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EVIDENCE FOR A CONTRIBUTION BY PURINES TO THE NEUROGENIC RESPONSE OF THE GUINEA-PIG URINARY BLADDER *

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In order to determine if ATP contributes as an excitatory transmitter in the guinea-pig bladder, experiments were conducted with $ANAPP_3$, a photoaffinity analogue of ATP, which is an antagonist of adenine nucleotides in several other smooth muscles. With or without photoactivation with visible light, $ANAPP_3$ antagonized contractile responses of in vitro strips of bladder to exogenous ATP. The antagonism was specific in that responses to acetylcholine and KCl were not affected by $ANAPP_3$. Responses of strips of bladder to transmural electrical stimulation were not antagonized by $ANAPP_3$ and were relatively insensitive to atropine. However, combined treatment with $ANAPP_3$ and atropine produced a marked antagonism of the neurogenic response. In experiments with bladders obtained from animals pretreated with 6-hydroxydopamine, the $ANAPP_3$-sensitive component of the neurogenic response was absent. These results suggest that acetylcholine, released from cholinergic nerves, and a purine, released from 6-hydroxydopamine-sensitive nerves, are both involved in motor transmission in this tissue.

Purinoceptor antagonist Dual transmitters Acetylcholine 6-Hydroxydopamine ATP $ANAPP_3$

1. Introduction

The smooth muscle of the body of the mammalian urinary bladder is a tissue which presents a confusing picture in terms of its excitatory innervation. As reviewed by Taira (1972), the classical view is that the bladder received excitatory cholinergic innervation and, indeed, exogenous acetylcholine mimics the motor response evoked by transmural electrical stimulation. However, in spite of profound antagonism of the response of the bladder to acetylcholine by the muscarinic cholinoceptor antagonist atropine, the neurogenic response of the bladder exhibits considerable resistance to antagonism by atropine. Consequently, there have been numerous suggestions that excitatory transmission to the bladder is partly, or perhaps entirely, noncholinergic (Ambache and Zar, 1970; Dumsday, 1971; Burnstock et al., 1972, 1978a; Raezer et al., 1973; DeGroat and Saum, 1972; Downie and Dean, 1977; Dean and Downie, 1978; Krell et al., 1981 and others).

The suggestion has been made that ATP or a congener may be involved in neurogenic responses of the bladder (Burnstock et al., 1972, 1978a; Downie and Dean, 1978). Consistent with this proposal are the findings that exogenously applied ATP produces contraction of the bladder and transmural stimulation of superfused bladder strips results in the appearance of ATP in the superfusate (Burnstock et al., 1978a,b). The chief imped-

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iment to acceptance of the notion that purines act as transmitters or co-transmitters in the bladder has been the unavailability of a specific pharmacological antagonist of responses to ATP (see, for example, Campbell and Gibbons, 1979). Although a number of compounds have been investigated in this regard, including 2-2′-pyridylisatogen, 2-2′-methoxyphenylisatogen and quinidine, the antagonism afforded by these drugs in guinea-pig bladder is nonspecific, i.e., in concentrations sufficient to antagonize response to ATP and transmural electrical stimulation, the responses to other stimulatory agonists are equivalently reduced (Weetman and Turner, 1977).

Recent work in our laboratory (Hogaboom et al., 1980) has indicated that 3′-O-[3[N-(4-azido-2-nitrophenyl)amino]propionyl] adenosine 5′-triphosphate ($ANAPP_3$), a photoaffinity analogue of ATP (Jeng and Guillory, 1975), antagonizes contractile responses of the guinea-pig vas deferens to adenine nucleotides but does not antagonize responses to norepinephrine, acetylcholine, histamine or KCl. In the present study we have examined the influence of $ANAPP_3$ on responses of guinea-pig bladder strips to agonists and transmural electrical stimulation. The results indicate that in bladder strips, as in the vas deferens (Hogaboom et al., 1980; Fedan et al., 1981), $ANAPP_3$ is a specific antagonist of responses to ATP and the motor response evoked by transmural electrical stimulation is mediated in part by a purine. A preliminary account of this work has been presented (Westfall et al., 1980).

2. Materials and methods

All experiments were performed with bladders obtained from male albino guinea-pigs (Hilltop Lab Animals, Inc., Scottdale, PA or Camm Research Lab Animals, Wayne, NJ). The animals, which ranged in weight from 250 to 525 g, were killed by a blow on the head and the bladders removed. From each bladder, two equivalent strips were prepared as described in detail by Ambache and Zar (1970).

