Chapter 14

Use of Photoaffinity Labels as P_2-Purinoceptor Antagonists

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I. INTRODUCTION

In recent years there has been considerable interest in the possible role of adenine nucleotides, such as ATP, as neuromodulators (Su, 1977; De Mey *et al.*, 1979; Katsuragi and Su, 1982), neurotransmitters (Burnstock *et al.*, 1970; Burnstock, 1979), or cotransmitters (Westfall *et al.*, 1978; Fedan *et al.*, 1981; Sneddon *et al.*, 1982a). The chief impediment to accepting the notion that adenine nucleotides act as neuromodulators or neurotransmitters 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, quinidine, apamin, and 2-substituted imidazolines, the antagonism afforded by these drugs in several autonomic nerve-smooth muscle preparations is nonspecific; i.e., in concentrations sufficient to antagonize responses to ATP, the responses to other agonists are also reduced (Weetman and Turner, 1977; Burnstock, 1979, 1983).

In 1980, it was reported that 3′-*O*-{3[*N*-(4-azido-2-nitro-phenyl)amino] propionyl}adenosine 5′-triphosphate ($ANAPP_3$), a photoaffinity analog of ATP,

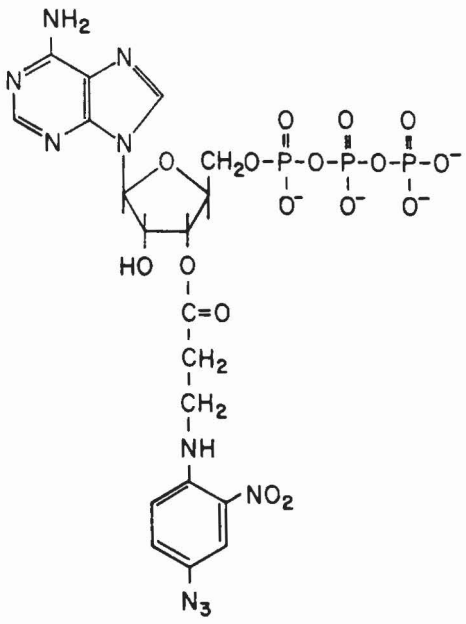

Figure 1. Structure of 3′-O-{3[*N*-(4-azido-2-nitrophenyl)amino]propionyl} adenosine 5′-triphosphate or $ANAPP_3$.

antagonized contractile responses of the guinea-pig vas deferens to adenine nucleotides, but not to a number of other agonists, including norepinephrine, acetylcholine, histamine, or KCl (Hogaboom *et al.*, 1980). Subsequent studies with a number of smooth muscle preparations have confirmed the specificity of antagonism by $ANAPP_3$, and, further, have demonstrated the utility of this substance as an antagonist of P_2 purinoceptors. The goal of this chapter is to provide a guide for the use of this photoaffinity label to produce a blockade of P_2 purinoceptors in isolated, intact tissue preparations.

II. THEORETICAL ASPECTS OF PHOTOAFFINITY LABELING

A thorough discussion of the theory of photoaffinity labeling is beyond the scope of this chapter. However, there are a number of general reviews that discuss in considerable detail the theoretical basis of this technique. Included among these are the following: Cooperman (1976); Bayley and Knowles (1977); Chowdry and Westheimer (1979); Fedan *et al.* (1983a,b); Guillory and Jeng (1983).

Photoaffinity labels are analogs of parent compounds that have an inherent affinity for a binding site and that also contain a light-sensitive grouping that, when irradiated with light, are capable of forming a covalent bond at or near the binding site. The light-sensitive moiety of $ANAPP_3$ (Figure 1) is an arylazide that is connected to the 3′-hydroxyl grouping of the parent ATP molecule through a β-alanine bridge. The rationale for the use of this compound to study P_2 puri-

noceptors is that the ATP portion of the molecule directs the binding of the compound to the receptors, and photolysis of the compound that is bound to the receptor results in the formation of a reactive nitrene intermediate that is capable of forming covalent bonds at or near the receptor. The result is a specific pharmacological antagonism of responses mediated via the receptors for ATP. Furthermore, the covalent attachment of $ANAPP_3$ to the receptors provides a means for biochemical characterization (Fedan *et al.*, 1983b) and isolation of the receptors.

