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COMPARISON OF PHOTOAFFINITY LABELING OF P_2-PURINERGIC RECEPTORS OF ISOLATED GUINEA-PIG VAS DEFERENS BY ARYLAZIDO AMINOPROPIONYL ATP AND BY ARYLAZIDO AMINOBUTYRYL ATP *

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Two chemically related arylazido photoaffinity analogs of ATP (arylazido aminopropionyl ATP ($ANAPP_3$) and arylazido aminobutyryl ATP ($ANABP_3$)), which have been reported in the literature to differ in their ability to inhibit myosin ATPase, were compared for their ability to antagonize contractile responses of the isolated guinea-pig vas deferens to ATP. During photolysis in organ chambers the photoconversion ($\Delta A_{260}/\Delta t$) of $ANAPP_3$ occurred with greater than first order kinetics or was multiexponential and $t_{1/2} = 7.5$ min, while $\Delta A_{260}/\Delta t$ for $ANABP_3$ was first order and $t_{1/2} = 2.25$ min. After photolysis of these compounds in the presence of the guinea-pig vas deferens, using irradiation periods which caused 80% consumption of the compounds, $ANABP_3$ was 2-3 times more potent than $ANAPP_3$ in antagonizing contractions to ATP, which are mediated by P_2-purinergic receptors. A comparison of concentration-response curves obtained for nonphotolyzed $ANAPP_3$ and $ANABP_3$ used as agonists suggested that the greater antagonism produced by photolyzed $ANABP_3$ is not attributable to a greater potency. The results suggest that the longer 3′-hydroxyl-arylazide bridge length of $ANABP_3$ places the arylnitrene intermediate in a position at or near the P_2-receptor which is more favorable for covalent insertion.

P_2-purinergic receptors Arylazido aminopropionyl ATP Arylazido aminobutyryl ATP

1. Introduction

Jeng and Guillory (1975) described the synthesis of arylazido photoaffinity analogs of ATP and their ability to inhibit myosin ATPase. One analog, $ANAPP_3$ (arylazido aminopropionyl ATP; 3′-O-(3-[N-(4-azido-2-nitrophenyl)amino]propionyl)-ATP), produces following its photolysis in organ baths containing the vas deferens a specific and irreversible antagonism of adenine nucleotide-induced responses (Hogaboom et al., 1980; Frew and Lundy, 1981). The compound demonstrated the existence of P_2-purinergic receptors which have been described by Burnstock (Burnstock, 1979).

Jeng and Guillory (1975) observed that the extent of inhibition of myosin ATPase activity by these analogs was related to the distance of the arylazido group from the 3′-hydroxyl of ribose. From their fig. 5 (p. 460), half-maximal inhibition of activity by $ANAPP_3$ occurred at an extrapolated value of ca. 100 μM. A second compound, $ANABP_3$ (arylazido aminobutyryl ATP; 3′-O-(4-[N-(4-azido-2-nitrophenyl)amino]butyryl)ATP), produced following an identical photolysis period half-maximal inhibition of activity at ca. 15 μM. $ANABP_3$ contains one more carbon than $ANAPP_3$

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in the 3′-hydroxyl-arylazide bridge.

In the present study $ANAPP_3$ and $ANABP_3$ were compared to determine whether a relationship also exists between bridge distance and P_2-purinergic receptor antagonism when the compounds are photolyzed in the presence of the guinea-pig vas deferens.

2. Materials and methods

$ANAPP_3$ and $ANABP_3$ were synthesized as described by Jeng and Guillory (1975).

The time courses of photoconversion of the compounds were determined as follows. $ANAPP_3$ (10^{-4} M) or $ANABP_3$ (10^{-4} M) were dissolved in the dark in modified Krebs-Henseleit solution (see O'Donnell et al. (1981) for composition). Solutions were placed in a 3 ml glass, water-jacketed (37°C) organ chamber and aerated vigorously (95% O_2-5% CO_2). A 0.1 ml sample was placed in a foil-wrapped tube and saved for analysis. The chamber was then irradiated continuously with a tungsten-halogen projecter bulb (DYH, 600 W, 3,200°K) housed in a Dyna-Lume heat projector (Cole-Parmer Instrument Co., Chicago, IL). The filament was 15 cm from the center of the chamber. Samples (0.1 ml) were removed at intervals and protected from light. 0.9 ml of Krebs-Henseleit solution was added to each sample (final concentrations of $ANAPP_3$ and $ANABP_3$ were 10^{-5} M) and the solutions were scanned (200-575 nm) against Krebs-Henseleit solution with a spectrophotometer. The time-dependent absorbance change at 260 nm was measured to obtain kinetic information.