The isolated bladder strips were placed in a small volume (approximately 2 ml) water-jacketed tissue chamber in the presence of a physiological salt solution which was maintained at 37°C and continually bubbled with 95% O_2, 5% CO_2. The composition of the salt solution was as follows (mM): NaCl 113; KCl 4.8; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; glucose, 5.5. The construction of the tissue chamber was such that the bladder strips could be washed intermittently or continuously suffused from bottom to top (at a rate of 3.5 ml/min) with fresh physiological salt solution. One end of the tissue was fixed and the other attached by thread to a Grass force displacement transducer (FT.03) for monitoring tension on a Grass polygraph. Approximately 0.5 g of resting tension was applied to the tissues.

After a 1 h equilibration period, responses of the bladder strips were elicited by addition of agonists, acetylcholine, potassium chloride or ATP, or by transmural electrical stimulation via platinum ring electrodes with pulses (0.5 ms, supramaximal voltage) from a Grass S9 stimulator. Unless otherwise specified, full dose-response curves were obtained noncumulatively by a stepwise increase in concentration of agonist. Each concentration was washed out before the next higher concentration was given. The peak tension development following each dose was used as the response in the construction of the dose-response curve. There were 8 min intervals between successive doses of agonists. Neurogenic responses of bladder strips were elicited in separate experiments with geometrically increasing frequencies starting at 1 Hz and continuing until maximum responses were established. The duration of the stimulus period was 30 s with 5 min allowed to elapse before the next greater frequency was applied. Responses were not obtained in the presence of tetrodotoxin (10^{-6} M), and were not mediated, therefore, by direct electrical stimulation of the muscle.

In order to assess the influence of potential antagonists on the agonist-induced or neurogenic responses, a second and in some cases a third dose-response curve or frequency-response curve was generated in the presence (or after treatment with $ANAPP_3$, vide infra) of the antagonist. Thirty min elapsed between frequency-response or dose-response determinations. Each tissue served as its own control, therefore. In addition, for each series

of experiments a second dose-response curve or frequency-response curve and, when appropriate, a third curve was generated in one of the bladder strips without drug intervention. In no case was the subsequent dose-response curve or the frequency-response curve depressed relative to the initial control.

As described previously (Hogaboom et al., 1980; Fedan et al., 1981), $ANAPP_3$ when present in the tissue chamber antagonizes responses of the guinea-pig vas deferens to ATP and transmural electrical stimulation and, if the compound is washed out, the antagonism is reversible. If, however, tissues are irradiated with visible light (hv) in the presence of $ANAPP_3$ and the compound then washed out, the antagonism persists. Because $ANAPP_3$ is an arylazido photoaffinity label, photolysis activates the compound to a reactive nitrene intermediate which forms covalent bonds at or near the P_2-receptor. In the present studies, therefore, two types of experiments were performed. In one, the potential antagonism was tested by adding $ANAPP_3$ to the bathing solution 5 min prior to and allowing it to remain during the development of the subsequent dose- or frequency-response curve. The effect of atropine was investigated in a similar manner with the exception that atropine was added 20 min prior to the initiation of the subsequent curve. In the second approach, $ANAPP_3$ was added and 3 min later the tissue was irradiated in the tissue chamber for 20 min with a tungsten halogen projector lamp (DVY, 650 W, 3400°K) as previously described (Hogaboom et al., 1980). At the end of the photoactivation period, the bath solution was continuously replaced at a rate of 3.5 ml/min and the subsequent curve generated. Whenever photoactivation was employed, control experiments consisting of a 20 min irradiation in the absence of $ANAPP_3$ were performed.

In one series of experiments, the neurogenic response was investigated with bladder strips obtained from animals pretreated with 6-hydroxydopamine. Treatment consisted of two injections 24 h apart via the dorsal vein of the penis. The first dose was 100 mg/kg, and the second was 250 mg/kg. The animals were killed 1 day after the second dose. In separate experiments, the norepinephrine concentration of the bladders from animals pretreated with 6-hydroxydopamine was analyzed spectrophotofluorometrically as previously described (Westfall et al., 1972). The endogenous norepinephrine concentration of the bladder was reduced from a control level of 0.17 ($\pm$0.02) μg/g wet weight (n = 8) to 0.04 ($\pm$0.001) μg/g (n = 4) by 6-hydroxydopamine pretreatment (S. Wong and R.L. Robinson, unpublished results).