III. SYNTHESIS OF $ANAPP_3$

At the present time, $ANAPP_3$ is not commercially available, thus, synthesizing the compound is necessary. The original synthesis of $ANAPP_3$ was described by Jeng and Guillory (1975). In the Appendix to this chapter, an expanded working protocol for the preparation of $ANAPP_3$ is provided.

IV. PHOTOLABELING AND BLOCKADE OF P_2 RECEPTORS

In this section, we summarize the procedures that have been used to establish a blockade of P_2 receptors in isolated smooth muscles. Among the preparations in which this has been accomplished are the vas deferens, urinary bladder, and taenia coli of several species (Hogaboom *et al.*, 1980; Westfall *et al.*, 1982; Westfall *et al.*, 1983). The approaches we have used should be applicable, with some modification, to other muscle and nonmuscle preparations. This discussion begins with general concepts and then amplifies certain details.

When possible, we treat preparations with $ANAPP_3$ under conditions in which the function of the tissue can be monitored; i.e., for smooth muscle preparations this would be in an organ bath so that tension can be recorded. (The reason for this is that $ANAPP_3$ is a P_2-receptor agonist and may therefore evoke responses when initially added.) After an appropriate equilibration period, $ANAPP_3$ is added to the bath, in *near darkness*, and allowed to incubate with the tissue for as long a period as is required for the compound to reach binding equilibrium with the receptor. This is taken as the time required for the response to $ANAPP_3$ to stabilize. At this point, the organ chamber, containing the tissue bathed in $ANAPP_3$-containing solution, is irradiated with high-intensity visible light for a predetermined period of time to induce photoactivation or photolysis of receptor-bound $ANAPP_3$ and the formation of covalent bonds. At the end of the photolysis period, $ANAPP_3$ is washed from the bath and the analysis of the effect of P_2-receptor antagonism is begun.

A. Light Sensitivity of $ANAPP_3$: Storage; Stability

Because $ANAPP_3$ is light sensitive, all preparative procedures, i.e., weighing, preparing solutions, and so on, and the organ bath procedures, until photolysis

is begun, should be done in near darkness. The compound is extremely soluble in water. It should be stored dessicated at −20°C when in powder form and solutions should be frozen. When it is stored in this fashion, we have observed no appreciable degradation of the compound over periods of several months.

B. Photolysis

$ANAPP_3$ can be photolyzed by visible or UV light. Glass quenches UV frequencies and therefore photolysis is more easily achieved with glass organ chambers using visible light. As a light source, DYH and DVY projector lamps, available at most photography suppliers, are quite satisfactory. It is likely that other bulbs can be used also, but the kinetics of photoactivation need to be established (see below). Visible lamps such as the DYH and DVY generate a great deal of heat so that one must insure that during photolysis the temperature in the organ bath is not elevated. Water-jacketed organ chambers or the immersion of chambers near the wall of a glass, water-filled aquarium can be used to maintain temperature at levels that will not disrupt the tissue. Chilling the preparation is less desirable than maintaining temperature at near-physiological levels, because active transport processes and the affinity of receptors for ligands may be compromised at low temperature. If the organ chamber assembly has rubber or plastic tubing, these should be covered with aluminum foil to protect them from the heat of the lamp. A fan to help dissipate heat is also quite useful.

UV light (254 nm) will cause the photolysis of $ANAPP_3$, so that a UV light source (Mineral Lite) could be used rather than the high-intensity projector bulbs. However, a quartz organ chamber would need to be used.

The intensity of light contacting the tissue and the time course of photolysis of $ANAPP_3$ will be a function of several factors:

1. Wattage and spectral characteristics of the lamp. Light intensity and wattage are directly related. The visible light bulb must emit blue wavelengths, since $ANAPP_3$ has an absorption peak at 460–480 nm, that is, in the blue range.
2. Composition and geometry of the lamp housing. A home-made apparatus for irradiating tissues, such as a desk lamp fitted with a projector bulb, can work quite satisfactorily. The amount of light reaching the tissue will be increased if, rather than a desk lamp, an apparatus is used that has a metallic parabolic reflector such as a Dyna Lume Heat Projector (Cole Parmer, Chicago).
3. Glass and water-jacket thickness, chamber diameter, and degree of gassing of the physiological solution. These factors will vary in different laboratories but should be standardized for a given apparatus.
4. Distance of the bulb *filament* from the tissue. Light intensity declines with the inverse square of distance. Therefore, the bulb alignment must be made carefully and consistently. A balance must be struck so as to maximize light intensity and to minimize heat delivery.

