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MECHANISM OF PHOTOAFFINITY LABELING OF P_2-PURINERGIC RECEPTORS BY ARYLAZIDO AMINOPROPIONYL ATP IN ISOLATED GUINEA-PIG VAS DEFERENS

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Summary

Following photolysis in the presence of the isolated guinea-pig vas deferens, arylazido aminopropionyl ATP ($ANAPP_3$), a photoaffinity label analog of ATP, produces an irreversible and specific pharmacological antagonism of contractile responses to adenine nucleotides. Experiments were performed to determine whether the antagonism follows the photolysis-dependent formation of nitrene intermediates at occupied receptors (true photoaffinity labeling) or if the reactive intermediate is photogenerated in solution prior to diffusion to the receptor and formation of covalent bonds (pseudo-photoaffinity labeling). When present during photolysis, para-aminobenzoic acid (PABA; 10 mM), a scavenger for nitrenes generated in solution, did not prevent the antagonism of ATP-induced responses by $ANAPP_3$. However, the absorption spectrum of $ANAPP_3$ photolyzed in the presence of PABA was different from that obtained when PABA was not present. This evidence for the formation of additional photolysis products suggests that $ANAPP_3$ had inserted into PABA during photolysis. Thus, covalent bonds arise from true photoaffinity labeling of the receptor. An analysis of the pharmacological effects of PABA indicated that responses to ATP, KCl and acetylcholine were unaffected either in the presence of, or after a 23 min incubation, with 10 mM PABA. In contrast, PABA produced a substantial, but reversible, antagonism of histamine- and norepinephrine-induced contractions. Irradiation of tissues in the presence of 10 mM PABA produced a slight potentiation of responses to ATP. Thus, information on the mechanisms of photoaffinity labeling may be obtained from functional studies on intact tissues. However, the pharmacological effects of agents used to define these mechanisms should be evaluated as well.

Introduction

The compound arylazido aminopropionyl ATP* ($ANAPP_3$), a photoaffinity label analog of ATP, was described by Jeng and Guillory (1) and by Russell

*Abbreviations used are: arylazido aminopropionyl ATP or $ANAPP_3$, 3'-O-{3-[N-4(azido-2-nitrophenyl)amino]propionyl}adenosine 5'-triphosphate; PABA, para-aminobenzoic acid.

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et al. (2) to produce a photolysis-dependent inhibition of myosin and mitochondrial F_1-ATPases. Using the reasoning that photoaffinity labels might be useful probes in more complex systems, e.g., in intact, isolated smooth muscle preparations, we (3,4) evaluated the ability of $ANAPP_3$ to antagonize contractions of guinea-pig vasa deferentia and urinary bladder to exogenous ATP. These responses are mediated by a specific, P_2-purinergic (5,6) receptor. $ANAPP_3$, following its photolysis in the presence of these and other isolated tissues, produced an irreversible and specific antagonism of adenine nucleotide-induced responses, thereby allowing an evaluation of the role of ATP or related compounds in neurotransmission in the presence of a postjunctional receptor blockade (4,7).

Photolysis of $ANAPP_3$ generates an aryl nitrene intermediate which is highly reactive and which can insert covalently into organic bonds (8). That nonphotolyzed $ANAPP_3$ has affinity for the P_2-receptor in the guinea-pig vas deferens is shown by the fact that it mimics ATP pharmacologically (3). Thus, nitrenes produced by photolysis insert covalently at or near the P_2-receptor. The resulting receptor blockade is irreversible after photolysis and preventable if the competing ligand ATP is also present during irradiation (3). If the nitrene is formed from $ANAPP_3$ while it occupies a receptor, and if insertion takes place before the compound dissociates from the receptor, then true photoaffinity labeling will have occurred (8,9). Alternately, covalent insertion could take place following the interaction with the receptor of $ANAPP_3$-nitrene which is formed in the biophase and which diffuses, during its lifetime ($t_{1/2} \sim 10^{-3}$ sec; 10), to the receptor. The latter type of mechanism is referred to as pseudo-photoaffinity labeling (8,9). These possibilities have been distinguished in other systems by evaluating photoaffinity labeling in the presence of a free radical scavenger such as PABA or soluble protein (8,9) to provide nonspecific insertion sites for nitrene formed in free solution.

