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Purines and pyrimidines are not involved in NANC relaxant responses in the rabbit vaginal wall

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1 Non-adrenergic non-cholinergic (NANC) relaxant responses were elicited by electrical field stimulation (EFS) in rabbit vaginal wall strips after treatment with guanethidine and scopolamine and raising smooth muscle tone with phenylephrine. Under these conditions treatment with NOS inhibitors revealed a non-nitrergic NANC relaxant response. The possible role of purines and pyrimidines in these non-nitrergic NANC responses was investigated.

2 Exogenous application of ATP, ADP, adenosine, UTP, or UDP (all at 0.03–10 mM) induced concentration-dependent relaxant responses.

3 Responses to exogenous application of ATP were reduced by the general P2 antagonist cibacron blue (500 μM), but not by suramin (100 μM) and were unaffected by L-NAME (500 μM), ω-conotoxin GVIA (ω-CTX, 500 nM) or tetrodotoxin (TTX, 1 μM).

4 Responses to exogenous application of adenosine were reduced by the A_{2A} antagonist ZM-241385 (30 μM).

5 ATP- and ADP-induced responses were unaffected by the G-protein inhibitor pertussis toxin (100 ng ml^{-1}), whilst ADP- but not ATP-induced responses were reduced by GDPβS (100 μM), which stabilizes G-proteins in their inactive state.

6 EFS-induced non-nitrergic NANC relaxant responses were unaffected by suramin, cibacron blue, ZM-241385, pertussis toxin or GDPβS, but were completely inhibited by TTX.

7 Exogenous application of ATP (10 mM) and adenosine (10 mM) increased intracellular cyclic adenosine-3′, 5′-monophosphate (cAMP). However, non-nitrergic NANC responses were not associated with increased cAMP. Neither non-nitrergic NANC responses nor responses to ATP or adenosine were associated with increased intracellular cyclic guanosine-3′, 5′-monophosphate (cGMP) concentrations.

8 These results suggest that adenosine A_{2A} receptors and P2 receptors are present in the rabbit vaginal wall, but that they are not involved in non-nitrergic NANC relaxant responses.
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Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; cAMP, cyclic adenosine-3′, 5′-monophosphate; cGMP, cyclic guanosine-3′, 5′-monophosphate; CRC, concentration-response curve; ω-CTX, ω-conotoxin GVIA; DETA NONOate, (2,2′-hydroxynitrosohydrazino)*bis*-ethanamine; EFS, electrical field stimulation; GDPβS, guanosine 5′-O-(2-thiodiphosphate); L-NAME, N^G-nitro-L-arginine methyl ester; NANC, non-adrenergic non-cholinergic; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PTX, pertussis toxin; TTX, tetrodotoxin; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; VOCC, voltage-operated calcium channel

Introduction

Relaxation of the vaginal wall smooth muscle leads to increased blood flow into this organ and also enlarges the vaginal canal (Park *et al*., 1997). Like most of the other organs in the urogenital tract, the tone of vaginal smooth muscle is regulated by adrenergic, cholinergic, and non-adrenergic, non-cholinergic (NANC) neurotransmitters. When adrenergic and cholinergic pathways are blocked *in vitro* and the tissue tone is raised, electrical field stimulation (EFS) reveals an inhibitory NANC relaxant response (Gillespie, 1972; Cellek *et al*., 1999).

Nitric oxide (NO) is a well-characterized neurotransmitter in the central and peripheral nervous system. In many tissues of the urogenital system, including those of the anococcygeus, the clitoral corpora cavernosae and the penile corpora cavernosae, NO mediates NANC relaxant responses (Gillespie *et al*., 1989; Li & Rand, 1989; Hobbs & Gibson, 1990; Ignarro *et al*., 1990; Cellek & Moncada, 1997; 1998). These responses are mediated by NO activating soluble guanylate cyclase, causing production of the second messenger cyclic guanosine-3′, 5′-monophosphate (cGMP). We have previously shown that NANC relaxant responses in the vaginal wall are partly due to NO (Ziessen *et al*., 2002). However, the identity of the mediator of non-nitrergic NANC relaxant responses in the vaginal wall remains unknown.

The purines ATP, ADP, and adenosine are involved in signalling processes in neural, vascular and non-vascular smooth muscle preparations (for review see Ralevic &

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Burnstock, 1998), influencing target cells through receptors, originally classified as P1 and P2 purinoceptors (Burnstock, 1978). P1 purinoceptors (also known as adenosine receptors) are specific for adenosine as their natural ligand and have been sub-classified as A_1, A_{2A}, A_{2B}, and A_3 subtypes (Fredholm *et al*., 1997). A_{2A} receptors have been shown to be involved in mediating inhibitory responses in smooth muscle structures *via* stimulation of adenylate cyclase (Haynes, 2000). A_{2B} receptors also stimulate adenylate cyclase, however, the physiological roles for this receptor are currently difficult to elucidate due to a lack of commercially available antagonists, although the synthesis of selective antagonists has been recently reported (Kim *et al*., 2000). A_1 and A_3 receptors are also coupled to adenylate cyclase, but inhibit its activity rather than stimulate it.

ATP acts *via* interaction with purinoceptors, which are either ligand-gated ion channels (P2X-purinoceptors), or G protein-coupled receptors (P2Y-purinoceptors) (Burnstock & Kennedy, 1985). The discovery that pyrimidines as well as purines can act through these receptors (von Kugelgen *et al*., 1987) has led to a change in the nomenclature and these receptors are now known as P2X or P2Y receptors (Fredholm *et al*., 1997). The purpose of our current study was to examine the possibility that the purines ATP, ADP or adenosine or the pyrimidines UTP or UDP may be involved in non-nitrergic NANC relaxations in the vaginal wall.

Methods

Tissue preparation

Female New Zealand white rabbits (3.47 ± 0.04 kg, range 3.0–4.2 kg, $n=49$, Harlan, U.K.) were killed by an overdose of pentobarbitone (Euthatal, Rhône Merieux, U.K.) injected into the ear marginal vein. The vaginal canal including the clitoris was excised down to the pubic bone and transferred to modified Krebs' solution consisting of (mM): NaCl 136.9, KCl 2.7, $MgSO_4$ 0.6, $NaHCO_3$ 11.9, KH_2PO_4 0.5, $CaCl_2$ 1.8, glucose 12.5, dexamethasone 0.01, indomethacin 0.01. The inducible isoform of NOS (iNOS) can be induced by trace amounts of endotoxin in the buffer (Rees *et al*., 1990). Since we wanted to study nNOS in isolation, dexamethasone was added to the Krebs' solution to prevent induction of iNOS. We also added the cyclooxygenase inhibitor indomethacin to prevent synthesis of prostaglandins, since these can cause non-neurogenic relaxations (Daniel *et al*., 1979). The modified Krebs' solution was kept at room temperature and gassed with 95% O_2/5% CO_2. The vaginal canal was carefully opened, the clitoral body removed and four longitudinal strips of vaginal wall (2×8 mm) were dissected free of connective tissue. The ends of the strips were tied with silk suture and mounted horizontally between two platinum electrodes in superfusion chambers continually perfused at 1 ml min^{-1} with modified Krebs' solution at 37°C as described previously (Ziessen *et al*., 2002).