As demonstrated for $ANAPP_3$ (Hogaboom *et al.*, 1980), photoaffinity labels bind in an equilibrium fashion prior to photolysis and, because of the formation of covalent bonds, irreversibly after photolysis. The latter is a time-dependent process (see below). It is important that the binding of $ANAPP_3$ to the P_2 receptor be at equilibrium prior to photolysis. If not, then three time-dependent processes will be occurring during photolysis, i.e. establishment of equilibrium binding of native $ANAPP_3$, time course of photolysis, and time course of covalent bond formation.

Experiments should be done in which tissues are irradiated in the absence of $ANAPP_3$. This must be done to control for the possible effect of irradiation *per se*. Although the DVY and DYH lamps used in our studies have not produced an effect by themselves, irradiation with light of other wavelengths is known to affect smooth muscle function (Somylo and Somylo, 1970; Burnstock and Wong, 1978; Hogaboom *et al.*, 1980; Galardy and LaVorgna, 1981).

In order to produce a consistent antagonism in each experiment, the same fraction of P_2 receptors should be blocked. For a given concentration of $ANAPP_3$, the extent of receptor blockade is a function of two processes: the time course of photolysis (nitrene intermediate formation) and the time course of covalent insertion. While the time courses of these events are somewhat different, they are related in that the second is obviously dependent on the first. It should be recognized that after a certain point, however, further photolysis will not increase appreciably the extent of antagonism, because the photoaffinity label that is in solution will have been consumed during the process. This is unlike the case with conventional irreversible antagonists, such as phenoxybenzamine, in which the extent of antagonism increases the longer the preparation is exposed to the antagonist.

It is necessary to assess the time course of photoactivation in order to know the extent to which $ANAPP_3$ is photolyzed during irradiation. Such information can be obtained using spectrophotometric methods to assess changes in absorption of samples removed at various times during irradiation. An example is shown in Figure 2 in which is depicted the native spectrum of $ANAPP_3$ prior to photolysis and after extensive photolysis of the compound to cause completion of the photolysis reaction. The spectrum is altered in a characteristic fashion. Figure 2 also illustrates the absorbance changes at 260 nm obtained from spectra of samples removed at various intervals during photolysis. The curve provides an indication of the extent of photolysis of $ANAPP_3$. Thus, photolysis of $ANAPP_3$ in our 3-ml organ chambers for 15 min with a DYH bulb, the filament of which was 15 cm from the center of the chamber, results in photolysis of 80% of the compound. This degree of photolysis would not necessarily occur if different conditions were used, e.g., a bulb of different wattage, a different distance of the filament from the chamber, and so on. Since 80% of the $ANAPP_3$ is photolyzed in 15 min under the conditions stated above, little more is gained by irradiating for longer time periods. If a more substantial P_2-receptor blockade is desired than that produced by a 15 min photolysis period, an approach would be to add additional fresh $ANAPP_3$ and photolyze again.

