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INTERACTION OF [^{3}H]ARYLAZIDO AMINOPROPIONYL ATP ([^{3}H]ANAPP$_3$) WITH P$_2$-PURINERGIC RECEPTORS IN THE SMOOTH MUSCLE OF THE ISOLATED GUINEA-PIG VAS DEFERENS *

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Following its photolysis in the presence of the isolated guinea-pig vas deferens, the ATP photoaffinity label ANAPP$_3$ produces a specific antagonism of adenine nucleotide-induced contractile responses which are mediated by P$_2$-purinergic receptors. To characterize the site of covalent photoincorporation of ANAPP$_3$, intact vasa deferentia were treated with [^{3}H]ANAPP$_3$ and samples of homogenate, cytosol and a crude membrane fraction were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Photolysis of [^{3}H]ANAPP$_3$ (10^{-5} M; 3.0 μCi/ml) resulted in the incorporation of radioactivity into cellular components with apparent molecular weights of 54-66 and 43-57 kilodaltons. The photoincorporation of [^{3}H]ANAPP$_3$ was associated with the crude membrane fraction and not the cytosol, was reduced in the presence of ATP in an ATP-concentration-dependent manner, was lessened following pretreatment of the tissues with photolyzed nonradiolabeled ANAPP$_3$, and was unaffected by the nucleoside transport inhibitor, dipyridamole. In tension studies on the same tissues the presence of ATP resulted in a concentration-dependent reduction in the initial contractile response to [^{3}H]ANAPP$_3$ the response to ^{3}H was antagonized in tissues which had been pretreated with nonradiolabeled ANAPP$_3$, and dipyridamole had no effect on the contractile response to [^{3}H]ANAPP$_3$. According to several criteria these findings indicate that the antagonism by photolyzed ANAPP$_3$ of adenine nucleotide-induced responses is a direct result of the covalent insertion at or near the recognition site of cell-surface P$_2$-purinergic receptors.

P$_2$-purinergic receptors   [^{3}H]Arylazido aminopropionyl ATP ([^{3}H]ANAPP$_3$)   Photoaffinity labeling
Vas deferens   Guinea pig

1. Introduction

ANAPP$_3$, an ATP photoaffinity label (Jeng and Guillory, 1975; Russell et al., 1976; Guillory and Jeng, 1983), produces, following its photolysis in the presence of the isolated guinea-pig vas deferens and other smooth muscles, a specific antagonism of ATP-induced contractile responses (Hogaboom et al., 1980; Frew and Lundy, 1982a, b; Fedan et al., 1982a, b; Fedan et al., 1983; Westfall et al., 1982, 1983). The specific P$_2$-purinergic receptor (Burnstock, 1971, 1978; Burnstock and Brown, 1981) blockade afforded by ANAPP$_3$ following its photolysis has allowed an evaluation of the role of ATP in neurotransmission

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in isolated guinea-pig vas deferens, urinary bladder and taenia coli (Fedan et al., 1981; Sneddon et al., 1982; Westfall et al., 1982, 1983), and in the in situ cat urinary bladder following intra-arterial administration of nonphotolyzed $ANAPP_3$ (Theobald, 1982, 1983).

Previous studies obtained using agarose-ATP beads as an agonist have suggested that P_2-receptors of the guinea-pig vas deferens are located on the cell surface; the finding that contractile responses to agarose-ATP beads were antagonized following treatment of the tissue with photolyzed $ANAPP_3$ was taken as strong evidence that the antagonism by this agent occurred at this site (Head et al., 1983). Since $ANAPP_3$ incorporates covalently upon photolysis into cell surface sites, the present experiments were undertaken with $[^3H]ANAPP_3$ to obtain information on the biochemical nature of the P_2-purinergic receptor.

2. Materials and methods

2.1. General

Protocols involving treatment of isolated guinea-pig vas deferens with $[^3H]ANAPP_3$ were virtually identical to those employed previously when the pharmacological antagonism of P_2-receptor-mediated responses was evaluated (Fedan et al., 1982a, b; O'Donnell et al., 1983).

