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COMPARISON OF THE EFFECTS OF APAMIN, A Ca^{2+}-DEPENDENT K^+ CHANNEL BLOCKER, AND ARYLAZIDO AMINOPROPIONYL ATP ($ANAPP_3$), A P_2-PURINERGIC RECEPTOR ANTAGONIST, IN THE GUINEA-PIG VAS DEFERENS *

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Apamin, which blocks Ca^{2+}-dependent increases in K^+ permeability, antagonizes ATP-induced relaxation of several smooth muscles. The ATP photoaffinity label arylazido aminopropionyl ATP ($ANAPP_3$), following its photolysis in the presence of the guinea-pig vas deferens, antagonizes contractile responses to ATP. This study was conducted to determine whether apamin antagonizes ATP-induced responses in the guinea-pig vas deferens, and also to evaluate whether $ANAPP_3$ antagonizes responses to ATP by interfering with Ca^{2+}-dependent K^+ permeability changes. Apamin (10^{-6} M) potentiated ATP-induced contractions. This potentiation was nonspecific in that responses to norepinephrine, histamine and acetylcholine also were enhanced; responses to KCl were unaffected. To evaluate the possible interactions between the two agents at the same cellular site, the effect of apamin was examined in $ANAPP_3$-treated tissues. In such tissues apamin did not potentiate the residual responses to ATP; however, apamin was nevertheless able to potentiate responses of $ANAPP_3$-treated tissues to norepinephrine, histamine and acetylcholine, and responses to KCl remained unaffected. These studies provide additional support for the view that $ANAPP_3$ antagonizes ATP-induced responses of the guinea-pig vas deferens by blocking P_2-purinergic receptors. The antagonism by $ANAPP_3$ is not attributable to a blockade of Ca^{2+}-dependent K^+ permeability changes.

Arylazido aminopropionyl ATP ($ANAPP_3$) | P_2-purinergic receptors | Vas deferens | Apamin
Ca^{2+}-dependent K^+ channels | Guinea-pig

1. Introduction

Apamin, a potent neurotoxin from bee venom (Habermann, 1972), blocks Ca^{2+}-dependent increases in K^+ conductance (Meech, 1978; Moolenaar and Spector, 1979; Barrett et al., 1982; De Peyer et al., 1982) in several cell types (Burgess et al., 1981; Hugues et al., 1982a; Maruyama et al., 1983) via its interaction with the Ca^{2+}-dependent K^+ channel (Cavey et al., 1979; Hugues et al., 1982b,c,d). The polypeptide antagonizes relaxation and associated membrane hyperpolarization responses of gastrointestinal smooth muscle preparations evoked with ATP and with stimulation of nonadrenergic, noncholinergic inhibitory nerves; these responses are often converted to contraction accompanied by depolarization (Mackenzie and Burnstock, 1980; Brown and Burnstock, 1981; Banks et al., 1979; Vladimirova and Shuba, 1978; Shuba and Vladimirova, 1980; Maas and Den Hertog, 1979; Maas et al., 1980). Apamin has been

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a useful agent to determine whether responses to adenine compounds are mediated by P_2-purinergic receptors (Vladimirova and Shuba, 1978; Shuba and Vladimirova, 1980; Brown and Burnstock, 1981), even though responses to agonists other than adenine nucleotides also are reduced if a similar cellular mechanism is involved (Banks et al., 1979; Maas and Den Hertog, 1979; Maas et al., 1980; Kitabgi and Vincent, 1981; Jodal et al., 1981; Burgess et al., 1981).

The ATP photoaffinity label $ANAPP_3$ produces, following its photolysis in the presence of isolated muscle preparations, a specific antagonism of adenine nucleotide-induced responses in vas deferens, urinary bladder and taenia coli preparations (Hogaboom et al., 1980; Westfall et al., 1982, 1983; Frew and Lundy, 1981). The likelihood that the P_2-receptor is a cell-surface receptor, and that $ANAPP_3$ exerts its antagonistic effect at this site, has been supported by the finding that contractions elicited with agarose-ATP beads were reduced following $ANAPP_3$ treatment of vas deferens (Head et al., 1983). However, this evidence does not exclude the possibility that the antagonism of P_2-mediated responses by $ANAPP_3$ occurs by an intracellular effect of the compound.