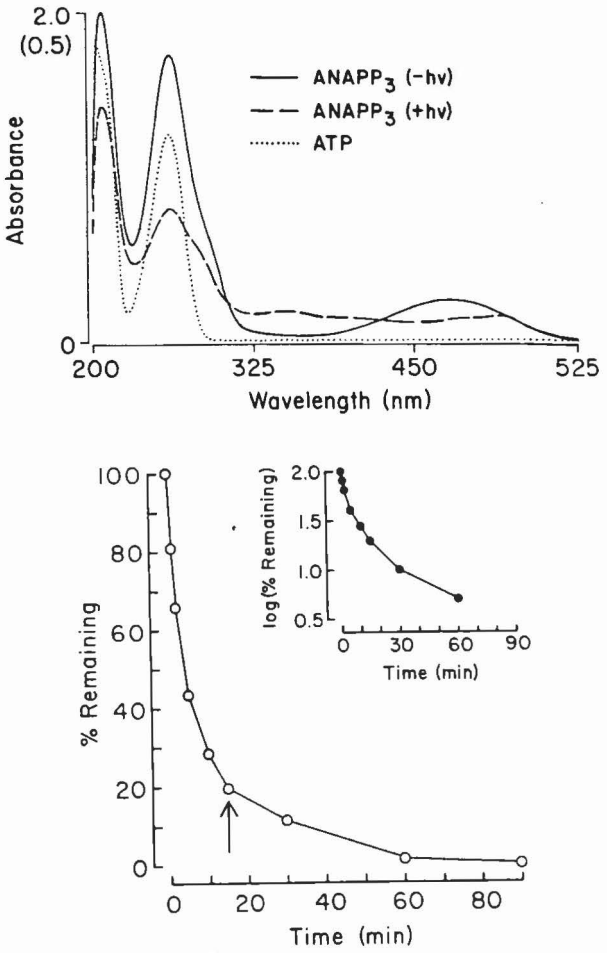

Figure 2. Upper panel: Spectra of $ANAPP_3$ ($10^{-4}M$) before ($-h\nu$) and 30 min after ($+h\nu$) photolysis of the compound in 20-ml organ chambers. A DVY bulb was used for irradiation and the $ANAPP_3$ was dissolved in modified Krebs–Henseleit solution. The reference cuvette contained modified Krebs–Henseleit solution. For comparison, the spectrum of ATP (2×10^{-5} *M*) is also shown (0.5 full-scale absorbance). Photolysis of $ANAPP_3$ produced a characteristic alteration in its spectrum, as shown, from which information on the time course of photolysis may be obtained, i.e., from the reduction in A_{260}. From Hogaboom *et al.* (1980). Copyright 1980 by the American Association for the Advancement of Science. Lower panel: Time course of reduction in A_{260} for 10^{-4} *M* $ANAPP_3$ (dissolved in modified Krebs–Henseleit solution) during photolysis with DYH bulb of the compound in 3-ml organ chambers. The reference cuvette contained modified Krebs–Henseleit solution. The bulb filament was 15 cm from the center of the organ chamber. Percent remaining was determined from the ΔA_{260} obtained at sampling intervals compared to the total ΔA_{260} between $t = 0$ and when the reaction reached completion (90 min), i.e., $100\% - [(A_{t=0} - A_{t=i})/A_{total} \times 100]$ where i = time of sample. The arrow indicates that 15 min was required to produce 80% of the maximum ΔA_{260}. This period (15 min) is used routinely in our experiments as the standard photolysis period. Inset: Logarithmic transform of the data. The plot yields a curve instead of a straight line. Photolysis of $ANAPP_3$ thus is not a simple exponential or first-order process, but proceeds with order 1.5. From O'Donnell *et al.* (1983), with permission.

C. Specificity of Antagonism

When $ANAPP_3$ is used as a tool to investigate P_2 receptors, the specificity of its antagonism needs to be evaluated. Although the evidence to date indicates a high degree of specificity, it remains possible that the reactive nitrene intermediate that is generated by photolysis of $ANAPP_3$ could react with sites in the preparation other than P_2 receptors. In our studies, the effects of $ANAPP_3$ treatment on responses to agonists that act via receptors other than purinoceptors has been investigated. For example, in several different smooth muscle preparations, photolysis of $ANAPP_3$ results in antagonism of ATP-induced responses, but not

those to norepinephrine, acetylcholine, isoproterenol, or KCl (Hogaboom *et al.*, 1980; Westfall *et al.*, 1982, 1983). Thus it appears that $ANAPP_3$ does not antagonize responses mediated by α- or β-adrenergic receptors or muscarinic receptors. Additionally, $ANAPP_3$ does not antagonize responses induced by KCl-induced membrane depolarization.

