. In the presence of 3 μM captopril bradykinin-induced contraction was enhanced, but the corresponding concentration-response curves remained shallow; moreover, this enhancement was apparently largely due to an effect at lower bradykinin concentrations whereas the response to the highest agonist concentration remained unaffected (Figure 1). Therefore, all further experiments were performed in the presence of captopril. Both the B1 receptor antagonist [Leu⁸,des-Arg⁹]-bradykinin (3 μM) and the B2 receptor antagonist icatibant (1 μM) inhibited bradykinin-induced contraction but the concentration-response curves remained shallow; as expected for competitive receptor antagonists, this inhibition did not involve a significant reduction of the response to 10 μM bradykinin (Figure 1).

Having established the receptor subtypes involved in the bradykinin response, we have explored the role of various signaling pathways, starting with PLC, i.e. the pathway of primary interest in this study. The PLC inhibitor U 73,122 did not significantly affect the bradykinin response at a concentration of 3 μM but caused significant inhibition at a concentration of 10 μM; however, this inhibition was no longer statistically significant when only effects against the highest bradykinin concentration were analyzed (Figure 2). However, its inactive analogue U 73,343 at 10 μM caused similar or even greater
inhibition of bradykinin-induced contraction, and its inhibition was also significant when only effects against 10 µM bradykinin were analyzed (Figure 2), indicating that the effect of U 73,122 cannot be interpreted as being due to PLC inhibition. The PLD inhibitor butan-1-ol relative to its inactive control butan-2-ol (0.3% each) significantly attenuated the bradykinin responses, but this inhibition did not reach statistical significance when only responses to the highest bradykinin concentration were analyzed (Figure 2). The cytosolic PLA2 inhibitor AACOCF3 (300 µM) markedly inhibited bradykinin-induced bladder contraction (Figure 3); the effect of AACOCF3 was mimicked by the cyclooxygenase inhibitor indomethacin (10 µM; Figure 3). Both effects remained statistically significant when only inhibition of the response to 10 µM bradykinin were analyzed.

Other than phospholipases, Ca2+-channels are known to be important in regulating smooth muscle tone. The L-type Ca2+-channel blocker, nifedipine (10-100 nM) concentration-dependently caused strong inhibition of bradykinin-induced bladder contraction yielding an almost complete abolishment at the highest concentration (Figure 4). In contrast, SK&F 96,365 (10 µM), an inhibitor of receptor-operated Ca2+-channels, only slightly but significantly attenuated the bradykinin-induced bladder contraction (Figure 4). Neither Ca2+-channel inhibitor significantly affected the response to 10 µM bradykinin.

Several protein kinases are potentially involved in the second step of signal transduction involved in the control of smooth muscle tone. However, three protein kinase C inhibitors
did not yield a conclusive picture. Thus, strong inhibition was seen with 10 μM bisindolylmaleimide I, somewhat less with 1 μM calphostin C, whereas 10 μM chelerythrin did not cause significant inhibition; the response to 10 μM bradykinin was only significantly inhibited by bisindolylmaleimide I (Figure 5). The rho kinase inhibitor Y 27,632 (1-10 μM) caused concentration-dependent inhibition of the bradykinin response yielding an almost complete inhibition at the highest concentration; this inhibition was also significant for the 3 and 10 μM concentration when only effects against the highest bradykinin concentration were analyzed (Figure 5).
DISCUSSION

Based on an ongoing discussion on the role of PLC in mediating bladder contraction, we have investigated this and other signaling pathways in bradykinin-stimulated contraction of isolated rat bladder strips.

Critique of methods

Studies in guinea pigs (Calixto, 1995), rabbits (Nakahata, et al., 1987) and pigs (Ribeiro, et al., 2014) have reported strong bradykinin-induced detrusor or bladder neck contraction. In contrast, other studies in rabbits (Butt, et al., 1995), in humans (Meini, et al., 2000;Sjuve, et al., 2000) and in healthy rats (Meini, et al., 1998;Meini, et al., 2000;Sjuve, et al., 2000;Chopra, et al., 2005;Michel and Sand, 2009) have reported that bradykinin-induced detrusor contraction is only weak, as confirmed in the present experiments. However, bradykinin-induced bladder contraction increases markedly under conditions of tissue stress such as extended periods in an organ bath (Butt, et al., 1995;Sjuve, et al., 2000), cyclophosphamide-induced cystitis (Meini, et al., 1998;Lecci, et al., 1999;Chopra, et al., 2005) or diabetes (Pinna, et al., 1992;Cardozo, et al., 2002), indicating a stronger pathophysiological than physiological role of bradykinin in the bladder. Such enhancement appears to largely reflect an increased B1 receptor-mediated contraction, which involves up-regulation of B1 receptor mRNA and protein expression (Butt, et al., 1995;Meini, et al., 1998;Lecci, et al., 1999;Chopra, et al., 2005;Forner, et al., 2012). The weak and variable contraction universally observed in healthy rat detrusor
poses a technical challenge, and the resulting limitations in data interpretation should be considered. This also is the reason why inhibitor effects were tested against the overall bradykinin response and not against its isolated B1 and/or B2 components. Thus, our study provides information on signaling in response to a physiological/pathophysiologial agonist but does not allow conclusions specific for one of the two receptor subtypes.