Vasa deferentia from adult albino guinea pigs, killed by a blow to the head, were prepared for the measurement of isometric contractile responses. They were mounted in the 3 ml organ chambers described above which contained aerated Krebs-Henseleit solution (37°C). The tissues were equilibrated for 1 h under 0.2-0.4 g resting tension prior to the experiment.

To evaluate the antagonisms by $ANAPP_3$ (10^{-4} M) and by $ANABP_3$ (10^{-4} M), the compounds were photolyzed in the organ baths in the presence of the tissues (see above) following a 3 min incubation in the dark. Contralateral control tissues were irradiated in the absence of any agent. ATP was added noncumulatively with stepwise increases in concentration beginning 10 min after the end of irradiation. Each concentration was present for 3 min and then washed out. Ten min elapsed between additions. When nonphotolyzed $ANAPP_3$ and $ANABP_3$ were used as agonists to compare concentration-related contractions with those elicited by ATP in the contralateral vas deferens, 1 ml water-jacketed organ chambers were used and the experiments were carried out in near-total darkness.

Responses were normalized in terms of the tissue's maximum response, obtained by adding 120 mM KCl at the end of the experiment. The data were evaluated for differences with Student's t-test using paired and unpaired analysis where appropriate. The 0.05 level of probability was accepted as significant. n is the number of separate experiments. The results are presented as means ± S.E.M.

3. Results

3.1. Agonist actions of nonphotolyzed $ANAPP_3$ and $ANABP_3$

$ANAPP_3$ causes a contractile response of the guinea-pig vas deferens when initially added to the bath, and no irreversible antagonism of ATP-induced responses occurs if the compound is not photolyzed (Hogaboom et al., 1980). Responses to ATP and to nonphotolyzed $ANAPP_3$ and $ANABP_3$ were compared. The $ANAPP_3$ concentration-response curve resembled that for ATP in that it was biphasic (fig. 1). A hypothesis explaining biphasicity of these curves has been advanced (Fedan et al., 1982a,b). $ANAPP_3$ appeared essentially equipotent with ATP, but the two curves were not superimposable. The maximum response to $ANAPP_3$ was slightly but significantly less than that to ATP ($P < 0.05$; paired analysis). Individual responses to $ANAPP_3$ in lower ($\sim 10^{-4}$ M) concentrations were prolonged compared to ATP and were characterized by an accentuated tonic component, while higher concentrations gave re-

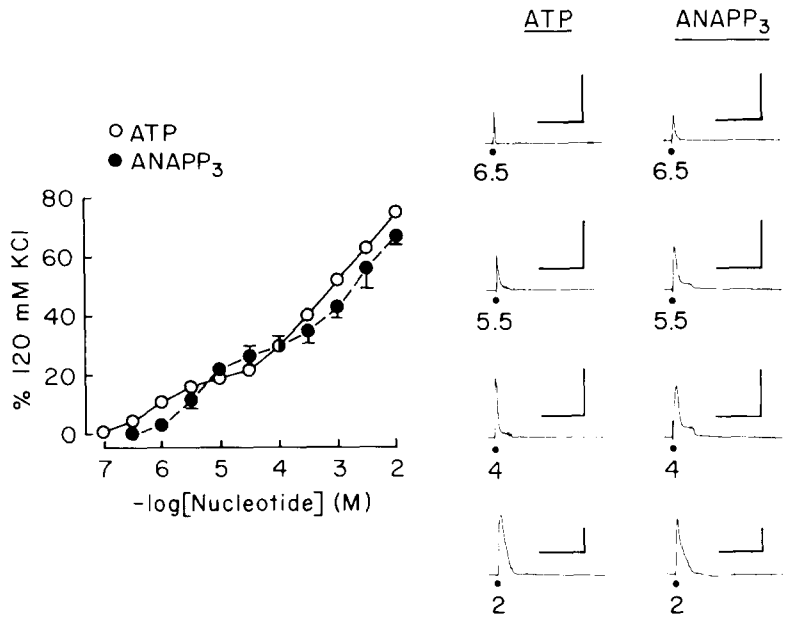

Fig. 1. Comparison of ATP and nonphotolyzed $ANAPP_3$ concentration-response curves and representative individual responses. n = 4. The horizontal calibration shows 3 min and the vertical calibration shows 2 g tension.

