University of Bristol - Explore Bristol Research
General rights

1 **Selective reduction of neurotransmitter release by cAMP-dependent pathways**

2 **in mouse detrusor**

3

4 Basu Chakrabarty[1], Marcus J. Drake[1,2,3], Anthony J. Kanai[4], Christopher H. Fry[1]

5

6 School of Physiology, Pharmacology, and Neuroscience, University of Bristol, Bristol,

7 UK[1], Translational Health Sciences, Bristol Medical School, University of Bristol,

8 Bristol, UK[2], Bristol Urological Institute, Southmead Hospital, Bristol, UK[3],

9 Departments of Medicine and Pharmacology & Chemical Biology, University of

10 Pittsburgh, Pittsburgh, Pennsylvania, USA[4]

11

12 **Running Head:** Selective attenuation of neuronal ATP release from detrusor

13

Corresponding Author: Basu Chakrabarty

Corresponding Address: School of Physiology, Pharmacology, and Neuroscience

                              Faculty of Life Sciences

                              University of Bristol

                              Biomedical Sciences Building

                              University Walk

                              Bristol BS8 1TD

                              United Kingdom

Email Address: basu.chakrabarty@bristol.ac.uk

Phone Number: +44 (0)117 331 1565

14

24

25 **Supplementary Material**

26 Supplemental   Material   available   at   URL:
27 https://figshare.com/s/42c3169843f6cdfd3e68,   DOI:
28 https://doi.org/10.6084/m9.figshare.21253557.

Abstract

Parasympathetic nerve-mediated contractions of detrusor smooth muscle are generated by ATP and ACh release from efferent nerve terminals. In humans, ACh is responsible for detrusor contractions in normal human bladders, whereas ATP has an additional role in overactive bladder pathologies. The ATP metabolite, adenosine, relaxes nerve-mediated contractions, with a potential action via presynaptic adenosine A_1 receptor activation and subsequent suppression of neuronal ATP release. We investigated the effect of A_1 receptor activation and downstream cAMP-dependent pathways on nerve-mediated ATP and ACh release, and detrusor contraction in mouse detrusor. Bladders from male C57BL/6 mice (12 weeks) were used for *in vitro* experiments. Upon electrical field stimulation of intact preparations (detrusor and mucosal layers), ATP or ACh release was measured simultaneously with tension recordings. Activation of A_1 receptors by adenosine or exogenous agonists reduced the lower frequency component of nerve-mediated contractions, and neuronal ATP release. The A_1 receptor antagonist abolished these effects. A_1 receptor activation inhibits AC activity and cAMP generation. The effect of A_1 receptor activation was mimicked by a PKA antagonist, but not by modulators of exchange proteins activated by cAMP, demonstrating that modulation of nerve-mediated ATP release is via PKA. Adenosine had no effect on ACh release or the higher frequency component of nerve-mediated contractions. Differential regulation of neurotransmitter release is possible at the detrusor nerve-muscle junction, as demonstrated by A_1 receptor activation, and downstream inhibition of AC, cAMP generation and PKA. The ability to specifically attenuate ATP release offers a potential to target purinergic motor pathways enhanced in overactive bladder pathologies.

Introduction

The purinergic system regulates bladder function through the action of ATP and its metabolites. ATP release from bladder efferent nerves plays a role in autonomic neurotransmission (1-3). It has been identified in sympathetic and parasympathetic varicosities, where ATP is co-released with either noradrenaline or ACh (4). The contribution of ATP to parasympathetic nerve-mediated contractions in detrusor smooth muscle has been extensively studied and has a role in most animal bladders. However, in humans, ATP is a functional neurotransmitter only in pathological situations, such as overactive bladder (OAB) syndrome, detrusor overactivity (DO) or bladder outlet obstruction. Detrusor contractions from normal human bladders are solely supported by ACh (5-12). In consequence, the ability to selectively attenuate nerve-mediated ATP release offers itself as an attractive therapeutic target to manage clinical conditions.

Extracellular ATP is rapidly metabolized, ultimately to adenosine, by extracellular endonucleotidases (13). Adenosine itself may bind to surface receptors (A_1, A_{2A}, A_{2B}, and A_3), but has other fates such as translocation to the cytoplasm, or conversion to inosine by adenosine deaminase, or AMP by adenosine kinase (14-16): all of which have important roles in the urinary tract (17, 18). Actions of adenosine receptor activation are mediated by G-protein coupled intracellular pathways to modulate AC activity and hence cAMP generation; A_1 and A_3 receptors inhibit AC activity, whereas A_{2A} and A_{2B} receptors stimulate AC activity. Downstream cAMP pathways include intermediates, such as PKA and exchange protein activated by cAMP (EPACs) (19). One action of adenosine is to regulate neurotransmission at synapses or neuroeffector junctions where ATP participates as a co-transmitter, in addition to the fact that adenosine may itself be a neuromodulator (20, 21).

94   Overall, adenosine attenuates nerve-mediated contraction of detrusor from mice

95   (20), rats (22, 23), guinea pigs (1, 24), and humans (6, 25). With human detrusor,

96   this action is greater in tissue from patients with neurogenic DO (NDO) compared to

97   normal stable bladders and is mimicked by the selective A_1 receptor agonist N^6-

98   cyclopentyladenosine (CPA) (6). Moreover, with tissue from NDO bladders, there is

99   a greater reduction of force by A_1 receptor agonists at lower frequencies of

100   stimulation (1-4 Hz), with a greater dependence on ATP release, compared with

101   higher frequency contractions where ACh release is dominant (6). This has been

102   interpreted as A_1 receptor activation having a relatively selective action on nerve-

103   mediated ATP release. However, immunolocalisation confocal microscopy has

104   demonstrated A_1 receptors to be colocalized with vesicular ACh transporter

105   (VAChT)-positive cholinergic nerve terminals and adenosine, or its stable analogues,

106   have been reported to reduce nerve-evoked ACh release (26).

107   An aim of this study was to measure directly in mouse detrusor the effect of A_1

108   receptor agonists on nerve-mediated ATP and ACh release, as well as tension

109   generation, to determine any differential effect on neurotransmitter release. A further

110   aim was to characterize downstream cAMP-dependent pathways involved in any

111   such actions. The motivation of the study was to identify potential drug targets that

112   may selectively attenuate release of transmitters associated with DO in humans.

Materials and Methods

Tissue samples and ethics approval

All animal care and experimental procedures followed the University of Bristol Ethics Committee guidelines and were in accordance with UK legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039) and the principles of the United States National Institutes of Health (NIH). Young (12 weeks) male C57BL/6 mice (Harlan UK Ltd) were used for experiments. The animal model was chosen to conform with previous experiments where transmitter release methods were validated (27, 28) and according to the stipulations of the funding authority (NIH).

Measurement of contractile function *in vitro*

Mice were killed by CO_2 asphyxiation and the bladder was removed through a midline laparotomy. The whole bladder was bisected and bladder strips from the bladder dome (detrusor with mucosa intact, 4-5-mm length, 1-2 mm width) were tied in a horizontal trough between a hook and an isometric force transducer. Preparations were superfused with Tyrode' solution at 37°C. Contractions generated by electrical field stimulation (EFS; 0.1 ms pulses, 1-40 Hz, 3-s train every 90 s) were inhibited by tetrodotoxin (TTX, 1 µM). Drugs were added to the superfusate, with appropriate vehicle and time controls, and the effects on nerve-mediated contraction amplitude measured. Tension (mN) was normalized to preparation weight ($mN.mg^{-1}$) to avoid confounding experimental variability due to preparation dimensions.

Measurement of nerve-mediated neurotransmitter release

136 Superfusate samples (100 µl) were taken from a fixed point near the preparation

137 (two-thirds downstream along the tissue length and 1 mm lateral to the horizontally

138 mounted preparation), ensuring minimal mechanical disturbance. Samples were

139 taken before EFS, and 2 s after the initiation of EFS, with nerve-mediated release

140 taken as the difference between these two values. Samples were stored on ice

141 before assay of released ATP or ACh. In separate experiments, EFS-mediated ATP

142 and ACh release was completely inhibited by TTX (1 µM) or 2% lignocaine (n=6

143 each).

144 ***Measurement of sampled ATP.*** ATP release was measured using a luciferin-

145 luciferase assay where emitted light was a positive function of ATP concentration.

146 The complete Sigma ATP assay mix (FLAAM, Sigma-Aldrich, Dorset, UK) was

147 diluted with an assay buffer supplied, as per the manufacturer's instructions.

148 Luminescence intensity was read using a luminometer (Glomax 20/20, Promega)

149 and calibrated with an ATP standard on the day of each experiment, with

150 luminescence being a linear function of concentration on a log-log plot over the

151 range of 100 fM to 1 µM. A log-log plot was chosen to linearize the calibration curve

152 over the wide range of calibration solution concentrations. The detection limit of the

153 system was 100 fM ATP. ATP release was measured across the EFS frequency

154 range to elicit contractions.

155 ***Measurement of sampled ACh.*** ACh release was measured with a choline/ACh

156 quantification assay (MAK056, Sigma-Aldrich, Dorset, UK) using the fluorescence

157 method following the manufacturer's instructions. Reaction mixes were added to

158 collected samples (50 µl) – one with AChE added to the reaction mix, which

159 hydrolyses ACh to choline and acetate to determine total choline, and the other

160 without AChE to determine free, background choline levels. The difference between

161   the two was equivalent to the quantity of ACh from nerve-mediated stimulation.

162   Fluorescence intensity (λ_{ex}=535/λ_{em}=587 nm) was read using a fluorescence

163   multiwell plate reader (CLARIOstar Plus, BMG Labtech). The system was calibrated

164   with a choline standard on the day of each experiment, with fluorescence a linear

165   function of concentration over the range of 0-250 pM choline.

