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Release and inhibitory effects of prostaglandin D_2 in guinea pig urinary bladder and the role of urothelium

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ABSTRACT

Background: While studying a urothelium-derived inhibitory factor in guinea pig urinary bladders we observed considerable release of prostanoids, including PGD_2-like activity. The present study was carried out to identify the prostanoids and to study their roles in modulating guinea pig urinary bladder motility.
Methods: Release of PGE_2 and PGD_2 in isolated guinea pig urinary bladder preparations was analyzed by high performance liquid chromatography (HPLC) combined with bioassay on bladder strips. Isolated urothelium-intact (UI) or -denuded (UD) bladder strips were subjected to electrical field stimulation (EFS) and applications of PGE_2 and PGD_2.
Results: A resting release of 95 ± 9 (n = 5) ng g tissue^{-1} h^{-1} PGE_2-like activity and 210 ± 34 (n = 4) ng g tissue^{-1} h^{-1} PGD_2-like activity was found, where PGD_2-like was subject to marked spontaneous inactivation during isolation. Prostanoids release was decreased by 70–90% by the cyclo-oxygenase inhibitor diclofenac in UI preparations. Urothelium removal decreased prostanoids release by more than 90%. PGE_2 increased basal tone and spontaneous contractions, whereas PGD_2 had little or no effect on these. Exogenous PGE_2 enhanced and PGD_2 inhibited contractile responses to EFS, exogenous acetylcholine- and ATP, whereas PGD_2 caused marked dose-dependent inhibition. PGE_2 and PGD_2 effects were more pronounced in diclofenac-treated UD tissues.
Conclusions: PGD_2 and PGE_2 are released from guinea pig bladder urothelium and PGD_2 has inhibitory effects on bladder motility, mainly through a postjunctional action on smooth muscle responsiveness.
General significance: The release and inhibitory effects merit further studies in relation to normal biological function as well as overactive bladder syndrome.

1. Introduction

Overactive bladder (OAB) syndrome is a clinical condition with symptoms such as urgency to void, urge incontinence, usually with frequency and nocturia [1]. Poor understanding of the underlying mechanisms and incomplete knowledge of bladder motility regulation is one reason behind ineffective treatment and management [24]. The urothelium serves as a barrier to separate underlying tissues from urine but it has come into focus also as a source of stimulatory and inhibitory mediators that could modulate bladder motility [8,10]. Bladder contractile motility has been found to be activated by two major neurotransmitters, acetylcholine and ATP, released from autonomic nerves in the bladder smooth muscle where the ATP-mediated part makes out the atropine-resistant component [8,11,25,54]. In addition neuropeptides from sensory nerves and ATP released from the urothelium can stimulate the bladder to contract [47,48]. The urothelium has also been shown to decrease the action of the contractile neuropeptide substance P [34] and this has led to investigations into whether the urothelium might release a relaxant or inhibitory factor. A seminal paper showed the existence of a diffusible urothelium-derived inhibitory factor (UDIF) in pig urinary bladder by using sandwich-type bioassay experiments [26]. Urothelium-dependent inhibitory influences, suggestedly due to the release of UDIF, were also observed in mouse urinary bladder by using electrophysiology [38] and quantitative analysis of contractile responses in human urinary bladder [12]. In addition to this, studies using co-axial close-proximity bioassay have demonstrated the release of a non-urothelium dependent relaxing factor in rat urinary bladder [9,21,22]. Thus, when considering regulation of bladder motility both excitatory and inhibitory as well as relaxing factors have to be considered as mediators.

Disturbed release of mediators within the urinary bladder wall including the urothelium is among candidate mechanisms in OAB. Prostanoids make up one such group that has roles in modulating the

Abbreviations: ACh, acetylcholine; EFS, electrical field stimulation; HPLC, high performance liquid chromatography; OAB, overactive bladder syndrome; UD, urothelium-denuded; UI, urothelium-intact; UDIF, urothelium-derived inhibitory factor

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detrusor motility [42]. It is well established that these prostanoids are produced locally within the bladder smooth muscle and mucosa in the human and other species [3,5,17,29]. Their production increases under various stimuli under pathological conditions such as bladder outlet obstruction [35,41], spinal cord injury induced bladder overactivity [36] and inflammation [43,50,51]. The production of prostanoids also responds to physical stretch [18,28] and electrical field stimulation (EFS) of urinary bladders strips in vitro [4].

Although the prostanoid effects differ slightly between different species, prostaglandin E and F series and thromboxanes contract the bladder strips and increase the spontaneous activities during in vitro experiments [2,6,40,49]. In in vivo experiments, urodynamic tests showed an increased detrusor pressure and reduced bladder capacity after intravesical administration of prostaglandin E_2 [27,45,46].

In addition to the release of the prostaglandins E and F series, some release studies also showed significant release of PGD_2 from chopped or minced normal rat urinary bladder [32,52] and from guinea pig urinary bladder after ovalbumin sensitization or dietary fat supplementation [43,52]. Whether PGD_2 also plays a role in regulating bladder muscle contractility has not been reported [42]. In the present experiments, initiated during a chemical search for a urothelium-derived inhibitory factor, we wished to measure the release of biologically active compounds from the urinary bladder and in addition to PGE_2 found a significant PGD_2-like release which was urothelium-derived. We found PGD_2 to have a potent and powerful inhibitory modulatory effect on guinea pig detrusor contractile responses.

2. Methods

2.1. Tissue preparation

The experiments were approved by the Stockholm North local animal ethics committee (Dnr N148/08, N178/11). All animals were used and cared for in accordance with EEC/EU guidelines (Directive 86/609/EEC and Directive 2010/63/EU) on the protection of animals used for scientific purposes. Guinea pigs of either sex weighing 350 to 550 g were anesthetized and exsanguinated. The abdominal aorta was perfused with warm saline 30–40 mL to achieve blood-free tissues. Two kinds of preparations were made. First, for the release experiments, the bladder was spirally cut into 2–3 cm long strips and nylon threads were tied to both ends. Second, for bioassay and pharmacological studies, one single bladder dome was cut into several 8 × 2 mm strips. These strips (6 to 8 from each animal) were tied at both ends with thin cotton threads. In some experiments, the bladder urothelium was removed as much as possible by fine dissection with iris scissors and forceps under the microscope. Preparations were placed in a storage bath for 30 min in Tyrode's solution (136.9 mM NaCl, 4.8 mM KCl, 23.8 mM $NaHCO_3$, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 2.5 mM $CaCl_2$, and 5.5 mM glucose) aerated with 5% CO_2 in O_2.

