79

Fry C[1], McLatchie L[1]
1. *University of Surrey*

THE FUNCTION AND REGULATION OF STRESS-INDUCED ACETYLCHOLINE RELEASE FROM THE BLADDER UROTHELIUM

Hypothesis / aims of study

Acetylcholine (ACh), and other functional transmitters such as ATP and prostaglandins, are released from the bladder mucosa when subjected to stretch or changes of transmural pressure. However, for ACh its precise cellular origin, routes of release and its function after release have not been fully characterised. The study aimed to quantify and characterise ACh release from freshly isolated urothelial cells when subjected to changes of shear stress .

Study design, materials and methods

Guinea-pigs were euthenised by cervical dislocation, the bladder was immediately removed and the mucosa dissected free of the detrusor layer by blunt dissection. Incubation of the mucosa in Trypsin EDTA (0.5g.l^{-1} Trypsin/ 0.2g.l^{-1} EDTA, Sigma) for 40 min at 37°C followed by gentle trituration, liberated urothelial cells. Cells were labelled with cytokeratin-20 (CK-20) to indicate their urothelial origin. An improved Neubauer haemocytometer stage was used to count cell number in a suspension and the radius of cells was measured from light micrographs of cell suspensions (x40). For experiments cells were resuspended at room temperature in Ca-free Tyrode's solution (pH 7.4 buffered with 24 mM $NaHCO_3$/5% CO_2) and incubated for at least 90 minutes before use. Mechanical stimuli were applied by pipetting 20 µl of the 45 µl cell suspension a set number of times and immediately removing and freezing 20 µl of the suspension for later analysis. ACh was measured with an Amplex®Red ACh/acetylcholinesterase assay kit (Invitrogen). Fluorescence (590 nm) was measured after excitation at 544 nm using a BMG FLUOstar Omega multi-mode plate reader. ACh standards were included in each plate. ATP was measured with a luciferin-luciferase assay (FL-AAM, Sigma) and a luminometer (GloMax 20/20; Promega) against a calibration curve over the range 10^{-13} – 10^{-7} M. All agents were form Sigma UK and all interventions were tested against vehicle controls. Data are mean±SD and differences in data sets were examined using Student's *t*-tests; the null hypothesis was rejected at $p<0.05$

Results

Isolated cell viability was tested by Trypan Blue exclusion (90.2±0.4% excluded, *n*=121) and nearly all cells (95±2, n=4) labelled for CK-20. Suburothelial and detrusor cells did not label for CK-20 in bladder sections. Cell size was conveniently described by three Gaussian distributions with diameters of: 10.9±0.5 µm (basal cells); 18.5±0.6 µm (intermediate cells); 30.6±2.5 µm (umbrella cells). Imposition of shear stress led to ACh and ATP release, however, the pattern of this release was very different (Fig 1): the amount of ACh release was smaller and maximal after just one intervention, whereas ATP was a continuous function of the number of interventions. Basal levels (x0, fig 1) of ACh and ATP were 45.5±13.2 pmol/10^5 cells and 413±228 pmol/10^5 cells, respectively (n=3).

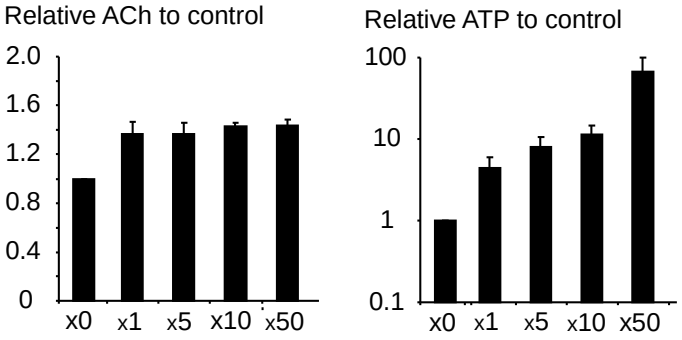

Figure 1. ACh and ATP release from isolated urothelial cells after 1-50 shear stress interventions. Note the linear ordinate on the ACh relation (left) and the logarithmic ordinate for the ATP relation (right)

Shear-stress evoked ACh release was unaffected by brefeldin-A (10^{-5} g.ml^{-1}; *n*=9) that attenuates vesicle-mediated release and the gap junction blocker, carbenoxolone (50 µM; *n*=13). Additional experiments showed that brefeldin and carbenoxolone were effective in reducing shear-stress induced ATP release (to 0.75±0.08 and 0.71±0.07 of control (=1.0), respectively).