166   **Data and statistical analyses**

167   The frequency-dependent percentage reduction of tension data, *T(f)*, by

168   interventions (e.g. Figure 1B) were fitted to equation 1a.

169   Equation 1a: $T(f) = T_{Lf} - \left(\left(\frac{(T_{Lf} - T_{Hf}) \cdot f^m}{f^m + k^m} \right) + T_{Hf} \right)$

170   T_{Lf} and T_{Hf} are the maximum and minimum force reductions respectively at low and

171   high frequencies, *m* and *k* are constants.

172   The frequency-dependence of peak tension or ATP release (e.g. Figures 1C and 1D)

173   were fitted to a linear two-component function, equation 1b, as this yielded a

174   significantly better fit than a one-component function (28): *Y(f)* is equivalent to either

175   *T(f)* or *[ATP](f)*.

176   Equation 1b: $Y(f) = \frac{Y_{Lf,max} \cdot f^m}{f_{*,Lf}^m + f^m} + \frac{Y_{Hf,max} \cdot f^m}{f_{*,Hf}^m + f^m}$

177   Y_{max} are the maximum estimated values of low (*Lf*) or high (*Hf*) components and f_*

178   are the frequencies at which the two components each reach $Y_{max/2}$; *m* is a constant.

179   The T_{max} is the sum of the T_{max} values of *Lf* and *Hf* components, and $f_{1/2}$ is the

180   frequency at half-maximal tension, $T_{max/2}$ – each of which is reported in Tables 2 and

181   3.

182   Frequency-dependent ACh data (e.g. Figure 3B) were fitted to a one-component

183   function, equation 1c; there was no statistical advantage by fitting ACh data to a two-

184   component model (28).

185   Equation 1c: $[ACh](f) = \frac{Y[ACh]_{max} \cdot f^m}{f_{\frac{1}{2}}^m + f^m}$

186   Y_{max} is the maximum estimated value of Y and $f_{1/2}$ is the frequency required to

187   achieve $Y_{max}/2$; m is a constant.

188   Data fits were performed with an iterative, least-squares Levenberg-Marquardt

189   algorithm (KaleidaGraph, v4.5, Synergy software, CA, USA). Data are mean ± SD

190   and differences between data sets were tested with Student's paired t-tests,

191   repeated measures one-way ANOVA followed by parametric *post hoc* tests, and

192   repeated measures two-way ANOVA followed by parametric *post hoc* tests where

193   appropriate; the null hypothesis was rejected at $p<0.05$. n values refer to the number

194   of preparations, one each from separate animals.

195   Values of $Y_{Lf,max}$ (Equation 1b) were used to analyze specifically the effect of

196   interventions on purinergic contractions without involvement of nerve-mediated ACh

197   release (e.g. Figure 3A). This component was dominant over the frequency range

198   over 1-2 Hz ($f_{*,Lf} = 0.7±0.2$ Hz, $n=48$), where ACh release was negligible (e.g. Figure

199   3B). An association between two variables, r, was calculated as a Pearson

200   correlation coefficient. All statistical analyses were undertaken using GraphPad®

201   Prism 7 (GraphPad Software Inc., CA, USA; GraphPad Prism). The number of

202   repeats in each control and intervention set was based on a power calculation to

203   reject the null hypothesis at $p<0.05$ and a power of 80%, with variance of data based

204   on previous experimental data with these methods. The data and statistical analyses

205 comply with the guidelines for reporting statistics in journals published by the

206 American Physiological Society (29).

207 **Materials**

208 Tyrode's solution was composed of (mM): NaCl, 118; $NaHCO_3$, 24; KCl, 4.0;

209 NaH_2PO_4, 0.4; $MgCl_2$, 1.0; $CaCl_2$, 1.8; glucose, 6.1; Na pyruvate, 5.0; 5% CO_2, 95%

210 O_2, pH 7.4.

211 The concentration of all stock solutions was between 1.0 and 10 mM. Adenosine,

212 atropine, cyclic 3',5'-(hydrogenphosphorothioate) triethylammonium (cAMPS-Rp),

213 N^6-monobutyryladenosine 3':5'-cyclic monophosphate sodium salt (6-MB-cAMP), 8-

214 (4-chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclicmonophosphate

215 acetoxymethyl ester (007-AM), and α-[2-(3-chlorophenyl)hydrazinylidene]-5-(1,1-

216 dimethylethyl)-b-oxo-3-isoxazolepropanenitrile (ESI-09) were dissolved in distilled

217 water. CPA, 5'-(N-ethylcarboxamido)adenosine (NECA), and 8-cyclopentyl-1,3-

218 dipropylxanthine (DPCPX) were dissolved in DMSO. Forskolin was dissolved in

219 ethanol. Stock solutions were diluted with Tyrode's solution to the final concentration

220 as indicated. Adenosine, atropine, CPA, NECA, DPCPX, forskolin, 6-MB-cAMP and

221 ESI-09, were from Sigma-Aldrich (Dorset, UK), and cAMPS-Rp and 007-AM were

222 from Tocris (Abingdon, UK). The mechanism of action of drugs and the

223 concentrations used in this study are listed in Table 1.

224 **Results**

225 **Adenosine on nerve-mediated contractions and neurotransmitter release**

226 Adenosine (1 mM) reduced nerve-mediated contractions, but the effect was

227 frequency-dependent, with a reduction by about 30% at 1 Hz, but absent at 20 Hz

228 (Figure 1A-B). The frequency-dependent effect was quantified in two ways. Firstly,

229 by plotting the percentage reduction of force as a function of frequency (Figure 1B)

230 and fitting the data with Equation (1a), Materials and Methods, to show a maximal

231 reduction of 31.8 ± 7.2% at low frequencies and a half-maximal reduction at 6.7 ± 2.6

232 Hz. The second approach was to generate separate force-frequency relations using

233 Equation (1b) (Figure 1C) to obtain parameters that are shown in Table 2: the

234 estimated T_{max} at high frequencies; and the frequency to achieve $T_{max}/2$, $f_{1/2}$.

235 Adenosine had no effect on T_{max}, but increased $f_{1/2}$; consistent with a preferential

236 reduction of force at low frequencies. The first approach is illustrated in subsequent

237 figures, where relevant. However, because quantitative data were not possible when

238 there was no effect of the intervention; the second approach provided parameterized

239 data to demonstrate an effect, or otherwise, of an intervention (Table 2).

240 Neurotransmitter release was measured directly by measuring [ATP] or [ACh] near

241 the preparation, at the same time as contractions were recorded. Control

242 experiments showed that ATP and ACh release, as well as tension, were completely

243 abolished by 1 µM tetrodotoxin at all frequencies used (n=6) and were therefore

244 designated nerve-mediated phenomena. ATP release occurred over the entire

245 frequency range (1-40 Hz) and adenosine reduced ATP release at all frequencies –

246 e.g. at 8 Hz from 73.6±16.3 to 44.2±9.3 fmol.μl^{-1}.mg^{-1}; a 35.2±6.5% reduction (n=12;

247 Figure 1D). The proportional reduction was similar at all stimulation frequencies

248 (Figure 1E).

249   ACh release occurred over a different frequency range (>4 Hz to a maximum at 20-

250   40 Hz; Figure 3B). Adenosine had no effect on ACh release; release values at 20 Hz

251   stimulation were 143±65 $fmol.\mu l^{-1}.mg^{-1}$ in comparison to 140±62 $fmol.\mu l^{-1}.mg^{-1}$ at

252   control; n=6, p=0.696 (Figure 1F). Thus, ATP and ACh release occur over different

253   frequency domains; at low frequencies (<4 Hz) ATP release is dominant, with

254   increasing ACh release at higher frequencies. Because adenosine predominantly

255   reduced nerve-mediated tension at lower frequencies, it may be hypothesized that

256   this results from a differential effect on nerve-mediated ATP over ACh release.

257   Henceforth, with adenosine and subsequent interventions, changes to nerve-

258   mediated tension and ATP release will used averaged data at 1 and 2 Hz stimulation

259   (Figure 3A), and data for any actions on ACh release will be reported at 20 Hz. Thus,

260   adenosine reduced tension by 29.8±6.6% and ATP release by 34.1±6.8%, with no

261   significant effect on ACh release (Table 3).

262   **A_1 receptor modulation; nerve-mediated contractions and ATP/ACh release**

263   The selective A_1 receptor antagonist, DPCPX (1 µM), alone had no effect on tension

264   or on ATP release at any frequency (Tables 2 and 3). Furthermore, in the presence

265   of DPCPX, there was no effect of adenosine on nerve-mediated contractions and

266   ATP release (Tables 2 and 3). This is consistent with involvement of the A_1 receptor

267   whereby adenosine suppresses nerve-mediated tension and ATP release.

268   The selective A_1 receptor agonist, CPA (10 µM), and the less-selective A_1/A_2

269   receptor agonist, NECA (10 µM) also reduced nerve-mediated contractions –

270   predominantly at low frequencies, with a maximal reduction of 54.3 ± 6.13% and

271   73.8 ± 10.5%, respectively (Figure 2A-C). The half-maximal reduction was at 10.0 ±

272   3.5 Hz for CPA, and 10.4 ± 3.6 Hz for NECA (Figure 2A). With both receptor

273 agonists there was no effect on the T_{max}, and an increase of the $f_{1/2}$ values (Table 2),

274 as well as reduction of low-frequency nerve-mediated contractions and ATP release,

275 but no effects on ACh release (Table 3).

276 Downregulation of cAMP-dependent signaling pathways; nerve-mediated

277 contractions and ATP/ACh release

278 Activation of adenosine A_1 receptors reduces AC activity to reduce intracellular

279 cAMP levels, and it was further hypothesized that this mediates the above A_1-

280 receptor actions on nerve-mediated tension and ATP/ACh release. PKA and EPAC

281 are targets for cAMP and the roles of each were investigated with selective

282 modulators. The PKA antagonist, cAMPS-Rp (10 µM), had overall effects similar to

283 adenosine or CPA. There was a selective reduction of low frequency contractions

284 (Figure 2D), as well as an increase of $f_{1/2}$ (Table 2), and in this case, a small but

285 significant increase of T_{max}. In addition, low-frequency contractions and nerve-

286 mediated ATP release was attenuated with no effect on ACh release (Table 3).