2.2. Superfusion and collection of the superfusate of the urinary bladder

The superfusion system consisted of a water-jacketed superfusion chamber maintained at 37 °C. The chamber was preceded by a thermostated 2 × 35 cm^3 Sep-Pak t C_{18} cartridge (Waters Inc., Milford, MA, USA) through which aerated (5% CO_2 in O_2) Tyrode's solution was pumped at 1.5 mL min^{-1} by a peristaltic pump and then led onto the urothelium-intact or urothelium-denuded tissue via a suspending nylon ligature. This was connected to an isometric transducer (FT03, Grass Technologies, Warwick, RI, USA), and the initial tension of the bladder was adjusted to 10 mN. The superfusate was for 60 min collected as 15 mL fractions in ice-cold test tubes with acetic acid (2% final) for HPLC analysis as per section 2.3, whereafter tissue wet weight was determined at the end of each experiment. In sets of experiments, diclofenac (10^{-7} M) was added to the superfusing solution in order to inhibit prostanoid production, or the urothelium was removed as described above. In these experiments samples from 3 tissues were pooled for each assay, due to the low amount of bioactive material released.

2.3. Separation and purification of superfusate

2.3.1. Concentration and initial separation by gradient elution (^{1st}HPLC)

The collected cold superfusate was filtered through a Nylon filter (Millex-HN 0.45 μm; Millipore) and directly applied by 7.5 mL repeated injections to a reversed phase HPLC column (ReproSil-Pur C_{18} AQ, 150 mm × 8 mm, particle size 5 μm; Dr Maisch GmbH, Ammerbuch-Entringen, Germany) in a total of 14 injections before each gradient HPLC run. The gradient profile was from water to 50% methanol in 10 min, 50 to 80% methanol in 60 min and 80 to 100% methanol in 10 min with 1% acetic acid throughout. The flow rate was 1.6 mL min^{-1}. The eluate was continuously monitored at 265 nm and 290 nm by two separate UV detectors in series with a scanning diode array UV absorbance detector (Waters 991, monitoring 190–500 nm at 5 s intervals). The eluate from the HPLC was collected in 1.5 min fractions which were dried by overnight vacuum centrifugation and then immediately bioassayed on bladder smooth muscle preparations or analyzed further in a second step of HPLC. Standards of PGE_2 and PGD_2 were analyzed in the HPLC system for the comparison of retention times (see below).

2.3.2. Purification and isolation by isocratic reversed phase chromatography (^{2nd}HPLC)

The second purification step was by reversed phase HPLC in isocratic elution mode, using a Kromasil C_{18} column (Kromasil 100, 150 mm × 2 mm, particle size 5 μm; Dr Maisch GmbH) according to a published method [33]. The mobile phase was 36% acetonitrile and 1% acetic acid at a flow rate of 0.45 mL min^{-1}. UV absorbance was monitored at 290 nm by a single wavelength detector and the scanning Waters diode array detector. Fractions (0.5 min) were freeze-dried overnight and then immediately bioassayed on bladder preparations. For calculations of recovery of prostaglandins in HPLC, external authentic standards of PGE_2 (20 nmol) and PGD_2 (5 nmol) were either injected separately or mixed and applied together into HPLC and subjected to the same procedures as biological samples. Similar recoveries were obtained by the two methods of external standards introduction.

2.4. Bioassay of superfusate fractions from the HPLC

Urothelium-denuded bladder strips were mounted vertically in 3.5 mL organ baths with one end fixed to a hook at the bottom of the bath and the top end connected to an isometric transducer, and initial tension was adjusted to 10 mN. EFS was applied by means of two 1 cm platinum electrodes in the walls of the organ baths (50 V, single pulses of 0.2 ms every 30 s). Evoked contractions were recorded by a computerized acquisition system (MP100, Biopac Systems, Goleta, CA, USA). When stable contractions developed, diclofenac 10^{-6} M was given and after 15 min the tissues were washed and then diclofenac was reapplied and was present throughout experiment. After 1 hour freeze-dried HPLC sample fractions, dissolved in 25–50 μL of distilled water, were applied and then PGE_2 and PGD_2 dose–response curves for calibration were obtained. Bioactivity was quantified by interpolation between the closest prostaglandin standards. In some experiments, differential assay was performed also on guinea pig colon longitudinal strips prepared as previously described [39].

2.5. Investigation of PGE_2 and PGD_2 bioactivities

2.5.1. Effects of PGD_2 and PGE_2 on EFS-induced contractile responses

The same type of preparations was used as in the bioassay experiments, but using both urothelium-denuded and -intact tissues. PGE_2 and PGD_2 were applied to the tissues cumulatively in half-log

increments from 10^{-9} M up to 3×10^{-7} M at 8–10 min intervals. Log concentration–response curves were constructed of the percentage comparison between control contractile responses before agonist and at 8 min of application of each agonist concentration. The prostaglandin applications were either done on naïve preparations or after pretreatment with diclofenac, where a wash was performed 15 min into the treatment, followed by reintroduction of diclofenac, to facilitate endogenous prostaglandin removal.

2.5.2. Effects of PGD_2 and PGE_2 on acetylcholine and ATP induced contractions

Urothelium-denuded similar bladder strips as used before were studied in the absence of EFS after pretreatment with diclofenac (10^{-6} M). When tissues were stable, acetylcholine (3×10^{-6} M) or ATP (10^{-5} M) were applied for 2.5 min and then washed out. The procedure was repeated at 10 min intervals until contractile responses were reproducible. Thereafter PGD_2 (10^{-8} M) or PGE_2 (10^{-8} M) were administered before the next acetylcholine or ATP application, to compare the contractile agonist effect in the absence and presence of PGE_2 and PGD_2, respectively.