2.2. Preparation of isolated vasa deferentia

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, and attached to a force-displacement transducer to allow measurement of isometric contractile responses. A 1 h equilibration period, during which the tissues were washed at 15 min intervals with fresh Krebs-Henseleit solution, was allowed prior to beginning the experiment. Resting tension was 0.2-0.4 g.

2.3. Synthesis of $ANAPP_3$ and $[^3H]ANAPP_3$

$ANAPP_3$ was synthesized as described by Jeng and Guillory (1975). Azido-2-nitrophenyl-β-alanine, N-4[alanine-^{3}H(N)] (39.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA) and used for the synthesis (by S.J.J. and R.J.G.) of $[^3H]ANAPP_3$ (306 μCi/mmol).

2.4. Photoaffinity labeling with $[^3H]ANAPP_3$

Following the 1 h equilibration period $[^3H]$-$ANAPP_3$ (3.0 μCi/ml) was added, in near-darkness, to the organ chambers. Contractile responses, attributable to the fact that nonphotolyzed $ANAPP_3$ is an agonist (O'Donnell et al., 1983), were recorded. Following a 3 min incubation the preparations were irradiated for 15 min with a high-intensity visible light (DYH bulb, 600 W; 3200°K; mounted in a Dyna-Lume housing (Cole-Parmer, Chicago, IL)). The bulb filament was 15 cm from the center of the organ chambers. At the end of irradiation the preparations were washed twice, at 5 min intervals, with fresh Krebs-Henseleit solution, and the tissues were processed for electrophoresis (vide infra).

2.4.1. Photoaffinity labeling in the presence of ATP

The antagonism by photolyzed $ANAPP_3$ of ATP-induced contractile responses is diminished if ATP is present during photolysis (Hogaboom et al., 1980). To establish that photoincorporation of $[^3H]ANAPP_3$ occurs at a pharmacologically relevant site, and to discriminate the site-specific insertion of $[^3H]ANAPP_3$ from possible nonspecific interactions, one vas deferens of a pair (the control) was incubated with 10^{-5} M $[^3H]ANAPP_3$ and irradiated, as described in section 2.5. The contralateral tissue was incubated with 10^{-3} M ATP for 3 min prior to the addition of 10^{-5} M $[^3H]ANAPP_3$, and then irradiated in the continued presence of 10^{-3} M ATP.

2.4.2. Photoaffinity labeling in the presence of dipyridamole

One vas deferens of a pair was incubated with the nucleoside transport inhibitor dipyridamole (10^{-5} M) for 30 min prior to and during the addition and photolysis of 10^{-5} M $[^3H]ANAPP_3$.

2.4.3. Photoaffinity labeling with [^{3}H]ANAPP$_3$ after treatment of tissues with nonradiolabeled ANAPP$_3$

One vas deferens of a pair was incubated for 3 min in near-darkness with nonradiolabeled ANAPP$_3$ (10^{-4} M) and then irradiated for 15 min. The contralateral control tissue was not exposed to ANAPP$_3$ but was irradiated. At the end of the irradiation period both preparations were washed twice with fresh Krebs-Henseleit solution at 5 min intervals. [^{3}H]ANAPP$_3$ (10^{-5} M) was then added to both preparations, incubated for 3 min, photolyzed and washed out as in section 2.4.

2.4.4. Nonphotolyzed [^{3}H]ANAPP$_3$

One vas deferens of a pair was incubated with 10^{-5} M [^{3}H]ANAPP$_3$ and photolyzed as in section 2.4. The contralateral tissue was incubated in 10^{-5} M [^{3}H]ANAPP$_3$ for 18 min and kept in darkness. Both tissues were then washed twice with fresh Krebs-Henseleit solution.