However, the CFTR channel inhibitor, $CFTR_{inh}$-172 did reduce ACh release, in a dose-dependent manner, with a maximum effect to reduce release to 0.60±0.11 (*n*=13) of control, with an IC_{50} of 3.4±0.7 µM.

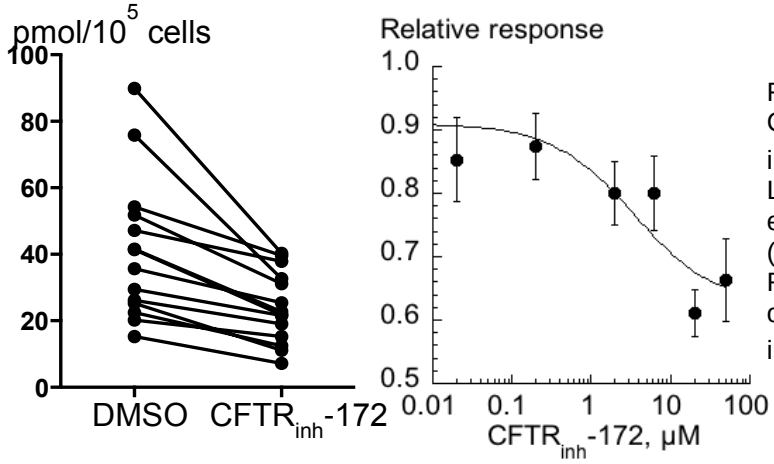

Figure 2. The effect of $CFTR_{inh}$-172 on stress-induced ACh release.
Left: paired results of 13 experiments in the vehicle (DMSO) or CFTRinh-172. Right: dose response curve of $CFTR_{inh}$-172 on stress-induced ACh release.

The effect of muscarinic receptor agonists and antagonists on shear-stress induced ATP release was tested. Carbachol (10 µM, *n*=6) increased shear-stress induced ATP release to 1.42±0.20 of control. By contrast, the M_2-selective antagonist methoctramine reduced ATP release in a dose-dependent manner to a maximum of 0.73±0.06 (n=7) of control and with a pIC_{50} of 6.60±0.25, *n*=5 (IC_{50}=-logIC_{50}, mean IC_{50} = 250 nM). The M3-selective antagonist 4-DAMP also reduced ATP release but to a smaller maximal effect; 0.87±0.04 (*n*=5) of control; pIC_{50} = 7.01±0.21; mean IC_{50} 99 nM; *n*=5. $CFTR_{inh}$-172 (20 µM) also significantly reduced ATP release to 0.60 ± 0.10 of control (*n*=8).

Interpretation of results

Acetylcholine is released in significant quantities from freshly-isolated, viable urothelial cells. Moreover, maximum ACh release is achieved by smaller external physical forces than ATP release. The route of ACh release is different from that of ATP release, a major fraction of ACh release is via a CFTR-like channel, whilst brefeldin-A and carbenoxolone affect ATP, but not ACh, release. The actions of carbachol, antimuscarinic agents and a CFTR channel inhibitor on ACh release has permitted the proposal of a hypothesis namely: stress-induced ACh release from urothelial cells regulates further release of ATP. This mechanism has a high gain, and regulation of the ACh fraction of the transmitter release pathway confers a high sensitivity to the overall release of stress-induced urothelial transmitters. The pIC_{50} values of methoctramine and 4-DAMP for their action on ATP release reflect a profile nearer to the M_2 rather than the M_3 receptor [1], but a more detailed subtype profiling is required.

Concluding message

Acetylcholine is released from urothelial cells when subjected to shear stress by a novel route, different from ATP release. It is hypothesised that ACh thus released controls the release of ATP from urothelial cells. This pathway may contribute to the action of antimuscarinic agents in their ability to reduce symptoms of LUTS and painful bladder syndrome through attenuation of sensory afferent pathways.

References

1. Choppin A, Eglen RM. Br J Pharmacol 2001; 133: 1035–1040.

Disclosures

Funding: EU FP7 consortium grant **Clinical Trial:** No **Subjects:** ANIMAL **Species:** Guinea-pig **Ethics Committee:** University of Surrey