2.6. Data analysis and chemicals

All the data are presented as mean ± standard error mean (SEM). Student's *t*-test was used to compare prostaglandin release and to compare effect outcomes. A p-value less than 0.05 was considered significant. EC50, IC50 and maximal effect values were calculated by Prism software (GraphPad Software Inc., La Jolla, CA, USA). Diclofenac, acetylcholine, ATP, tetrodotoxin, acetonitrile, acetic acid and methanol were purchased from Sigma-Aldrich. Prostaglandin E_2 and Prostaglandin D_2 were generous gifts from Professor Ernst Oliw, Uppsala. PGE_2 and PGD_2 stock solutions (10^{-3} M) were dissolved in ethanol and fresh further dilutions were made in water based medium by an initial further at least 10-fold dilution, and final organ bath concentration of ethanol was ≤0.03%, appropriate solvent controls having no effects on bladder strips.

3. Results

3.1. Release of prostaglandin E_2 and D_2

When superfusate was analyzed by reversed phase gradient HPLC a multitude of peaks appeared as determined by UV detection and it soon became clear that these to a large extent emanated from several non-biological sources such as buffer salts (despite these being of highest quality), oxygenation gas and tubings (data not shown). The most effective way to minimize this was the inclusion of a large preadsorbing C_{18} column immediately before the superfused tissue (see Methods), and the resulting relatively quiet background pattern from a run with a sham tissue is shown in Fig. 1 (panel A, lower trace). When tissue superfusates were analyzed by HPLC several additional peaks appeared, and when the HPLC fractions were bioassayed an excitatory activity was observed (Fig. 2). The release of excitatory activity was sensitive to cyclo-oxygenase inhibition, appeared at 48 ± 2 min, and was without any evident accompanying UV absorbing peak. The retention time for the bioactivity coincided with that for prostaglandin E_2, and was later determined to coincide also with prostaglandin D_2 (Fig. 1).

Isocratic separation of major smooth muscle excitatory prostaglandins is feasible in a single run [33]. We therefore subjected excitatory fractions from the gradient elutions to this kind of separation (Fig. 1B). Subsequent bioassay on isolated urothelium-denuded bladder tissues revealed an excitatory activity corresponding to the retention time of authentic PGE_2 (Fig. 2, lower panel, and Fig. 3), and an inhibitory activity corresponding to the retention time of PGD_2 (Fig. 3). PGE_2 recovery after two steps of HPLC, determined by external standard, was 36.5% while

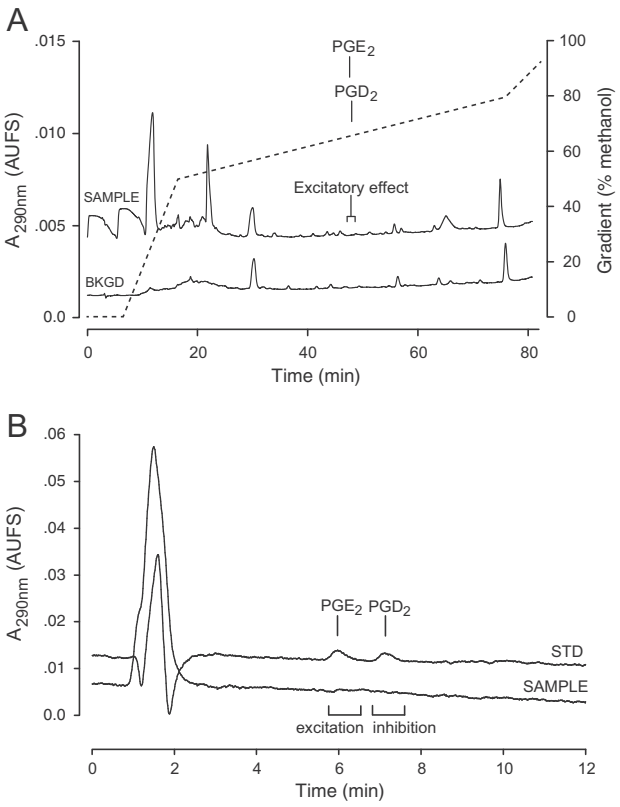

Fig. 1. Isolation and purification of excitatory and inhibitory prostaglandin-like bioactivity from guinea-pig urinary bladder. (A) HPLC chromatograms with absorbance detection at 290 nm showing elution profile of on-column accumulated material applied by repeated direct injections of guinea pig urinary bladder superfusate collected from an urothelium intact urinary bladder (SAMPLE) and elution profile of a run with a sham tissue (BKGD). Indicated are the retention times of standard PGE_2 and PGD_2 around 48 min under the same elution profile. Fractions of the HPLC eluent were bioassayed on contractile responses induced by electrical field stimulation of guinea pig urothelium-denuded detrusor, and excitatory activity was found to elute at 47.8–49.3 min. The stationary phase was a C_{18} column (150 mm × 8 mm, 5 μm Reprosil-Pur C_{18}-AQ). The mobile phase started with A) 1% acetic acid in water to B) methanol with 1% acetic acid at a flow rate of 1.6 mL min^{-1}. The dotted line indicates the gradient used which started at 8 min and was from 0% B to 50% B in 10 min, from 50% B to 80% B in 60 min, 80% B to 100% B in 10 min. (B) Purification and isolation by isocratic reversed phase chromatography of excitatory activity fractions obtained at 48 min from first step HPLC as in panel A (SAMPLE) and compared with standard PGE_2 and PGD_2 (STD) monitored at 290 nm. The stationary phase was a C_{18} column (150 mm × 2 mm, 5 μm Kromasil 100). The mobile phase was 36% acetonitrile and 1% acetic acid at a flow rate of 0.45 mL min^{-1}. Indicated two peaks in curve STD denote the retention times of standard PGE_2 and PGD_2. Excitatory and inhibitory activities, detected by bioassay in guinea pig detrusor, were eluted in the fractions as indicated in the tracing labeled SAMPLE.

the loss of PGD_2 amounted to almost 99%. After adjusting for recovery, the biological productions of PGE_2- and PGD_2-like bioactivity from urothelium-intact bladder tissue were 95 ± 9 ng g $tissue^{-1}$ h^{-1} (n = 5) and 210 ± 34 ng g $tissue^{-1}$ h^{-1} (n = 4), respectively. Removal of the urothelium abolished 95% of PGE_2-like production, reducing it to 4 ng g $tissue^{-1}$ h^{-1}. PGD_2-like bioactivity was by the present method undetectable in superfusates from urothelium-denuded bladder tissue. When superfusates from urothelium-intact bladders pretreated for 1 h with diclofenac (10^{-7} M) were carried through the two HPLC separations and bioassayed, the diclofenac pretreatment abolished more than 68% of PGE_2-like and 94% of PGD_2-like release, yielding 30 ng g $tissue^{-1}$ h^{-1} (n = 3) PGE_2 and 12 ng g $tissue^{-1}$ h^{-1} (n = 3) PGD_2 bioactivity (Fig. 4). The data thus indicated that cyclo-oxygenase in the urothelium was the major source of PGE_2- and PGD_2-like bioactivity, although a full blockade with diclofenac (10^{-7} M) was obviously not obtained. The use of a higher diclofenac concentration (10^{-6} M) resulted in column overload in the first HPLC separation step.