2.4.5. Analysis of photolyzed and nonphotolyzed [^{3}H]ANAPP$_3$ in solution

[^{3}H]ANAPP$_3$ (10^{-5} M; 0.5 μCi/ml) was dissolved in water or in Krebs-Henseleit solution. One ml aliquots were placed in organ chambers and irradiated for 15 min, as in section 2.4, or kept in near-darkness. The samples were then collected for analysis.

The Krebs-Henseleit bathing solutions from vas deferens preparations which were irradiated in the presence of [^{3}H]ANAPP$_3$ (10^{-5} M; 3.0 μCi/ml) (section 2.4) or merely incubated in darkness with [^{3}H]ANAPP$_3$ (section 2.4.4) were saved for analysis in some experiments.

2.5. Preparation of vas deferens for electrophoresis

Following the organ bath procedures the vasa deferentia were blotted, weighed and minced. The mince was suspended in 100 – 125 volumes per g wet weight (gww) of buffer (50 mM Tris · HCl, pH 7.4, 0°C) and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) set at half speed by delivering four 10 s bursts; there were 10 s rests between bursts. The homogenate was centrifuged (4°C) at $800 \times g_{av}$ for 10 min. The supernatant fluid was saved and the pellet was resuspended in 75 volumes of buffer/gww. The $800 \times g_{av}$ supernatant fluid and the resuspended pellet were combined and centrifuged at $105\,000 \times g_{av}$ for 1 h. The resulting supernatant fluid, referred to as 'Cytosol', was saved for electrophoresis. The $105\,000 \times g_{av}$ pellet, referred to as 'Membranes', was suspended in 50 volumes/gww of buffer for electrophoresis.

Protein was assayed using the method of Bradford (1976) using human gamma globulin as standard.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples (1.4 mg) and molecular weight markers (Bio-Rad Laboratories, Rockville Center, NY) were solubilized by heating (100°C; 5 min) in a solution containing 300 mM Tris · HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 36% glycerol, 0.008% bromophenol blue tracking dye and 4% β-mercaptoethanol. Each sample was subjected to SDS-PAGE using a modification of the discontinuous buffer system described by Laemmli (1970) and 11 mm internal diameter glass tubes. The 1.2 cm stacking gel contained 5% acrylamide : bis (30.0 : 0.86), 62.5 mM Tris · HCl (pH 6.8), 0.1% SDS, 0.05% TEMED and 0.1% ammonium persulfate. The 8.6 cm separating gel contained 12% acrylamide : bis (30.0 : 0.86), 450 mM Tris · HCl (pH 8.8), 0.1% SDS, 5% glycerol, 0.0167% TEMED and 0.05% ammonium persulfate. The electrode buffer contained 50 mM Tris · HCl (pH 8.8), 380 mM glyine and 0.1% SDS. The samples were electrophoresed at 7-8 mA per tube until, in most cases, the tracking dye ran off the separating gel. The gels containing the molecular weight standards were stained in a solution containing 0.1% Coomassie brilliant blue R-250, 10% acetic acid and 50% methanol, destained in a solution containing 45% methanol plus 10% acetic acid and stored in 10% acetic acid for R_f measurements. The sample gels were cut into 2 mm slices and dried overnight (55-60°C) in scintillation vials. The slices were solubilized by adding 1 ml of 40% H_2O_2 + 1% NaOH to each vial and heating (55-60°C) overnight prior to the addition of scintillation counting solution.

3. Results

3.1. Experiments on vas deferens homogenates; evaluation of the formation of photoproducts

[^{3}H]ANAPP$_3$ (10^{-5} M) produced a contraction when added to the organ bath (fig. 1). In the contralateral vas deferens the presence of ATP (10^{-3} M) antagonized the response. This ability of ATP to antagonize responses to adenine nucleotides (Dean and Downie, 1978; Hayashi et al., 1978; Hogaboom et al., 1980; Fedan et al., 1981; Meldrum and Burnstock, 1983; Lukascko and Krell, 1981, 1982; Okwuasaba et al., 1977) reflects an autoinhibitory state the cellular basis of which is unknown. Insofar as this antagonistic effect of ATP is readily reversed by washing, and since the antagonism by ANAPP$_3$ is reduced if photolysis is conducted in the presence of ATP (Hogaboom et al., 1980), the blockade of the ANAPP$_3$-induced contractile response by ATP and the ability of ATP to protect against the incorporation of [^{3}H]-ANAPP$_3$ are criteria to be used in establishing the identity of the insertion site(s) for photolyzed ANAPP$_3$.