Bradykinin-induced bladder contraction in our and most (Pinna, et al., 1992; Meini, et al., 1998; Sjuve, et al., 2000; Forner, et al., 2012) but not all previous studies (Meini, et al., 2000; Kubota, et al., 2003; Ribeiro, et al., 2014) was characterized by shallow concentration-response curves not allowing meaningful estimates of maximum response or agonist potency. Inhibition of bradykinin metabolism by captopril enhanced bradykinin-induced contraction in the present and previous studies (Butt, et al., 1995; Sjuve, et al., 2000) and, therefore, was included in all further experiments. Nevertheless, the bradykinin concentration-response curve remained shallow in the present and some previous studies (Meini, et al., 1998; Sjuve, et al., 2000). Therefore, the effects of inhibitors had to be determined by two-way ANOVA testing for overall treatment effects. Moreover, we have used considerably more experiments per condition as compared to our studies with muscarinic agonists to maintain a robust analysis in the face of weaker contractile responses.

Bradykinin receptors are expressed not only in the smooth muscle but also in the urothelium (Chopra, et al., 2005) (Ochodnicky, et al., 2013) (Ribeiro, et al., 2014) but the
present study did not discriminate between bradykinin receptors in smooth muscle and urothelium.

Role of receptor subtypes and signaling pathways

The present and all previous in vitro and in vivo studies demonstrate that B2 receptor antagonists such as icatibant can inhibit bradykinin-induced bladder contraction in rats (Meini, et al., 2000), rabbits (Butt, et al., 1995), pigs (Ribeiro, et al., 2014) and humans (Meini, et al., 2000). Accordingly, bladder contractions can also be elicited by B2-selective agonists (Meini, et al., 1998). Although the shallow concentration-response curves in the present study did not allow formal calculations of antagonist potency, the magnitude of shift by icatibant appears to be in the same order as reported by others (Meini, et al., 2000). In contrast, the effect of B1-selective agonists (Butt, et al., 1995; Meini, et al., 1998; Lecci, et al., 1999; Sjuve, et al., 2000; Chopra, et al., 2005; Forner, et al., 2012) or antagonists (Butt, et al., 1995; Lecci, et al., 1999; Forner, et al., 2012; Ribeiro, et al., 2014) showed a limited role in the healthy but a more prominent one in the diseased bladder. In the present study the B1 antagonist [Leu^8,des-Arg^9]-bradykinin produced some inhibition of bradykinin-induced bladder contraction, indicating that both B1 and B2 receptors are involved in the bradykinin response under our experimental conditions, possibly reflecting the presence of bladder stones in some of our rats. In vivo studies in a rat spinal cord injury model reported that both B1 and B2 receptors are involved in the detrusor overactivity of this model (Forner, et al., 2012).
Hence, our data relate to bradykinin in the bladder in general rather than to a specific subtype of bradykinin receptors.