$ANAPP_3$ was synthesized in our laboratories according to the method of Jeng and Guillory (1975). Other drugs and chemicals were obtained from commercial sources.

3. Results

Shown in fig. 1 are mean dose-response curves for acetylcholine, ATP and potassium chloride in guinea-pig bladder strips after irradiation (+hv) in the absence and presence of 10^{-4} M $ANAPP_3$. Following irradiation, the tissues were continuously suffused with physiological salt solution which did not contain $ANAPP_3$. Exposure to $ANAPP_3$ resulted in a substantial displacement of the dose-response curve for ATP to the right of control. $ANAPP_3$ treatment, however, produced no significant antagonism of the response of the bladder to acetylcholine or to potassium chloride.

Responses of the bladder strips to ATP were also reduced when $ANAPP_3$ was used as a conventional antagonist, that is, without photoactivation but continuously present in the bath. This is illustrated in fig. 2 along with a demonstration of the reversibility of the antagonism by $ANAPP_3$ when not photolyzed, the specificity of the antagonism by $ANAPP_3$ for responses to ATP and the specificity of the antagonism by atropine for responses to acetylcholine. In this study, approximately equi-effective concentrations of acetylcholine and ATP were administered. In the presence of 10^{-4} M $ANAPP_3$, the response to ATP was reduced by approximately 65% whereas the magnitude of the response to acetylcholine was unaffected. Following wash-out of $ANAPP_3$, but in the presence of 10^{-6} M atropine, the response to ATP was restored to control levels whereas the response to acetylcholine was virtually abolished.

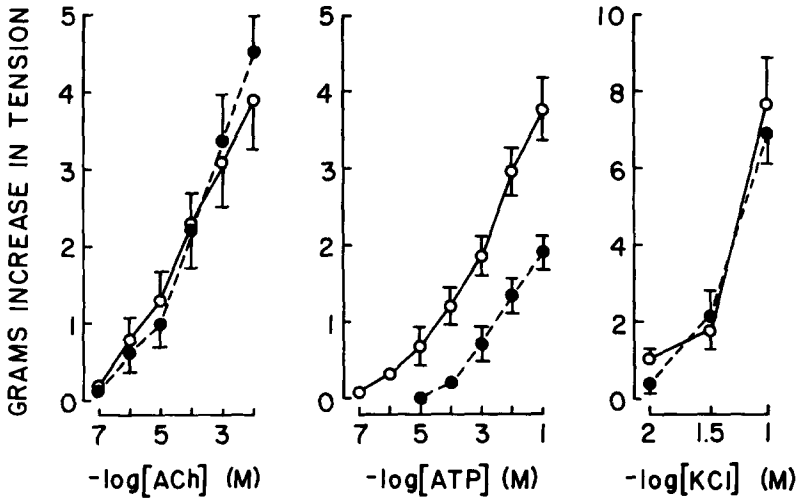

Fig. 1. Mean dose-response curves for acetylcholine (ACh), ATP and KCl in strips of guinea-pig bladder. Control (○) refers to responses which were obtained after irradiation but without $ANAPP_3$. $ANAPP_3$-treated (●) refers to responses which were obtained after irradiation in the presence of 10^{-4} M $ANAPP_3$ followed by washout with $ANAPP_3$-free bathing solution. Each curve represents the mean of 4 experiments. In this, and subsequent figures, vertical bars represent standard errors of the mean.

The effect of atropine on the full dose-response curve for acetylcholine was also studied, the results of which are shown in fig. 3. At 10^{-6} M, atropine produced a profound antagonism of the response to acetylcholine. In spite of this antagonism of exogenously administered acetylcholine, and consistent with previous observations, atropine produced only modest antagonism of the neurogenic response of the bladder (fig. 3).

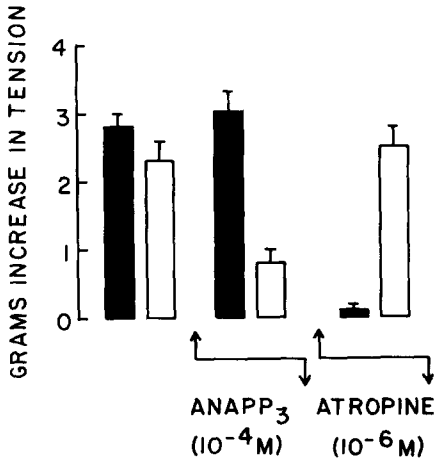

Fig. 2. Mean responses (n = 6) of guinea-pig bladder strips to 10^{-5} M acetylcholine (■) and 10^{-3} M ATP (□) in the absence of $ANAPP_3$ or atropine (first two histograms), in the presence of 10^{-4} M $ANAPP_3$ (second two histograms) and following wash-out of $ANAPP_3$ but in the presence of 10^{-6} M atropine (third two histograms).