The distribution of radioactivity on SDS-polyacrylamide gels from homogenate samples, following photolysis of [^{3}H]ANAPP$_3$, is shown in fig. 2.

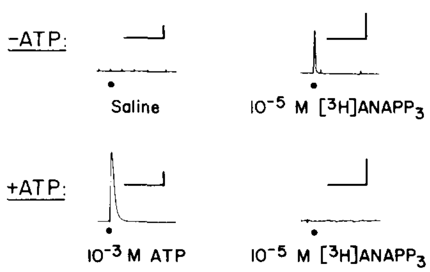

Fig. 1. Effect of ATP (10^{-3} M) on the initial contractile response to 10^{-5} M [^{3}H]ANAPP$_3$. Top panels: in the absence of ATP, the addition of [^{3}H]ANAPP$_3$ in near darkness initiated a contractile response (right). Bottom panels: 10^{-3} M ATP elicited a characteristic response in the contralateral tissue (left); the response to nonphotolyzed [^{3}H]ANAPP$_3$, added 3 min later in the presence of ATP, was antagonized (right). In this and in other figures in which tissue responses are shown, the horizontal calibration is 1 min and the vertical calibration is 1 g. The small deflections in the top panels are air bubble-induced artifacts.

[^{3}H]ANAPP$_3$ became incorporated into two cellular components with relative molecular weights of ca. 66 and 57 kD. In the contralateral vas deferens, photolysis of [^{3}H]ANAPP$_3$ in the presence of 10^{-3} M ATP resulted in a marked reduction in the incorporation of radioactivity. The findings shown in fig. 2 (left panels) are representative of three separate experiments with qualitatively but not quantitatively similar results; owing to the low specific activity of the [^{3}H]ANAPP$_3$ the peak heights in the other experiments were smaller (vide infra). No peaks of radioactivity corresponding to those seen following photolysis were observed if the preparations were not irradiated (fig. 2, right panels). However, a peak with a relative molecular weight of ca. 40 kD was seen in one of three experiments (fig. 2, right panels). Its presence suggested that the peaks evident after photolysis (fig. 2, left panels) could be, in part or entirely, related artifactually to the presence of nonphotolyzed [^{3}H]ANAPP$_3$ and/or its photoproducts generated in the bathing medium and carried through the homogenization procedure. This possibility was assessed by electrophoresing samples of the Krebs-Henseleit solution ('Bath') from the organ baths in which the tissues had been treated with photolyzed and nonphotolyzed [^{3}H]ANAPP$_3$. The results are shown in fig. 3. A very broad peak of radioactivity appeared with a maximum at ca. 25 kD. This peak was not reduced if ATP was present during photolysis, and its appearance was photolysis-dependent. Analysis of bathing solution samples taken at the end of exposure to irradiated and nonirradiated [^{3}H]ANAPP$_3$ showed that protein had been released into the bath: the values obtained were 27.2 ± 3.9 μg/ml (n = 8), or 560 ± 67 μg protein/gww (n = 8). This protein may have originated from the cut ends of the tissues or from leakage from damaged surface cells. If ATPases or kinases were among the released proteins, then they might have been photolabeled by [^{3}H]-ANAPP$_3$ (Jeng and Guillory, 1975; Russell et al., 1976; Haley, 1983). Alternatively, [^{3}H]ANAPP$_3$ might have inserted nonspecifically into other released proteins. To evaluate these possibilities [^{3}H]ANAPP$_3$ was photolyzed in Krebs-Henseleit solution lacking tissues and in water. After photolysis in either Krebs-Henseleit solution (fig. 4) or