To test our main research question, i.e. the involvement of PLC in bradykinin-induced bladder contraction, we have used U 73,122 which effectively inhibits PLC in the bladder (Schneider, et al., 2004b) and had proven superior to others inhibitors (Frazier, et al., 2007). Nevertheless, it can also have effects unrelated to PLC (Altmann, et al., 2003) including inhibition of calcium influx (Wang, 1996). Therefore, we have used its analog U 73,343, which does not inhibit PLC, to control for non-specific effects. U 73,122 inhibited bradykinin-induced bladder contraction only weakly in a concentration where it completely inhibited PLC activation in rat bladder in a previous study (Schneider, et al., 2004b). Moreover, its inactive analogue U 73,343 caused at least similar inhibition of bladder contraction. A possible reason for the shared moderate inhibition by the PLC-active U 73,122 and the PLC-inactive U 73,343 could be their effect on L-type Ca\(^{2+}\) channels (Macrez-Lepretre, et al., 1996; Wang, 1996). Therefore, these data demonstrate that not only M\(_3\) muscarinic but also bradykinin receptors cause bladder contraction in a largely PLC-independent manner. Of note the recent data on PLC-involvement in P2Y6 receptor-mediated bladder contraction relied on a high concentration of U 73,122 (50 \(\mu\)M) and did not include a negative control (Yu, et al., 2013). Work in guinea pig trachea also supports the idea that bradykinin-induced smooth muscle contraction may occur independent of PLC (Schlemper, et al., 2005). Therefore, additional experiments were designed to explore which other signaling pathways may be involved in bradykinin-induced bladder contraction.
The PLD inhibitor butan-1-ol relative to its inactive control butan-2-ol had a roughly similar effect of butan-1-ol against bradykinin as against M₃ receptor-mediated contraction of rat and human bladder (Schneider, et al., 2004b; Schneider, et al., 2004a), indicating that PLD plays a quantitatively similar role for both receptor systems. Based upon the strong role of rho kinase in bradykinin-induced bladder contraction (see below) and the finding that rho kinase can mediate bradykinin-induced PLD stimulation in other cell types (Meacci, et al., 1999), we speculate that the role of PLD in the bladder may occur secondary to a rho kinase activation.

Bradykinin activates PLA₂ and prostaglandin formation in the urinary bladder of rats and rabbits (Nakahata and Nakanishi, 1988; Nakahata, et al., 1987; Pinna, et al., 1992; Meini, et al., 1998) and cyclooxygenase inhibitors such as indomethacin attenuate bradykinin-induced bladder contraction in rats (Pinna, et al., 1992; Meini, et al., 1998; Kubota, et al., 2003), rabbits (Nakahata, et al., 1987; Nakahata and Nakanishi, 1988) and humans (Sjuve, et al., 2000) as confirmed in the present study. In contrast to all of these studies, indomethacin did not inhibit but rather enhance bradykinin-induced contraction in porcine bladder neck (Ribeiro, et al., 2014). On the other hand, studies with PLA₂ inhibitors have yielded less consistent results. In one study the cytosolic PLA₂ inhibitor AACOCF₃ did not affect bradykinin responses in rat bladder (Kubota, et al., 2003), while our study found a strong inhibition by AACOCF₃. Our findings are in line with the inhibitory effect of the PLA₂ inhibitor mepacrine in the rabbit bladder (Nakahata, et al., 1987). Studies with bromoenol lactone, an inhibitor of Ca²⁺-independent PLA₂, did not
report inhibition in rat bladder (Kubota, et al., 2003), and the lipoxygenase inhibitor caffeic acid had no effect in rabbits (Nakahata, et al., 1987). The sensitivity to inhibitors of cyclooxygenase, and perhaps, PLA$_2$, differentiates bradykinin-induced from muscarinic receptor-mediated bladder contraction in rats and humans (Schneider, et al., 2004b;Schneider, et al., 2004a).

Muscarinic and bradykinin receptor stimulation elevates intracellular Ca$^{2+}$ concentrations in the urinary bladder (Nakahata and Nakanishi, 1988;Hashitani, et al., 2004;Ohtake, et al., 2004;Chopra, et al., 2005). Dihydropyridine-sensitive, voltage-operated Ca$^{2+}$-channels play a major role in muscarinic receptor-mediated bladder contraction (Wegener, et al., 2004;Schneider, et al., 2004b;Schneider, et al., 2004a). Bradykinin-induced guinea pig bladder contraction is fully abolished by Ca$^{2+}$-free medium and strongly inhibited by nicardipine (Calixto, 1995); similar data were recently reported for porcine bladder neck using Ca$^{2+}$-free medium or nifedipine (Ribeiro, et al., 2014). We extend these findings to the rat by demonstrating a strong inhibition of bradykinin-induced bladder contraction by nifedipine. In contrast, SK&F 96,365, an inhibitor of receptor-operated Ca$^{2+}$-channels, caused only much less inhibition in rat detrusor, which is in agreement with our previous findings with muscarinic receptors (Schneider, et al., 2004b;Schneider, et al., 2004a), but strong inhibition in porcine bladder neck (Ribeiro, et al., 2014).