A question that has not been completely answered is whether $ANAPP_3$ antagonizes responses mediated by P_1 purinoceptors, although the evidence to date indicates that it does not. Part of the difficulty in answering this question resides in the fact that in a number of preparations in which the effects of $ANAPP_3$ have been investigated, ATP, the prototype P_2 agonist, and adenosine, the prototype P_1 agonist, produce qualitatively dissimilar effects. For example, in the guinea pig vas deferens, ATP, ADP, and a number of nucleotide analogs, produce contraction. Adenosine, however, does not produce contraction, so that the potential antagonism by $ANAPP_3$ cannot be assessed. In a preparation in which ATP and adenosine produce similar effects, the results are equivocal. Experiments conducted with the guinea-pig taenia coli show that $ANAPP_3$ produces an approximate 6-fold antagonism of relaxation responses to ATP, but only a 2-fold antagonism of relaxation responses to adenosine (Westfall *et al.*, 1982). One interpretation of this finding is that $ANAPP_3$ antagonizes responses mediated by P_1 receptors, although somewhat less effectively than those mediated by P_2 receptors. However, it is also possible that a component of the response to adenosine is mediated by its interaction with P_2 receptors, thereby accounting for the antagonism by $ANAPP_3$.

In some preparations, responses to ATP are not antagonized by $ANAPP_3$. For example, Sneddon *et al.* (1982b) have found that relaxations of the rabbit anococcygeus muscle produced by ATP and adenosine were not blocked by $ANAPP_3$. ATP and adenosine were nearly equipotent, and, on the basis of other experiments, it was determined that $ANAPP_3$ was ineffective against ATP because responses to the nucleotide followed its conversion to adenosine, which actually mediated the response. Similarly, Frew and Lundy (1982a) reported that $ANAPP_3$ was unable to antagonize ATP-induced relaxation of the fundus of the guinea-pig stomach. Additional studies indicated that these responses are mediated by P_1 receptors because they are antagonized by 8-phenyltheophylline (Frew and Lundy, 1982b); this implies that a conversion of ATP to adenosine occurs. The cumulative evidence obtained by us and others therefore indicates that the antagonism by $ANAPP_3$ is selective for the P_2-receptor.

D. Effects of Nonphotolyzed $ANAPP_3$

Nonphotolyzed $ANAPP_3$ is an agonist and, upon initial application, produces responses that resemble those produced by ATP. If $ANAPP_3$ is allowed to remain in the organ bath during subsequent exposure to ATP, the responses to ATP are reduced, whereas those to chemically unrelated agonists are not (Hogaboom *et al.*, 1980; Fedan *et al.*, 1981; Westfall *et al.*, 1983).

The effects of nonphotolyzed $ANAPP_3$ on responses of the guinea-pig vas deferens to ATP are illustrated in Figure 3. (Also shown in Figure 3 are the effects

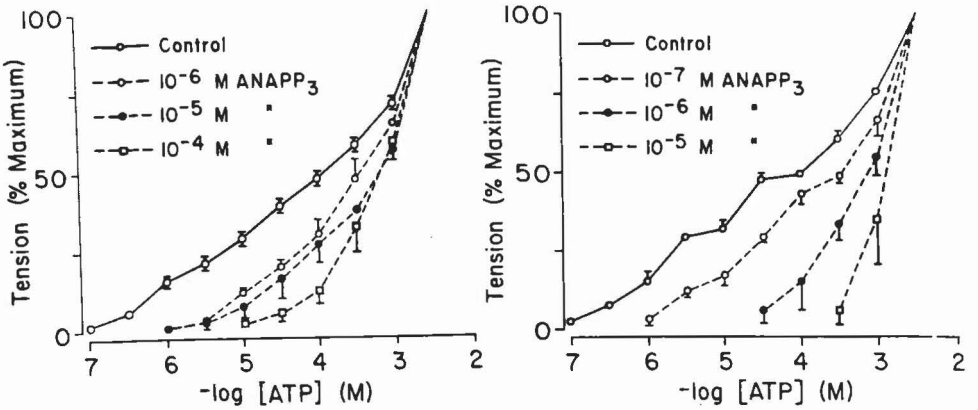

Figure 3. Left panel: Effect of several concentrations of $ANAPP_3$, following its photolysis (DVY bulb, 30-min irradiation period) in organ chamber containing the guinea-pig vas deferens, on contractile responses of the tissue to ATP. The $ANAPP_3$ was no longer present during the ATP concentration–response determinations. Right panel: Effect of nonphotolyzed $ANAPP_3$ on ATP concentration–response curves of isolated guinea-pig vas deferens. In these experiments, which were performed in the dark, the $ANAPP_3$ was continually present during the additions of ATP. From Hogaboom et al. (1980). Copyright 1980 by the American Association for the Advancement of Science.

of photolyzed $ANAPP_3$ on responses to ATP.) This ATP-specific antagonism by nonphotolyzed $ANAPP_3$ is reversed readily by washing out the $ANAPP_3$; i.e., covalent bonds are not formed if the compound is not photolyzed. A similar phenomenon occurs *in situ*. Nonphotolyzed $ANAPP_3$ administered intraarterially to the cat urinary bladder produces a contraction followed by an antagonism of intraarterially administered adenine nucleotides (Theobald, 1982, 1983).