In the present studies a comparison was made between the pharmacological effects of apamin and of $ANAPP_3$ in the guinea-pig vas deferens. Evidence was sought for the ability of apamin to exert an antagonistic effect at P_2-receptors, and for $ANAPP_3$ to modify responses in a way which would be indicative of an interaction with Ca^{2+}-dependent K^+ channels.

2. Materials and methods

Guinea pigs (350-600 g; Camm Research Institute, Inc., Wayne, NJ) were killed by a blow to the head and bled. Vasa deferentia were removed, placed in modified Krebs-Henseleit solution (see O'Donnell et al., 1981 for composition) and cleaned. Each tissue was tied at one end to a holder, placed in a separate 1 ml glass, water jacketed (37 °C) organ chamber containing modified Krebs-Henseleit bathing solution, and attached to a transducer for the measurement of isometric contractile responses. Resting tension was 0.2-0.4 mg. A 1 h equilibration period, during which the tissues were washed every 15 min with fresh bathing medium, was allowed prior to the experiment. Concentration-response relationships were obtained following the stepwise-increasing, noncumulative addition of agonists to the bath. Each concentration was present for 3 min, and was followed with two washes of the tissues with fresh bathing at 5 min intervals prior to the next drug addition. At the end of the experiments, except when KCl was used, the tissues were contracted with 120 mM KCl. Contractile responses shown in the figures are normalized as a percentage of the response to 120 mM KCl (% 120 mM KCl) or as grams developed tension per gram wet tissue weight (g tension/gww) when the comparisons include KCl concentration-response curves.

$ANAPP_3$ was synthesized as described by Jeng and Guillory (1975). The effect of $ANAPP_3$ (10^{-4} M) on agonist-induced responses was evaluated following a 3 min incubation of the tissues in the organ chambers with the compound in near-darkness, after which the preparations were photolyzed for 15 min (in the continued presence of $ANAPP_3$) with a DYH (600 W, 3,200 °K) bulb mounted in a Dyna-Lume lamp housing (Cole-Parmer, Chicago, IL). The bulb filament was 15 cm from the center of the organ chamber. Following photolysis the preparations were washed twice at 5 min intervals with fresh bathing solution prior to the addition of agonists. Contralateral control tissues for these experiments were not exposed to $ANAPP_3$ but were irradiated for 15 min.

When present, apamin (Sigma Chemical Co.; St. Louis, MO) was added to untreated or $ANAPP_3$-treated preparations 20 min prior to the addition of agonists, and it remained present during the concentration-response determinations.

Each tissue was used for only one concentration-response determination. The data were evaluated for differences using Student's t-test for paired samples. A probability less than 0.05 was considered significant. The results are presented as means $\pm$ S.E.M.; n is the number of separate experiments. EC_{50} values were obtained from linear regression analyses of probit-transformed data.

3. Results

3.1. Effects of apamin on ATP-induced responses in control ('untreated') tissues

Resting tension was not changed upon the addition of apamin to the organ bath. The morphology of responses to agonists was not noticeably affected by any of the apamin concentrations used in this study (not shown).

Low concentrations (0.5-100 nM of apamin had either no effect on ATP-induced contractile responses or produced a small but significant potentiation (0.5, 5 and 50 nM apamin) of the maximum responses. A higher concentration of apamin (1 000 nM), which is the reference concentration used for the remainder of these studies, produced a significant potentiation of responses to ATP, i.e., a 2- to 10-fold shift of the ATP concentration-response curve to the left of control (fig. 1). The maximum response was not affected. The ATP concentration-response curve was biphasic both in the absence and presence of apamin.

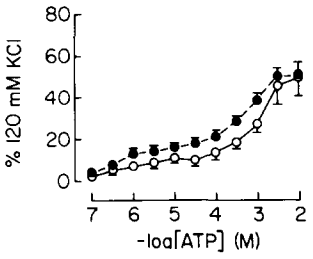

Fig. 1. Effect of apamin (1 000 nM) on responses of untreated guinea-pig vas deferens to ATP. n = 4. ○ Control; ● 1 000 nM apamin.