287 A role for EPAC was explored using the inhibitor, ESI-09 (20 µM). There was no

288 effect on magnitude or frequency-dependent of nerve-mediated contractions, nor on

289 corresponding ATP or ACh release (Tables 2 and 3). Thus, the principal target for

290 altered intracellular cAMP levels to regulate tension or transmitter release is on this

291 evidence via PKA.

292 Figure 3A summarizes the above experiments with adenosine, CPA, NECA and

293 cAMPs-Rp to demonstrate a significant (r=0.97, p=0.001) relationship between

294 reduction of both tension and ATP release at low stimulation frequencies (Table 3).

295 Included, also are data from the EPAC activator, 007-AM (see below) that exerted no

296 effect on either variable. There was also a significant order of potency for the A_1-

297 receptor agonists, with NECA>CPA>adenosine. These data are consistent with an

298 adenosine A_1 receptor mediated-pathway via PKA to suppress selectively both

299 nerve-mediated ATP release and low-frequency contractions.

300 The selective adenosine A_1 receptor agonist, CPA, reduced atropine (1 µM)-

301 resistant, purinergic contractions by 26-44% across the frequency range (1-40 Hz,

302 n=6). Similarly, CPA resulted in a 30-45% reduction of ATP release across the same

303 frequencies (n=6). It is worth noting that this proportional reduction of atropine-

304 resistant contractions and nerve-mediated ATP release was consistent at all

305 frequencies – i.e., they were not frequency-dependent, similar to the inhibitory effect

306 of adenosine on nerve-mediated ATP release (Figure 1E).

307 **Upregulation of cAMP-dependent signaling pathways; nerve-mediated**

308 **contractions and ATP/ACh release**

309 By contrast, interventions designed to up-regulate cAMP signaling has no significant

310 effect on nerve-mediated tension or ATP/ACh release. The cAMP analogue, 6-MB-

311 cAMP (100 µM), a PKA activator, had no effects on nerve-mediated contractions or

312 on nerve-mediated ATP release (Tables 2 and 3). In addition, the EPAC activator,

313 007-AM (10 µM), also had no effects on contraction magnitude or ATP/ACh release

314 at any frequency (Tables 2 and 3). However, in both cases, subsequent addition of

315 adenosine in the continuous presence of 6-MB-cAMP or 007-AM, produced

316 comparable reductions of contraction magnitude and ATP release as generated by

317 adenosine alone (Tables 2 and 3).

318 The AC activator, forskolin (1 µM), reduced nerve-mediated contractions, with a

319 maximal reduction of 61.6 ± 12.4% at low frequencies and a half maximal reduction

320 at 13.2 ± 4.6 Hz (Figure 4A). However, it had no effect on nerve-mediated ATP or

321 ACh release (Figure S6, Supplemental Material available at URL:

322 https://figshare.com/s/42c3169843f6cdfd3e68, DOI:

323 https://doi.org/10.6084/m9.figshare.21253557). Adenosine in the presence of

324 forskolin produced an additional effect, further reducing low-frequency contractions

325 (Figure 4A) and nerve-mediated ATP release (Table 3). Forskolin was the only

326 intervention that also reduced the resting baseline tension, by 25.5 ± 17.4%

327 (Student's paired t-test). This effect was significant in comparison to other

328 interventions, for example, adenosine, had no effect on resting baseline tension (8.9

329 ± 14.4%, two-way ANOVA, Figure S6). Finally, forskolin directly reduced purinergic

330 contractions (Figure 4B). Atropine (1 µM) reduced nerve-mediated contractions, with

331 a reduction of T_{max} and $f_{1/2}$ values, and these atropine-resistant contractions were

332 further reduced by forskolin (Table 2).

Discussion

Adenosine receptor pathways in nerve-mediated tension and neurotransmitter release

Adenosine reduces nerve-mediated contractions in the detrusor of many species including those from mice (20) and humans (6). In humans, adenosine had a greater reduction on detrusor nerve-mediated contractions from patients with DO in comparison to those from normal bladders. In addition, there was a greater effect at lower stimulation frequencies in these pathological bladders (6). Nerve-mediated contractions are generated by ATP and ACh release from efferent nerves; with dominant roles for the purinergic component of release at lower frequencies of stimulation and the cholinergic component at higher frequencies (27, 30). The selective A_1 receptor agonist, CPA, had a similar effect to that of adenosine, and it has been inferred that adenosine acting at adenosine A_1 receptors suppresses ATP release (21), an action directly confirmed here for the first time. These effects of adenosine were inhibited by the A_1 receptor antagonist, DPCPX. Adenosine and CPA also reduce the amplitude of excitatory junction potentials (EJPs) in mouse detrusor bladder preparations, mediated by A_1 receptors and suggest a presynaptic inhibitory effect of A_1 receptor activation on evoked ATP release (31).

The A_1/A_2 receptor agonist, NECA, had a similar effect to CPA, with no suppression of contractions by high-frequency stimulation, a uniform proportional suppression of nerve-mediated ATP release over the range of stimulation frequencies and no effect on ACh release. There is a differential distribution of A_1 and A_2 receptors in detrusor, with A_2 receptors more abundant on detrusor muscle (20, 32, 33). The similarity of actions with CPA and NECA, are consistent with their major actions being on pre-

357 junctional A_1 receptors, and consistent, under the conditions of these experiments,

358 with only minor direct actions of adenosine on detrusor via A_{2A} and A_3 receptors (6).

359 However, a more complex role for adenosine and A_1 receptors has been suggested

360 in addition to reduction of nerve-mediated ATP release, namely to regulate nerve-

361 mediated ACh release as judged from colocalisation of A_1 receptors with vesicular

362 ACh transporters on cholinergic nerve terminals (26). In detailed studies, it was

363 proposed that β_3-adrenoceptor agonists indirectly mediate adenosine release from

364 detrusor smooth muscle, via the equilibrative nucleoside transporter 1, to activate A_1

365 receptors on cholinergic nerves and reduce neuronal ACh release (34, 35). These

366 varying effects of adenosine on nerve-mediated ACh release may be due to the

367 difference in the concentration of adenosine and the different conditions from the

368 experiments reported here. The above observation is important as a potential

369 additional route whereby β_3-adrenoceptor agonists may relax detrusor smooth

370 muscle.

371 Adenosine may also exert actions at other sites in the urinary bladder and contribute

372 to its overall effects. Spontaneous contractions in isolated detrusor smooth muscle

373 are accompanied by similar ATP release transients from motor nerves (36) and may

374 represent leakage from the two vesicular pools measured in this study. However, A_1

375 receptor involvement was not supported by the fact that neither adenosine nor CPA

376 altered spontaneous contractions or accompanying EJPs (31). In addition, the

377 mucosa is another source of ATP, evoked by mechanical or chemical stimuli (37,

378 38), in turn increased in tissue from patients with OAB (39). The neurotoxin, TTX,

379 completely abolished EFS-induced ATP release at all frequencies in these

380 experiments. With rat mucosal strips, EFS-induced ATP release was unaffected by

381 TTX, except small effects at 20 and 40 Hz and suggests a minor role for mucosal

382 ATP release in these experiments (38).

383 All adenosine receptors are expressed in the urothelium; however, an adenosine A_1

384 receptor agonist is the most potent stimulator of umbrella cell exocytosis, whilst the

385 A_1 receptor antagonist, DPCPX, was most effective at inhibiting adenosine-induced

386 changes in capacitance. This suggests that the A_1 receptor is the predominant

387 adenosine receptor regulating transmitter exocytosis at the mucosal surface (40).

388 However, adenosine reduces distension-induced ATP release, and A_1 receptor

389 antagonism enhanced urothelial ATP release (41). In cystometry studies, the

390 adenosine A_1 receptor agonist reduced threshold pressure, and intercontraction

391 intervals, which is reversed by DPCPX (42). Intravesical administration of adenosine

392 A_1 receptor agonist also has an inhibitory effect on micturition reflex but

393 administration of adenosine A_2 receptor agonists had no effect (43), suggesting a

394 potential role for adenosine A_1 receptors in stretch-activated urothelial ATP release

395 and targeting pathological purinergic sensory pathways.

396 **Cellular pathways mediating A_1 receptor activation**

397 Adenosine A_1 receptors use the prototypical transduction pathway for the G_i/G_o

398 protein family, inhibiting AC activity and decreasing cAMP levels (19), to reduce the

399 purinergic component of nerve-mediated contractions and neuronal ATP release

400 (Figure 5). Downstream intracellular pathways involving cAMP signaling include

401 intermediates like PKA and EPACs (19). In this study, the cAMP analogue/PKA

402 activator, 6-MB-cAMP, had no effect on neuronal ATP release, and on any

403 parameters of nerve-mediated contractions. The actions of adenosine on nerve-

404 mediated contractions and neuronal ATP release were also mimicked by the PKA

405 inhibitor, cAMPs-Rp. The addition of the EPAC activator, 007-AM, or EPAC inhibitor,

406 ESI-09, had no effect on neuronal ATP release or nerve-mediated contractions.

407 Although inhibiting EPACs reduces the inhibitory effect of forskolin on ACh release

408 (35), inhibiting PKA or EPACs had no direct effect on neuronal ACh release

409 measured in this study. Modulators of cAMP effectors PKA and EPACs have been

410 shown to regulate P_2X receptors and consequently atropine-resistant, purinergic

411 mediated contractions of detrusor (44, 45). This study suggests a role for

412 downstream PKA signaling, and not signaling via EPACs, in the regulation of

413 neuronal ATP release, with subsequent effects on detrusor (Figure 5). The presence

414 of activators of PKA or EPAC signaling did not have an impact on the ability of

415 adenosine to reduce ATP release from efferent nerve terminals and the low-

416 frequency, purinergic component of nerve-mediated contractions of detrusor smooth

417 muscle in the mouse bladder.