The influence of $ANAPP_3$ on the frequency-response curve of bladder strips was investigated. $ANAPP_3$ (10^{-4} M) failed to alter the frequency-response curve. This is shown in fig. 4 for nonphotolyzed $ANAPP_3$. Identical results were obtained (5 experiments) following photolysis of $ANAPP_3$ (not shown). A higher concentration (10^{-3} M; data not shown) also failed to significantly influence the frequency-response curve. Interestingly, however, combined treatment with atropine and $ANAPP_3$ produced a marked inhibition of the neurogenic response of the bladder. This is illustrated in fig. 4 for experiments with nonphotolyzed $ANAPP_3$. Also shown in fig. 4, for comparative purposes, are the effects of atropine and $ANAPP_3$ administered alone. A similar pattern occurs if $ANAPP_3$ is photolyzed, i.e., combined treatment with $ANAPP_3$ and atropine produces a greater antagonism than treatment with either agent alone. These results are illustrated in the left panel of fig. 5.

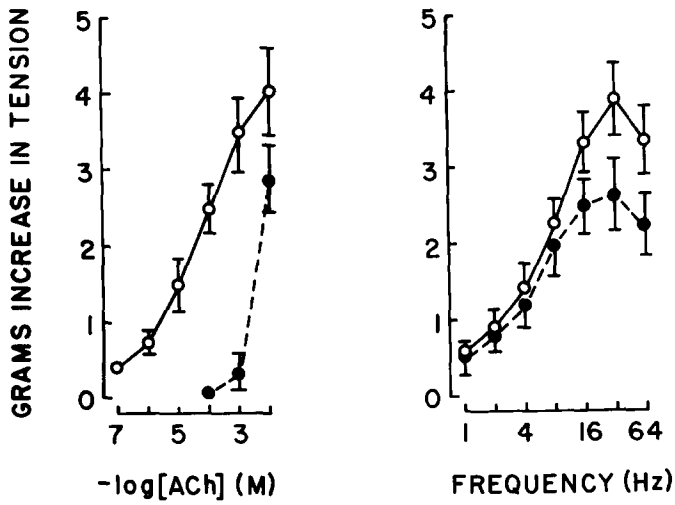

Fig. 3. Mean dose-response curves for acetylcholine (ACh), *left panel*, and frequency-response curves, *right panel*, in the absence (○) and presence of 10^{-6} M atropine (●). The curves are the mean of 4–6 experiments.

Also shown in fig. 5 are the effects of atropine and atropine plus $ANAPP_3$ on the neurogenic response of bladder strips from animals pretreated with 6-hydroxydopamine. Treatment with 6-hydroxydopamine had a rather dramatic effect in that the magnitude of the neurogenic response, relative to the response of bladder strips from untreated animals, was significantly less. In these preparations, atropine alone became a more effective antagonist. In addition, after 6-hydroxydopamine treatment, the $ANAPP_3$-sensitive component of the neurogenic response was absent, i.e.,