3.2. Effect of apamin on responses of untreated tissues to other agonists

As shown in fig. 2, 1 000 nM apamin produced a marked potentiation of responses of the tissues to norepinephrine (NE), histamine (Hist) and acetylcholine (ACh). The EC_{50} values for NE and Hist were not affected; however, the EC_{50} for ACh was reduced significantly (6.6-fold) in the presence of apamin. In contrast, apamin (1 000 nM) had no effect on responses to KCl.

3.3. Effect of apamin on ATP-induced responses in ANAPP$_3$-treated tissues

As has been reported previously (Hogaboom et al., 1980: Fedan et al., 1982a,b), $ANAPP_3$, following its photolysis and washout, produced an antagonism of ATP-induced responses (fig. 3, left panel). $ANAPP_3$ treatment resulted in a conversion

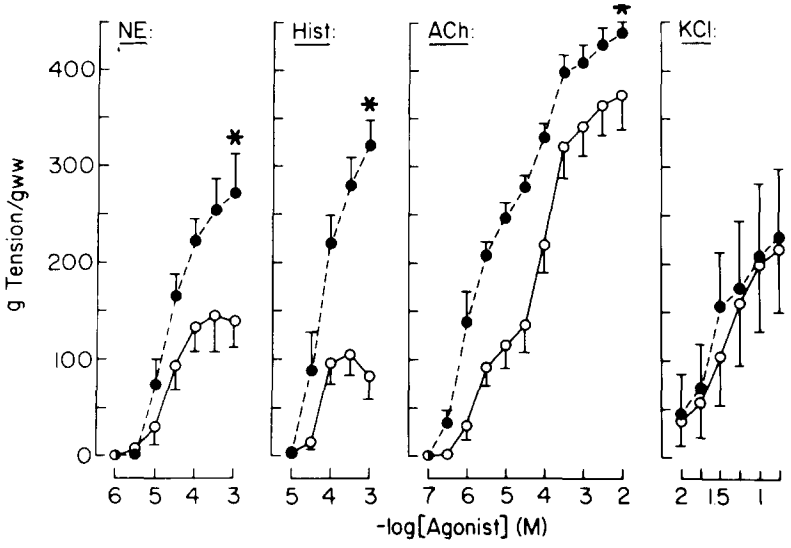

Fig. 2. Effect of apamin (1 000 nM) on responses of untreated guinea-pig vas deferens to norepinephrine (NE; n = 4), histamine (Hist; n = 6), acetylcholine (ACh; n = 5) and KCl (n = 5). * Significantly different from control. ○ Control; ● 1 000 nM apamin.

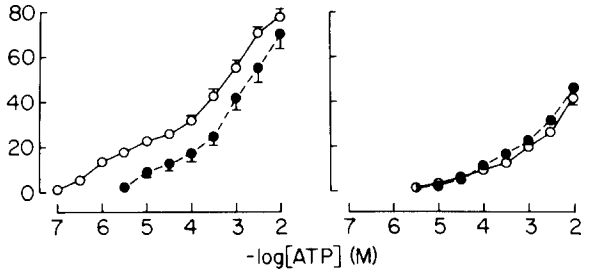

Fig. 3. *Left panel*: Effect of 10^{-4} M $ANAPP_3$ following photolysis ($+h\nu$) in the presence of the guinea-pig vas deferens on responses of the tissue to ATP. n = 6. ○ Control ($+h\nu$); ● $ANAPP_3$ (10^{-4} M, $+h\nu$). *Right panel*: Effect of apamin (1000 nM) on ATP-induced responses of guinea-pig vas deferens which had been treated with $ANAPP_3$ (10^{-4} M; $+h\nu$), as in the left panel. In these experiments $ANAPP_3$-treated tissues (open circles) served as the control for the effect of apamin on $ANAPP_3$-treated tissues (closed circles). n = 6. *Ordinates*: % 120 mM KCl.

of the biphasic ATP concentration-response curve into a monophasic one; the maximum response to ATP was not affected.