Measurement of responses

One end of the preparation was tied to a Grass FT03C force-displacement transducer connected to a Linearcorder WR3101 (Graphtec, U.K.) for measurement of isometric changes in tension. The mechanical responses were also recorded on a computer running specialized software (Axotape, Axon Instruments, U.S.A.). The preparations were stretched to approximately their *in situ* length by applying tension of 0.4 g and allowed to equilibrate for 90 min without stimulation.

Eliciting NANC relaxant responses

After the equilibration period vaginal wall smooth muscle strips were stimulated with EFS (5 s trains of rectangular pulses of 50 V, 0.3 ms pulse duration, 5 Hz, delivered by Grass S88 stimulators). Sympathetic (noradrenergic) and cholinergic (muscarinic) responses were blocked by addition of guanethidine (10 μM) and scopolamine (10 μM) respectively, and the tissue tone was raised with a sub-maximal concentration of phenylephrine (1 μM), revealing NANC relaxant responses as previously reported (Ziessen *et al*., 2002).

Drugs were introduced either by addition to the reservoir feeding the superfusion chamber or, for ATP, ADP, adenosine, UTP, UDP, DETA NONOate or forskolin, by injection into the perfusate at a rate of 100 μl min^{-1} using a syringe pump (Harvard Apparatus Model '22', U.K.). Four minutes exposure time was used to apply purines and pyrimidines as this was found to evoke maximal relaxations.

Measurement of intracellular cyclic nucleotide concentrations

Modified perfusion chambers in which the tissues were accessible from above (Cellek *et al*., 1996; Ziessen *et al*., 2002) were used in experiments to evaluate intracellular concentrations of cyclic adenosine-3′, 5′-monophosphate (cAMP) or cGMP. The tissues were set up as above and, after incubation with guanethidine, scopolamine and phenylephrine, relaxant responses to EFS or drugs were elicited. After recovery to basal tone the tissues were incubated for 20 min with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 1 mM), which inhibited phenylephrine-induced tone, before being freeze-clamped either under basal conditions or at the time of the peak of their response to EFS or drugs as determined by the time-course of the relaxation elicited prior to incubation with IBMX. The samples were then homogenized in a stainless steel pestle and mortar on dry ice. Each sample was then incubated in 1 ml of 0.5 M perchloric acid for 1 h on ice then sonicated for 5 s at 4°C (18 μm using Soniprep-150). The samples were centrifuged (10 min, $10{,}000 \times$ g, 4°C), and supernatants used for cyclic nucleotide assays and for measuring soluble protein content (BCA protein assay kit, Pierce, U.S.A.). For measurement of intracellular cyclic nucleotide concentrations, 450 μl of the supernatant was neutralized with 300 μl of 1 M K_3PO_4. The sample was centrifuged (8000 g, 5 min, 4°C) and the supernatant was recovered, lyophilized and assayed for cAMP and cGMP content using specific enzyme immunoassay systems (Amersham Pharmacia, U.K.).

Chemicals

ZM-241385 was purchased from Tocris. Suramin, pertussis toxin, DETA NONOate and forskolin were purchased from

Calbiochem, U.K. All other chemicals were from Sigma, U.K. and were dissolved in modified Krebs' solution, with the exception of ω-conotoxin GVIA (1 mM in water), pertussis toxin (50 μg ml^{-1} in water), DETA NONOate (50 mM in water), forskolin (100 mM in DMSO), ZM-241385 (100 mM in DMSO), indomethacin (5 mg ml^{-1} in 5% $NaHCO_3$), dexamethasone (10 mg ml^{-1} in ethanol) and isobutylmethylxanthine (500 mM in DMSO). The hemisulphate salt of adenosine was used to improve solubility in Krebs' solution.

Analysis of the results

Concentration-response curves to agonists: Concentration-response curves (CRCs) were constructed by injecting each concentration of the agonist and comparing the response to an EFS-induced response prior to each injection. The relaxant effect of each concentration was expressed as a percentage of 5 Hz EFS-induced relaxation. The tissue was allowed to recover tone between successive concentrations of agonist.

Effect of antagonists on CRCs: When CRCs were constructed as above, repeated CRCs gave lower maximal responses, therefore in order to examine the effect of antagonists the second CRCs were constructed in the presence or absence of antagonists. In each tissue these CRCs were expressed as a percentage of their initial response to 3 mM agonist.

The effect of antagonists on EFS-induced relaxant responses: Over long periods, loss of phenylephrine-induced tone was observed in the vaginal wall. To enable us to compare responses over these periods, EFS-induced relaxations were measured as a percentage of the tone at the time of the relaxation. To compare responses in the presence of different drugs time-matched control experiments were performed in the absence of drugs.

Calculations of IC_{50} values (the concentrations of purines or pyrimidines that gave responses equal to 50% of the maximum for that agent) for relaxant responses to purines and pyrimidines were performed using Origin software version 6.1 (OriginLab Corporation, U.S.A.). The software used calculates the IC_{50} values from best-fit curves generated using iterative least squares regression using a logistical model with the equation:

$y = ((A_1 - A_2)/((1 + (x/x_0)^p)) + A_2$ where x_0 = center (IC_{50}), p = power, A_1 = initial y value and A_2 – final y value.

Tension changes in tissue strips were measured in mN.

Statistics

Results are expressed as mean values ± standard error of mean from a number (n) of tissue strips from a number (N) of animals. Statistical analyses were performed using Prism v3.0 software (GraphPad Software Inc, U.S.A.). Data were compared as appropriate by Student's unpaired t-test or for comparison of multiple means one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. P values of less than 0.05 were considered significant.

Results

Responses to purines and pyrimidines

After eliciting NANC relaxant responses EFS was terminated and CRCs were constructed for relaxant responses to ATP (0.03 – 10 mM), ADP (0.03 – 10 mM), adenosine (0.03 – 10 mM), UTP (0.03 – 10 mM) or UDP (0.03 – 10 mM). All these purines and pyrimidines caused concentration-dependent relaxant responses (Figure 1A, B). There was a high degree of variability in the responsiveness to the nucleotides and to adenosine. The maximum responses and potency of the different purines and pyrimidines (maximum response was measured as the relaxation induced by the highest concentra-