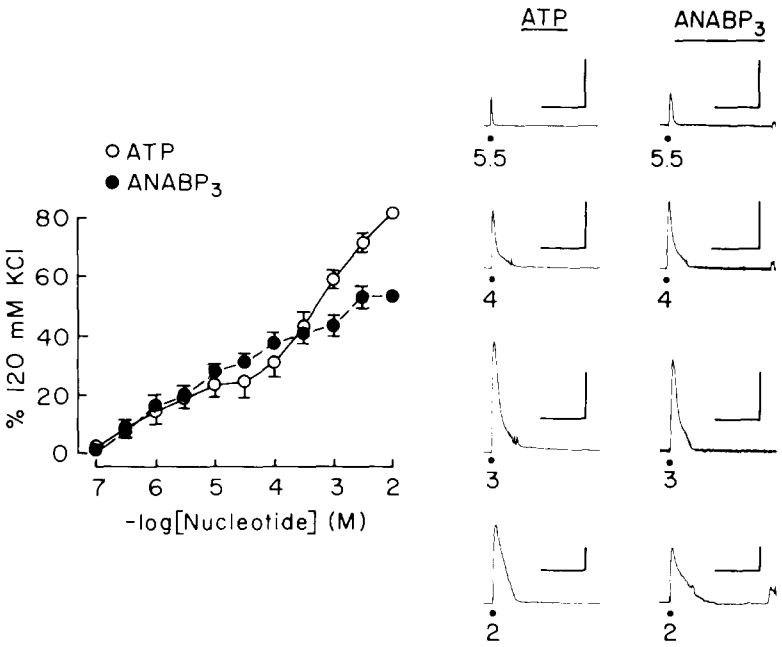

Fig. 2. Comparison of ATP and nonphotolyzed $ANABP_3$ concentration-response curves and representative individual responses. n = 4. Horizontal calibration: 3 min; vertical calibration: 2 g tension.

sponses similar to those elicited by ATP. The concentration-response curve for $ANABP_3$ (fig. 2) was monophasic, the curve was essentially superimposable in low concentrations with that of ATP, and the maximum response obtained was substantially and significantly less ($P < 0.05$; paired analysis) than the maximum response to ATP. As in the case of $ANAPP_3$, responses to low concentrations were prolonged with respect to ATP-induced responses; responses to high concentrations of $ANABP_3$ were similar to responses to ATP. It is somewhat remarkable that the addition of these bulky substituents at the 3′ position of ribose should be so well tolerated by the P_2-receptor. Compared to ATP, the alterations in the morphology of responses to low concentrations of $ANAPP_3$ and $ANABP_3$ are similar to those reported for a number of adenine nucleotides with phosphate chain modification which would be expected to render the compounds as lesser substrates for ectophosphohydrolase enzymes (Fedan et al., 1982a).

3.2. Kinetics of photoconversion of $ANAPP_3$ and $ANABP_3$

The native spectrum for nonphotolyzed $ANABP_3$ resembled that for $ANAPP_3$ (see Hogaboom et al., 1980). Photolysis of both compounds caused time-dependent reductions in absorbance at the 260 nm peak, the appearance of many peaks and troughs between 300-575 nm, and the gradual loss of the 450-460 nm broad peak (see Hogaboom et al., 1980 for example). The time course of absorbance changes at the 260 nm peak for both $ANAPP_3$ and $ANABP_3$ are shown in fig. 3. Both compounds appeared to have similar time courses for photoconversion. Log transformation of the data (insets) resulted in a straight line in the case of $ANABP_3$, indicative of a single exponential or first-order process. However, log transformation yielded a curve in the case of $ANAPP_3$, indicative of a multiexponential process or one which is greater than first order. Since the reactions for both agents, within the limits of detection, went to completion (no further $\Delta A_{260}/\Delta t$ was observed after 90 min for $ANAPP_3$