418 The pathways whereby A_1 receptor activation reduces neuronal ATP exocytosis, but

419 not ACh, is yet to be determined. Nerve-ending varicosities contain many vesicles

420 enclosing transmitters, that are released via Ca^{2+}-dependent exocytosis and Ca^{2+}

421 influx may be mediated by several channel types Ca^{2+} including N-type and P/Q-type

422 channels. With detrusor muscle it has been proposed that Ca^{2+} entry through N-type

423 channels is associated with ACh release, whilst P/Q-type channels regulate ATP

424 release (46-49). It is unclear if differential neurotransmitter release is from different

425 populations of nerves, or from different vesicles in the same varicosities. This study

426 suggests that ATP and ACh can be separately released from motor efferent nerves

427 that innervate bladder detrusor smooth muscle, and this is consistent with their

428 different frequency dependencies (28) and the ability to manipulate differential

429 release by modulation of cyclic nucleotides by PDE_5 inhibitors like sildenafil (27, 28),

430   or adenosine A_1 receptor activation. It has been demonstrated in several studies that

431   adenosine A_1 receptor activation can modulate neurotransmitter release (50),

432   suggesting a potential role for modulation of cyclic nucleotides and downstream

433   effects on protein kinases in the selective inhibition of purinergic neurotransmissions.

434   However, further studies are required to clarify the particular pathways that regulate

435   differential neurotransmitter release from efferent nerves.

436   Direct application of the AC activator, forskolin, had no effect on the amount of

437   neuronal ATP, nor ACh release, and is consistent with the lack of actions of the

438   cAMP analogue, 6-MB-cAMP on ATP release. However, forskolin inhibits the

439   atropine-resistant, purinergic component of nerve-mediated contractions in mouse

440   detrusor strips (44), also confirmed by this study. Forskolin also reduced resting

441   baseline tension, suggesting a direct effect on detrusor smooth muscle and

442   intracellular pathways involved in regulating tension. Detrusor contractions in the

443   mouse bladder induced by the P_2X receptor agonist, α,β-methylene ATP (α,β-me-

444   ATP), were inhibited by forskolin (44), suggesting a role for AC activation

445   downstream of ATP acting at P_2X receptors in detrusor smooth muscle. This was

446   further supported by the lack of effect on atropine-resistant nerve-mediated

447   contractions, and α,β-me-ATP-evoked contractions in the mouse detrusor by an AC

448   inhibitor, SQ22536 (44). Downstream intracellular pathways involving cAMP

449   signaling regulate activity of P_2X receptors (45). AC is also the target for

450   nonselective activators of G-protein coupled stimulatory pathways, via action on the

451   α subunit. For example, $β_3$-adrenoceptor agonists utilize the prototypical pathway for

452   the G_s protein family, modulating AC activity and increasing the level of cAMP (19),

453   and several studies have shown an effect of $β_3$-adrenoceptor activation on inhibiting

454   both purinergic and cholinergic contractions of the detrusor (44, 51). In this study,

455 activating AC with forskolin did not hinder the ability of adenosine to reduce neuronal

456 ATP release from efferent nerve terminals and further reduce the lower frequency,

457 purinergic component of nerve-mediated contractions of detrusor smooth muscle.

458 In conclusion, adenosine A_1 receptor agonism generated a frequency-dependent

459 attenuation of nerve-mediated contractions, with a greater effect at lower stimulation

460 frequencies where ATP release is more predominant, compared to ACh. This was

461 corroborated by direct measurement of reduced nerve-mediated ATP release. By

462 contrast, adenosine had no effect on ACh release or contractions at higher

463 stimulation frequencies. A selective adenosine A_1 receptor antagonist, DPCPX,

464 abolished the effects of adenosine, consistent with the hypothesis that adenosine

465 acts via A_1 receptor activation to regulate ATP release from efferent nerve terminals.

466 The main target for cAMP in mediating nerve-mediated ATP release is via PKA and

467 not by an EPAC route. Increasing cAMP levels had no effect, which implies normal

468 levels of intracellular cAMP are sufficient to maintain nerve-mediated ATP release.

469 This study demonstrates that differential regulation of transmitter release is possible

470 at the detrusor nerve-muscle junction. The ability to specifically attenuate ATP

471 release offers a novel therapeutic target, as ATP is associated with pathological

472 contractile function in the human bladder, whilst leaving physiological contractile

473 function unaffected. Adenosine selectively reduces ATP release from motor nerves

474 supplying detrusor smooth muscle, and the pathway of this action has been

475 characterized: initially by activation of an adenosine A_1 receptor, with downstream

476 inhibition of AC, cAMP generation, and PKA. Modulation of cyclic nucleotide levels,

477 such as cAMP, provides a novel target of pathological purinergic motor pathways

478 implicated in conditions like OAB.

479 **Perspectives and Significance**

480   The current first-line of treatment for OAB – antimuscarinics – have recognised

481   limitations, including uncertain efficacy as normal physiological pathways involved in

482   healthy bladder function are suppressed, and adverse side effects. Our study

483   demonstrated a novel finding in the bladder, where adenosine acting via adenosine

484   A_1 receptor activation, and downstream signaling of cAMP and PKA, selectively

485   inhibits ATP release from motor efferent nerves to detrusor smooth muscle, whilst

486   leaving ACh release intact. This is of clinical interest as pathological bladders in

487   humans are associated with these pathways – enhanced purinergic motor pathways.

488   Our findings offer the ability to differentially regulate neuronal transmitter release at

489   the neuromuscular junction, with the possibility to suppress pathological pathways,

490   rather than suppressing normal physiological pathways associated with ACh release,

491   as occurs at present with current therapeutic interventions.

References

1.   **Burnstock G, Cocks T, Crowe R, and Kasakov L**. Purinergic innervation of the guinea-pig urinary bladder. *Br J Pharmacol* 63: 125-138, 1978.

2.   **Burnstock G, Cocks T, Kasakov L, and Wong HK**. Direct evidence for ATP release from non-adrenergic, non-cholinergic ("purinergic") nerves in the guinea-pig taenia coli and bladder. *Eur J Pharmacol* 49: 145-149, 1978.

3.   **Burnstock G, Dumsday B, and Smythe A**. Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br J Pharmacol* 44: 451-461, 1972.

4.   **Campbell G**. Cotransmission. *Annu Rev Pharmacol Toxicol* 27: 51-70, 1987.

5.   **Bayliss M, Wu C, Newgreen D, Mundy AR, and Fry CH**. A quantitative study of atropine-resistant contractile responses in human detrusor smooth muscle, from stable, unstable and obstructed bladders. *J Urol* 162: 1833-1839, 1999.

6.   **Pakzad M, Ikeda Y, McCarthy C, Kitney DG, Jabr RI, and Fry CH**. Contractile effects and receptor analysis of adenosine-receptors in human detrusor muscle from stable and neuropathic bladders. *Naunyn Schmiedebergs Arch Pharmacol* 389: 921-929, 2016.

7.   **Fry CH, Bayliss M, Young JS, and Hussain M**. Influence of age and bladder dysfunction on the contractile properties of isolated human detrusor smooth muscle. *BJU Int* 108: E91-96, 2011.

8.   **Levin RM, Ruggieri MR, and Wein AJ**. Functional effects of the purinergic innervation of the rabbit urinary bladder. *J Pharmacol Exp Ther* 236: 452-457, 1986.

9.   **Yoshida M, Homma Y, Inadome A, Yono M, Seshita H, Miyamoto Y, Murakami S, Kawabe K, and Ueda S**. Age-related changes in cholinergic and purinergic neurotransmission in human isolated bladder smooth muscles. *Exp Gerontol* 36: 99-109, 2001.

10.   **McCarthy CJ, Ikeda Y, Skennerton D, Chakrabarty B, Kanai AJ, Jabr RI, and Fry CH**. Characterisation of nerve-mediated ATP release from bladder detrusor muscle and its pathological implications. *Br J Pharmacol* 176: 4720-4730, 2019.

11.   **Sjogren C, Andersson KE, Husted S, Mattiasson A, and Moller-Madsen B**. Atropine resistance of transmurally stimulated isolated human bladder muscle. *J Urol* 128: 1368-1371, 1982.

524 12. **Palea S, Artibani W, Ostardo E, Trist DG, and Pietra C**. Evidence for
525 purinergic neurotransmission in human urinary bladder affected by interstitial cystitis.
526 *J Urol* 150: 2007-2012, 1993.

527 13. **Cusack NJ, and Hourani SM**. Some pharmacological and biochemical
528 interactions of the enantiomers of adenylyl 5'-(beta, gamma-methylene)-
529 diphosphonate with the guinea-pig urinary bladder. *Br J Pharmacol* 82: 155-159,
530 1984.

531 14. **Deussen A**. Metabolic flux rates of adenosine in the heart. *Naunyn*
532 *Schmiedebergs Arch Pharmacol* 362: 351-363, 2000.

533 15. **Law WR, Conlon BA, and Ross JD**. The extracellular cardiac purine
534 metabolome in sepsis. *Shock* 28: 259-264, 2007.

535 16. **Lloyd HG, and Fredholm BB**. Involvement of adenosine deaminase and
536 adenosine kinase in regulating extracellular adenosine concentration in rat
537 hippocampal slices. *Neurochem Int* 26: 387-395, 1995.

538 17. **Hansen PB, and Schnermann J**. Vasoconstrictor and vasodilator effects of
539 adenosine in the kidney. *Am J Physiol Renal Physiol* 285: F590-599, 2003.