In the present study we evaluated whether photolysis of $ANAPP_3$ in the presence of PABA affects its ability to block P_2-receptors in the guinea-pig vas deferens. To ascertain whether mechanistically relevant scavenging by PABA can occur near the smooth muscle cell membrane, the pharmacological effects of PABA on other receptor systems was also determined.

Methods

Vasa deferentia from adult albino guinea pigs were prepared for the measurement of isometric contractile responses and mounted in separate, water-jacketed glass organ chambers containing modified Krebs-Henseleit solution (37°C), as described previously (11). The tissues were equilibrated for 1 hr under 0.2-0.4 g resting tension before the experiment was begun.

$ANAPP_3$ was synthesized according to Jeng and Guillory (1). Treatment of the tissues with photolyzed $ANAPP_3$ was as follows. $ANAPP_3$ (10^{-4} M) was added to the organ bath containing the vas deferens and, after a 3 min incubation in the dark, the preparation was irradiated continuously for 15 min with a Dyna-Lume lamp which housed a DYH (600 W; 3,200° K) tungsten-halogen projector lamp. The filament was 15 cm from the center of the chamber. Bath temperature was maintained at 37° C with an external heating/cooling circulator. At the end of irradiation the tissue was washed twice with fresh Krebs-Henseleit solution lacking $ANAPP_3$. Concentration-response determinations were begun 10 min later. Control preparations were irradiated in the absence of $ANAPP_3$.

To evaluate its effect on the antagonism by photolyzed $ANAPP_3$, PABA (10 mM) was added to the baths of control and test preparations 5 min before

the incubation of the test tissue in the dark with $ANAPP_3$. The PABA remained present during the irradiation step prior to its washout. Thus, the tissues were in contact with PABA for 23 min. The effect of irradiation of tissues in the presence of PABA alone on responses to MgATP was evaluated following the incubation of one tissue of a pair in 10 mM PABA for 8 min prior to irradiation of control and test tissues for 15 min. To determine if transient incubation of the tissues with PABA *per se* could modify the pharmacological properties of the tissues, some preparations were incubated with 10 mM PABA for 23 min, followed by washout, before agonists were added. The contralateral control vasa deferentia received no treatment. In a separate series of experiments PABA (10 mM) was added 10 min prior to, and remained present during, concentration-response determinations. Contralateral control preparations were not exposed to PABA.

Concentration-response relationships were obtained following the stepwise, noncumulative addition of agonists to the baths. Each concentration was present for 3 min, followed by two washes at 5 min intervals prior to the next addition.

The data were evaluated for significant differences with Student's *t*-test. A probability less than 0.05 was considered significant. *n* is the number of separate experiments. Means are presented ± S.E.M.

Results

Under the conditions described in Methods, photolysis of $ANAPP_3$ for 15 min converts 80% of the compound to unknown products (manuscript in preparation). The effect of PABA on the resulting antagonism of ATP-induced contractions is shown in Figure 1. Compared to the contralateral control tissues irradiated in the presence of PABA, the concentration-response curve for the $ANAPP_3$-treated preparations was shifted substantially to the right.

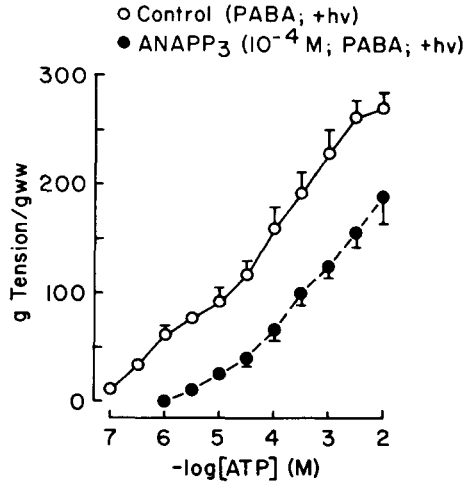

FIG. 1

Effect of $ANAPP_3$ (10^{-4}M) photolyzed (+hv) in the presence of 10 mM PABA on the MgATP concentration-response relationship of guinea-pig vas deferens. Controls were irradiated in the presence of PABA. See Methods for details. g Tension/gww is developed tension divided by grams wet tissue weight. *n*=6.