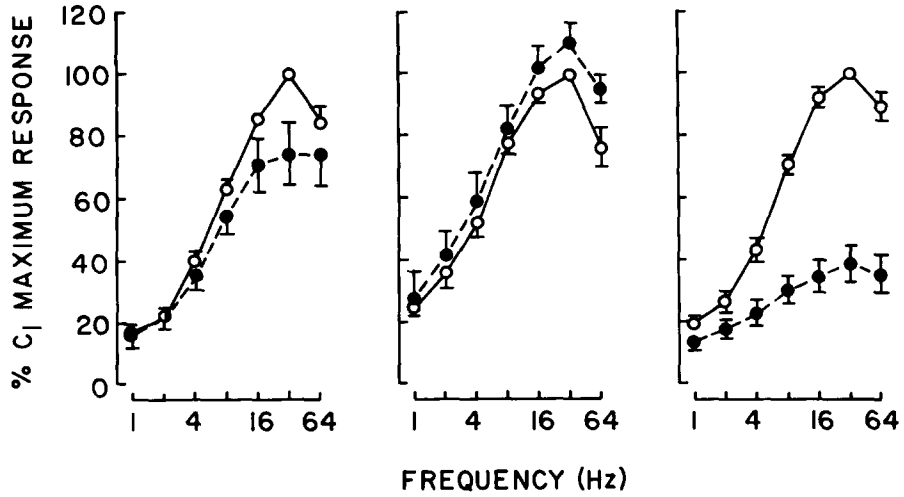

Fig. 4. Mean frequency-response curves of guinea-pig bladder strips. Responses are plotted as % of maximum response obtained during the first (C_1) frequency-response curve. *Left panel*: two consecutive curves, the first control (○) and the second obtained in the presence of 10^{-6} M atropine (●). *Middle panel*: two consecutive curves, the first control (○) and the second obtained in the presence of 10^{-4} M $ANAPP_3$ (●). *Right panel*: two consecutive curves, the first control (○) and the second obtained in the combined presence of 10^{-6} M atropine and 10^{-4} M $ANAPP_3$ (●). Each curve represents the mean of 5–7 experiments.

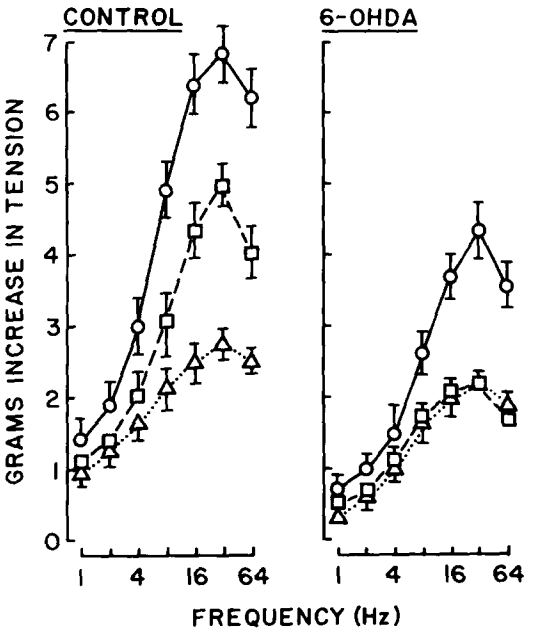

Fig. 5. Mean frequency-response curves of bladder strips from untreated (*left panel*) or 6-hydroxydopamine pretreated (*right panel*) guinea-pigs. Each panel illustrates three consecutive curves: the first, control (○); the second obtained in the presence of atropine (10^{-6} M) and after irradiation (□); the third, also in the presence of atropine (10^{-6} M), and after irradiation in the presence of 10^{-4} M $ANAPP_3$ followed by washout with $ANAPP_3$-free bathing solution (△). Each curve is the mean of 5–6 experiments.

atropine and $ANAPP_3$ together produced no greater antagonism than atropine alone.

4. Discussion

$ANAPP_3$, a photoaffinity analogue of ATP, has been shown to antagonize the contractile response of the guinea-pig vas deferens to adenine nucleotides but does not antagonize responses of this tissue to norepinephrine, acetylcholine, histamine or potassium chloride (Hogaboom et al., 1980). In the present study, $ANAPP_3$ was also found to act as an antagonist of the effects of ATP in the guinea-pig bladder, and further, the antagonism was specific in that responses to acetylcholine and potassium chloride were not affected (figs. 1 and 2). Because of the specificity of its action, we utilized $ANAPP_3$ to examine the suggestion (Burnstock, 1972; Burnstock et al., 1978a) that the atropine-resistant neurogenic response of the guinea-pig bladder may be mediated by ATP.

As shown in fig. 4, the frequency-response curve of guinea-pig bladder strips was unaffected by treatment with $ANAPP_3$. Interestingly, however, combined treatment of bladder strips with atropine, which by itself produces only modest depression of the neurogenic response, and $ANAPP_3$ produced a profound depression of the frequency-response curve (figs. 4 and 5). It appears from the results, therefore, that both acetylcholine and ATP, or a congener, can contribute as excitatory transmitters in the bladder.