540 18. **Klinger M, Freissmuth M, and Nanoff C**. Adenosine receptors: G protein-
541 mediated signalling and the role of accessory proteins. *Cell Signal* 14: 99-108, 2002.

542 19. **Alexander SP, Christopoulos A, Davenport AP, Kelly E, Mathie A, Peters**
543 **JA, Veale EL, Armstrong JF, Faccenda E, Harding SD, Pawson AJ, Southan C,**
544 **Davies JA, Abbracchio MP, Alexander W, Al-Hosaini K, Back M, Barnes NM,**
545 **Bathgate R, Beaulieu JM, Bernstein KE, Bettler B, Birdsall NJM, Blaho V,**
546 **Boulay F, Bousquet C, Brauner-Osborne H, Burnstock G, Calo G, Castano JP,**
547 **Catt KJ, Ceruti S, Chazot P, Chiang N, Chini B, Chun J, Cianciulli A, Civelli O,**
548 **Clapp LH, Couture R, Csaba Z, Dahlgren C, Dent G, Singh KD, Douglas SD,**
549 **Dournaud P, Eguchi S, Escher E, Filardo EJ, Fong T, Fumagalli M, Gainetdinov**
550 **RR, Gasparo M, Gerard C, Gershengorn M, Gobeil F, Goodfriend TL, Goudet C,**
551 **Gregory KJ, Gundlach AL, Hamann J, Hanson J, Hauger RL, Hay DL,**
552 **Heinemann A, Hollenberg MD, Holliday ND, Horiuchi M, Hoyer D, Hunyady L,**
553 **Husain A, AP IJ, Inagami T, Jacobson KA, Jensen RT, Jockers R,**
554 **Jonnalagadda D, Karnik S, Kaupmann K, Kemp J, Kennedy C, Kihara Y,**
555 **Kitazawa T, Kozielewicz P, Kreienkamp HJ, Kukkonen JP, Langenhan T, Leach**
556 **K, Lecca D, Lee JD, Leeman SE, Leprince J, Li XX, Williams TL, Lolait SJ, Lupp**
557 **A, Macrae R, Maguire J, Mazella J, McArdle CA, Melmed S, Michel MC, Miller**

LJ, Mitolo V, Mouillac B, Muller CE, Murphy P, Nahon JL, Ngo T, Norel X, Nyimanu D, O'Carroll AM, Offermanns S, Panaro MA, Parmentier M, Pertwee RG, Pin JP, Prossnitz ER, Quinn M, Ramachandran R, Ray M, Reinscheid RK, Rondard P, Rovati GE, Ruzza C, Sanger GJ, Schoneberg T, Schulte G, Schulz S, Segaloff DL, Serhan CN, Stoddart LA, Sugimoto Y, Summers R, Tan VP, Thal D, Thomas WW, Timmermans P, Tirupula K, Tulipano G, Unal H, Unger T, Valant C, Vanderheyden P, Vaudry D, Vaudry H, Vilardaga JP, Walker CS, Wang JM, Ward DT, Wester HJ, Willars GB, Woodruff TM, Yao C, and Ye RD. THE CONCISE GUIDE TO PHARMACOLOGY 2021/22: G protein-coupled receptors. *Br J Pharmacol* 178 Suppl 1: S27-S156, 2021.

20.   **Acevedo CG, Contreras E, Escalona J, Lewin J, and Huidobro-Toro JP**. Pharmacological characterization of adenosine A1 and A2 receptors in the bladder: evidence for a modulatory adenosine tone regulating non-adrenergic non-cholinergic neurotransmission. *Br J Pharmacol* 107: 120-126, 1992.

21.   **Gallo-Rodriguez C, Ji XD, Melman N, Siegman BD, Sanders LH, Orlina J, Fischer B, Pu Q, Olah ME, van Galen PJ, and et al.** Structure-activity relationships of N6-benzyladenosine-5'-uronamides as A3-selective adenosine agonists. *J Med Chem* 37: 636-646, 1994.

22.   **King JA, Huddart H, and Staff WG**. Purinergic modulation of rat urinary bladder detrusor smooth muscle. *Gen Pharmacol* 29: 597-604, 1997.

23.   **Parija SC, Raviprakash V, and Mishra SK**. Adenosine- and alpha,beta-methylene ATP-induced differential inhibition of cholinergic and non-cholinergic neurogenic responses in rat urinary bladder. *Br J Pharmacol* 102: 396-400, 1991.

24.   **Fry CH, Ikeda Y, Harvey R, Wu C, and Sui GP**. Control of bladder function by peripheral nerves: avenues for novel drug targets. *Urology* 63: 24-31, 2004.

25.   **Husted S, Sjogren C, and Andersson KE**. Direct effects of adenosine and adenine nucleotides on isolated human urinary bladder and their influence on electrically induced contractions. *J Urol* 130: 392-398, 1983.

26.   **Silva-Ramos M, Silva I, Faria M, Magalhaes-Cardoso MT, Correia J, Ferreirinha F, and Correia-de-Sa P**. Impairment of ATP hydrolysis decreases adenosine A1 receptor tonus favoring cholinergic nerve hyperactivity in the obstructed human urinary bladder. *Purinergic Signal* 11: 595-606, 2015.

27.   **Chakrabarty B, Ito H, Ximenes M, Nishikawa N, Vahabi B, Kanai AJ, Pickering AE, Drake MJ, and Fry CH**. Influence of sildenafil on the purinergic

components of nerve-mediated and urothelial ATP release from the bladder of normal and spinal cord injured mice. *Br J Pharmacol* 176: 2227-2237, 2019.

28.	**Chakrabarty B, Aitchison K, White P, McCarthy CJ, Kanai AJ, and Fry CH**. Frequency-dependent characteristics of nerve-mediated ATP and acetylcholine release from detrusor smooth muscle. *Exp Physiol* 2022.

29.	**Curran-Everett D, and Benos DJ**. Guidelines for reporting statistics in journals published by the American Physiological Society. *Am J Physiol Regul Integr Comp Physiol* 287: R247-249, 2004.

30.	**Searl TJ, and Silinsky EM**. Modulation of purinergic neuromuscular transmission by phorbol dibutyrate is independent of protein kinase C in murine urinary bladder. *J Pharmacol Exp Ther* 342: 312-317, 2012.

31.	**Searl TJ, Dynda DI, Alanee SR, El-Zawahry AM, McVary KT, and Silinsky EM**. A1 Adenosine Receptor-Mediated Inhibition of Parasympathetic Neuromuscular Transmission in Human and Murine Urinary Bladder. *J Pharmacol Exp Ther* 356: 116-122, 2016.

32.	**Dixon AK, Gubitz AK, Sirinathsinghji DJ, Richardson PJ, and Freeman TC**. Tissue distribution of adenosine receptor mRNAs in the rat. *Br J Pharmacol* 118: 1461-1468, 1996.

33.	**Nicholls J, Hourani SM, and Kitchen I**. Characterization of P1-purinoceptors on rat duodenum and urinary bladder. *Br J Pharmacol* 105: 639-642, 1992.

34.	**Silva I, Costa AF, Moreira S, Ferreirinha F, Magalhaes-Cardoso MT, Calejo I, Silva-Ramos M, and Correia-de-Sa P**. Inhibition of cholinergic neurotransmission by beta3-adrenoceptors depends on adenosine release and A1-receptor activation in human and rat urinary bladders. *Am J Physiol Renal Physiol* 313: F388-F403, 2017.

35.	**Silva I, Magalhaes-Cardoso MT, Ferreirinha F, Moreira S, Costa AF, Silva D, Vieira C, Silva-Ramos M, and Correia-de-Sa P**. beta3 Adrenoceptor-induced cholinergic inhibition in human and rat urinary bladders involves the exchange protein directly activated by cyclic AMP 1 favoring adenosine release. *Br J Pharmacol* 177: 1589-1608, 2020.

36.	**McCarthy CJ, Marangos C, Fry CH, and Ikeda Y**. ATP transients accompany spontaneous contractions in isolated guinea-pig detrusor smooth muscle. *Exp Physiol* 104: 1717-1725, 2019.

625　37.　**Ferguson DR, Kennedy I, and Burton TJ**. ATP is released from rabbit

626　urinary bladder epithelial cells by hydrostatic pressure changes--a possible sensory

627　mechanism? *J Physiol* 505 (Pt 2): 503-511, 1997.

628　38.　**Sadananda P, Shang F, Liu L, Mansfield KJ, and Burcher E**. Release of

629　ATP from rat urinary bladder mucosa: role of acid, vanilloids and stretch. *Br J*

630　*Pharmacol* 158: 1655-1662, 2009.

631　39.　**Munoz A, Smith CP, Boone TB, and Somogyi GT**. Overactive and

632　underactive bladder dysfunction is reflected by alterations in urothelial ATP and NO

633　release. *Neurochem Int* 58: 295-300, 2011.

634　40.　**Yu W, Zacharia LC, Jackson EK, and Apodaca G**. Adenosine receptor

635　expression and function in bladder uroepithelium. *Am J Physiol Cell Physiol* 291:

636　C254-265, 2006.

637　41.　**Dunning-Davies BM, Fry CH, Mansour D, and Ferguson DR**. The

638　regulation of ATP release from the urothelium by adenosine and transepithelial

639　potential. *BJU Int* 111: 505-513, 2013.

640　42.　**Prakasam HS, Herrington H, Roppolo JR, Jackson EK, and Apodaca G**.

641　Modulation of bladder function by luminal adenosine turnover and A1 receptor

642　activation. *Am J Physiol Renal Physiol* 303: F279-292, 2012.

643　43.　**Kitta T, Chancellor MB, de Groat WC, Kuno S, Nonomura K, and**

644　**Yoshimura N**. Roles of adenosine A1 and A2A receptors in the control of micturition

645　in rats. *Neurourol Urodyn* 33: 1259-1265, 2014.