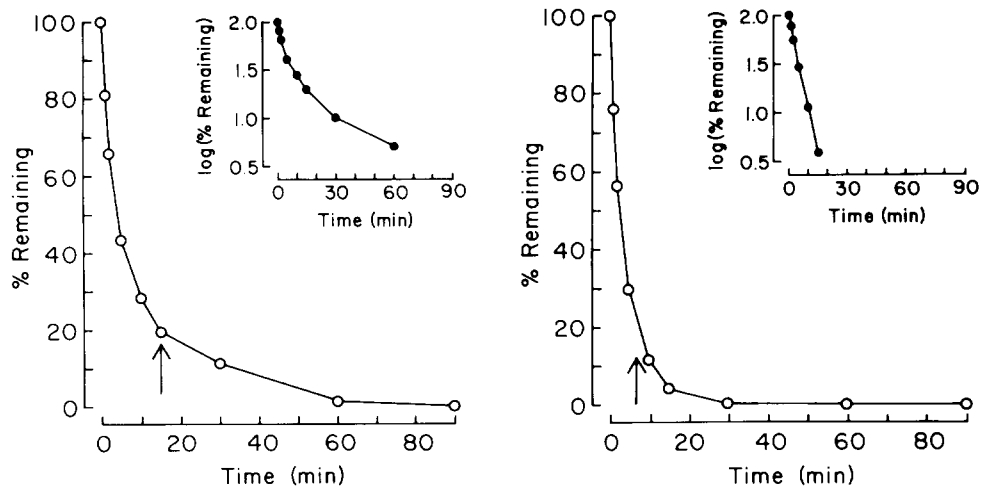

Fig. 3. Time courses of reduction in absorbance at 260 nm for 10^{-4} M $ANAPP_3$ (left panel) and 10^{-4} $ANABP_3$ M (right panel) during continuous photolysis of the compounds in organ chambers. '% Remaining' was determined from the change in absorbance observed at sampling intervals compared to the total absorbance change produced between t = 0 and when the reactions reached completion (90 min for $ANAPP_3$, 60 min for $ANABP_3$), i.e., $100\% - [(A_{t=0} - A_{t=i})/\Delta A_{total} \times 100]$ where i = time of sample. The arrows indicate that different photolysis periods were needed to produce 80% of the maximum ΔA_{260}. These periods were used in the experiments shown in fig. 4. *Insets*: Logarithmic ordinate plots to evaluate exponential behavior of $\Delta A/\Delta t$.

and after 60 min for $ANABP_3$) no attempt was made to resolve the composite curve for $ANAPP_3$ into component exponential functions by the process of 'curve stripping'. While it may be that $\Delta A_{260}/\Delta t$ for $ANAPP_3$ is a multiexponential process, the data were also evaluated assuming that $\Delta A_{260}/\Delta t$ might occur with an order different than unity, viz., that (% Remaining)^x = t and $x \neq 1$, where x is the order of the reaction. A plot of log t vs. log(% Remaining) using the 1, 2, 5, 10, 15 and 30 min % Remaining values produced an essentially linear function, and linear regression of the relationship log t vs. xlog(% Remaining) gave a value for x of 1.500 (correlation coefficient = 0.99).

The time required to produce 50% of the maximum ΔA_{260} ($t_{1/2}$) was determined by graphic approximation to be 7.5 min for $ANAPP_3$. For $ANABP_3$, linear regression of the logarithmic plot gave $t_{1/2} = 2.25$ min. No information is available at present to explain the chemical basis for these differences. Additional work will be necessary to ascertain in more detail the kinetic characteristics of $\Delta A_{260}/\Delta t$ for $ANAPP_3$.

3.3. Effects of photolyzed $ANAPP_3$ and $ANABP_3$ on the ATP concentration-response relationship

The above findings indicated that a comparison of the pharmacological properties of photolyzed $ANAPP_3$ and $ANABP_3$ must take into account the different kinetic characteristics of $\Delta A_{260}/\Delta t$ in designing the experimental protocol. Thus, Jeng and Guillory (1975) observed a greater inhibitory potency of photolyzed $ANABP_3$ compared to $ANAPP_3$, but the *same* photolysis period (2 min) was used for each agent. It is clear from fig. 1 that, before the reactions go to completion, a greater fraction of total $ANABP_3$ would be photoconverted in a given photolysis period than occurs with $ANAPP_3$. Therefore, in the present experiments, $ANAPP_3$ and $ANABP_3$ were photolyzed in the presence of the tissues using irradiation periods which resulted in the consumption of 80% of starting material (e.g., 15 min for $ANAPP_3$ and 7.25 min for $ANABP_3$) as determined from the above kinetic data.

The concentrations of $ANAPP_3$ and $ANABP_3$ used (10^{-4} M) were equieffective ($P > 0.05$; unpaired analysis) in their ability to cause contraction as judged from full concentration-response curves for agonist activity (figs. 1 and 2) and from the contractile responses elicited by the agents upon addition to the organ bath (117.6 ± 12.1 and 119.7 ± 5.0 g developed tension per gram wet tissue weight for $ANAPP_3$ (n = 5) and $ANABP_3$ (n = 6), respectively). Thus, precautions were taken to achieve equivalent fractional receptor occupancies by the nonphotolyzed compounds prior to photolysis, and the above results suggest strongly that this was accomplished.