This shift is in agreement with that observed previously (3) in experiments in which $ANAPP_3$ was photolyzed in the absence of PABA. That is, the antagonism by $ANAPP_3$ was not reduced by irradiating the compound in the presence of the scavenger. These findings are opposite those obtained by

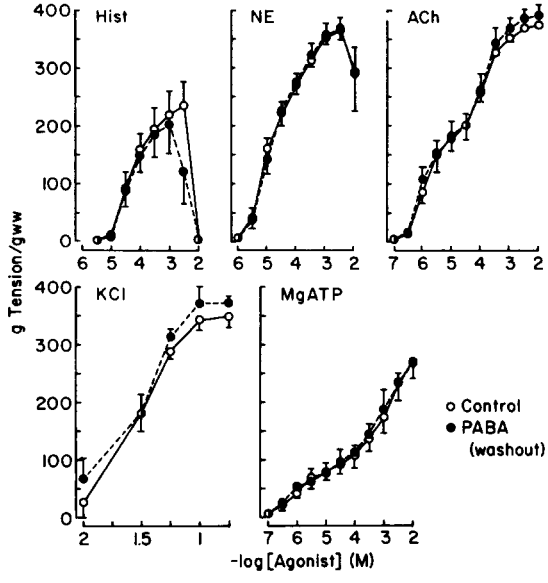

FIG. 2

Concentration-response relationships of various agonists obtained in the absence of PABA and 10 min after a 23 min transient incubation in 10 mM PABA. n=4 for each agonist.

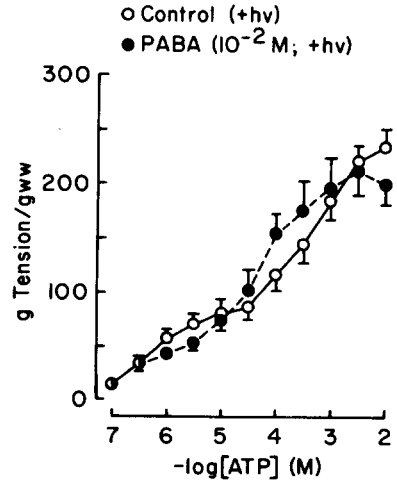

FIG. 3

Effect of irradiation in the presence of 10 mM PABA on the MgATP concentration-response relationship. Controls were irradiated in the absence of PABA. n=5.

Ruoho et al. (9), who observed that PABA prevented inhibition of erythrocyte acetylcholinesterase by a quarternary ammonium aryl azide, and who concluded that pseudo-photoaffinity labeling of the enzyme had occured.

In this series of experiments the maximum response to MgATP was reduced significantly by $ANAPP_3$. This finding has not been observed previously (3 and additional unpublished findings). Experiments were performed to determine if the reduced maximum response observed here could be attributable to the presence of PABA during irradiation or to some other pharmacological effect of the scavenger. First, the effect of a transient 23 min incubation with PABA was examined. Figure 2 shows that the concentration-response curves for histamine, norepinephrine, acetylcholine, KCl and MgATP were not modified 10 min after the incubation in PABA. These findings suggested that the reduced maximum response to MgATP following treatment with photolyzed $ANAPP_3$ was not explained by an irreversible action of nonirradiated PABA per se.