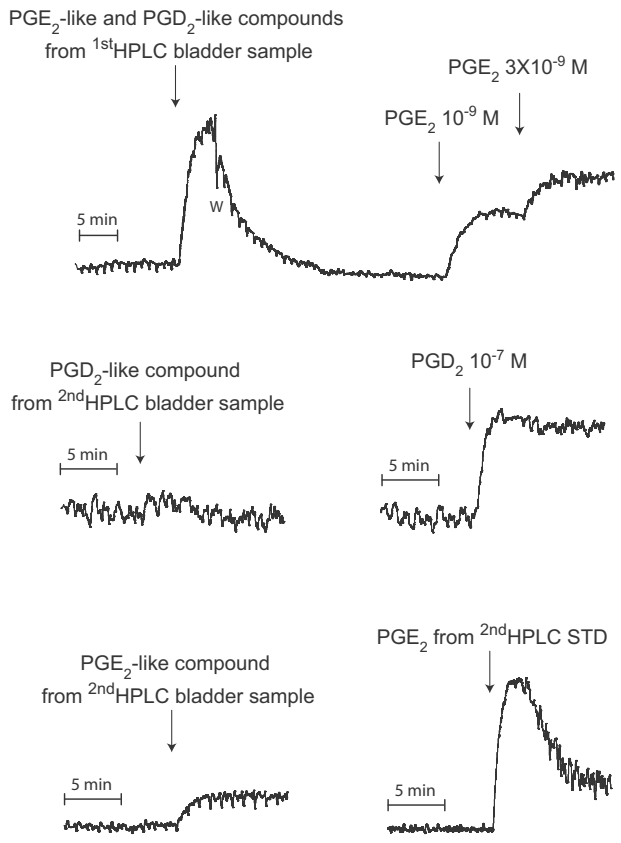

Fig. 2. Guinea pig isolated colon longitudinal muscle preparations. Original experimental recordings showing bioassay of superfusates from guinea pig bladder obtained as fractions from ^{1st}HPLC (gradient elution) and further purified by the subsequent ^{2nd}HPLC (isocratic elution), and for comparison effects of authentic PGE_2 and PGD_2 and external standard PGE_2 (STD) which had been subjected to the same two-step purification. PGE_2, PGD_2, PGE_2-like and PGD_2-like fractions of bladder released material all caused contractile responses on colon muscle strips. W denotes wash.

3.2. Effects of prostaglandin E_2 and D_2 on bladder contractile responses evoked by nerve stimulation

Electrical field stimulation induced regular contractions (Fig. 5) which were nerve-mediated as indicated by their abolishment with tetrodotoxin (5×10^{-7} M, data not shown), whereas concomitant spontaneous activity was unaffected by tetrodotoxin. The responses to EFS were partially cholinergic and 50 ± 8% ($n = 6$; $p < 0.01$) remained after atropine 10^{-6} M. The tissues were studied under 4 different conditions, urothelium-intact and -denuded, and in the absence or presence of the cyclo-oxygenase inhibitor diclofenac. In the urothelium-intact non-pretreated tissues the spontaneous activity was most evident, and the effects of prostaglandins were minor or sometimes even absent (Fig. 5A). In urothelium-denuded tissues the responses to prostaglandins became more evident, and even more so with the combination of urothelium removal and treatment with diclofenac. Thus, in urothelium-denuded tissues, the amplitudes of the contractile responses were reduced by about 25% by diclofenac (10^{-6} M) and spontaneous contractions decreased or ceased, especially if a tissue wash was introduced and diclofenac reapplied (Fig. 5B). Prostaglandin effects already in the nanomolar concentration range now appeared and were more pronounced than before pretreatment (Fig. 5B). Diclofenac 10^{-6} M combined with wash inhibited the contractile responses to EFS by 41 ± 7% in urothelium-intact tissues ($p < 0.01$; $n = 8$) and by 24 ± 6% in urothelium-denuded tissues ($p < 0.01$; $n = 9$). With PGE_2 three major effects were found. First, the EFS induced contraction amplitude was enhanced dose-dependently by PGE_2. Second, the tissue tone was increased and, third, spontaneous contractions appeared or were increased. Under the same conditions PGD_2 markedly inhibited the nerve-induced contractile responses but had no effect on tone or spontaneous contractions (Fig. 5C). Dose–response curves were obtained (Fig. 6A and C) and when EC50 values were calculated the pEC_{50} for PGE_2 in UD + diclofenac treated tissues was 8.0 ± 0.38 ($n = 6$) and for the inhibitory effect by PGD_2 the pIC_{50} was 7.6 ± 0.23 ($n = 8$). At PGE_2 3×10^{-7} M and PGD_2 3×10^{-7} M near-maximal effects were attained (Fig. 6).

An analysis was made for the 4 different conditions of prostaglandin applications by plotting the dose–response data for the different conditions, expressing the data as percent of the EFS-induced contractile response immediately before prostaglandin application (Fig. 6 and Supporting Information Table S2). Although a seemingly left-ward shift of the dose–response curves was obtained in urothelium-denuded diclofenac-treated tissues this was not statistically significant and was mainly a result of an upward shift of the dose–response curves as demonstrated by significantly increased maximal effects (Supporting Information Table S2).