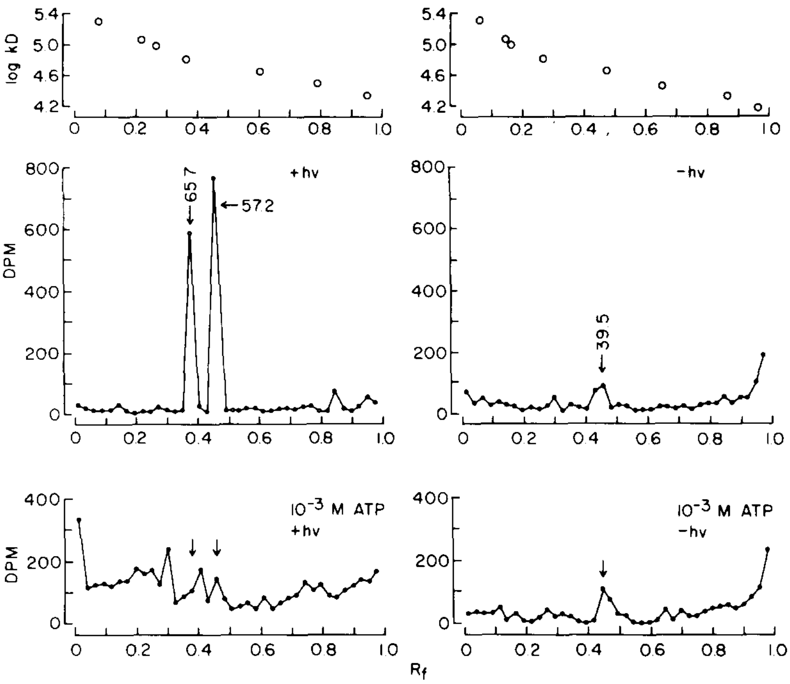

Fig. 2. Distribution of ^{3}H on SDS-polyacrylamide gels of homogenate samples. The top panels in this and in similar figures show the location of molecular weight standards (in kilodaltons, kD) from Coomassie blue-stained tube gels which were electrophoresed in the same chambers as the vas deferens samples: myosin (200000), β-galactosidase (116250), phosphorylase B (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500) and lysozyme (14400). Left half of figure: paired tissues from the same animal were incubated in 10^{-5} M [^{3}H]ANAPP$_3$ and irradiated (+hv) in the absence (middle panel) or presence (bottom panel) of 10^{-3} M ATP. Tension responses of these tissues are shown in fig. 1. Right half of figure: paired tissues were incubated in the absence (middle panel) or presence (bottom panel) of 10^{-3} ATP M but not irradiated (−hv). The arrows in this and in similar figures show interpolated molecular weight values (in kD).

in water (fig. 5), a broad peak of radioactivity (maximum occurring at ca. 19-20 kD) was observed. The peak was not seen in nonphotolyzed samples in either medium, and it was not affected by the presence of 10^{-3} M ATP. The results of figs. 3 to 5 would suggest that there is appreciable insertion of photolyzed [^{3}H]ANAPP$_3$ into the proteins released by the tissue, and that high molecular weight photoproducts were formed as well. However, the R_f values of these peaks were different from those obtained when the tissue was present.

3.2. Localization of [^{3}H]ANAPP$_3$ insertion sites: membranes vs. cytosol

In the absence of ATP two peaks of radioactivity (ca. 57 and 49 kD) were observed in the crude membrane fraction (fig. 6). Their magnitude was reduced in the contralateral vas deferens which was photolyzed in the presence of ATP (10^{-3} M). A peak of radioactivity (ca. 46 kD in fig. 6) was consistently observed in the cytosol; its magnitude, however, was not reduced by irradiating in the presence of ATP. These results are representative of three separate experiments.