The reversible antagonistic effects of nonphotolyzed $ANAPP_3$ could result from a conventional equilibrium-competitive antagonism of P_2 receptors. Alternatively, because a similar antagonistic effect occurs after administration of ATP itself, the antagonism by nonphotolyzed $ANAPP_3$ may be a manifestation of an autoinhibition phenomenon known to be induced by adenine nucleotides in smooth muscle (Ambache and Zar, 1970; Dean and Downie, 1978; Baer and Frew, 1979; Meldrum and Burnstock, 1983). Definitive evidence to support either possibility is not available. However, because the agonistic potency of nonphotolyzed $ANAPP_3$ and ATP are virtually identical (O'Donnell *et al.*, 1983), whereas nonphotolyzed $ANAPP_3$ is more effective than ATP in reducing ATP-induced responses (Hogaboom *et al.*, 1980), it would seem that part of the antagonism produced by nonphotolyzed $ANAPP_3$ results from a true competitive antagonism.

E. Structural Considerations in the Design of P_2-Receptor Photoaffinity Labels.

The potency of photoaffinity labels under equilibrium binding conditions (i.e., before photolysis) and the extent of photolabeling upon photolysis will be influenced by the location of the photolabile moiety on the ATP molecule. Information

on where the photosensitive grouping can be added to ATP without reducing potency at the P_2 receptor can be obtained *a priori* from the ability of ATP analogs to induce responses. This information also provides a useful comparison to the structural requirements of ligands for the P_1 receptor.

In general, additions of varying size to the adenine ring at N^1, N^6, and C^8 all reduce agonist activity. The replacement of the N^6 amino group of ATP with a hydroxyl (e.g., inosine triphosphate) reduces agonist activity substantially. It seems clear that the adenine ring is involved in receptor recognition and even slight modifications reduce activity (Fedan *et al.*, 1982). An ATP photoaffinity label used widely in biochemical studies is 8-azido ATP (Haley, 1975; Pomerantz *et al.*, 1975; Owens and Haley, 1978). While we have had no direct experience with this compound, it would be predicated that 8-azido ATP would be less useful than $ANAPP_3$ as a P_2 antagonist, based on the finding that other 8-substituted compounds, such as 8-bromo ATP and 8-(6-aminohexyl) amino ATP, are less potent than ATP in the guinea-pig vas deferens.

Modification of ribose are better tolerated than modifications of the adenine moiety. While there is some loss of potency with 2′-deoxy ATP, two other modifications, e.g., 3′-deoxy-ATP and 9-β-D-ribofuranosyl ATP yield compounds that are nearly equivalent to ATP. $ANAPP_3$ is a 3′ derivative of ATP, as is arylazido aminobutyryl ATP ($ANABP_3$), another photoaffinity analog; these compounds have agonist potency similar to ATP (O'Donnell *et al.*, 1983).

The replacement of 5′-phosphates with sulfate, amidate, morpholidate, or other groups results in compounds with little agonist activity. In terms of structure–activity relationships, therefore, an intact adenine and the presence of 5′-phosphates are critical for receptor recognition, whereas modifications of the ribose are better tolerated. These structural features are quite different from those for P_1 receptors, where N^6 and 5′ hydroxyl additions confer receptor specificity without loss of potency. From this analysis, it seems that for P_2 receptor interaction the best location for a photolabile moiety is on the 3′ hydroxyl of ribose, which is where ATP is derivitized in the case of $ANAPP_3$.