3.3. Effects of prostaglandin E_2 and D_2 on acetylcholine and ATP induced contractions

In diclofenac-pretreated (10^{-6} M) urothelium-denuded bladder strips with EFS turned off, acetylcholine (3×10^{-6} M) caused contractions which were sustained until wash and were reproducible when repeated (Fig. 7). Application of PGE_2 (10^{-8} M) increased the tone and also enhanced the contractile response to exogenous acetylcholine (Fig. 7, upper trace). When PGD_2 (10^{-8} M) was similarly applied there was no effect on basal tone but responses to exogenous acetylcholine were significantly inhibited (Fig. 7, lower trace and Fig. 8A). ATP (10^{-5} M) elicited a fast twitch-contraction in the guinea pig urinary bladders treated with cyclo-oxygenase inhibitor, as reported by others [7]. PGE_2 (10^{-8} M) enhanced the ATP induced contraction while PGD_2 (10^{-8} M) inhibited the ATP-induced contractions, in similarity with their effects on the acetylcholine-induced contractile responses (Fig. 8B).

4. Discussion

The major findings of this study were that a urothelium-dependent release of prostaglandin like material from guinea-pig urinary bladder was found by combining HPLC separation with bioassay, that significant amounts of PGD_2-like activity was found when further purifying an excitatory PGE_2-like bioactivity, and that PGD_2 unexpectedly had potent inhibitory effects on bladder motility measured as effects on contractile responses to EFS. The observed effects of authentic PGD_2 suggest that it exerts its action mainly by a postjunctional inhibitory effect.

A major reason for performing the present studies was to investigate whether an inhibitory factor, UDIF, could be isolated by HPLC from the urinary bladder. The found inhibitory PGD_2-like bioactivity does not constitute the unknown bladder-derived relaxing factor nor UDIF, since these are not blocked by cyclo-oxygenase inhibition [22,26]. However, the developed method for on-column accumulation and gradient HPLC separation of bioactive material should be useful in further search for UDIF. Importantly, the finding in the second HPLC step of an inhibitory PGD_2-like bioactivity hidden within an excitatory PGE_2-like activity as observed in the first gradient HPLC underpins the need to perform bioassays in every step during purification of biologically active principles as in the search for UDIF.

When searching for bioactive principles it is very important to be able to minimize background interference in e.g. UV detection and other sensing methods. We were surprised to find that there were so many sources contributing to this background, including tubes and aeration gas. The solution we found effective, inclusion of a C_{18} in-line column cleaning the superfusion fluid immediately before the releasing

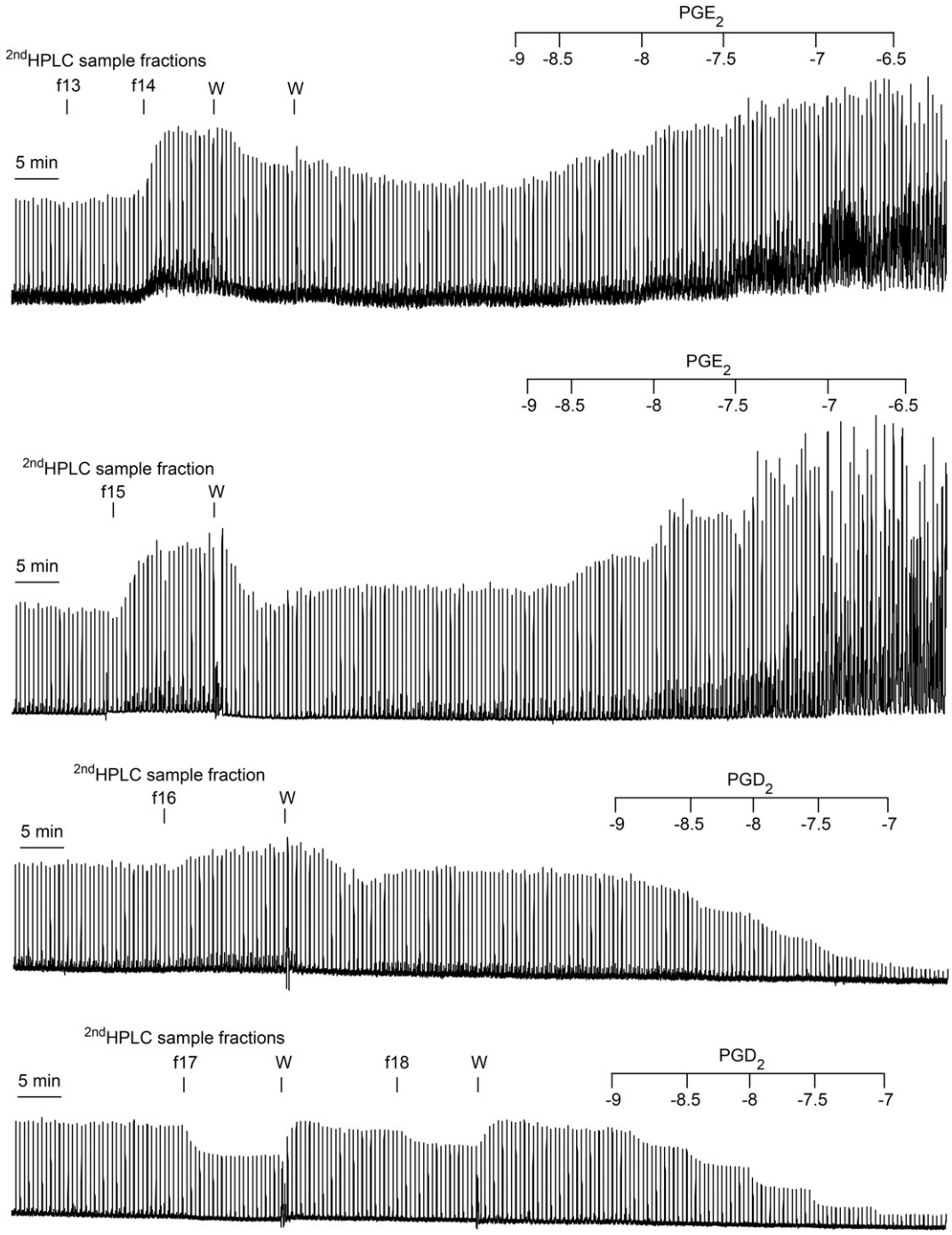

Fig. 3. Original experiments recordings showing bioassay results of guinea pig bladder release fractions after ^{2nd}HPLC performed in parallel on 4 guinea pig urothelium-denuded bladder strips. All the bladder strips were pretreated with diclofenac 10^{-6} M, and were made to contract by electrical field stimulation (EFS). After bioassay of HPLC fractions, PGE_2 or PGD_2 cumulative dose–response curves were obtained on the same tissue. Authentic PGE_2 and HPLC fractions f14, f15, and f16 caused enhancement of EFS-induced contractions in bladder strips while PGD_2 and HPLC fractions f17 and f18 inhibited EFS-induced contractions. W denotes wash.

tissue, we believe might be useful in general in attempts on purification of bioactive compounds from isolated tissues.