3.3. Effect of dipyridamole on the photoincorporation of radioactivity

As ANAPP$_3$ is a substrate for F_1-mitochondrial and myosin ATPases, the sequential degradation of the compound by ectophosphohydrolase enzymes might form an arylazido aminopropionyl adenosine derivative of [^{3}H]ANAPP$_3$ in the organ

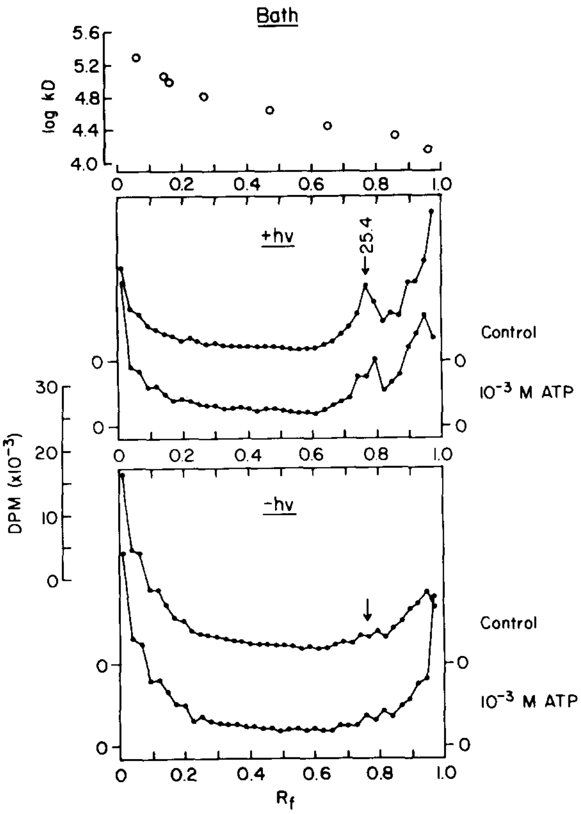

Fig. 3. Distribution of ^{3}H on SDS-polyacrylamide gels of samples of Krebs-Henseleit solution from organ baths ('Bath') in which vasa deferentia were incubated with 10^{-5} M [^{3}H]ANAPP$_3$ (3.0 μCi/ml) in the absence or presence of 10^{-3} M ATP and irradiated ('+hv') or not irradiated ('−hv'). Middle panel: irradiated in the absence (upper curve) and presence (lower curve) of ATP; bottom panel: not irradiated. The results are plotted in each panel with the ordinate zero values of the upper gel offset for clarity: the upper distributions are for control (no ATP present) while the bottom ones show the results obtained when ATP was present.

bath. The transport of this substance into the cell by a nucleoside transporter prior to and during photolysis of [^{3}H]ANAPP$_3$ could result in the photolabeling of the transport carrier as well as intracellular enzymes associated with adenosine metabolism. To determine whether any of the observed peaks in radioactivity were associated with these processes the effect of dipyridamole, a nucleoside transport inhibitor, on the photoincorporation of [^{3}H]ANAPP$_3$ into membranes and cy-

