Bradykinin Contracts Rat Urinary Bladder Largely Independently of Phospholipase C

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ABSTRACT

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 explored the role of PLC in rat bladder contraction by bradykinin. The PLC inhibitor U 73,122 [1-(6-[[17β]-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl)-1H-pyrrole-2,5-dione] did not affect the bradykinin response to a significantly greater degree than its inactive analog U 73,343 [10 μM each; 1-(6-[[17β]-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl)-1H-pyrrole-2,5-pyrrolidinedione], whereas the phospholipase D inhibitor butan-2-ol relative to its inactive control butan-2-ol caused a weak but significant inhibition (0.3% each). The cystolic 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 SKF 96,365 [10 μM; 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole HCl], 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; trans-4-[[1R]-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide] 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 physiologic mechanism to induce urinary bladder contraction is the stimulation of muscarinic receptors (Abrams et al., 2006; Hegde, 2006). Similarly 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 physiologic 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 Ca2+ from intracellular stores (Caulfield and Birdsaal, 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 Ca2+ channels and rho kinase appear to be key mediators of muscarinic receptor–induced bladder contraction (Frazier et al., 2008). On the other hand, it was recently proposed that PLC mediates 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 levels, 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, not only muscarinic but also bradykinin receptors in the bladder couple to activation of a PLC (Nakahata and Nakanishi, 1988; Butt et al., 1995; Bellucci et al., 2007) and thereby 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 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 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 μM. Nevertheless, bradykinin responses remained highly variable and, therefore, about twice the number of experiments was performed per condition as compared with 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 (Fig. 1), no curve fitting was performed. Rather, the effect of a given inhibitor was determined by two-way analysis of variance (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

![Fig. 1. Bradykinin-induced bladder contraction.](image-url)
were performed with the Prism program (version 6.0; GraphPad Software, LaJolla, CA).

**Chemicals.** Bradykinin, its antagonists icatibant (also known as Hoe 140) and [Leu<sub>8</sub>,des-Arg<sub>9</sub>]-bradykinin acetate, carbachol HCl, nifedipine, SK&F 96,365 [1-β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole HCl], U 73,122 [1-β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole HCl], U 73,343 [1-β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole HCl], aPLD inhibitor butan-2-ol (0.3% each) significantly attenuated the bradykinin-induced bladder contraction (Fig. 3); the effect of AACOCF<sub>3</sub> was mimicked by the cyclooxygenase inhibitor indomethacin (10 μM; Fig. 3). Both effects remained statistically significant when only inhibition of the response to 10 μM bradykinin was analyzed.

Other than phospholipases, Ca<sup>2+</sup> channels are known to be important in regulating smooth muscle tone. The L-type Ca<sup>2+</sup> 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 (Fig. 4). In contrast, SK&F 96,365 (10 μM), an inhibitor of receptor-operated Ca<sup>2+</sup> channels, only slightly but significantly attenuated the bradykinin-induced bladder contraction (Fig. 4). Neither Ca<sup>2+</sup> 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 (Fig. 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 concentrations when only effects against the highest bradykinin concentration were analyzed (Fig. 5).

Discussion

Based on an ongoing discussion on the role of PLC in mediating bladder contraction, we 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, 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 physiologic role of bradykinin in the bladder. Such enhancement appears to largely reflect an increased B1 receptor-mediated contraction, which involves upregulation 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 physiologic/pathophysiological agonist but does not allow conclusions specific for one of the two receptor subtypes.

