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SHORT COMMUNICATIONS

Cell surface localization of P_2-purinergic receptors in vas deferens*

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The concentration–response curve for ATP-induced contraction of the guinea-pig vas deferens is biphasic [1, 2]. $ANAPP_3$,† a photoaffinity analog of ATP [3], produces a specific pharmacological antagonism of ATP-induced contractions following photolysis in the presence of the isolated smooth muscle [1]. This agent appears to block the first phase of the ATP concentration–response curve but has no effect on the maximum response. We have suggested that the antagonism is receptor mediated, that is, ATP-induced contraction occurs in a manner consistent with agonist occupation of cell-surface P_2-purinergic [4, 5] receptors.

The mechanism of action of ATP in inducing contraction might also involve one or more unrelated mechanisms, i.e. carrier-mediated intracellular transport [6, 7] of ATP, the generation of a permeant or nonpermeant pharmacologically active metabolite [8], membrane premeability changes [9–12] or obligatory hydrolysis of ATP *per se*.‡ It is possible that $ANAPP_3$ could have caused blockade by interfering with one of these alternative mechanisms. To determine the site of action of ATP and $ANAPP_3$, we sought derivatives of ATP which would induce a contractile response in the guinea-pig vas deferens but be less susceptible to cellular penetration and extracellular enzyme rearrangement than ATP. Therefore, we evaluated the ability of ATP covalently linked to 4% agarose beads to evoke responses.

Vasa deferentia were removed from guinea pigs and prepared for the measurement of isometric contractions *in vitro* as described previously [13]. Concentration–response relationships were determined for three different preparations of ATP linked covalently to agarose beads. The beads as provided by the manufacturer (P-L Biochemicals Inc., Milwaukee, WI) were pelleted by centrifugation (1300 $g \times 10$ min), resuspended in 50 vol. % of Krebs–Henseleit solution [13], and washed five times by centrifugation to remove preservatives. Washed beads suspended (50 vol. %) in Krebs–Henseleit solution (37°) were added to the organ bath and maintained in suspension by aeration (95% O_2-5% CO_2) of the Krebs–Henseleit physiological solution. The three preparations of agarose-ATP tested differed in the site of attachment of ATP to the hexane bridge connecting the ATP moiety to the agarose bead. For AGATP-4, ATP is bound at the ribose hydroxyl group; for AGATP-3, ATP is bound at the C^8 position of adenine; for AGATP-2, ATP is bound at the N^6 position of adenine. The molar equivalents of ATP added to the bath were calculated from the manufacturer's determination of incorporation of ATP into the agarose beads. The bead suspensions added to the organ bath remained present for 3 min. After washout 10 min elapsed between administrations. Responses are expressed in terms of the maximum response of the tissue to 120 mM KCl, which was determined at the end of the experiment.

To evaluate the effect of $ANAPP_3$ treatment on responses to AGATP, test vasa deferentia in glass, water-jacketed organ chambers were preincubated for 3 min in the dark with 10^{-4} M $ANAPP_3$. Control (no $ANAPP_3$ present) and test preparations were then irradiated (+hv) for 15 min with a tungsten–halogen projector lamp (DYH; filament 15 cm from center of chamber). The $ANAPP_3$ was washed from the bath prior to the addition of AGATP 10 min later.

Microsomes and cytosol from vasa deferentia were obtained as described previously [14]. Mg^{2+}-ATPase activity was assayed in media (0.4 ml) containing Tris·Cl, pH 7.4 (37°), 6 mM $MgCl_2$, 0.1 mM EGTA and 1 mM ATP or AGATP (0.7, 0.7 or 1.4 "mM" ATP equivalents for AGATP-2, AGATP-3 and AGATP-4 respectively). Preservative in the AGATP suspensions were eliminated prior to the assays by centrifugation (1300 $g \times 10$ min; five times) of the beads which had been suspended (50 vol. %) in distilled water. When ATP was substrate, reactions were begun with ATP; when AGATP was substrate, reactions were begun with protein (microsomes: 9 μg; cytosol: 135 μg). Inorganic phosphate (P_i) measurement and additional assay details have been described [15].

The addition of AGATP-4 to the organ bath elicited "concentration"-related monotonic contractile responses in the vas deferens (Fig. 1) similar to that well-documented for ATP in this preparation. The responses to AGATP-4 were reduced significantly following photolytic treatment of the tissues with 10^{-4} M $ANAPP_3$ (Fig. 1), a specific P_2-purinergic receptor antagonist in isolated smooth muscle preparations [1, 16, 17]. The addition of AGATP-3 (N = 8) and agarose not covalently bound to ATP (N = 7) to the organ bath failed to contract the vasa deferentia. In one out of eight preparations, AGATP-2 produced a modest contraction of the tissue at the largest concentration employed (10^{-3} M equivalents of ATP).