V. CONCLUDING COMMENTS

There are both disadvantages and advantages to the use of the photoaffinity label $ANAPP_3$ in the study of P_2 receptors. The most obvious disadvantage is that $ANAPP_3$ cannot be used as an irreversible antagonist *in vivo*, because there is no mechanism, at present, to irradiate the compound under these conditions. Related to this is the potential difficulty of photolyzing $ANAPP_3$ *in vitro* if relatively thick tissue preparations are being investigated. The novelty of dealing with photoaffinity compounds, as well as the unusual apparatus, such as the lamps that are required, may seem to be a disadvantage as well. In spite of these difficulties, however, $ANAPP_3$ is an important tool for investigators interested in the pharmacology and physiology of adenine nucleotides and nucleosides. $ANAPP_3$ has the advantage of being the only specific antagonist of responses mediated through P_2 receptors. Thus, the compound is of considerable value in

probing the potential physiological role of ATP and in helping to distinguish between responses mediated by ATP and by adenosine.

APPENDIX: PROCEDURE FOR PREPARATION OF ANAPP$_3$ (based on Jeng and Guillory, 1975)

Step I. Preparation of 4-fluoro-3-nitrophenyl azide

Supplies

Acetone, sodium nitrite, dry ice, sodium azide, hydrochloric acid, petroleum ether, 4-fluoro-3-nitroaniline.

Equipment

Filter flask and funnel (3) two 250 ml, one 1000 ml; 50–100 ml beaker; Pasteur pipettes and bulbs; stir bars and stir rods (glass); 100-ml 3-neck round bottom flask (2); stir/heat plate; ice bucket; 2-liter Erlenmeyer flask; two acetone-dry Ice Baths (any small cold-proof bowl will suffice); filter paper.

Procedure

Heat gently 30-ml conc. HCl/5 ml H_2O on a stir/heat plate in a hood. Place 4.4 g of 4-fluoro-3-nitroaniline in the 100-ml beaker and stir well for 10 min at 40–50°C. While heating, cool a 100-ml 3-neck round bottom flask to −20° to −30°C in an acetone/dry ice bath. After heating/stirring the 4-floro-3-nitroaniline, filter through a filter flask/funnel and discard the black residue on the filter paper. Place the light brown filtrate in the 100-ml round bottom (RB) flask and maintain the slush bath at −20 to −30°C while stirring. At this time dissolve 2.4 g of sodium nitrite in 5 ml of water and 2.2 g of sodium azide in 8 ml of water. Also cool the 250-ml filter flask in a second acetone/dry ice bath to −20° to −30°C. Set up this apparatus for filtering. With a pasteur pipette, add slowly (about a drop a second) the sodium nitrite to the cold (−20°C) well-stirred 4-floro-3-nitroanline/acid medium. Stir for 10 min and keep at −20°. Filter the mixture into the −20°C filter flask and cool another 100-ml RB flask (3 neck) to −20°C in a slush bath. Discard the residue and place the light brown filtrate into the −20°C RB flask. Cool the mixture to −20°C and add the sodium azide slowly (about a drop every 5 sec). Stir well with a medium-sized stir bar. After about a minute, stir the mixture with a glass rod as an insoluble layer forms over the top of the reaction mixture. This layer must be dispersed to insure even mixing. Repeat this until the azide has been added. Keep the dry ice/acetone bath at −20°C to −30°C at all times. Filter the light brown mixture. Rinse the residue in the RB flask with ice-cold distilled water. Wash the solid with about 2 liters of cold distilled H_2O. Take the light brown solid on the filter paper and dry by vacuum dessication. When absolutely dry, recrystallize with hot petroleum ether. Allow the crystals to form in a cool dark dry area over night. (*The reactions were carried out in a lab room with no*

outside lighting. The lights were dimmed in the room. These were the only precautions taken. However, there is a constant danger of the azides exploding.)

Step II: Preparation of *N*-4-Azide-2-nitrophenyl-β-alanine

Supplies

β-Alanine, ether, sodium carbonate, pH paper, ethanol (95%), sodium chloride, hydrochloric acid, magnesium sulfate, 4-fluoro-3-nitrophenyl azide.

Equipment

Oil bath with heating device and rheostat, small beakers, 100-ml round-bottom flask, cooling condenser, rotating evaporator (roto-vap), separatory funnel (500 ml), 500-ml round-bottom flask (2), filter funnel and paper.