The PGE_2- and PGD_2-like bioactivities found to be released from the urinary bladder we find strong reasons to consider identified as PGE_2 and PGD_2. First, they co-eluted in two different HPLC separations with authentic PGE_2 and PGD_2. Second, they had bioactivities on the urinary bladder identical with those of authentic PGE_2 and PGD_2. Third, in differential bioassay on guinea pig ileum and colon they behaved as PGD_2 and PGE_2 (Fig. 2 and Supporting Information Table S1). Fourth, their release was strongly inhibited by cyclo-oxygenase inhibition. Finally, the second isocratic HPLC separation we performed is identical with that previously reported for the two prostaglandins [33] where, at the expected retention times, these prostaglandins were identified by mass-spectrometry.

When quantifying the bladder release of PGE_2-like and PGD_2-like activity it was evident that there was a large loss of PGD_2 during the isolation and separation process. Therefore the exact amount of release of PGD_2-like has to be interpreted with caution although we feel it reasonable to conclude that it was at least comparable to PGE_2 and safe to conclude it was derived from the urothelium (Fig. 4). Although we did not investigate it, one possible explanation for the low recovery of PGD_2 could be its spontaneous conversion by dehydration to PGJ_2 metabolites which have much less smooth muscle activities [23,44].

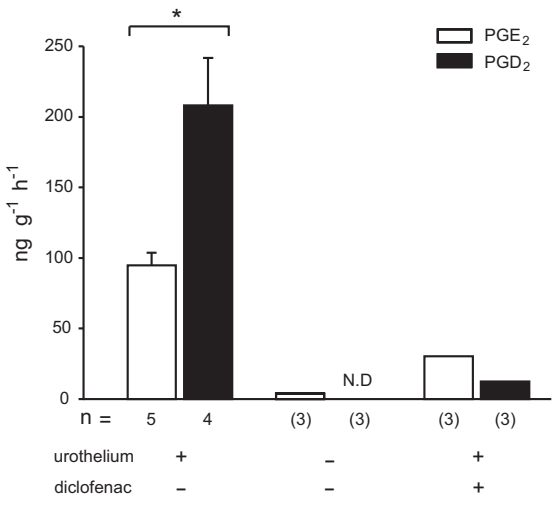

Fig. 4. Quantification of PGE_2- and PGD_2-like material released from urothelium-intact guinea pig urinary bladders determined by bioassay of pooled HPLC fractions with excitatory and inhibitory activity obtained after two-step purification as in Fig. 1 and corrected for recovery of external authentic prostaglandin standards. Denoted below x-axis is the presence and absence of urothelium and/or diclofenac treatment, respectively. Y-axis indicates how many ng PGE_2 or PGD_2 was produced per g guinea pig urinary bladder in 1 h. Data were acquired from three to five animals in each group. * denotes $p < 0.05$ by Student's *t*-test. In order to increase the detection, samples in the urothelium denuded and diclofenac treaded groups were pooled from 3 guinea pig bladders, denoted by numbers within parentheses and therefore no error bar is shown in these two groups. N.D. indicates not detected.

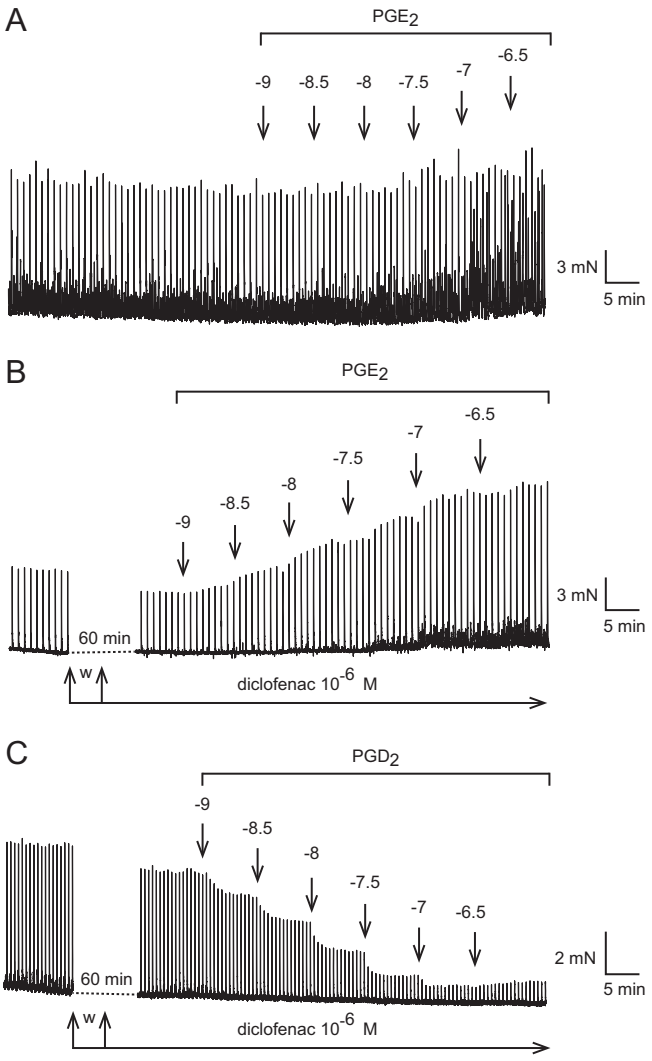

Fig. 5. Original experimental recordings of PGE_2 and PGD_2 effects on isometric contractile responses evoked by electrical field stimulation of guinea pig detrusor strips. Panel (A) shows an urothelium-intact, non-pretreated bladder strip tracing. Panels (B) and (C) are the recordings of urothelium-denuded diclofenac treated bladder strips, where diclofenac 10^{-6} M was applied to the tissues after stable contractions had developed and as indicated by arrows below tracings. 10 min after diclofenac application the tissues were washed and diclofenac 10^{-6} M was reapplied and remained throughout the experiments in panels (B) and (C). Note the inhibitory effect of diclofenac on contractile responses. Arrows above tracings indicate the administrations of PGE_2 and PGD_2 cumulatively in half-log increments from 10^{-9} M up to 3×10^{-7} M.