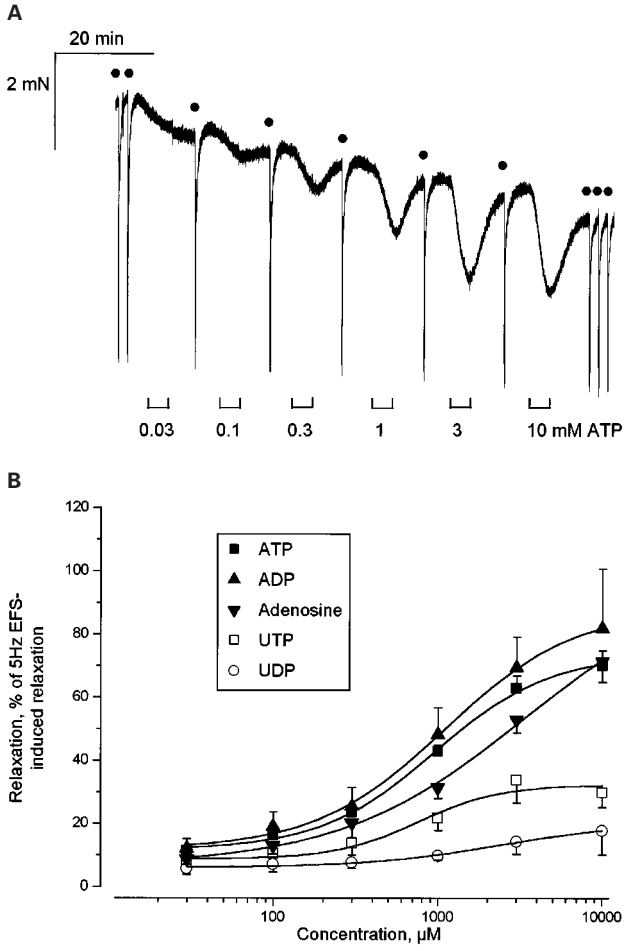

Figure 1 (A) Exogenous application of ATP causes concentration-dependent relaxant responses. The tissue was stimulated by EFS (5 Hz, 50 V, 0.3 ms pulse duration, 5 s train, indicated by dots) between successive applications of ATP. The mechanogram is an original recording of a single tissue preparation and is representative of all the experiments in this series ($n/N = 8/4$). (B) Concentration-response curves showing the relaxant effect of ATP ($n/N = 8/4$), ADP ($n/N = 8/4$), adenosine ($n/N = 10/4$), UTP ($n/N = 8/4$) and UDP ($n/N = 6/4$). Relaxations are expressed as a percentage of 5 Hz EFS-induced relaxations in the absence of nucleotides. Data points represent mean ± s.e.mean.

tion of nucleotide or nucleoside compared to that induced by 5 Hz EFS-induced relaxation and potency was represented by IC_{50} values) are compared in Table 1.

Effect of A_{2A} and P2 antagonists on purine-induced responses The role of A_{2A} receptors was investigated by repeating the CRC to adenosine in the presence of the A_{2A} antagonist ZM-241385 (30 μM). This significantly inhibited relaxant responses to adenosine ($n/N=6/3$, Figure 2C).

To investigate the role of P2 receptors in mediating the relaxant responses to ATP, the CRC to ATP was repeated in the presence of the general P2 antagonists cibacron blue (500 μM) or suramin (100 μM). Cibacron blue significantly inhibited the response to ATP ($n/N=6/4$, Figure 2A and Figure 3) whereas suramin potentiated the response to ATP ($n/N=6/3$, Figure 2B). To assess the effect of the P2 antagonist PPADS 500 μM ATP-induced relaxant responses were induced, then repeated in the presence of 50 μM PPADS. ATP-induced responses were not inhibited in the presence of PPADS with $103.8\pm2.5\%$ of control responses remaining after incubation with the inhibitor ($P>0.05$, $n/N=3/3$).

Effect of P1 and P2 antagonists on NANC relaxant responses: Tissue tone was slightly reduced by cibacron blue with $83.7\pm1.8\%$ of tone prior to incubation with the inhibitor remaining after 35 min ($n/N=5/3$), but EFS-induced NANC relaxant responses were unaffected by cibacron blue ($94.2\pm2.5\%$ of time-matched control, $P>0.05$, $n/N=6/4$, Figure 3) or suramin ($97.6\pm1.6\%$ of time-matched control, $P>0.05$, n/N = 6/3). ZM-241385 also caused a reduction in tissue tone with $75.7\pm2.2\%$ of tone prior to addition of the antagonist remaining after 35 min incubation, however, it had no effect on EFS-induced relaxant responses ($94.0\pm4.4\%$ of time-matched control, $P>0.05$, $n/N=6/3$). Tissue tone was unaffected by PPADS (50 μM). Furthermore EFS-induced relaxant responses were unaffected by PPADS (50 μM), with $94.0\pm1.4\%$ of control responses remaining ($P>0.05$, $n/N=3/3$).

Effect of L-NAME and neurotoxins

Relaxant responses induced by adenosine, ATP, ADP, UTP and UDP were unaffected by treatment with 500 μM L-NAME (Figure 4 for adenosine and ADP and Figure 5). In the presence of L-NAME (500 μM), exogenous application of a single, sub-maximal dose of adenosine (500 μM) caused relaxant responses that were abolished in the presence of ZM-241385 (30 μM, $n/N=4/4$, Figure 6). Relaxant responses induced by 1 mM ATP were unaffected by treatment of vaginal wall strips with a combination of L-NAME (500 μM), ω-CTX (100 nM) and TTX (1 μM) ($n/N=8/4$, not shown).

Effect of G protein inhibitors

After eliciting relaxant responses to ATP or ADP (both 1 mM) vaginal wall strips were incubated with 100 μM GDPβS (which stabilizes G proteins in their inactive state) for 30 min. GDPβS caused a slight decrease in tone, and significantly inhibited relaxant responses to subsequent application of exogenous ADP ($P<0.05$, $n/N=4/4$, Figure 5). Relaxant responses to exogenous application of ATP were not significantly attenuated in the presence of GDPβS ($n/N=4/4$, Figure 5).

Incubation of vaginal wall strips with pertussis toxin (100 ng ml^{-1}) for 60 min, after eliciting an initial relaxant response to ATP or ADP (1 mM), failed to inhibit relaxant responses to subsequent application of ATP or ADP ($n/N=4/4$, Figure 5).

Measurement of changes in intracellular cyclic nucleotide concentrations

Vaginal wall strips were frozen under basal conditions, when stimulated by EFS in the absence and presence of L-NAME (500 μM), in the presence of ATP or adenosine (both at 10 mM), the NO donor DETA NONOate (250 μM) or the adenylate cyclase activator forskolin (10 μM). Measurement of changes in the intracellular concentrations of cyclic nucleotides showed that ATP or adenosine significantly increased concentrations of cAMP, although not to forskolin-induced cAMP levels. EFS did not cause any change in intracellular cAMP concentration. cGMP levels were unaltered except when stimulated by EFS, or in the presence of DETA NONOate. EFS-induced increases in cGMP were completely abolished by L-NAME ($n/N=5/5$ for basal, DETA NONOate and forskolin treatment, $n/N=4/4$ for all others; Figure 7).