Bradykinin-induced bladder contraction in our and most previous studies (Pinna et al., 1992; Meini et al., 1998; Sjuve et al., 2000; Forner et al., 2012), but not all (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 used considerably more experiments per condition as compared with 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 used U 73,122, which effectively inhibits PLC in the bladder (Schneider et al., 2004b) and had proven superior to other inhibitors (Frazier et al., 2007). Nevertheless, it can also have effects unrelated to PLC (Altman et al., 2003), including inhibition of calcium influx (Wang, 1996). Therefore, we used its analog U 73,343, which does not inhibit PLC, to control for nonspecific effects. U 73,122 inhibited bradykinin-induced bladder contraction only weakly in a concentration at which it completely inhibited PLC activation in rat bladder in a previous study (Schneider et al., 2004b). Moreover, its inactive analog 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 μM) 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 independently 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 M_3 receptor-mediated contraction of rat and human bladder (Schneider et al., 2004a,b), indicating that PLD plays a quantitatively similar role for both receptor systems. Based on 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 PLA_2 and prostanoid formation in the urinary bladder of rats and rabbits (Nakahata et al., 1987; Nakahata and Nakaniishi, 1988; Pinna et al., 1992; Meini et al., 1998), and cyclooxygenase inhibitors such as indomethacin attenuates bradykinin-induced bladder contraction in rats (Pinna et al., 1992; Meini et al., 1998; Kubota et al., 2003),

Fig. 5. Bradykinin-induced bladder contraction. (Top) Effects of the protein kinase C inhibitors chelerythin (10 μM), calphostin C (1 μM), and bisindolylmaleimide I (10 μM). (Bottom) Effects of the rho-kinase inhibitor Y 27,632 (1 μM). Data are means ± S.E.M. of 6–11 experiments. In a two-way ANOVA the overall treatment effect of calphostin C and bisindolylmaleimide versus their vehicle DMSO and of 3 and 10 μM Y 27,632 versus its vehicle H_2O 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).
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 enhanced bradykinin-induced contraction in porcine bladder neck (Ribeiro et al., 2014). On the other hand, studies with PLAr inhibitors have yielded less consistent results. In one study, the cytosolic PLAr inhibitor AACOCF3 did not affect bradykinin responses in rat bladder (Kubota et al., 2003), whereas our study found a strong inhibition by AACOCF3. Our findings are in line with the inhibitory effect of the PLAr inhibitor mapacrine in the rabbit bladder (Nakahata et al., 1987). Studies with bromoeno lactone, an inhibitor of Ca2+-independent PLAr, 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 PLAr may differentiate bradykinin-induced from muscarinic receptor-mediated bladder contraction in rats and humans (Schneider et al., 2004a,b).

Muscarinic and bradykinin receptor stimulation elevates intracellular Ca2+ 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 Ca2+ channels play a major role in muscarinic receptor-mediated bladder contraction (Wegener et al., 2004; Schneider et al., 2004a,b). Bradykinin-induced guinea pig bladder contraction is fully abolished by Ca2+-free medium and strongly inhibited by nicardipine (Calixto, 1995); similar data were recently reported for porcine bladder neck using Ca2+-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 Ca2+ channels, caused only much less inhibition in rat detrusor, which is in agreement with our previous findings with muscarinic receptors (Schneider et al., 2004a,b), 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 nonspecific effects (Davies et al., 2000, 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 tested three different inhibitors in effective concentrations with the predefined 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 that 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 stimuli 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 (Peters et al., 2006; Frazier et al., 2008). In the present study the rho-kinase inhibitor Y 27,632 almost completely abolished bradykinin-induced bladder contraction, an observation that is consistent with all available studies on muscarinic receptors in the detrusor of several species, although a negative study has recently been reported for porcine bladder neck (Ribeiro et al., 2014). Taken together our data demonstrate that bradykinin-induced bladder contraction shares some but not all signaling pathways with M3 receptors for causing bladder contraction (Fig. 6). Thus, both responses appear to occur largely independently of PLC, involve to a limited extent 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 PLAr.

![Fig. 6. Schematic of signaling pathways involved in contractile responses to bradykinin in rat detrusor smooth muscle. Solid and dashed lines represent activating pathways 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 Ca2+ channels and rho kinase and weaker for phospholipase D and receptor-operated Ca2+ channels. The role of phospholipase C and protein kinase C needs to be defined.](image-url)
Thus, multiple PLC-coupled receptors can cause bladder contraction largely independently 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 (Ochdnicki et al., 2012, 2013).

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Authorship Contributions

Participated in research design: Sand, Michel.
Conducted experiments: Sand
Performed data analysis: Sand, Michel.
Wrote or contributed to the writing of the manuscript: Sand, Michel.

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