Release of PGD_2 from urinary bladder tissue has been reported before. Thus, Saban et al. by GC/MS determination found a basal release of 9.8 ng g^{-1} h^{-1} from mucosa and 0.22 ng g^{-1} h^{-1} from detrusor in minced bladder tissues from ovalbumin sensitized guinea pigs, and the release was in parity with the basal release of PGE_2 [43]. A comparable release was by LC/MS found in minced bladders from safflower-supplemented animals and the PGD_2 release was increased by allergen challenge [52]. In incubates of urothelium-denuded rat detrusor muscle PGD_2 release was by radioimmunoassay found at 10–15% of that of PGE_2 and to be stimulated by ATP application [32]. In comparison the present results show a significantly higher release of prostaglandins from intact superfused bladders, suggesting that the release is not due to tissue damage, and that also under these conditions the urothelium is the major source of released prostaglandins with PGD_2 being comparable to PGE_2. It is in agreement with a marked localization of cyclo-oxygenase 1 to the urothelium [17] and merits further investigations on the physiological conditions favoring PGD_2 release. Stretch and several autacoids have been shown to stimulate E and F prostaglandins release as well as thromboxane release in urinary bladders [8] and might be considered also in the regulation of PGD_2 production and release.

A major and novel finding was the inhibitory effects of PGD_2 on contractile responses in the guinea pig bladder. There might be several reasons why this effect has not been reported before. First, the effects of PGD_2 were quite minor when urothelium was present. The basal urothelium release of PGD_2 might desensitize the bladder smooth muscle to low concentrations of exogenously added PGD_2 and the urothelium might offer a penetration block towards the smooth muscle. The urothelium might also release proteins, which are known to enhance the breakdown of PGD_2 also in vitro [20,33,44], and therefore might inactivate low concentrations of added PGD_2. Second, PGD_2 at relatively high concentrations has been reported to contract human bladder detrusor muscle [40]. Therefore a concomitant excitatory effect by PGD_2, if present in the guinea pig bladder, might counteract the inhibitory effect and thereby minimizing it to the extent that it might be overlooked. This should be addressed by future studies.

The enhancing effect by PGE_2 on responses to nerve activation by electrical field stimulation as well as on responses to the exogenous neurotransmitters ATP and acetylcholine are well in agreement with previous studies, suggesting that the mechanism is an enhancing effect on responsiveness to stimulation at the postjunctional effector level [8,11,16]. The enhancing effect has been shown to involve a depolarizing effect at the smooth muscle membrane [16]. Presently, PGD_2 in addition to its inhibitory effect on contractile responses to nerve stimulation also inhibited the responses to the neurotransmitters acetylcholine and ATP. It can therefore be concluded that PGD_2 mainly acts by a postjunctional inhibitory effect. Whether this involves changes in membrane potential or changes in second messengers or both remain to be studied, but PGD_2 has been shown to have both calcium-, cyclic AMP- and phosphoinositide-dependent actions [53]. Additional prejunctional effects by PGE_2 and PGD_2 cannot be excluded from the present results.

Considerable interest has been given to the role of prostaglandins, and especially the excitatory role of PGE_2 and its receptors, in bladder

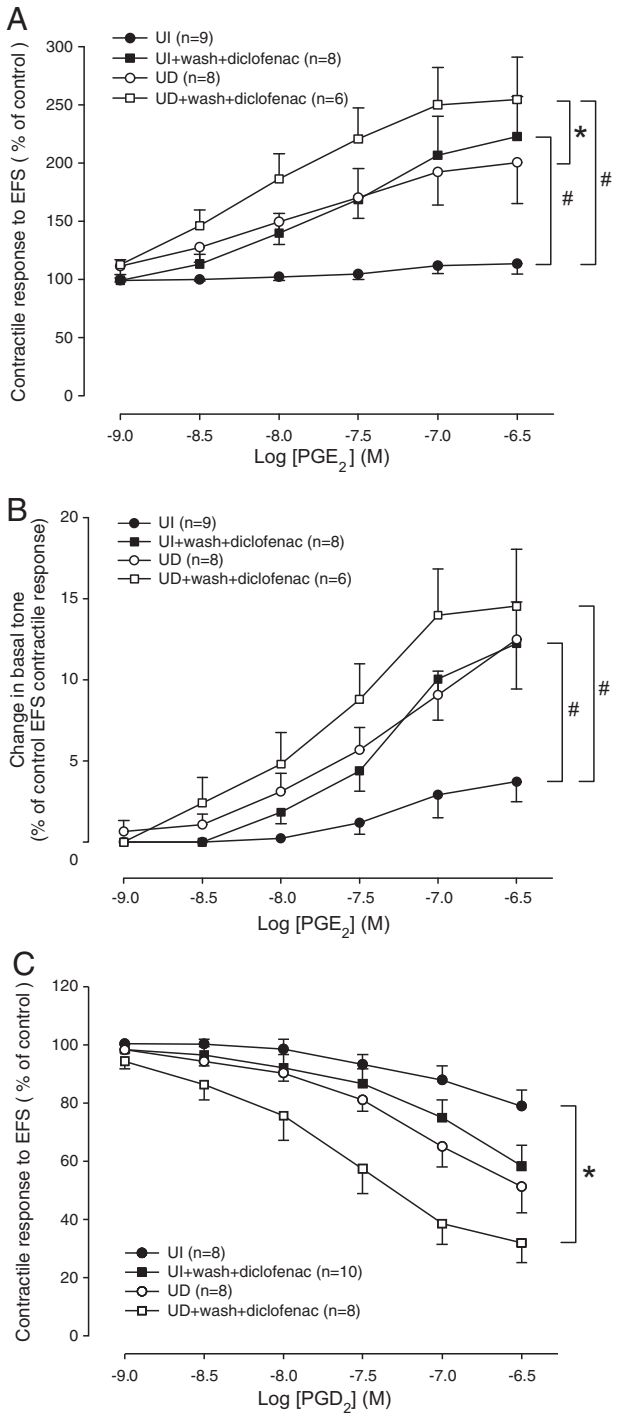

Fig. 6. Guinea pig urinary bladder strips subjected to electrical field stimulation (EFS). Dose–response curves of (A) PGE_2 enhancing effect on contractile responses to EFS, (B) PGE_2 effect on basal tone measured between contractile responses to EFS, and (C) PGD_2 inhibitory effect on contractile responses to EFS. Bladder strips were either urothelium intact (UI) or urothelium denuded (UD) with or without diclofenac treatment (10^{-6} M). Y-axis indicates the percentage changes after PGE_2 and PGD_2 applications compared with 100% control response to EFS induced contractions. Data were collected from 6 to 10 individual strips from at least 4 animals and presented as mean ± SEM. # denotes $p < 0.005$ and * denotes $p < 0.05$ for comparison of maximal response data between the different conditions as indicated by brackets and as further described in Supporting Information Table S2.