Discussion

A normal female sexual response is a multifactorial event consisting of desire, arousal, increased genital lubrication and orgasm (Kaplan, 1974). The physiological changes that occur during the arousal phase include genital vasocongestion leading to vaginal engorgement, increased lubrication and clitoral erection. These physiological processes are all associated with changes in smooth muscle tone. Neurogenic control of smooth muscle tone is mediated by noradrenergic, cholinergic and NANC transmitters. In the clitoral corpus cavernosum, NO is the neurotransmitter responsible for NANC relaxant responses. However, in the smooth muscle of the vaginal wall, NO only accounts for 30% of the NANC

Table 1 Efficacy and potency of purines and pyrimidines in producing relaxation responses in the rabbit vaginal wall

	Concentration range (mM)	*Maximum relaxation* (efficacy, per cent of 5 Hz EFS-induced relaxation ± s.e.)	*Maximum relaxation range* (per cent of 5 Hz EFS)	*Potency, IC_{50} ± s.e.* (mM)	n/N
ATP	0.3–10	70.1 ± 4.9	52.4–83.3	0.95 ± 0.10	8/4
ADP	0.3–10	81.9 ± 19.0	25.2–105.0	1.09 ± 0.15	8/4
Adenosine	0.3–10	71.5 ± 6.5	47.6–100.0	3.34 ± 0.26	10/4
UTP	0.3–10	29.9 ± 4.6	17.6–67.2	0.76 ± 0.31	8/4
UDP	0.3–10	17.8 ± 7.6	6.0–34.6	2.37 ± 0.95	6/4

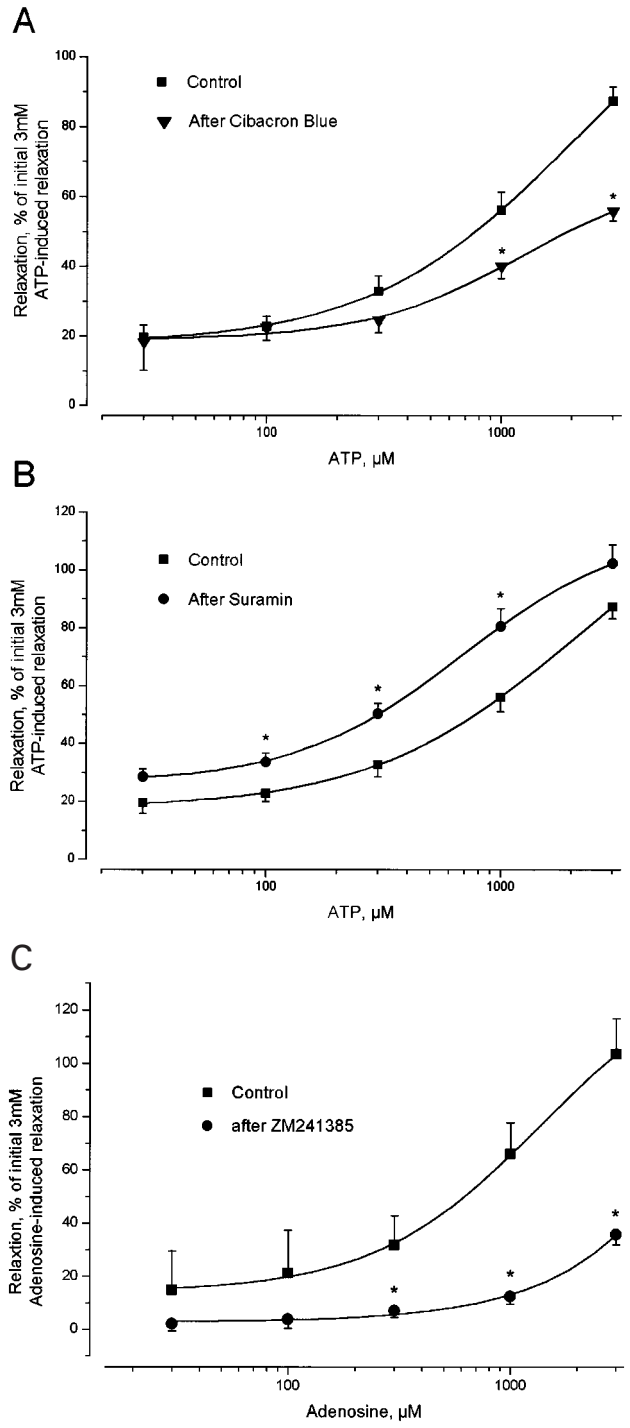

Figure 2 Relaxant responses elicited by exogenous application of ATP were (A) partially inhibited by cibacron blue (500 μM, $n/N=6/4$) but were (B) potentiated by the presence of suramin (100 μM, $n/N=6/3$). (C) Relaxant responses elicited by exogenous application of adenosine were partially inhibited by ZM-241385 (30 μM, $n/N=6/3$). Data points represent mean $\pm$ s.e.mean. *Significantly different from control, $P<0.05$. Statistical analyses performed using two population, unpaired Student's *t*-test.

relaxant responses (Ziessen *et al*., 2002). Other candidates for mediators of NANC neurotransmission are neuropeptides (such as VIP), purines (such as ATP), or pyrimidines (such as UTP). We have previously provided evidence suggesting that the neuropeptides known to be present in the vaginal wall are unlikely to be involved in non-nitrergic NANC relaxant responses (Ziessen *et al*., 2002). Therefore we investigated whether or not purines or pyrimidines were involved in these responses.

In this study we have demonstrated that purines (ATP, ADP and adenosine) and pyrimidines (UTP and, to a lesser extent UDP) are able to induce relaxant responses in vaginal smooth muscle. These responses were not due to an indirect action on intrinsic nerves leading to release of a secondary transmitter causing the smooth muscle to relax since inhibition of neurotransmission with the sodium channel blocker TTX and the N-type voltage-operated calcium channel (VOCC) inhibitor ω-CTX did not affect them.

ATP-induced activation of P2X and P2Y receptors in many smooth muscles has been shown to result in contraction and relaxation respectively (see Ralevic & Burnstock, 1998 for review), which would suggest that P2Y receptors are involved in the responses observed in the current study. However, it has recently been suggested that ATP may induce relaxations in the rat pylorus and ileum strips as well as in the mesenteric arterial bed *via* P2X receptors (Ishiguchi *et al*., 2000; Storr *et al*., 2000; Ralevic, 2001; 2002; Stanford *et al*., 2001). In the rat pylorus and ileum strips ATP-induced relaxant responses are inhibited by the PPADS, which has greater antagonism at P2X than P2Y receptors (Ishiguchi *et al*., 2000; Storr *et al*., 2000), however, in the current study ATP-induced responses were not inhibited by this antagonist. P2X receptors are thought to be principally activated by ATP as their natural ligand, whist P2Y receptors may be activated preferentially by any of the adenosine or uridine nucleotides dependent on the receptor subtype (see Ralevic & Burnstock, 1998 for review); indeed in rat ileum strips ADP, UTP and UDP are without relaxant effect (Storr *et al*., 2000). In the current study ATP was not the only nucleotide to induce relaxant responses with ADP being equally potent and giving equal maximum responses; UTP and UDP also induced relaxant responses but with lower maximal responses achieved. In the rat mesenteric arterial bed the suggested P2X-mediated ATP-induced relaxations are preceded by a contractile response, suggesting that even in this tissue P2X receptors are still involved in contracting the tissue (Ralevic, 2002). However, in the current study ATP-induced relaxations were not preceded by contractile responses, and ATP did not affect the resting tone in the rabbit vaginal wall (unpublished observations). Together these observations strongly suggest that the relaxant effects of the nucleotides are mediated *via* P2Y, rather than P2X receptors.