646　44.　**Fong Z, Griffin CS, Hollywood MA, Thornbury KD, and Sergeant GP**.

647　beta3-Adrenoceptor agonists inhibit purinergic receptor-mediated contractions of the

648　murine detrusor. *Am J Physiol Cell Physiol* 317: C131-C142, 2019.

649　45.　**Fong Z, Griffin CS, Large RJ, Hollywood MA, Thornbury KD, and**

650　**Sergeant GP**. Regulation of P2X1 receptors by modulators of the cAMP effectors

651　PKA and EPAC. *Proc Natl Acad Sci U S A* 118: 2021.

652　46.　**Maggi CA**. Omega conotoxin and prejunctional modulation of the biphasic

653　response of the rat isolated urinary bladder to single pulse electrical field stimulation.

654　*J Auton Pharmacol* 11: 295-304, 1991.

655　47.　**Zygmunt PM, Zygmunt PK, Hogestatt ED, and Andersson KE**. Effects of

656　omega-conotoxin on adrenergic, cholinergic and NANC neurotransmission in the

657　rabbit urethra and detrusor. *Br J Pharmacol* 110: 1285-1290, 1993.

658   48.   **Frew R, and Lundy PM**. A role for Q type Ca2+ channels in
659   neurotransmission in the rat urinary bladder. *Br J Pharmacol* 116: 1595-1598, 1995.

660   49.   **Waterman SA**. Multiple subtypes of voltage-gated calcium channel mediate
661   transmitter release from parasympathetic neurons in the mouse bladder. *J Neurosci*
662   16: 4155-4161, 1996.

663   50.   **Veggetti M, Muchnik S, and Losavio A**. Effect of purines on calcium-
664   independent acetylcholine release at the mouse neuromuscular junction.
665   *Neuroscience* 154: 1324-1336, 2008.

666   51.   **Griffin CS, Bradley E, Hollywood MA, McHale NG, Thornbury KD, and**
667   **Sergeant GP**. beta3-adrenoceptor agonists inhibit carbachol-evoked Ca(2+)
668   oscillations in murine detrusor myocytes. *BJU Int* 121: 959-970, 2018.

669

Figure legends

Figure 1. (A) Representative traces of nerve-mediated contractions under control

conditions and with adenosine (1 mM, n=12) at 1, 4 and 20 Hz stimulation. At 1Hz

stimulation, the response is measured from the final transient of the individual stimuli

(dotted line). **(B)** Percentage reduction of nerve-mediated contractions by adenosine.

Fits are from Equation (1a), Materials and Methods. **(C)** Force-frequency relationship

curves for nerve-mediated contractions in control and in the presence of adenosine.

Fits are from Equation (1b). Parameters measured include T_{max} (mN.mg^{-1}) and $f_{1/2}$

(Hz) and are included in Table 2. **(D)** ATP release-frequency relationship curves for

nerve-mediated transmitter release in control and in the presence of adenosine. Fits

are from Equation (1b). **(E)** Percentage reduction of nerve-mediated ATP release by

adenosine. Fits are from Equation (1a). **(F)** Adenosine had no effect on nerve-

mediated ACh release (fmol.µl^{-1}.mg^{-1}) at 20 Hz stimulation (n=6, *Student's paired t-*

test). Individual data points for data sets are illustrated in Figure S1.

Figure 2. (A) Percentage reduction of nerve-mediated contractions by CPA (10 µM,

n=6) and NECA (10 µM, n=6). Fits are from Equation (1a). Representative traces of

nerve-mediated contractions under control conditions and with **(B)** CPA and **(C)**

NECA, at 1, 2 and 20 Hz stimulation. **(C)** Percentage reduction of nerve-mediated

contractions by cAMPs-Rp (10 µM, n=6). Fits are from Equation (1a).

Figure 3. (A) Relationship between tension and ATP release at low stimulation

frequencies. Values of $Y_{Lf,max}$ from Equation (1b) were used to analyze the effect of

interventions on purinergic contractions without the involvement of nerve-mediated

ACh release, specifically at 1 and 2 Hz. There is a significant (r=0.97, p=0.001)

relationship between reduction of both tension and ATP release at low stimulation

frequencies. **(B)** At low frequencies, no nerve-mediated ACh release was recorded.

695   In this example, CPA (10 µM, n=6) had no effect on nerve-mediated ACh release

696   across the frequency range. Fits are from Equation (1c).

697   **Figure 4. (A)** Percentage reduction of nerve-mediated contractions by forskolin (1

698   µM) and adenosine (1 mM) in the presence of forskolin (n=6). **(B)** Percentage

699   reduction of nerve-mediated contractions by atropine (1 µM) and forskolin (1 µM) in

700   the presence of atropine (n=6). Fits are from Equation (1b).

701   **Figure 5.** Schematic diagram displaying the prototypical transduction pathway upon

702   the activation of adenosine A_1 receptors by adenosine. Activation of this receptor

703   inhibits AC activity and decreases cAMP levels. Downstream signaling pathways

704   involving cAMP signaling include PKA, which plays a role in reducing neuronal ATP

705   release (red) from efferent nerve terminals and the purinergic component of nerve-

706   mediated contractions. Activation of adenosine A_1 receptors had no effect on

707   neuronal ACh release (green) and the cholinergic component of nerve-mediated

708   contractions in the mouse detrusor.

709

710   **Table 1.** Mechanism of action of drugs used in this study.

Drug	Mechanism of action	Concentration used
Adenosine	Endogenous adenosine receptor agonist	1 mM
CPA	Selective adenosine A_1 receptor agonist	10 µM
NECA	Adenosine A_1/A_2 receptor agonist	10 µM
DPCPX	Selective adenosine A_1 receptor antagonist	1 µM
Forskolin	AC activator	1 µM
Atropine	Non-selective muscarinic receptor antagonist	1 µM
cAMPs-Rp	Selective PKA inhibitor (cell permeable)	10 µM
6-MB-cAMP	PKA activator	100 µM
007-AM	EPAC activator	10 µM
ESI-09	EPAC inhibitor	20 µM

711

Table 2. Force-frequency curve parameters, T_{max} and $f_{1/2}$, with modulation of cAMP-dependent signaling pathways. Mean data ± SD. *$p<0.05$; **$p<0.01$; ***$p<0.001$ with respect to control or immediately preceding intervention, exact p values are also shown (repeated measures ANOVA followed by parametric *post hoc* test, see Materials and Methods).

Intervention	T_{max} (mN.mg^{-1})	p	$f_{1/2}$ (Hz)	p
Control (n=12)	2.18 ± 0.66		5.1 ± 1.3	
+ adenosine	2.19 ± 0.61	0.893	7.5 ± 2.0 **	0.002
Control (n=6)	1.81 ± 0.41		4.0 ± 2.2	
+ DPCPX	1.96 ± 0.35	0.068	4.4 ± 1.4	0.218
+ DPCPX, aden.	2.01 ± 0.34	0.410	4.7 ± 0.8	0.423
Control (n=6)	1.92 ± 0.60		5.8 ± 1.5	
+ CPA	1.95 ± 0.67	0.077	9.0 ± 2.4 ***	0.0002
Control (n=6)	1.94 ± 0.56		5.8 ± 1.5	
+ NECA	2.12 ± 0.57	0.124	10.7 ± 2.3 **	0.001
Control (n=6)	1.99 ± 0.55		5.9 ± 1.4	
+ cAMPs-Rp	2.15 ± 0.54 **	0.006	7.4 ± 1.3 **	0.006
Control (n=8)	2.09 ± 0.30		6.6 ± 1.6	
+ ESI-09	2.22 ± 0.42	0.293	6.7 ± 1.1	0.826
Control (n=6)	2.24 ± 0.31		5.6 ± 0.8	
+ 6-MB-cAMP	2.26 ± 0.39	0.814	5.9 ± 1.4	0.730
+ 6-MB-cAMP, aden.	2.37 ± 0.36	0.150	8.5 ± 1.8 *	0.026
Control (n=6)	2.60 ± 0.72		6.6 ± 1.3	
+ 007-AM	2.86 ± 1.06	0.062	5.9 ± 1.4	0.479
+ 007-AM, aden.	2.73 ± 0.82	0.369	9.1 ± 1.7 *	0.0001
Control (n=6)	2.16 ± 0.17		5.5 ± 0.9	
+ forskolin (FSK)	2.30 ± 0.21	0.129	10.5 ± 3.1 *	0.019
+ FSK, aden.	2.20 ± 0.34	0.731	19.4 ± 7.7 **	0.008
Control (n=6)	1.84 ± 0.46		3.9 ± 0.4	
+ atropine	1.21 ± 0.32 **	0.003	2.4 ± 0.1 **	0.002
+ atropine, FSK	0.60 ± 0.17 **	0.002	2.2 ± 0.6 **	0.010

717   Individual data points in Figures S1-6 (Supplemental Material available at URL:

718   https://figshare.com/s/42c3169843f6cdfd3e68,   DOI:

719   https://doi.org/10.6084/m9.figshare.21253557).