With regard to second messenger-activated protein kinases protein kinase C inhibitors are notorious for their frequent non-specific effects (Davies, et al., 2000;Davis, et al., 2011).
Accordingly, previous studies with muscarinic receptors in rat or human bladder
(Fleichman, et al., 2004; Schneider, et al., 2004a) or bradykinin in guinea pig bladder
(Calixto, 1995) have reported inconsistent inhibitor effects. Therefore, we have tested
three different inhibitors in effective concentrations with the pre-defined understanding
that only consistent inhibition by all three inhibitors would provide convincing evidence
for a role of this enzyme. Inhibition of bradykinin-induced rat bladder contraction was
observed by some (bisindolylmaleimide I, calphostin) but not by other inhibitors
(chelerythrin). Interestingly, one of the positive studies in the field, evaluating a role of
protein kinase C in spontaneous and field stimulation-induced contraction of rabbit
bladder, has also been based on bisindolylmaleimide I (Hypolite, et al., 2013), indicating
that there may be something specific to this inhibitor which may or may not be related to
protein kinase C inhibition. Thus, the overall data remain inconclusive with regard to a
role of protein kinase C in bladder contraction, with differences in species and contractile
stimulus potentially contributing to the inconclusive data. With regard to bradykinin in
rat bladder, the present data do not support a role of protein kinase C.

Rho kinase plays an important role in muscarinic receptor-mediated bladder contraction
(Frazier, et al., 2008; Peters, et al., 2006). In the present study the rho-kinase inhibitor Y
27,632 almost completely abolished bradykinin-induced bladder contraction, an
observation which is consistent with all available studies on muscarinic receptors in the
detrusor of several species, although a negative study has recently been reported for
Taken together our data demonstrate that bradykinin-induced bladder contraction shares some but not all signaling pathways with M₃ receptors for causing bladder contraction (Figure 6). Thus, both responses appear to occur largely independent of PLC, to a limited extent involve PLD and receptor-operated Ca²⁺-channels but exhibit a major role for L-type Ca²⁺-channels and rho kinase. It can be speculated that these shared signaling pathways may represent drug targets in an attempt to inhibit bladder overactivity covering more than one mediator system. In contrast to muscarinic receptors, bradykinin receptors cause part of their bladder contraction via cyclooxygenase and, perhaps, cytosolic PLA₂. Thus, multiple PLC-coupled receptors can cause bladder contraction largely independent of this phospholipase. The role of bradykinin receptors in control of bladder function in vivo may be even more complex, as they are also expressed in the urothelium where they mediate release of mediators important for bladder function including ATP and nerve growth factor (Ochodnicky, et al., 2013; Ochodnicky, et al., 2012).
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Author contributions:

Participated in research design: Michel, Sand

Conducted experiments: Sand

Contributed new reagents or analytical tools: not applicable

Performed data analysis: Michel, Sand

Wrote or contributed to the writing of the manuscript: Michel, Sand
Reference List


Macrez-Lepretre N, Morel J-L and Mironneau J (1996) Effects of phospholipase C inhibitors on Ca^{2+} channel stimulation and Ca^{2+} release from intracellular stores evoked by α_{1A}- and α_{2A}-adrenoceptors in rat portal vein myocytes. Biochem Biophys Res Commun 218:30-34.


Wang JP (1996) U-73122, an aminosteroid phospholipase C inhibitor, may also block Ca$^{2+}$ influx through phospholipase C-independent mechanism in neutrophil activation. *Naunyn-Schmiedeberg's Arch Pharmacol* 353:599-605.


Footnotes:

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Address for correspondence:

Prof. Martin C. Michel
Dept. of Pharmacology
Johannes Gutenberg Universität
Obere Zahlbacher Str. 67
55101 Mainz, Germany
phone +49-6132-7799000
fax +49-6132-7299000
mail marmiche@uni-mainz.de
LEGENDS TO THE FIGURES

Figure 1: Bradykinin-induced bladder contraction. Upper panel: Representative tracing of bradykinin concentration-response curve in the presence of captopril. Middle panel: Effects of captopril (3 μM). Lower panel: Effects of the B1 receptor antagonist [Leu⁸,des-Arg⁹]-bradykinin (3 μM; des-Arg) and the B2 receptor antagonist icatibant (1 μM). Data are means ± SEM of 4-8 experiments. In a two-way ANOVA the overall treatment effect of all three inhibitors was significant at the p < 0.01 level. However, the response to 10 μM bradykinin was not significantly affected by any of the inhibitors in a one-way ANOVA.