So far seven mammalian P2Y receptors have been cloned and characterized: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ (Ralevic & Burnstock, 1998; Hollopeter *et al*., 2001; Communi *et al*., 2001). Three other P2Y receptors have been cloned from non-mammalian sources but these may be orthologues of mammalian receptors. The mammalian P2Y receptors have been reported to have a different selectivity for purines and pyrimidines as well as different selectivities for nucleotide di- and triphosphates. $P2Y_1$ receptors are selective for adenine nucleotides, and $P2Y_{11}$ receptors are selective for ATP (Nicholas *et al*., 1996; Communi *et al*., 1997). $P2Y_2$ receptors are activated by both purines and pyrimidines, but are selective for triphosphate nucleotides, and it has been

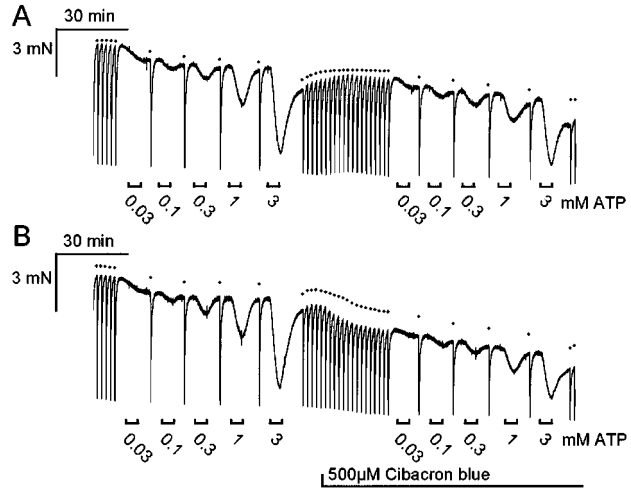

Figure 3 Concentration-dependent relaxant responses to exogenous application of ATP were repeated. The second CRC was obtained in the absence (A) or presence (B) of cibacron blue (500 μM). In the presence of cibacron blue, responses to ATP are reduced when compared to time-matched controls, whilst EFS-induced relaxant responses are unaffected by cibacron blue (500 μM, B). The mechanograms are original recordings of single tissue strips and are representative of all the experiments in this series ($n/N=6/4$).

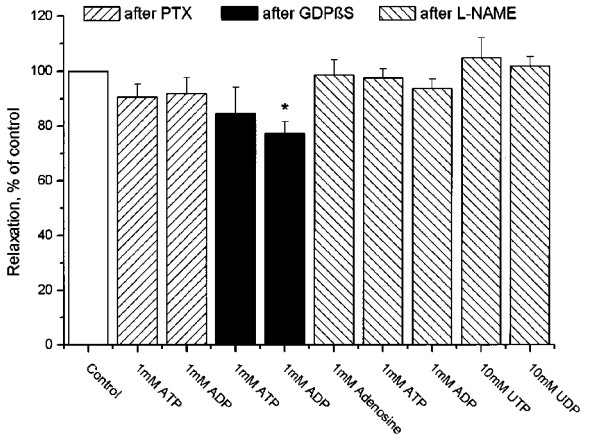

Figure 5 Relaxant responses to ATP (1 mM) are not significantly inhibited after incubation with pertussis toxin (PTX, 100 ng ml^{-1}, $P>0.05$, $n/N=4/4$) or GDPβS (100 μM, $P>0.05$, $n/N=4/4$). Relaxant responses to ADP (1 mM) were significantly reduced after incubation with GDPβS ($P<0.05$, $n/N=4/4$), but were unaffected by pertussis toxin ($P>0.05$, $n/N=4/4$). Relaxant responses to adenosine (1 mM, $n/N=6/3$), ATP (1 mM, $n/N=4/4$), ADP (1 mM, $n/N=4/4$), UTP (10 mM, $n/N=6/3$) and UDP (10 mM, $n/N=6/3$) were not inhibited by incubation with L-NAME (500 μM, $P>0.05$). Statistical analyses performed using Student's t-tests.

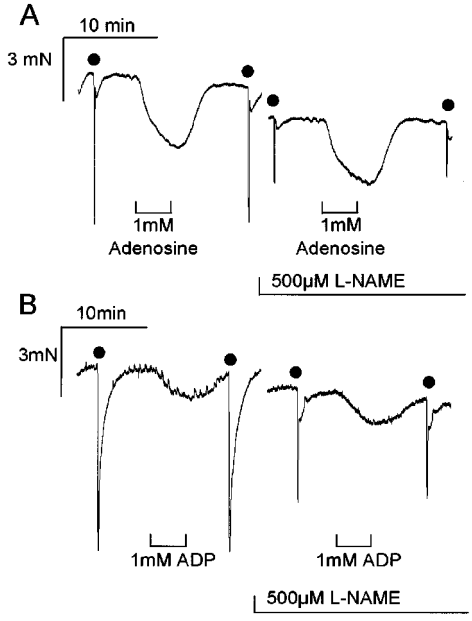

Figure 4 Relaxant responses to exogenous application of adenosine (1 mM, A) and ADP (1 mM, B) are not inhibited by incubation with L-NAME (500 μM). Responses to 5 Hz EFS are reduced by incubation with L-NAME. The mechanograms are original recordings of single tissue preparations and are representative of all the experiments in this series ($n/N=6/3$ for adenosine, $n/N=4/4$ for ADP).

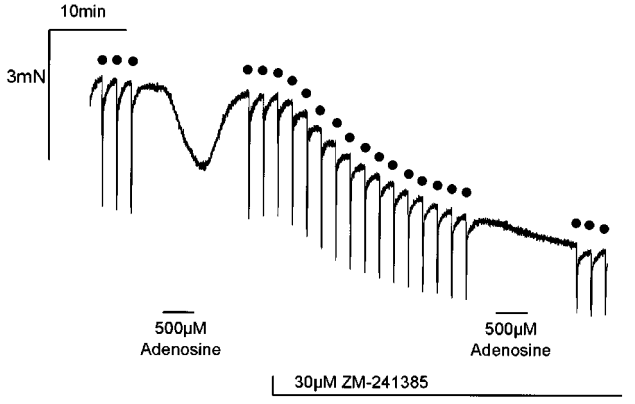

Figure 6 Relaxant response elicited by adenosine (500 μM) after incubation with L-NAME (500 μM) is completely inhibited by ZM-241385 (30 μM), whilst EFS-induced relaxations are not inhibited. The mechanogram is an original recording of a single tissue preparation and is representative of all the experiments in this series ($n/N=4/4$).

shown that UDP and ADP do not activate this receptor (Nicholas *et al*., 1996). $P2Y_4$ receptors show great species variability. The human $P2Y_4$ receptor shows strong selectivity for UTP over ATP, whilst the rat and mouse $P2Y_4$ receptors are activated equally by the nucleotides (Nicholas *et al*., 1996; Bogdanov *et al*., 1998; Lazarowski *et al*., 2001). However, neither ADP nor UDP activate this receptor. $P2Y_6$ receptors, on the other hand, respond preferentially to the nucleoside diphosphate UDP, whilst ADP, ATP and UTP are less effective (Nicholas *et al*., 1996; Communi *et al*., 1996a). $P2Y_{12}$ receptors are selectively activated by ADP, but are only found in platelets (Hollopeter *et al*., 2001). $P2Y_{13}$ receptors have a wide distribution, and whilst they are predominately expressed in the brain and spleen, they are also present in the uterus (Communi *et al*., 2001).