Table 3. Percentage reduction (red^n) of nerve-mediated contractions and ATP/ACh release, with modulation of cAMP-dependent signaling pathways. Reductions are referenced to control conditions, and in cases when adenosine is also added after an intervention, with reference to that preceding intervention. Tension and ATP values are the averaged reductions at 1 and 2 Hz stimulation, ACh values are as recorded at 20 Hz stimulation. Mean data ± SD, * $p<0.05$, ** $p<0.01$; *** $p<0.001$ with respect to control or immediately preceding intervention, exact p-values are also shown.
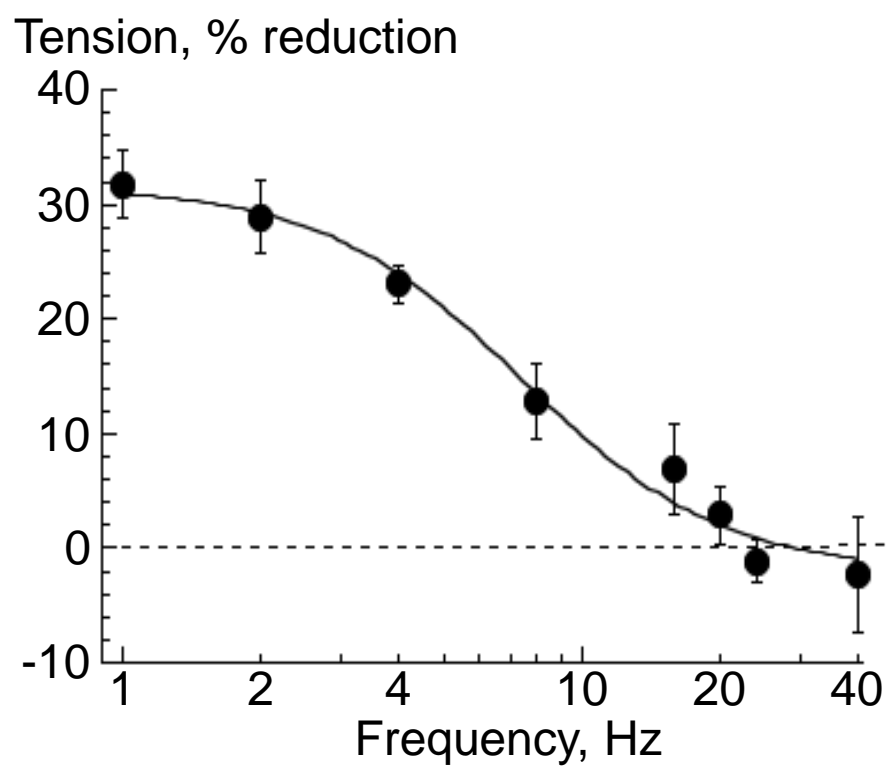
Intervention	n	Tension, %red^n 1, 2 Hz	p	ATP, %red^n 1, 2 Hz	p	ACh, %red^n 20 Hz	p
Adenosine	12; 6 ACh	29.8 ± 6.6 ***	<0.0001	34.1 ± 6.8 ***	<0.0001	-1.0 ± 10.5	0.697
DPCPX	6	-2.2 ± 6.7	0.539	-0.3 ± 1.7	0.469	Not recorded	
DPCPX, aden.		2.1 ± 12.1	0.524	2.2 ± 4.5	0.420		
CPA	6	50.7 ± 5.7 **	0.003	44.1 ± 4.4 ***	<0.0001	-5.6 ± 7.2	0.119
NECA	6	69.2 ± 9.1 ***	0.0005	51.8 ± 8.6 ***	<0.0001	-0.7 ± 10.2	0.983
cAMPs-Rp	6	46.4 ± 2.4 **	0.002	35.5 ± 3.2 ***	<0.0001	0.4 ± 5.1	0.469
ESI-09	6	0.1 ± 11.5	0.900	-1.2 ± 2.4	0.334	0.4 ± 9.9	0.962
6-MB-cAMP	6	3.1 ± 2.6	0.173	-3.0 ± 3.3	0.349	Not recorded	
6-MB-cAMP, aden.		41.4 ± 18.4 **	0.008	32.1 ± 4.4 ***	0.0002		
007-AM	6	4.3 ± 1.2	0.251	-0.7 ± 3.2	0.482	Not recorded	
007-AM, aden.		34.0 ± 2.8 **	0.004	30.3 ± 8.3 *	0.014		

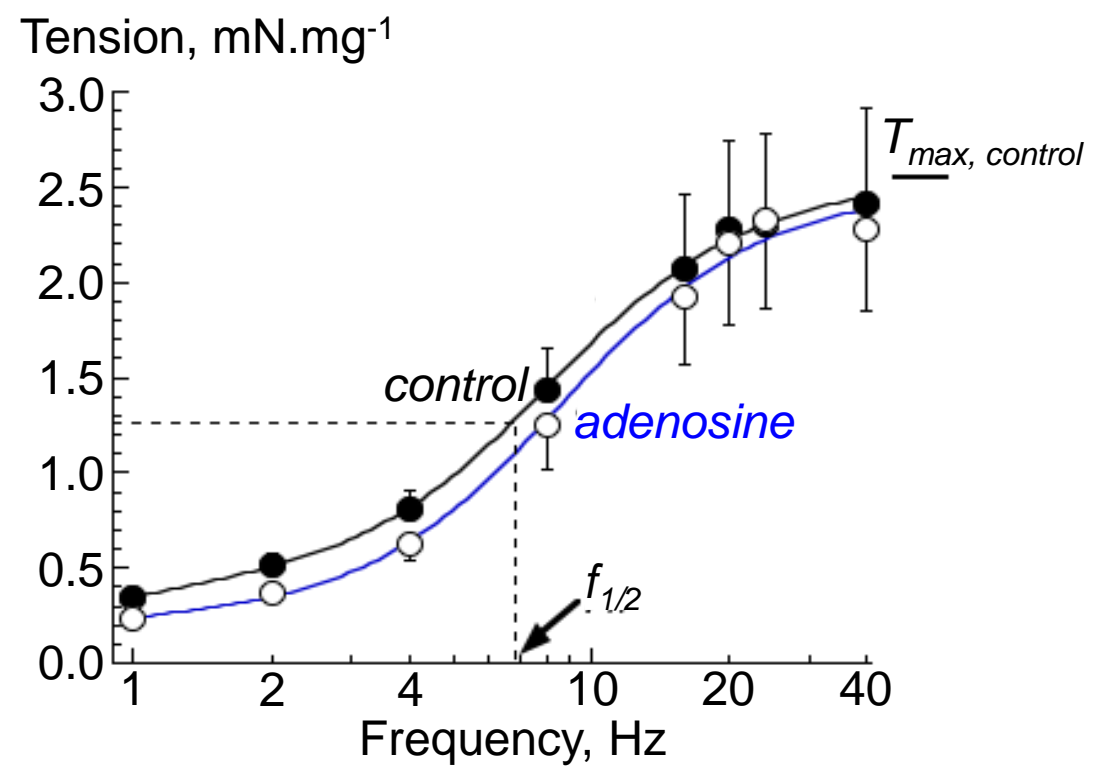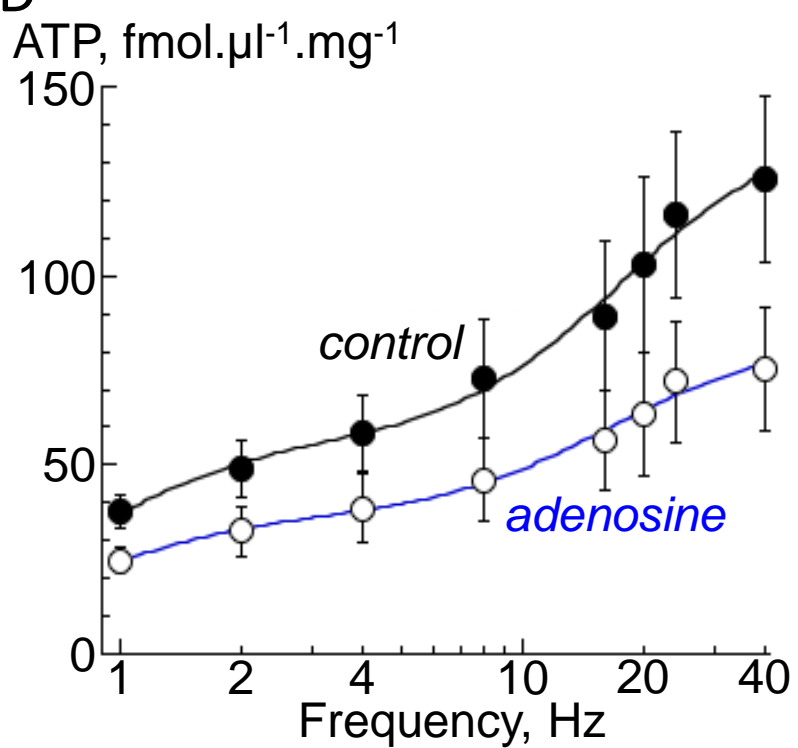Individual data points in Figures S1-6 (Supplemental Material available at URL: https://figshare.com/s/42c3169843f6cdfd3e68, DOI: https://doi.org/10.6084/m9.figshare.21253557).