The presence of nucleotides associated with, but not covalently bound to, the agarose beads was evaluated to determine if contaminants in the suspensions, rather than ATP bound to agarose *per se*, could have elicited the responses. Aliquots of post-6000 $g \times 15$ min supernatant fractions of beads washed in Krebs–Henseleit solution, as described for the organ bath experiments, were analyzed for nucleotide content using high performance liquid chromatography in conjunction with fluorescence detection [18]. In the 50 vol. % bead suspensions, soluble nucleotide contaminants were present (in pmoles/ml) as follows: in AGATP-2: ATP, 20 and ADP, 7; in AGATP-3: no nucleotides detected: in AGATP-4: ATP, 461. The addition of 10^{-3} and 3×10^{-3} "M" AGATP-4 to the organ baths was calculated to expose the tissues to 1.4×10^{-7} M and 4.1×10^{-7} M contaminant ATP respectively. These concentrations are at or above threshold for eliciting contraction [19]. Figure 1 illustrates that 10^{-3} and 3×10^{-3} "M" AGATP-4 produced responses which were 14 and 28% of that elicited with 120 mM KCl. A rigorous analysis of ATP

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† Abbreviations: $ANAPP_3$, arylazido aminopropionyl ATP (3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)aminol]propionyl}ATP); AGATP-2, agarose-ATP type 2; AGATP-3, agarose-ATP type 3; AGATP-4, agarose-ATP type 4; and EGTA, ethyleneglycol-bis-(β-amino-ethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

‡ J. S. Fedan, G. K. Hogaboom, D. P. Westfall and J. P. O'Donnell, unpublished observations.

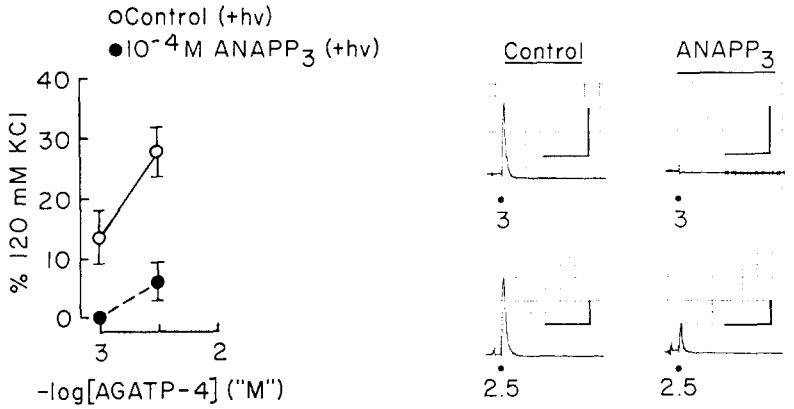

Fig. 1. Left panel: Effect of $ANAPP_3$ on isometric contractile responses of isolated guinea-pig vas deferens to AGATP-4. These results show the mean ± S.E. of four experiments. "M" indicates apparent molarity of ATP bound covalently to insoluble agarose beads. The responses were reduced significantly ($P < 0.05$; Student's *t*-test) by $ANAPP_3$. Right panel: Representative recordings of contractions to AGATP-4 in untreated and $ANAPP_3$-treated vasa deferentia. Concentrations of AGATP-4 are shown as negative log "molar". Horizontal calibration: 1 min; vertical calibration: 0.5 g.

concentration–response curves [19] has indicated, however, that 10^{-7} and 3×10^{-7} M soluble ATP yielded responses which were 2.0 ± 0.4 (N = 13) and 5.0 ± 0.6% (N = 13) of the maximum responses of the tissues to 120 mM KCl. It follows that the responses to AGATP-4 were only in small part due to contaminant ATP. Likewise, the marked antagonism of AGATP-induced responses by $ANAPP_3$ (Fig. 1) could not have resulted primarily from blockade of the component due to soluble ATP.

The preceding results indicate that ATP bound to agarose through the ribose hydroxyl moiety can cause contraction of the guinea-pig vas deferens *in vitro*, and that this contraction can be markedly attenuated by a P_2-receptor antagonist. The failure of AGATP-3 and AGATP-2 to elicit a similar response suggests that there exist distinct structural requirements for the ATP analogs at the site of initiation of responses to ATP and AGATP-4. The latter conclusion is entirely consistent with our previous findings which suggest that structural modifications of the ribose moiety of ATP are well-tolerated while modification of the adenine moiety decreased potency [19]. It follows that the agarose-bound derivatives of ATP share a similar structure–activity relationship with soluble ATP analogs in this tissue. A direct comparison of the potency of ATP with that of AGATP-4 was not possible due to the unavailability of an agarose bead preparation with sufficient ATP incorporation to permit estimation of the maximum response of the tissue to AGATP-4. However, it is apparent that the threshold for response to soluble ATP (*ca.* 10^{-7} M) is lower than that for AGATP-4 (10^{-3} "M"). The latter is not unexpected and probably reflects differences between the dispersion and tissue penetration of soluble ATP and agarose-bound ATP.