As well as having different agonist potencies the P2Y receptors have different affinities for antagonists. Thus $P2Y_2$ receptors have been reported to be sensitive to suramin (Charlton *et al*., 1996), a P2 receptor antagonist (Dunn & Blakeley, 1988), whilst $P2Y_4$ receptors are suramin-insensitive (Communi *et al*., 1996b). Another P2 receptor antagonist,

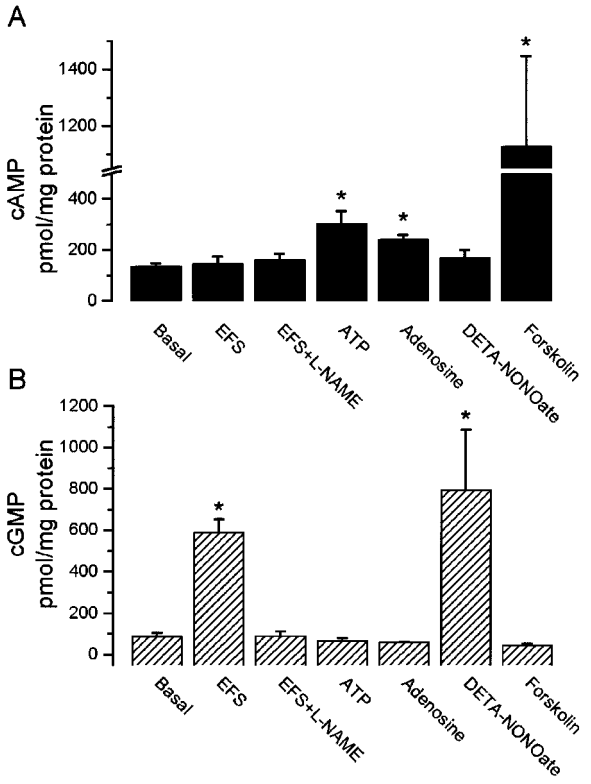

Figure 7 Intracellular concentrations of cAMP (A) and cGMP (B) in the rabbit vaginal wall. Tissues frozen during EFS (5 Hz) showed a marked increase in cGMP concentrations that was completely inhibited in the presence of L-NAME (500 μM). EFS did not cause an increase in cAMP concentrations. Exogenous application of ATP (10 mM) or adenosine (10 mM) caused no increase in either cGMP or cAMP. DETA NONOate (250 μM) and forskolin (10 μM) produced a significant increase in cGMP and cAMP concentrations respectively. *Significantly different from basal, $P<0.05$, $n/N=5/5$ for basal, DETA NONOate and forskolin, $n/N=4/4$ for all other columns. Statistical analyses performed using ANOVA followed by Dunnetts' test.

cibacron blue (Manzini *et al*., 1986), has greater antagonist potency than suramin at the $P2Y_6$ receptor (Robaye *et al*., 1997). In the current study ATP-induced relaxations were reduced by cibacron blue but enhanced by suramin.

None of the receptor subtypes have a profile that matches the responses seen in the rabbit vaginal wall. It is possible that a new subtype of P2Y receptor is present in this tissue, but it is far more probable that co-expression of multiple types of receptor is responsible for the pharmacological profile seen. In this case, a combination of $P2Y_4$ and $P2Y_6$ may account for our observed results. $P2Y_4$ is probably present as this would account for the efficacy of cibacron blue, and the activity of ATP and UTP. $P2Y_6$ is also likely to be present as this would account for the activity of ADP and UDP, as well as further supporting the greater antagonist potency of cibacron blue over suramin.

Further complicating matters, the pharmacological profiles of these P2Y receptors have shown variation in different biological systems. It is possible that the smooth muscle of the vaginal wall has both P2X and P2Y receptors, and that exogenous application of ATP causes conflicting signals to the muscle to both contract and relax, although only the dominant relaxant response is observed. Suramin may enhance the relaxant responses by inhibition of the P2X component. There is also another, more likely, explanation for suramin enhancing the responses, which is that suramin has been reported to be an ectoATPase inhibitor at concentrations below that used in this study (Crack *et al*., 1994; Yegutkin & Burnstock, 2000). If it is acting in this way in this system, the responses could be enhanced by an increase in the amount of agonist reaching the receptors. A high ectoATPase activity in the vaginal wall is also suggested by the high concentrations of nucleotides required to induce relaxations. Whatever the reason for these differences, the lack of effect of cibacron blue and suramin on EFS-induced NANC relaxations suggests that these relaxations are not mediated by nucleotides acting at P2Y receptors.

In this study, adenosine induced relaxant responses in the rabbit vaginal wall. These were mediated at least in part by A_{2A} adenosine receptors since the A_{2A} antagonist ZM-241385 (Poucher *et al*., 1995) abolished them. ZM-241385 caused a slight decrease in tone, but did not inhibit the EFS-induced relaxant responses, suggesting that the EFS-induced relaxations are not mediated by adenosine.

A_{2A} and P2Y receptors are G-protein coupled receptors. Adenosine receptors are coupled to adenylate cyclase and A_{2A} receptors are thought to induce relaxations *via* production of cAMP (Haynes, 2000). All P2Y receptors are G protein-coupled receptors, and have been shown to couple to various signal transduction pathways including adenylate cyclase (Communi *et al*., 1997; King *et al*., 2000), phospholipase C (Communi *et al*., 1997), Rho-dependent kinase (Sauzeau *et al*., 2000) and MAP kinase pathways (Sellers *et al*., 2001). In this study both ATP and adenosine increased cAMP levels, suggesting that this is the second messenger pathway stimulated by these agonists to induce relaxation. EFS-induced nitrergic and non-nitrergic relaxations on the other hand were not associated with increased cAMP, suggesting that neither ATP nor adenosine is involved in NANC relaxant responses in the vaginal wall.

As P2Y receptors are coupled to G proteins, GDPβS, which stabilizes G proteins in their inactive state, should inhibit responses mediated by these receptors (Eckstein *et al*., 1979). Indeed responses to ADP were significantly reduced, suggesting that these responses are mediated by P2Y receptors. Surprisingly we saw no significant inhibition of the responses to ATP when using GDPβS. The concentration used (100 μM) and the incubation time (20 min) is the same as those that inhibited relaxant responses to 1 mM ATP in the marmoset urinary bladder smooth muscle (McMurray *et al*., 1998). ATP- and ADP-induced relaxations were also resistant to inhibition by pertussis toxin. The inactivity of both these inhibitors may not indicate that the receptors responsible for the relaxant responses in this tissue are coupled to pertussis toxin-insensitive G proteins. Instead it may be that accessory proteins are present in this tissue that stabilize the receptor-G protein complex, making it insensitive to inhibition by these compounds, as has been suggested to be the case in other tissues (van der Ploeg *et al*., 1992).