Procedure

All of the following steps take place in a hood. Dissolve 0.534 g of β-alanine and 1.08 g of sodium carbonate in 5.4 ml of water using a 100-ml round-bottom flask. Add 0.9000 g of 4-fluoro-3-nitrophenyl azide and stir well. Add 6.75 ml of ethanol, 5.4 ml of water, and then 13.5 ml of ethanol. Cover with foil, place in a hood, and stir at 52–55°C overnight (12–18 hr). Reduce the volume to about 10 ml using a roto-vap apparatus. Extract with 45 ml of ether (twice) to remove the starting azide. Save the red aqueous layer and add 3 *N* HCl until the solution is a pH of 2 or less. The solution becomes somewhat opaque. Extract with 90 ml of ether (three times) and save the ether layer (clear orange-red). Wash the ether layer three times with 50 ml of saturated NaCl. Place the ether layer in a 500-ml round-bottom flask and add in magnesium sulfate (2–3 g) and allow to stand for 15–30 min. Filter the ether into a 500-ml round-bottom flask and evaporate to dryness using a roto-vap. Add in 20–40 ml of hot ethanol and heat until the residue is dissolved. Place the hot ethanol in a 100-ml beaker and set in the hood covered with foil until dry. The resulting dark red solid is used in the next step.

Step III: Preparation of 3′-*O*-{3[*N*-(4-Azido-2-nitrophenyl)amino-propionyl} adenosine 5′-triphosphate ($ANAPP_3$)

Supplies

Carbonyldiimidazole, acetone, N,N-dimethylformamide, n-butanol, Whatman 3MM Chromatography Sheets, Glacial Acetic acid.

Equipment

50-ml round-bottom flask (2) (*dry*), rotating evaporator, small beakers, 16 × 100 test tubes, table top centrifuge, vacuum dessicator, chromatography tank, Pasteur pipette and bulb, vortex.

Procedure

Dissolve carbodiimidazole (1.35 g) in 2.5 ml of dimethylformamide (dried over molecular sieves) in a 50-ml RB flask. Add and dissolve by stirring well 0.630 g of *N*-4-azide-2-nitrophenyl-β-alanine. The dark-red solution will turn into thick orange-red viscous material after about 5 min. Pour in 0.325 g of Na_2ATP dissolved in 12.5 ml of water. (A ratio of 5:1 water to dimethylformamide is essential). The solution will immediately become an orange foamy solution. Cover with foil, set in the hood, and stir well overnight (18–24 hr). Using a roto-vap, dry the solution to a dark-red residue. Add acetone (20 ml) to the 50-ml round-bottom flask. An orange precipitate will form in the red acetone solution. Pipette the solution and precipitate into 16 × 100 mm test tubes. Use as much acetone as needed to get the precipitated material out of the flask (usually 50 ml). Centrifuge the tubes 10 min at 2000 rpm. Aspirate out the clear red supernatant and discard. Add 2 ml of acetone to each tube and disperse the precipitate using a vortex and a small spatula. Combine the acetone mixture into two tubes. Disperse the precipitate very well in the acetone. Centrifuge again 2000 rpm for 10 min. Aspirate off the acetone. Add fresh acetone, disperse, centrifuge. Repeat this step 3–5 times until the acetone supernatant becomes a faint clear orange. Combine the dispersed precipitate–acetone mixture into one tube, centrifuge, aspirate the acetone, place a foil cap over the test tubes, and punch small holes in the foil. Tape the foil on the sides of the tube to keep the foil on and vaccuum dessicate overnight. *Once the dried solid is obtained, make attempts to keep the orange solid away from light.* Add 1 ml of distilled water to make a dark-red solution. Apply to Whatman 3MM chromatography sheets and chromatograph using *n*-butanol, water, and acetic acid (5:3:2) *in the dark.* Let the solvent front run to about an inch from the bottom and then *dry in the dark.* Cut out the orange band eluting at R_f = 0.30–0.50, cut the paper into fine strips (0.5 cm × 2 cm), and place in distilled water overnight *in the dark* at room temperature (50–100 ml water). Pour off the orange solution and filter twice through glass wool. Add 50 ml of water to the remaining strips and repeat as above. Lyophilize the solution *in the dark* to a resulting fluffy orange powder. *Store in the dark, dessicated at −20°C.*

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