Apamin (1000 nM) had no effect on the residual responses of $ANAPP_3$-treated vasa deferentia to ATP, nor did not modify the monophasic ATP concentration-response curve of these $ANAPP_3$-treated tissues (fig. 3, right panel).

3.4. Effects of apamin on responses of $ANAPP_3$-treated tissues to other agonists

The effects of apamin (1000 nM) in vasa deferentia which had been treated with $ANAPP_3$ (fig. 4) were similar to those seen in control preparations (fig. 2) in that responses to NE, and Hist and ACh were potentiated substantially by apamin, whereas contractions elicited with KCl were unaffected. The EC_{50} values for Hist and KCl were not affected, while that for ACh was decreased (3.0-fold) significantly in the presence of apamin. In contrast to the above findings (fig. 2), the maximum response of $ANAPP_3$-treated preparations to ACh was not increased, and apamin caused a modest (1.7-fold) but significant reduction in the EC_{50} for NE. The reason for these two discrepancies in the effects of apamin in control vs. $ANAPP_3$-treated tissues, and whether there is physiological significance to them, is unclear at present.

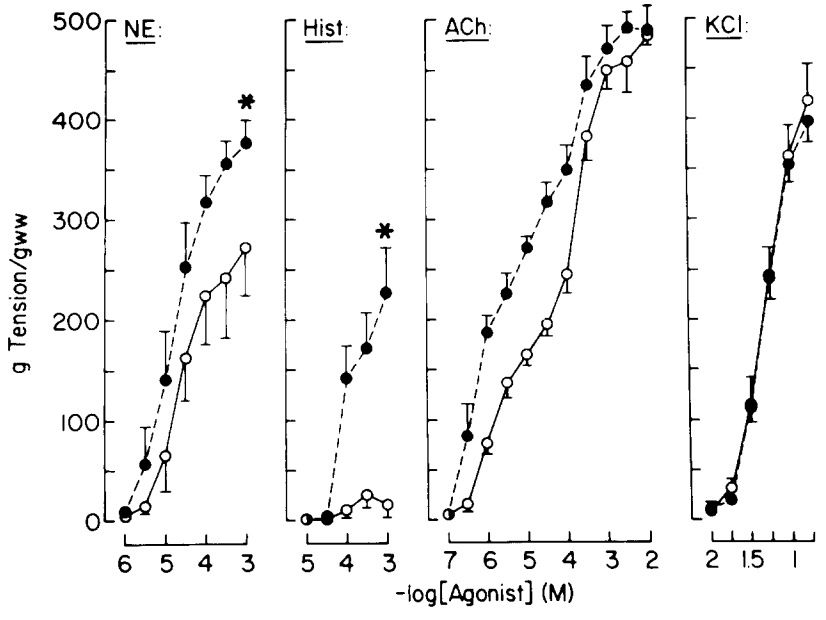

Fig. 4. Effect of apamin (1000 nM) on responses of $ANAPP_3$-treated (10^{-4} M; $+h\nu$) guinea-pig vas deferens to norepinephrine (NE; n = 4), histamine (Hist; n = 3), acetylcholine (ACh; n = 5) and KCl (n = 5). * Significantly different from contralateral, $ANAPP_3$-treated control. ○ Control; ● 1000 nM apamin.

4. Discussion

When used in concentrations equal to or greater than those which antagonize noncompetitively (Brown and Burnstock, 1981) adenine nucleotide-induced relaxation of gastrointestinal smooth muscle, i.e., 0.5-1 000 nM (vide supra), apamin did not antagonize contractile responses of the guinea-pig vas deferens to ATP. In contrast, apamin had very little effect in low concentrations (0.5 and 5 nM) but potentiated ATP-induced responses when present in a concentration (1 000 nM) well above that needed to block ATP-induced responses in gut preparations. This finding indicates that the ability of apamin to antagonize ATP-induced responses is tissue-specific, and it also may be related to the qualitative nature of the response, viz., contraction vs. relaxation. The effects of apamin in smooth muscles in which ATP causes contraction is apparently quite variable. Shuba and Vladimirova (1980) noted that contractions of bladder, uterus, anococcygeus, pulmonary artery and cerebral vessel preparations (species not indicated, but presumably these were guinea-pig tissues) to 10^{-3} M ATP were unaffected by apamin (concentration not indicated), whereas responses of the portal vein were, as described, 'increased somewhat'. In contrast, contractions of rat portal vein induced by ATP were unaffected by 50 000 nM apamin (Jodal et al., 1983).