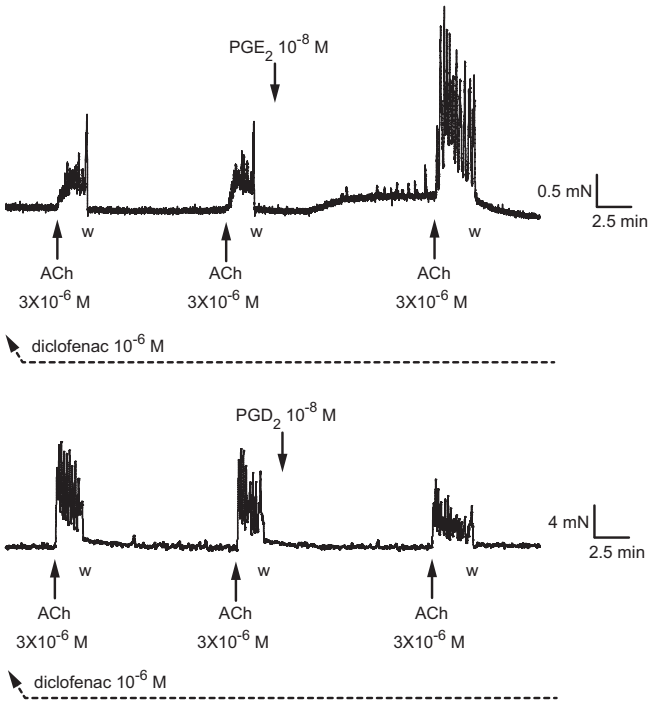

Fig. 7. Urothelium-denuded guinea pig urinary bladder strips monitored by isometric recording of muscle responses in the absence of EFS. Original experimental recordings of enhancing PGE_2 (upper trace) and inhibitory PGD_2 (lower trace) modulation of contractile responses induced by repeated applications of exogenous acetylcholine (ACh) with washings indicated by w. Tissues were pretreated with diclofenac 10^{-6} M, repeated after each wash.

overactivity [5,15,37,42,45]. Blockade of at least one of these receptors the EP_1 receptor, highly implicated in the biology of overactive bladder syndrome, failed to show clinical efficacy [13]. Although inhibition of prostaglandin synthesis has been considered as one possible treatment of overactive bladder syndrome it should be noted that inhibitors of prostaglandin formation have been reported both to modify and not modify contractile responses of urinary bladders [7,11,14,16,19,30,31]. The current findings, that prostaglandin D_2 is released and may have inhibitory effects, perhaps to a variable degree balancing the excitatory effects of prostaglandin E_2, might explain the variable reported effects of cyclo-oxygenase inhibition and should be considered in both studies on normal bladder biology and in overactive bladder syndrome where a decreased formation or release of inhibitory factors ought to be evaluated as an alternative explanation for bladder overactivity. We believe prostaglandin D_2 might be one such compound, deserving further studies concerning both its physiological and possible pathophysiological roles in bladder function.

5. Conclusions

We have found that isolated normal guinea pig urinary bladders release prostaglandin D_2 in significant amounts, comparable to prostaglandin E_2. The release is urothelium-derived and is sensitive to cyclo-oxygenase inhibitor. Prostaglandin D_2 can exert an inhibitory effect on bladder contractile responses by an action mainly on the smooth muscle. The inhibitory effect occurs in the nanomolar concentration range and probably balances the well-known excitatory effect of endogenous prostaglandin E_2 release. Prostaglandin D_2 deserves further investigations considering whether it is a major regulator of bladder function and whether this might have pathophysiological implications in e.g. overactive bladder syndrome.

Acknowledgements

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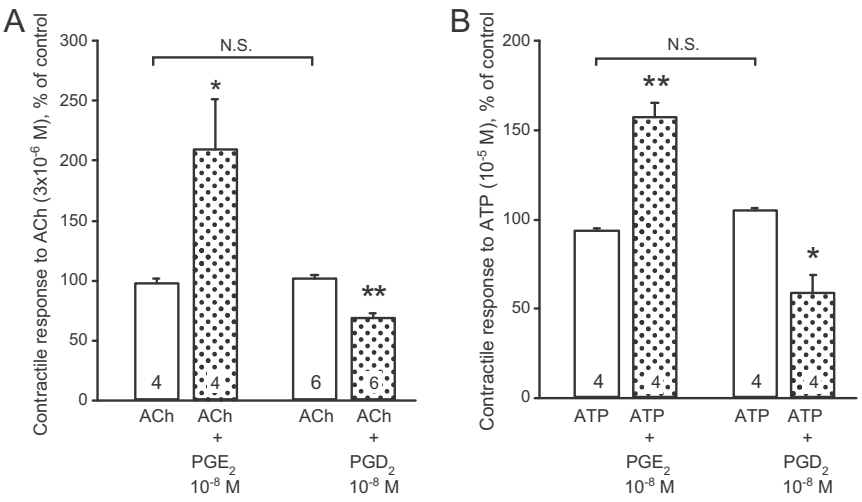

Fig. 8. Summary of PGE_2 enhancing and PGD_2 inhibitory modulation of guinea-pig urothelium-denuded diclofenac treated bladder strip contractile responses induced by applications of exogenous ACh (Panel A) and ATP (Panel B). Data were acquired from four to six animals in each group (indicated within each bar) and presented as mean ± SEM. * and ** denote $p < 0.05$ and $p < 0.01$ by Student's *t*-test. N.S. denotes not significant.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.09.010.

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