Female sexual dysfunction (FSD) is a common disorder occurring in 22–43% of women. This can have detrimental effects on the emotional and physical well being of the individual (Goldstein, 2000). One type of FSD is Female Sexual Arousal Disorder (FSAD), defined as persistent or recurrent inability to attain, or to maintain until completion

of the sexual activity, an adequate lubrication-swelling response of sexual excitement, causing personal distress (American Psychiatric Association, 2000). FSD arising from arousal disorders has symptoms that include diminished vaginal lubrication, decreased clitoral engorgement and lack of vaginal wall relaxation (Goldstein, 2000), all of which result from a lack of smooth muscle relaxation. We have demonstrated that receptors for purines and pyrimidines are present in vaginal wall smooth muscle and that they can cause relaxant responses. These may provide targets for treatment of FSD. However, these compounds have a low potency compared to the effects of purines and pyrimidines in many other central and peripheral systems (see Ralevic & Burnstock, 1998 for review), and thus a treatment strategy based on these compounds may have significant side effects. Furthermore the relatively low efficacy of the uridine nucleotides compared to the adenine nucleotides makes their use as a potential treatment for FSD even more unlikely. A better treatment strategy is one that enhances the peripheral neurogenic signal originating in the brain. Our evidence suggest that purines and pyrimidines are not involved in non-nitrergic NANC relaxant responses in this tissue, and thus do not provide a target for this type of treatment.

We have previously reported a non-nitrergic NANC relaxant response in the vaginal wall, and provided evidence suggesting that the mediator of this response was not one of the neuropeptides know to be present in the vaginal wall. This study provides evidence that this response is not mediated by purines or pyrimidines. Further work should lead to a full characterization of the neurotransmitters involved in regulating vaginal wall smooth muscle tone, and provide targets for the treatment of FSD.

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References

AMERICAN PSYCHIATRIC ASSOCIATION (2000). Female sexual arousal disorder. In *Diagnostic and Statistical Manual of Mental Disorders. Fourth edition, Text Revision (DSM-IV-TR)*. pp. 543 – 544. Washington D.C.: American Psychiatric Association.

BOGDANOV, Y.D., WILDMAN, S.S., CLEMENTS, M.P., KING, B.F. & BURNSTOCK, G. (1998). Molecular cloning and characterization of rat P2Y4 nucleotide receptor. *Br. J. Pharmacol.,* **124,** 428 – 430.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptor. In *Cell membrane receptors for drugs and hormones: A multidisciplinary approach*. ed. Straub, R.W., Bolis, L. pp. 107 – 118. New York: Raven Press.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P2-purinoceptor? *Gen. Pharmacol.,* **16,** 433 – 440.

CELLEK, S., KASAKOV, L. & MONCADA, S. (1996). Inhibition of nitrergic relaxations by a selective inhibitor of the soluble guanylate cyclase. *Br. J. Pharmacol.,* **118,** 137 – 140.

CELLEK, S. & MONCADA, S. (1997). Nitrergic control of peripheral sympathetic responses in the human corpus cavernosum: a comparison with other species. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 8226 – 8231.

CELLEK, S. & MONCADA, S. (1998). Nitrergic neurotransmission mediates the non-adrenergic non-cholinergic responses in the clitoral corpus cavernosum of the rabbit. *Br. J. Pharmacol.,* **125,** 1627 – 1629.

CELLEK, S., ZIESSEN, T.M. & MONCADA, S. (1999). Non-adrenergic non-cholinergic responses in the rabbit vaginal wall are mediated by nitrergic neurotransmission. *Br. J. Pharmacol.,* **126,** 98P.

CHARLTON, S.J., BROWN, C.A., WEISMAN, G.A., TURNER, J.T., ERB, L. & BOARDER, M.R. (1996). PPADS and suramin as antagonists at cloned P2Y- and P2U-purinoceptors. *Br. J. Pharmacol.,* **118,** 704 – 710.

COMMUNI, D., GONZALEZ, N.S., DETHEUX, M., BREZILLON, S., LANNOY, V., PARMENTIER, M. & BOEYNAEMS, J.M. (2001). Identification of a novel human ADP receptor coupled to G(i). *J. Biol. Chem.,* **276,** 41479 – 41485.

COMMUNI, D., GOVAERTS, C., PARMENTIER, M. & BOEYNAEMS, J.M. (1997). Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J. Biol. Chem.,* **272,** 31969 – 31973.

COMMUNI, D., MOTTE, S., BOEYNAEMS, J.M. & PIROTTON, S. (1996b). Pharmacological characterization of the human P2Y4 receptor. *Eur. J. Pharmacol.,* **317,** 383 – 389.

COMMUNI, D., PARMENTIER, M. & BOEYNAEMS, J.M. (1996a). Cloning, functional expression and tissue distribution of the human P2Y6 receptor. *Biochem. Biophys. Res. Commun.,* **222,** 303 – 308.

CRACK, B.E., BEUKERS, M.W., MCKECHNIE, K.C., IJZERMAN, A.P. & LEFF, P. (1994). Pharmacological analysis of ecto-ATPase inhibition: evidence for combined enzyme inhibition and receptor antagonism in P2X-purinoceptor ligands. *Br. J. Pharmacol.,* **113,** 1432 – 1438.

DANIEL, E.E., CRANKSHAW, J. & SARNA, S. (1979). Prostaglandins and tetrodotoxin-insensitive relaxation of opossum lower esophageal sphincter. *Am. J. Physiol.,* **236,** E153 – E172.

DUNN, P.M. & BLAKELEY, A.G. (1988). Suramin: a reversible P2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.,* **93,** 243 – 245.

ECKSTEIN, F., CASSEL, D., LEVKOVITZ, H., LOWE, M. & SELINGER, Z. (1979). Guanosine 5′-O-(2-thiodiphosphate). An inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. *J. Biol. Chem.,* **254,** 9829 – 9834.

FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DUBYAK, G.R., HARDEN, T.K., JACOBSON, K.A., SCHWABE, U. & WILLIAMS, M. (1997). Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol. Sci.,* **18,** 79 – 82.

GILLESPIE, J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmacol.,* **45,** 404 – 416.

GILLESPIE, J.S., LIU, X.R. & MARTIN, W. (1989). The effects of L-arginine and N^G-monomethyl-L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. *Br. J. Pharmacol.,* **98,** 1080 – 1082.

GOLDSTEIN, I. (2000). Female sexual arousal disorder: new insights. *Int. J. Impot. Res.,* **12** (Suppl. 4), S152 – S157.

HAYNES, J.M. (2000). A(2A) adenosine receptor mediated potassium channel activation in rat epididymal smooth muscle. *Br. J. Pharmacol.,* **130,** 685 – 691.

HOBBS, A.J. & GIBSON, A. (1990). L-N^G-nitro-arginine and its methyl ester are potent inhibitors of non-adrenergic, non-cholinergic transmission in the rat anococcygeus. *Br. J. Pharmacol.,* **100,** 749 – 752.

HOLLOPETER, G., JANTZEN, H.M., VINCENT, D., LI, G., ENGLAND, L., RAMAKRISHNAN, V., YANG, R.B., NURDEN, P., NURDEN, A., JULIUS, D. & CONLEY, P.B. (2001). Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature,* **409,** 202 – 207.