The effect of apamin was nonspecific insofar as responses of the vas deferens to NE, Hist and ACh were potentiated. The sensitivity of the tissues to ACh also was increased, as reflected in the reduction in its EC_{50}. KCl-induced contractions were, however, unaffected in the presence of apamin. This degree of nonspecificity is comparable to that now generally recognized to occur in gastrointestinal muscle in which responses to catecholamines, adenine nucleotides, 5-hydroxytryptamine, ACh and neurotensin are antagonized or modified by apamin (Maas and Den Hertog, 1979; Maas et al., 1980; Brown and Burnstock, 1981; Vladimirova and Shuba, 1978; Shuba and Vladimirova, 1980; Jodal et al., 1983; Banks et al., 1979; Mackenzie and Burnstock, 1980), while those to vasoactive intestinal polypeptide and adenosine are not (Mackenzie and Burnstock, 1980; Brown and Burnstock, 1981; Daniel et al., 1983). The nonspecificity in the potentiating effect of apamin indicates that this agent would be of little utility as a tool to discriminate ATP-mediated responses in the guinea-pig vas deferens (Fedan et al., 1981).

The results of the present study demonstrate that the specific antagonism by $ANAPP_3$ of ATP-induced responses of the guinea-pig vas deferens (Hogaboom et al., 1980) does not involve a physiological antagonism resulting from an interaction of the photoaffinity label with the Ca^{2+}-dependent K^+ channels in the manner reported for apamin (Banks et al., 1979; Burgess et al., 1981; Hugues et al., 1982b,c,d). Several lines of evidence support this conclusion. (1) Responses to ATP were potentiated by apamin but antagonized by $ANAPP_3$. (2) The potentiation by apamin was a relatively nonspecific effect in that responses to NE, Hist and ACh were affected as well as those to ATP, while the antagonism produced by $ANAPP_3$ is specific for adenine nucleotides in guinea-pig vas deferens, urinary bladder and taenia coli (Hogaboom et al., 1980; Westfall et al., 1982, 1983). (3) Apamin binds to a 28 kilodalton component of the Ca^{2+}-dependent K^+ channel, as demonstrated following covalent crosslinking of apamin with disuccinimidyl suberate to rat brain membranes (Hugues et al., 1982d). In contrast, [^{3}H]$ANAPP_3$ following photolysis incorporates covalently in guinea-pig vas deferens membrane sites into 55-70 and 47-60 kilodaltons moieties (Fedan et al., 1983). To the extent that the apamin-binding site in rat brain-membranes and guinea-pig vas deferens are similar, it would appear that apamin does not bind to P_2-receptors in guinea-pig vas deferens, nor does $ANAPP_3$ covalently insert into the Ca^{2+}-dependent K^+ channel. (4) An interaction of apamin and of $ANAPP_3$ at the same site might be suggested by the observation that apamin was no longer able to potentiate ATP-induced responses in tissues which were treated with photolyzed $ANAPP_3$, i.e., that the prior covalent insertion of $ANAPP_3$ into Ca^{2+}-dependent K^+ channels prevented the subsequent action of apamin. However, apamin was as effective in potentiating responses to NE, Hist and ACh in $ANAPP_3$-treated tissues as it was in untreated tissues (compare figs. 2 and 4). Thus, photolyzed $ANAPP_3$ did not interfere

with the effect of apamin under conditions in which responses to ATP were antagonized.