Figure 2: Bradykinin-induced bladder contraction. Upper panel: Effects the phospholipase C inhibitor U 73,122 and its inactive analogue U 73,343. Lower panel: Effects the phospholipase D inhibitor butan-1-ol relative to its inactive analogue butan-2-ol (0.3% each). Data are means ± SEM of 8-13 experiments. In a two-way ANOVA the overall treatment effect of 10 μM U 73,122 and U 73,343 (but not that of 3 μM U 73,122) vs. their vehicle DMSO and of butan-1-ol vs. butan-2-ol was significant at the level of p < 0.01. The response to 10 μM bradykinin was inhibited by U 73,343 (p < 0.01 in a one-way ANOVA) but not by either U 73,122 concentration or by butan-1-ol as compared to butan-2-ol.

Figure 3: Bradykinin-induced bladder contraction. Upper panel: Effects of the cytosolic phospholipase A₂ inhibitor AACOCF₃ (300 μM). Lower panel: Effects of the
cyclooxygenase inhibitor indomethacin (10 μM). Data are means ± SEM of 6-10 experiments. In a two-way ANOVA the overall treatment effect of AACOCF₃ and indomethacin vs. their vehicles DMSO and ethanol, respectively, was significant at the p < 0.01 level. The response to 10 μM bradykinin was also significantly reduced by both inhibitors (p < 0.01 in a t-test).

Figure 4: Bradykinin-induced bladder contraction. Upper panel: Effects of the voltage-operated Ca²⁺-channel inhibitor nifedipine (10-100 nM). Lower panel: Effects of the receptor-operated Ca²⁺-channel inhibitor SK&F 96,365 (10 μM). Data are means ± SEM of 9-10 experiments. In a two-way ANOVA the overall treatment effects nifedipine (all concentrations) and SK&F 96,365 vs. their vehicles DMSO and H₂O, respectively, was significant at the p < 0.01 level (except for 10 nM nifedipine). However, the response to 10 μM bradykinin was not significantly inhibited by any nifedipine concentration (one-way ANOVA) or by SK&F 96,365 (t-test).

Figure 5: Bradykinin-induced bladder contraction. Upper panel: Effects of the protein kinase C inhibitors chelerythrin (10 μM), calphostin C (1 μM) and bisindolylmaleimide I (10 μM). Lower panel: Effects of the rho-kinase inhibitor Y 27,632 (1-10 μM). Data are means ± SEM of 6-11 experiments. In a two-way ANOVA the overall treatment effect of calphostin C and bisindolylmaleimide vs. their vehicle DMSO and of 3 and 10 μM Y 27,632 vs. its vehicle H₂O was significant at the level of p < 0.01. When only inhibition of the response to 10 μM bradykinin was analyzed in a one-way ANOVA, effects were significant only for bisindolylmaleimide I and 3 and 10 μM Y 27,632 (p < 0.01).
Figure 6: Schematic drawing of signaling pathways involved in contractile responses to bradykinin in rat detrusor smooth muscle. Solid and dashed lines represent activating pathways which are considered proven and hypothetical, respectively; of note, the relationship between phospholipase D and rho kinase depicted here is very speculative as data in other models suggest the opposite, i.e. phospholipase D activation by rho kinase. The overall contribution to the contractile response appears strong for involvement of cyclooxygenase, L-type voltage-gated Ca\(^{2+}\) channels and rho kinase and weaker for phospholipase D and receptor-operated Ca\(^{2+}\) channels. The role of phospholipase C and protein kinase C needs to be defined.
Figure 1

Graph showing the effect of captopril and control on the contractile force of KCl/carbachol in response to bradykinin. The graph plots the percentage change in contractile force against the logarithm of the bradykinin concentration. Captopril treatment enhances the contractile response compared to control and other treatments.

Graph showing the effect of icatibant and des-Arg on the contractile force of KCl/carbachol in response to bradykinin. Icatibant and des-Arg also enhance the contractile response compared to control.
Figure 3

Δ contractile force % of KCl/carcabachol

[bradykinin], log M

○ vehicle
■ AACOCF₃

Δ contractile force % of KCl/carcabachol

[bradykinin], log M

○ vehicle
■ indomethacin
Figure 4

The graphs depict the effect of bradykinin on contractile force in the presence of different concentrations of nifedipine and SK&F 96,365.

- **Top graph:**
  - **Vehicle**
  - **10 nM nifedipine**
  - **30 nM nifedipine**
  - **100 nM nifedipine**

- **Bottom graph:**
  - **Vehicle**
  - **SK&F 96,365**

The contractile force is expressed as a percentage of KCl/carbachol. The x-axis represents the concentration of bradykinin in log M, while the y-axis shows the percentage change in contractile force.
Figure 6

Bradykinin → B1/B2 receptor → Phospholipase C → Protein kinase C → Ca^{2+} channel
Phospholipase D → Cyclooxygenase
Phospholipase A_2 → Rho kinase → contraction