Somewhat surprisingly, these results also indicate that if the action of either substance is antagonized, the other stimulant is capable of producing a contraction which is nearly, if not fully, equivalent to the neurogenic response in the absence of any antagonist. One might have expected some degree of antagonism by each antagonist when used alone and that simultaneous treatment with both antagonists might have produced additive effects as has been shown to occur with combined adrenoceptor and purinoceptor antagonists in the guinea-pig vas deferens (Fedan et al., 1981). While atropine alone produced some antagonism of the neurogenic response in the bladder, $ANAPP_3$ alone did not. The reason for this is not clear. It may be related to several factors including (1) the close proximity of postganglionic nerve endings and the smooth muscle cells, (2) a relatively small fractional receptor blockade by $ANAPP_3$ such that an antagonism of a purine-component is not evident until an overwhelming cholinergic input is offset by the presence of atropine, and (3) the fact that electric field stimulation of the in vitro preparation elicits neurogenic responses mediated by stimulation of all postganglionic fibers which release excitatory substances, rendering it difficult to evaluate the contribution of one component singly. In support of this latter view, $ANAPP_3$ administered intra-arterially has been shown recently to antagonize contractions of the cat urinary bladder in vivo which were elicited with stimulation of the hypogastric (i.e., sympathetic) nerve (R.J. Theobold, personal communication) under conditions

in which responses to injected adenine nucleotides were also antagonized (Theobald, 1982). Thus, under experimental conditions in which given excitatory fibers can be selectively stimulated while others are not, $ANAPP_3$ *alone* produces an effective antagonism. Nevertheless, it is clear that treatment with both a muscarinic cholinoceptor antagonist and a purinoceptor antagonist produces a significant reduction in the neurogenic response.

In view of our previous findings with the guinea-pig vas deferens (Fedan et al., 1981) that adrenergic nerves serve as the source of neurally-released purines, we conducted experiments designed to assess whether adrenergic nerves in the bladder also might be the source of the $ANAPP_3$-sensitive component of the neurogenic response. Guinea-pigs were pretreated with 6-hydroxydopamine, an agent which is known to produce a destruction of adrenergic nerves (Malmfors and Thoenen, 1971) but which does not affect quinicrine-staining nerves or nerves which contain large, opaque vesicles (Robinson et al., 1971; Burnstock, 1972; Burnstock et al., 1978a). The treatment schedule employed in these studies was sufficient to produce disruption of adrenergic nerves as judged by a 75% loss of endogenous norepinephrine from the bladder (see Materials and methods). Interestingly, the magnitude of the neurogenic response of bladder strips from 6-hydroxydopamine-treated animals was significantly less than the response of untreated bladders (fig. 5). This finding supports the idea that adrenergic nerves in the mammalian bladder elaborate an excitatory transmitter in addition to norepinephrine, an inhibitory transmitter (DeGroat and Theobald, 1976). Furthermore, after treatment with 6-hydroxydopamine, the $ANAPP_3$-sensitive component of the motor response was absent, that is, combined $ANAPP_3$ and atropine treatment produced no greater antagonism than atropine alone. Thus it appears that ATP, released from nerves which are sensitive to destruction by 6-hydroxydopamine, can contribute along with acetylcholine, released from cholinergic nerves, to the motor response of the transmurally stimulated guinea-pig bladder.

At first glance, the present results would appear to be inconsistent with previous findings made using this organ. We have evidence which supports the existence of separate nonadrenergic, noncholinergic nerves. Thus, Burnstock et al. (1978a) observed that 6-hydroxydopamine treatment had no effect on neurogenic response elicited *in the presence of guanethidine*, while we, having intentionally eliminated guanethidine, observed that 6-hydroxydopamine treatment *alone* reduced substantially responses to field stimulation. We, and not previous workers who routinely include guanethidine, have shown that the adrenergic nerves are also a source of purines. It is likely that the innervations responsible for responses in the presence of atropine and guanethidine seen by others, and those observed here in the presence of atropine and $ANAPP_3$ (or following treatment with photolyzed $ANAPP_3$) are the same. Since $ANAPP_3$ is ineffective in 6-hydroxydopamine-treated preparations stimulated in the presence of atropine, it may well be that the nonadrenergic, noncholinergic innervation is not 'purinergic' per se.

While we favor the hypothesis that purines from sympathetic nerves contribute via cotransmission to the development of neurogenic responses, alternative explanations should be considered. It is possible that, in the presence of atropine in control preparations, $ANAPP_3$ exerts a presynaptic action to interfere with neurotransmission. Since the effect of $ANAPP_3$ is not seen after 6-hydroxydopamine treatment, this could occur in control preparations by an effect on neurotransmitter release from sympathetic nerves. This possibility seems unlikely, however, for the reason that $ANAPP_3$ has no effect on norepinephrine release in the guinea-pig vas deferens (Fedan et al., 1981). If a prejunctional effect on another nerve type is responsible, than such an effect should have occurred when the adrenergic nerves are eliminated with 6-hydroxydopamine. Such was not the case, however.

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