Our findings indicate that the action of apamin is linked to receptor-operated cellular mechanisms in the guinea-pig vas deferens. The agent's inability to modify responses to KCl would indicate that it does not produce a partial membrane depolarization, as reported for taenia coli (Maas et al., 1980) because this effect results in an increased sensitivity of the muscle to excitatory agonists and to KCl (Fleming, 1980), and maximum responses to these agents are not elevated (Urquilla et al., 1978). The effect of apamin is clearly different. The possibility that apamin modifies the interaction of the agonists with their receptors is very unlikely because several neurotransmitters, neuropeptides and receptor antagonists do not alter the binding of apamin to its binding sites, which appears to be the Ca^{2+}-dependent K^+ channel (Cavey et al., 1979; Hugues et al., 1982a,b,c).

In gastrointestinal smooth muscle preparations, apamin inhibits ATP- and α-adrenoceptor-induced relaxation responses by preventing the membrane hyperpolarization which results from Ca^{2+}-dependent increases in K^+ permeability (Burgess et al., 1981; Banks et al., 1979; Maas and Den Hertog, 1980; Maas et al., 1981; Hugues et al., 1982c). The mechanism of potentiation of responses of the vas deferens by apamin may also involve an interaction of the agent with Ca^{2+}-dependent K^+ channels, which serve the purpose of action potential deactivation or membrane rectification (Vassort, 1981). All of the agonists used in the present study, including ATP (Sakai et al., 1979), induce depolarization and action potential firing. Action potential parameters may be affected by apamin in such a way as to prolong the excitatory signal. For example, in guinea-pig taenia coli apamin increased action potential amplitude and frequency, and reduced action potential decay time (Maas et al., 1981). Using this line of reasoning it would follow that excitation-contraction coupling involving voltage-dependent Ca^{2+} channels would be facilitated in the presence of apamin. It is puzzling why apamin did not modify KCl-induced contractions (figs. 2 and 4), since action potentials initiated with KCl would be expected to be modified similarly by apamin. However, the Ca^{2+}-dependent K^+ conductance of other tissues displays a dependence both on membrane potential and on extracellular K^+ concentration (Moolenaar and Spector, 1979; Barrett et al., 1982). The alteration in K^+ equilibrium potential upon the addition of KCl to the organ bath might perturb normal channel operation, rendering the binding of apamin to the channel silent.

It has been suggested that responses of the guinea-pig vas deferens to ATP may involve two, concentration-dependent interactions: one with two P_2-receptors, which are blocked by 10^{-4} M $ANAPP_3$, and a second, seen at higher ATP concentrations, whereby hydrolysis initiates the contractile response and which is not affected by 10^{-4} M $ANAPP_3$ (Fedan et al., 1982a,b;1983). The present results which show that the effect of apamin in untreated and in $ANAPP_3$-treated tissues is different supports this hypothesis. In studies employing the sucrose-gap technique, Sakai et al. (1979) observed that the membrane effects of exogenous ATP in the guinea-pig vas deferens are concentration-dependent: low concentrations (10^{-6} M) produced a transient depolarization and a few action potentials; high concentrations (10^{-4}-10^{-3} M) elicited a sustained depolarization, which persisted as long as the ATP was present, and repetitive action potential firing. These concentration ranges correspond to the two regions of the biphasic ATP concentration-response curve (Fedan et al., 1982a,b) which are $ANAPP_3$-sensitive (low concentrations) or $ANAPP_3$-resistant (high concentrations). There may exist an association between P_2-receptor activation and action potential formation on the one hand, and sustained depolarization and hydrolysis on the other. If after $ANAPP_3$-treatment responses to ATP involve only prolonged membrane depolarization and not action potential firing, then an action of apamin would not, in fact, be expected.

In summary, apamin is not an ATP antagonist in the guinea-pig vas deferens. Its nonspecific, agonist-potentiating effect could involve a similar interaction with Ca^{2+}-dependent K^+ channels which results in an antagonism in gastrointestinal smooth muscle. The antagonism of ATP-induced responses by $ANAPP_3$ is P_2-receptor mediated and does not involve an interaction with Ca^{2+}-dependent K^+ channels.

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