This experiment did not eliminate the possibility that PABA contributed to the decreased maximum response to MgATP as a result of irradiation of the agent in the presence of the tissue. This was examined in separate experiments by comparing the responses to MgATP following irradiation in the presence of 10 mM PABA with those obtained after irradiation in the absence

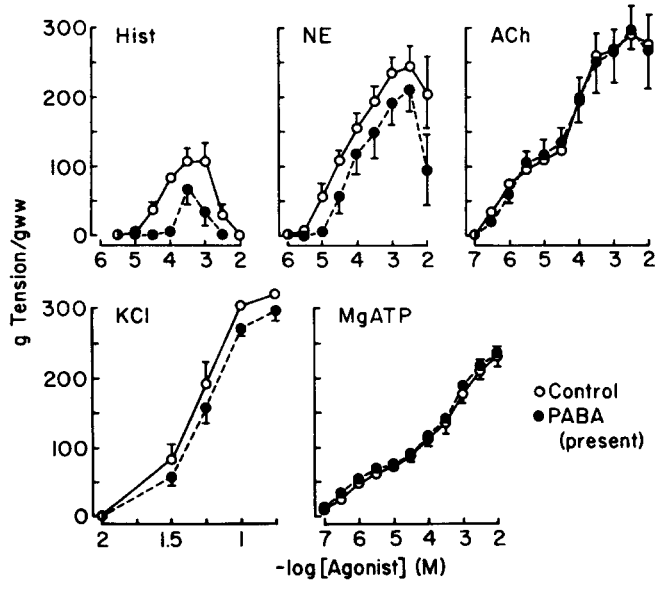

FIG. 4

Concentration-response relationships of various agonists obtained in the absence and in the continued presence of 10 mM PABA. n=6 for each agonist.

of the agent. PABA was essentially without effect on MgATP concentration-response curves under these conditions (Figure 3), although there was a modest potentiation of responses to higher concentrations (10^{-4} - 10^{-3} M) of MgATP. The reduced maximum response to MgATP observed in the presence of PABA (Figure 1) may have been a result, in part, of this potentiation in the

control preparations.

To further examine possible pharmacological effects of PABA and to obtain information as to whether it had access to the biophase where a scavenging effect would have been important in interpreting mechanisms, concentration-response relationships obtained in the absence and presence of 10 mM PABA were compared. The results are shown in Figure 4 which indicates that there was a substantial antagonism of histamine- and norepinephrine-induced responses. The concentration-response curves for acetylcholine, KCl and MgATP were not modified in the presence of PABA. These results are to be compared to Figure 2 in which it was shown that a transient incubation of the tissues with PABA had no residual pharmacological effect.

The preceding results might suggest that PABA failed to reduce the antagonism by $ANAPP_3$ (Figure 4) because no scavenging effect occurred in the organ bath. Therefore, evidence was sought to determine whether $ANAPP_3$ had inserted into PABA. Absorption spectra of $ANAPP_3$ (10^{-4} M) which was photolyzed in the absence and presence of 10 mM PABA were compared. Shown in Figure 5 is the fact that the spectrum of $ANAPP_3$ photolyzed in the presence of PABA was different from the one obtained when PABA was absent. This difference suggests that additional reaction products were formed as a result of a chemical interaction between $ANAPP_3$ and PABA, i.e., that PABA exerted a scavenging effect on photolyzed $ANAPP_3$ in free solution.