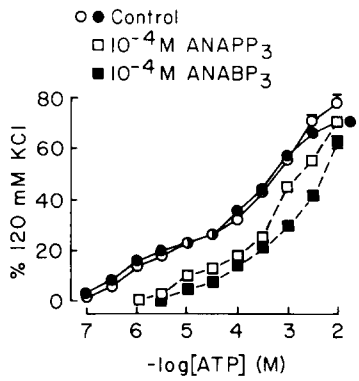

Fig. 4. Comparison of the antagonistic effects of 10^{-4} M $ANAPP_3$ (photolyzed for 15 min) and 10^{-4} M $ANABP_3$ (photolyzed for 7.25 min) on the ATP concentration-response curve. *Open circles*: irradiated contralateral controls for $ANAPP_3$ treatment. *Closed circles*: irradiated contralateral controls for $ANABP_3$ treatment. *Open squares*: $ANAPP_3$-treated (n = 5). *Closed squares*: $ANABP_3$-treated (n = 6). Values shown are means ± S.E.M.

Under these conditions the ATP concentration-response curve following $ANABP_3$ treatment was shifted 2-3 fold to the right of the curve describing the antagonistic effect of photolyzed $ANAPP_3$ (fig. 4), a finding in qualitative agreement with the greater inhibition of myosin ATPase by $ANABP_3$ which was seen by Jeng and Guillory (1975). That is, $ANABP_3$ was a more 'potent' antagonist than $ANAPP_3$. Neither compound affected the maximum response to ATP ($P > 0.05$; paired analysis).

4. Discussion

Two assumptions were made in this study, namely, (1) that the extent of photoconversion (i.e., nitrene formation) is proportional to $\Delta A_{260}/\Delta t$ and (2) that the extent of receptor blockade by these compounds is related to the time-dependent but not necessarily linear formation of nitrene intermediate, up until consumption has occurred (Kyte, 1981). If the affinities of nonphotolyzed $ANAPP_3$ and $ANABP_3$ for the receptor are the same, or if an equivalent fractional receptor occupancy by compound before photolysis is produced at 10^{-4} M, as the data suggests, then the relative 'potency' of photolyzed compound will be linked to the kinetics and extent of photoactivation, the efficiency of photoaffinity labeling, the availability of insertion sites, and the time-course of insertion (i.e., receptor blockade), which, in this system, is unknown. If the affinities of nonphotolyzed $ANAPP_3$ and $ANABP_3$ are in fact not very similar, then different fractional receptor occupancies would result at 10^{-4} M, and the greater (2- to 3-fold) antagonistic 'potency' of photolyzed $ANABP_3$ could reflect this difference. Present results suggest tentatively that this important theoretical consideration does not obtain, for there is little evidence to suggest (figs. 1 and 2) that nonphotolyzed $ANABP_3$ is 2 to 3 times more potent than $ANAPP_3$.

In the present study, the fractional photoactivation of $ANAPP_3$ and $ANABP_3$ was controlled. If our assessment of the relative affinities of $ANAPP_3$ and $ANABP_3$ prove accurate, then the variables which determine 'potency' thus reduce to efficiency of photoaffinity labeling and availability of insertion sites. Both factors would influence the time-course of covalent insertion into the receptor. The two compounds, upon photolysis, may insert covalently into different moieties at or near the receptor (Jeng and Guillory, 1975), or the longer 3′-hydroxyl-arylazide bridge length of $ANABP_3$ might place the aryl nitrene in a location in the receptor domain which increases the probability that insertion will take place. Should the latter occur, then the time-course of insertion into receptors would increase, and the greater 'potency' of photolyzed $ANABP_3$ would reflect a blockade of a greater number of receptors than produced by $ANAPP_3$, even though a shorter photolysis period was used.

In conclusion, comparisons of the antagonistic potency of photoaffinity labels should take into account the aforementioned variables. Under rigorously controlled conditions, photolyzed $ANABP_3$ was more potent than $ANAPP_3$ in antagonizing ATP-induced responses. This may have occurred because of a faster rate of receptor blockade. The precise reason for the difference will ultimately require knowledge of the actual relative affinities and efficacies of both agents for the P_2-purinergic receptor, of which nothing is known at present.

Acknowledgement

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