The possibility exists that, if ATP, bound to agarose beads, makes sufficient contact with the smooth muscle cells to interact with receptors, then the bound ATP may also be available to interact with plasmalemmal ectoATPase or other ectophosphohydrolase enzymes. Notwithstanding the above antagonism of contractile responses to AGATP-4 by a P_2-receptor antagonist, it is possible that hydrolysis of the phosphate chain of ATP bound to agarose could initiate the contractile response. Alternatively, the agarose-bound product of this hydrolysis may be implicated in the contractile response observed with AGATP-4. These notions are contigent upon the ability of the tissue to metabolize AGATP-4. To explore this possibility we examined the activities of the Mg^{2+}-ATPases present in microsomal and soluble cell fractions from homogenized guinea-pig vas deferens, using as substrates ATP and agarose-bound ATP. The results of these experiments are summarized in Table 1, where it may be seen that under optimal assay conditions all three agarose derivatives served as substrate for Mg^{2+}-ATPase present in cytosol. This observation indicates that soluble Mg^{2+}-ATPase has access to and can liberate inorganic phosphate from ATP bound covalently to agarose. In contrast, ATP bound to agarose was a poor substrate for the membrane-bound microsomal Mg^{2+}-ATPase (Table 1). The foregoing results would suggest that particle-bound ATP but not soluble ATP has limited access to Mg^{2+}-ATPase localized in microsomal membranes. It should be noted that the interaction of agarose-bound ATP with microsomal Mg^{2+}-ATPase is at best an approximation of the interaction of the substrate with ecto-enzyme in membranes in the intact tissue. Since cells do not normally release Mg^{2+}-ATPase [20], however, the results of these experiments could suggest that minimal hydrolysis of AGATP-4 occurs in the intact tissue, i.e. in the organ bath.

The diameter of the agarose beads (60–140 microns*) is much greater than the minor (2–4 microns) or major (3–8 microns) cross-sectional axes of smooth muscle fibers in the guinea-pig vas deferens [21]. It is not tenable that AGATP-4 caused responses by an intracellular action fol-

Table 1. Comparison of ATP and AGATP as substrates for soluble cytosolic and membrane-bound microsomal Mg^{2+}-ATPase of guinea-pig vas deferens*

	P_i [μmoles · mg^{-1} · (60 min)$^{-1}$]	
Substrate	Microsomes	Cytosol
ATP	8.03	0.313
AGATP-2	0.33	0.094
AGATP-3	0.00	0.081
AGATP-4	1.20	0.223

* Values shown are means of triplicate determinations.

* Information provided by Pharmacia, Inc., Piscataway, NJ, U.S.A.

lowing internalization of the beads. The significance of our observation, that ATP bound to agarose can promote a contractile response in the guinea-pig vas deferens that is blocked by $ANAPP_3$, is several-fold. First, it strengthens the concept that in this tissue there exists cell surface receptors for ATP and that occupation of these receptors promotes smooth muscle contraction. To this extent it implies that the ATP-mediated contraction of the guinea-pig vas deferens adheres, in part, to the pattern well-documented for other smooth muscle agonists. Second, it provides evidence that the blockade of AGATP-4-induced responses, and ATP-induced responses as well, by $ANAPP_3$ occurs at a receptor level. Since ATP will protect against the blockade [1], the effect of $ANAPP_3$ is not the result of an intracellular action of this antagonist. Third, it offers a methodological approach whereby the pharmacological and biochemical actions of ATP in the extracellular space of tissues can be explored free from the constraints that apply to soluble ATP.

In summary, ATP covalently linked to agarose beads via ribose, but not via N^6 or C^8, caused contractions of the vas deferens which were antagonized by $ANAPP_3$, a specific ATP antagonist. Responses of the vas deferens to adenine nucleotides were mediated, at least in part, by a cell surface P_2-purinergic receptor. The antagonism by $ANAPP_3$ occurred at this site.

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Inhibition of normal and leukemic lymphocyte proliferation by compound 48/80*

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Polycations of various types have proven to be extremely useful pharmacological and biochemical agents whose full potential is still being evaluated. Basic polyamino acids promote the leakage of small molecules from cells [1, 2], cause the degranulation of mast cells [3], serve as effective drug carriers into cells [4–6], and can both stimulate and inhibit the mitogen stimulation of lymphocytes [7, 8]. Increasing the molecular weight (and the degree of polymerization) results in increased toxicity [9] and leakage of small molecules [1, 2]. Usually, significant activity is not observed until molecular weights greater than 10,000 have been achieved; lower molecular weights are usually inactive.

Considerable research has also been done uitilizing the low molecular weight polycation, compound 48/80. This

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