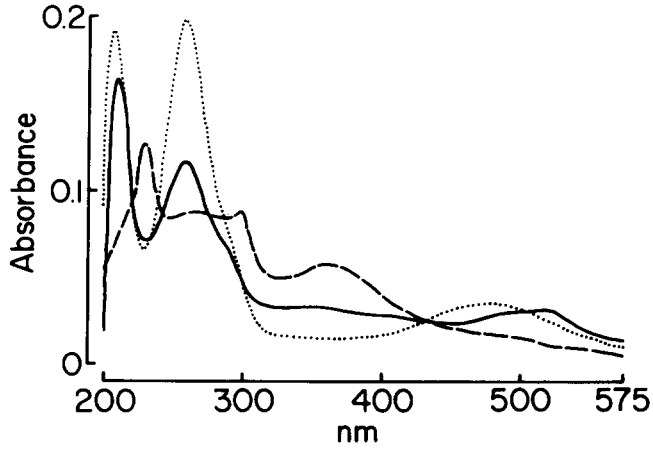

FIG. 5

Spectra of $ANAPP_3$ photolyzed in organ chambers in the absence and presence of PABA (10 mM). Solid line: Spectrum of $ANAPP_3$ (in Krebs-Henseleit solution) following photolysis (15 min) in the absence of PABA. The $ANAPP_3$ concentration was 10^{-4} M during photolysis; for scanning, the $ANAPP_3$ concentration was diluted to 10^{-5} M. The reference cuvette contained Krebs-Henseleit solution. Dashed line: Spectrum of $ANAPP_3$ (in Krebs-Henseleit solution) following photolysis in the presence of PABA. $[ANAPP_3]_{chamber}$ was 10^{-4} M; $[ANAPP_3]_{cuvette}$ was 10^{-5} M. The reference cuvette contained 10 mM PABA in Krebs-Henseleit solution. Photolysis (15 min) had no effect on the spectrum of PABA (data not shown). Dotted line: Spectrum of nonphotolyzed $ANAPP_3$ (in Krebs-Henseleit solution; 10^{-5} M in cuvette) for comparison. The reference cuvette contained Krebs-Henseleit solution. Baselines were corrected electronically.

Discussion

The mechanistic information which can be obtained by studying photoaffinity labeling in the presence of a scavenger has been reviewed (8-10,12). The degree of reversible binding of a photoaffinity label to a specific site is determined, in part, by the affinity of the parent molecule for the site. However, the insertion of the aryl nitrene into C-H bonds is nonselective. It may occur at regions in or distant from the binding site. Nonselective labeling of regions outside the site can be reduced and estimated in the presence of a scavenger (9). The degree of labeling of nonspecific, non-receptor sites by photolyzed $ANAPP_3$ in the intact vas deferens is currently unknown. Nonselective photoaffinity labeling by $ANAPP_3$, if it occurs, has no effect on the pharmacological characteristics of the tissue (3). An indication of nonselective incorporation of photolyzed $ANAPP_3$ in the guinea-pig vas deferens may be given by the effects of scavengers on the binding of an isotopic form of $ANAPP_3$ rather than by their effects on functional responses of the tissue. Jeng and Guillory (1) observed negligible nonselective labeling by $[^{14}C]$-$ANAPP_3$ of myosin ATPase. It is thus possible that very little nonselective labeling occurs in the isolated vas deferens.

Information was gained in this study regarding whether the antagonism of MgATP-induced responses by photolyzed $ANAPP_3$ resulted from true photoaffinity labeling or from pseudo-photoaffinity labeling. The inability of PABA to lessen the antagonism by photolyzed $ANAPP_3$ of ATP-induced responses (Figure 1) indicates that the antagonism resulted from true photoaffinity labeling of the P_2-receptor. This conclusion is supported by the evidence (Figure 5) that $ANAPP_3$, in free solution, had inserted upon photolysis into PABA. Pseudo-photoaffinity labeling at a receptor level should have been prevented under these conditions. The pharmacological consequences of photoaffinity labeling by either mechanism, namely, the antagonism of ATP-induced contraction, would be expected to be the same, however.

The conclusion that $ANAPP_3$ is antagonistic as a result of true photoaffinity labeling was also contingent upon knowledge of the pharmacological effects of PABA, *per se*. For example, had PABA been totally innocuous pharmacologically, then it would have been questionable whether PABA and $ANAPP_3$ could interact at a receptor level. However, that PABA did gain entry into the biophase was indicated by its antagonistic effect on histamine-and norepinephrine-induced contractions (Figure 4). PABA, at a 10 mM concentration, appears to have interacted with α-adrenoceptors and H_1-histamine receptors, perhaps because it contains an amine grouping. Fortunately, this antagonism was readily reversed following washout of PABA from the bath (Figure 2). A residual pharmacological effect would have complicated the interpretation of its effect when present during the photolysis of $ANAPP_3$.

A large number of photoaffinity labels have been utilized as probes in biochemical studies. When they are used, certain criteria must be satisfied (8,10,12,13). These criteria should and have been satisfied (3,11) when they are applied in functional studies on intact tissues such as smooth muscle. In general, the present study indicates that mechanisms related to photoaffinity labeling theory may also be evaluated in more complex systems. Because of possible interactions with cellular processes unrelated to the mechanism under study, the pharmacological effects of those agents used to define mechanisms, such as scavengers, also need to be evaluated.

Acknowledgements

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