IGNARRO, L.J., BUSH, P.A., BUGA, G.M., WOOD, K.S., FUKUTO, J.M. & RAJFER, J. (1990). Nitric oxide and cyclicGMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. *Biochem. Biophys. Res. Commun.,* **170,** 843 – 850.

ISHIGUCHI, T., TAKAHASHI, T., ITOH, H. & OWYANG, C. (2000). Nitrergic and purinergic regulation of the rat pylorus. *Am. J. Physiol. Gastrointest. Liver. Physiol.,* **279,** G740 – G747.

KAPLAN, H.S. (1974). *The new sex therapy: active treatment of sexual dysfunctions*. London: Baillière Tindall.

KIM, Y.C., JI, X., MELMAN, N., LINDEN, J. & JACOBSON, K.A. (2000). Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A(2B) adenosine receptors. *J. Med. Chem.,* **43,** 1165 – 1172.

KING, B.F., BURNSTOCK, G., BOYER, J., BOEYNAEMS, J.M., WEISMAN, G.A., KENNEDY, C., JACOBSON, K.A., HUMPHRIES, R.G., ABBRACCHIO, M.P., GACHET, C. & MIRAS-PORTUGAL, M.T. (2000). P2Y receptors. In *The IUPHAR compendium of receptor characterization and classification,* 2nd edition. ed. Girdlestone, D. pp. 306 – 320. London: IUPHAR Media.

LAZAROWSKI, E.R., ROCHELLE, L.G., O'NEAL, W.K., RIBEIRO, C.M., GRUBB, B.R., ZHANG, V., HARDEN, T.K. & BOUCHER, R.C. (2001). Cloning and functional characterization of two murine uridine nucleotide receptors reveal a potential target for correcting ion transport deficiency in cystic fibrosis gallbladder. *J. Pharmacol. Exp. Ther.,* **297,** 43 – 49.

LI, C.G. & RAND, M.J. (1989). Evidence for a role of nitric oxide in the neurotransmitter system mediating relaxation of the rat anococcygeus muscle. *Clin. Exp. Pharmacol. Physiol.,* **16,** 933 – 938.

MANZINI, S., HOYLE, C.H. & BURNSTOCK, G. (1986). An electrophysiological analysis of the effect of reactive blue 2, a putative P2-purinoceptor antagonist, on inhibitory junction potentials of rat caecum. *Eur. J. Pharmacol.,* **127,** 197 – 204.

McMURRAY, G., DASS, N. & BRADING, A.F. (1998). Purinoceptor subtypes mediating contraction and relaxation of marmoset urinary bladder smooth muscle. *Br. J. Pharmacol.,* **123,** 1579 – 1586.

NICHOLAS, R.A., LAZAROWSKI, E.R., WATT, W.C., LI, Q., BOYER, J. & HARDEN, T.K. (1996). Pharmacological and second messenger signalling selectivities of cloned P2Y receptors. *J. Auton. Pharmacol.,* **16,** 319 – 323.

PARK, K., GOLDSTEIN, I., ANDRY, C., SIROKY, M.B., KRANE, R.J. & AZADZOI, K.M. (1997). Vasculogenic female sexual dysfunction: the hemodynamic basis for vaginal engorgement insufficiency and clitoral erectile insufficiency [published erratum appears in Int J Impot Res 1998 Mar; 10(1):67]. *Int. J. Impot. Res.,* **9,** 27 – 37.

POUCHER, S.M., KEDDIE, J.R., SINGH, P., STOGGALL, S.M., CAULKETT, P.W., JONES, G. & COLL, M.G. (1995). The in vitro pharmacology of ZM 241385, a potent, non-xanthine A2a selective adenosine receptor antagonist. *Br. J. Pharmacol.,* **115,** 1096 – 1102.

RALEVIC, V. (2001). Mechanism of prolonged vasorelaxation to ATP in the rat isolated mesenteric arterial bed. *Br. J. Pharmacol.,* **132,** 685 – 692.

RALEVIC, V. (2002). The involvement of smooth muscle P2X receptors in the prolonged vasorelaxation response to purine nucleotides in the rat mesentaeric arterial bed. *Br. J. Pharmacol.,* **135,** 1988 – 1994.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413 – 492.

REES, D.D., CELLEK, S., PALMER, R.M. & MONCADA, S. (1990). Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock. *Biochem. Biophys. Res. Commun.,* **173,** 541 – 547.

ROBAYE, B., BOEYNAEMS, J.M. & COMMUNI, D. (1997). Slow desensitization of the human P2Y6 receptor. *Eur. J. Pharmacol.,* **329,** 231 – 236.

SAUZEAU, V., LE JEUNE, H., CARIO-TOUMANIANTZ, C., VAILLANT, N., GADEAU, A.P., DESGRANGES, C., SCALBERT, E., CHARDIN, P., PACAUD, P. & LOIRAND, G. (2000). P2Y(1), P2Y(2), P2Y(4), and P2Y(6) receptors are coupled to Rho and Rho kinase activation in vascular myocytes. *Am. J. Physiol. Heart Circ. Physiol.,* **278,** H1751 – H1761.

SELLERS, L.A., SIMON, J., LUNDAHL, T.S., COUSENS, D.J., HUMPHREY, P.P. & BARNARD, E.A. (2001). Adenosine nucleotides acting at the human P2Y1 receptor stimulate mitogen-activated protein kinases and induce apoptosis. *J. Biol. Chem.,* **276,** 16379 – 16390.

STANFORD, S.J., GITLIN, J.M. & MITCHELL, J.A. (2001). Identification of two distinct vasodilator pathways activated by ATP in the mesenteric bed of the rat. *Br. J. Pharmacol.,* **133,** 825 – 832.

STORR, M., FRANCK, H., SAUR, D., SCHUSDZIARRA, V. & ALLESCHER, H.D. (2000). Mechanisms of alpha,beta-methylene ATP-induced inhibition in rat ileal smooth muscle: involvement of intracellular Ca^{2+} stores in purinergic inhibition. *Clin. Exp. Pharmacol. Physiol.,* **27,** 771 – 779.

VAN DER PLOEG, I., PARKINSON, F.E. & FREDHOLM, B.B. (1992). Effect of pertussis toxin on radioligand binding to rat brain adenosine A1 receptors. *J. Neurochem.,* **58,** 1221 – 1229.

VON KUGELGEN, I., HAUSSINGER, D. & STARKE, K. (1987). Evidence for a vasoconstriction-mediating receptor for UTP, distinct from the P2 purinoceptor, in rabbit ear artery. *Naunyn. Schmiedebergs. Arch. Pharmacol.,* **336,** 556 – 560.

YEGUTKIN, G.G. & BURNSTOCK, G. (2000). Inhibitory effects of some purinergic agents on ecto-ATPase activity and pattern of stepwise ATP hydrolysis in rat liver plasma membranes. *Biochim. Biophys. Acta.,* **1466,** 234 – 244.

ZIESSEN, T., MONCADA, S. & CELLEK, S. (2002). Characterisation of the non-nitrergic NANC relaxation responses in the rabbit vaginal wall. *Br. J. Pharmacol.,* **135,** 546 – 554.

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