A

control

adenosine

1 Hz 4 Hz 20 Hz

0.5 mN.mg^{-1}

1 s

B

Tension, % reduction

Frequency, Hz

C

Tension, mN.mg^{-1}

$T_{max, control}$

control

adenosine

$f_{1/2}$

Frequency, Hz

D

ATP, fmol.µl^{-1}.mg^{-1}

control

adenosine

Frequency, Hz

E

ATP, % reduction

Frequency, Hz

F

ACh, fmol.µl^{-1}.mg^{-1}

p=0.696

control adenosine

A

Tension, % reduction

NECA

CPA

Frequency, Hz

B

control

CPA

0.5

|mN.mg^{-1}

C

control

NECA

0.5

|mN.mg^{-1}

1 Hz

2 Hz

20 Hz

1 s

D

Tension, % reduction

cAMPs-Rp

Frequency, Hz

A

Tension, % reduction

NECA

CPA

cAMPs-Rp

adenosine

007-AM

ATP, % reduction

B

ACh, fmol^{-1}·µl^{-1}·mg^{-1}

+CPA

control

Frequency, Hz

A

Tension, % reduction

FSK + adenosine

FSK

Frequency, Hz
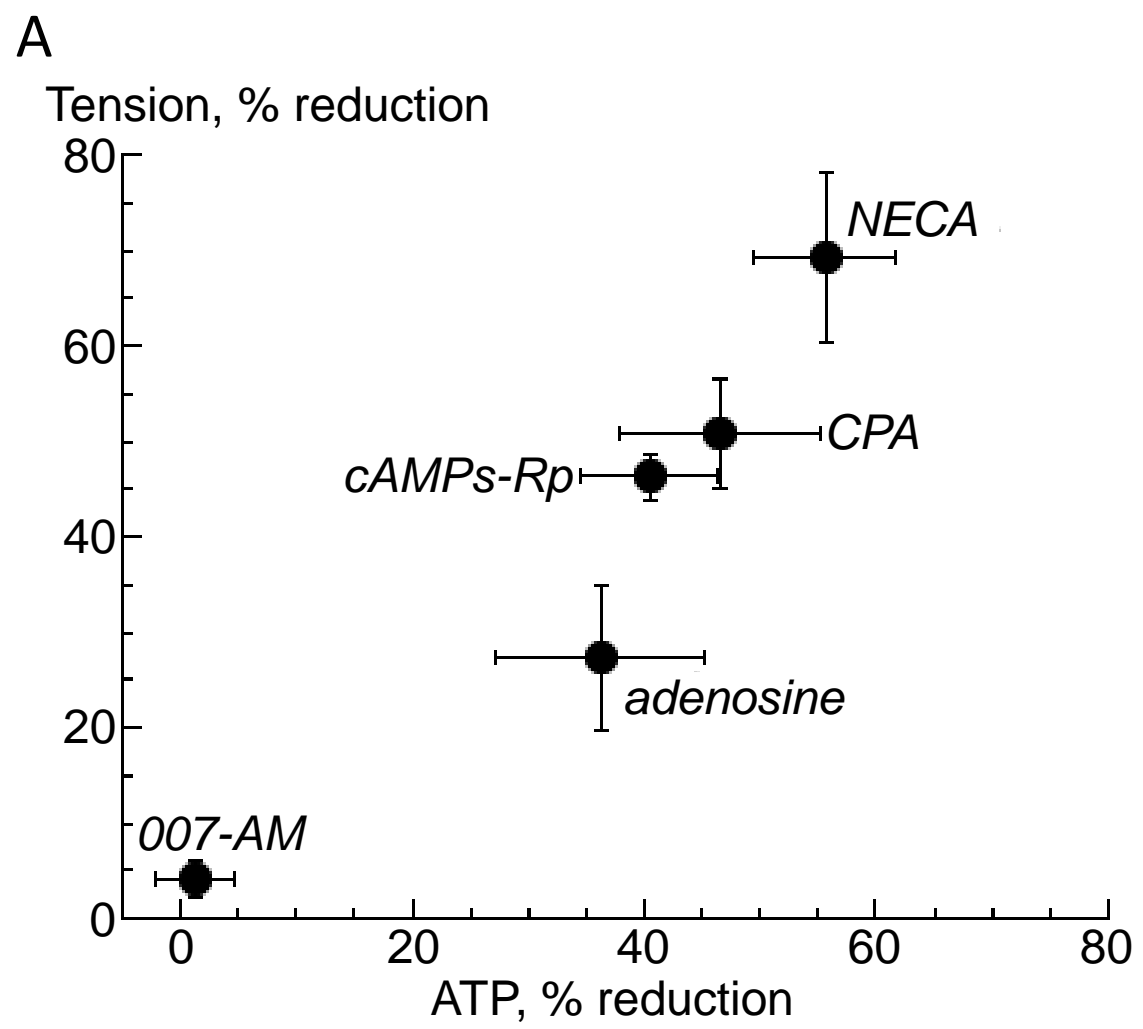
B

Tension, % reduction

Atropine + FSK

Atropine

Frequency, Hz

1   **Table 1.** Mechanism of action of drugs used in this study.

Drug	Mechanism of action	Concentration used
Adenosine	Endogenous adenosine receptor agonist	1 mM
CPA	Selective adenosine A_1 receptor agonist	10 µM
NECA	Adenosine A_1/A_2 receptor agonist	10 µM
DPCPX	Selective adenosine A_1 receptor antagonist	1 µM
Forskolin	AC activator	1 µM
Atropine	Non-selective muscarinic receptor antagonist	1 µM
cAMPs-Rp	Selective PKA inhibitor (cell permeable)	10 µM
6-MB-cAMP	PKA activator	100 µM
007-AM	EPAC activator	10 µM
ESI-09	EPAC inhibitor	20 µM

2

Table 2. Force-frequency curve parameters, T_{max} and $f_{1/2}$, with modulation of cAMP-dependent signaling pathways. Mean data ± SD. *$p<0.05$; **$p<0.01$; ***$p<0.001$ with respect to control or immediately preceding intervention, exact p values are also shown (repeated measures ANOVA followed by parametric *post hoc* test, see Materials and Methods).

Intervention	T_{max} (mN.mg^{-1})	p	$f_{1/2}$ (Hz)	p
Control (*n*=12)	2.18 ± 0.66		5.1 ± 1.3	
+ adenosine	2.19 ± 0.61	0.893	7.5 ± 2.0 **	0.002
Control (*n*=6)	1.81 ± 0.41		4.0 ± 2.2	
+ DPCPX	1.96 ± 0.35	0.068	4.4 ± 1.4	0.218
+ DPCPX, aden.	2.01 ± 0.34	0.410	4.7 ± 0.8	0.423
Control (*n*=6)	1.92 ± 0.60		5.8 ± 1.5	
+ CPA	1.95 ± 0.67	0.077	9.0 ± 2.4 ***	0.0002
Control (*n*=6)	1.94 ± 0.56		5.8 ± 1.5	
+ NECA	2.12 ± 0.57	0.124	10.7 ± 2.3 **	0.001
Control (*n*=6)	1.99 ± 0.55		5.9 ± 1.4	
+ cAMPs-Rp	2.15 ± 0.54 **	0.006	7.4 ± 1.3 **	0.006
Control (*n*=8)	2.09 ± 0.30		6.6 ± 1.6	
+ ESI-09	2.22 ± 0.42	0.293	6.7 ± 1.1	0.826
Control (*n*=6)	2.24 ± 0.31		5.6 ± 0.8	
+ 6-MB-cAMP	2.26 ± 0.39	0.814	5.9 ± 1.4	0.730
+ 6-MB-cAMP, aden.	2.37 ± 0.36	0.150	8.5 ± 1.8 *	0.026
Control (*n*=6)	2.60 ± 0.72		6.6 ± 1.3	
+ 007-AM	2.86 ± 1.06	0.062	5.9 ± 1.4	0.479
+ 007-AM, aden.	2.73 ± 0.82	0.369	9.1 ± 1.7 *	0.0001
Control (*n*=6)	2.16 ± 0.17		5.5 ± 0.9	
+ forskolin (FSK)	2.30 ± 0.21	0.129	10.5 ± 3.1 *	0.019
+ FSK, aden.	2.20 ± 0.34	0.731	19.4 ± 7.7 **	0.008
Control (*n*=6)	1.84 ± 0.46		3.9 ± 0.4	
+ atropine	1.21 ± 0.32 **	0.003	2.4 ± 0.1 **	0.002
+ atropine, FSK	0.60 ± 0.17 **	0.002	2.2 ± 0.6 **	0.010

6   Individual data points in Figures S1-6 (Supplemental Material available at URL:

7   https://figshare.com/s/42c3169843f6cdfd3e68,   DOI:

8   https://doi.org/10.6084/m9.figshare.21253557).

Table 3. Percentage reduction (red^n) of nerve-mediated contractions and ATP/ACh release, with modulation of cAMP-dependent signaling pathways. Reductions are referenced to control conditions, and in cases when adenosine is also added after an intervention, with reference to that preceding intervention. Tension and ATP values are the averaged reductions at 1 and 2 Hz stimulation, ACh values are as recorded at 20 Hz stimulation. Mean data ± SD, * $p<0.05$, ** $p<0.01$; *** $p<0.001$ with respect to control or immediately preceding intervention, exact p-values are also shown.

Intervention	n	Tension, %red^n 1, 2 Hz	p	ATP, %red^n 1, 2 Hz	p	ACh, %red^n 20 Hz	p
Adenosine	12; 6 ACh	29.8 ± 6.6 ***	<0.0001	34.1 ± 6.8 ***	<0.0001	-1.0 ± 10.5	0.697
DPCPX	6	-2.2 ± 6.7	0.539	-0.3 ± 1.7	0.469	Not recorded	
DPCPX, aden.		2.1 ± 12.1	0.524	2.2 ± 4.5	0.420		
CPA	6	50.7 ± 5.7 **	0.003	44.1 ± 4.4 ***	<0.0001	-5.6 ± 7.2	0.119
NECA	6	69.2 ± 9.1 ***	0.0005	51.8 ± 8.6 ***	<0.0001	-0.7 ± 10.2	0.983
cAMPs-Rp	6	46.4 ± 2.4 **	0.002	35.5 ± 3.2 ***	<0.0001	0.4 ± 5.1	0.469
ESI-09	6	0.1 ± 11.5	0.900	-1.2 ± 2.4	0.334	0.4 ± 9.9	0.962
6-MB-cAMP	6	3.1 ± 2.6	0.173	-3.0 ± 3.3	0.349	Not recorded	
6-MB-cAMP, aden.		41.4 ± 18.4 **	0.008	32.1 ± 4.4 ***	0.0002		
007-AM	6	4.3 ± 1.2	0.251	-0.7 ± 3.2	0.482	Not recorded	
007-AM, aden.		34.0 ± 2.8 **	0.004	30.3 ± 8.3 *	0.014		

Individual data points in Figures S1-6 (Supplemental Material available at URL: https://figshare.com/s/42c3169843f6cdfd3e68, DOI: https://doi.org/10.6084/m9.figshare.21253557).