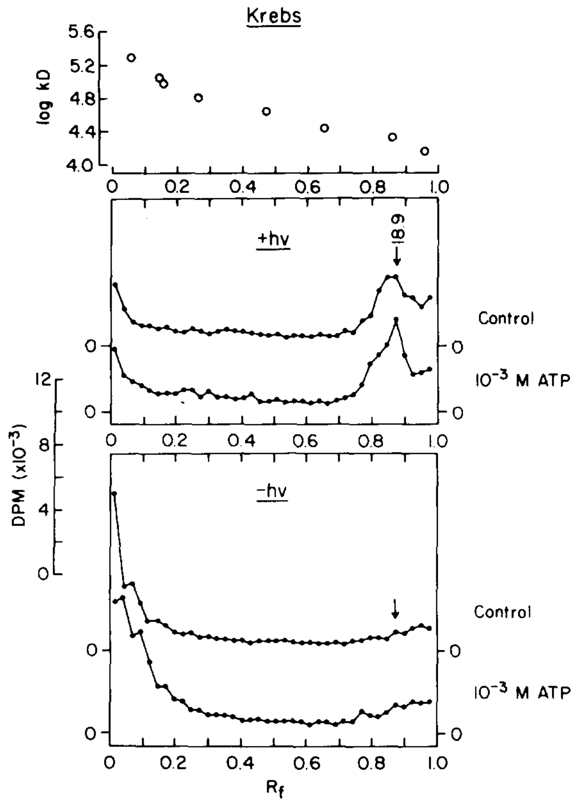

Fig. 4. Distribution of ^{3}H on SDS-polyacrylamide gels of samples of Krebs-Henseleit solution containing 10^{-5} M [^{3}H]ANAPP$_3$ (0.5 μCi/ml) which were irradiated in the organ chambers ('+hv') or not irradiated ('−hv'). Middle panel: irradiated in the absence (upper curve) or presence (lower curve) of 10^{-3} M ATP; bottom panel: not irradiated.

tosol was evaluated. Representative results of four experiments are shown in fig. 7. Compared to the membranes and cytosol from control tissues, the presence of dipyridamole (10^{-5} M) during photolysis of [^{3}H]ANAPP$_3$ had no effect on the peaks of interest (in fig. 7, ca. 54 and 43 kD for membranes; ca. 47 kD for cytosol). In addition, dipyridamole had no effect on the initial response to 10^{-5} M [^{3}H]ANAPP$_3$ (not shown).

3.4. Effect of prior treatment of tissues with nonradiolabeled photolyzed ANAPP$_3$ on the subsequent photoincorporation of [^{3}H]ANAPP$_3$

Since the nitrene intermediate generated upon photolysis of ANAPP$_3$ is highly reactive, photo-

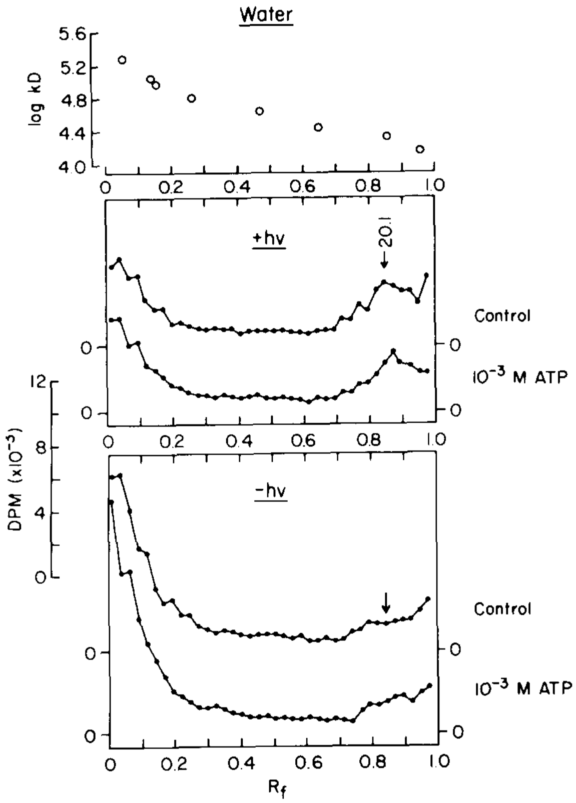

Fig. 5. Distribution of ^3H on SDS-polyacrylamide gels of samples of water containing 10^{-5} M [^{3}H]ANAPP$_3$ (0.5 μCi/ml) which were irradiated in the organ chambers ('+hv') or not irradiated ('−hv'). Middle panel: irradiated in the absence (upper curve) or presence (lower curve) of 10^{-3} M ATP; bottom panel: not irradiated.

labeling of nonspecific sites unrelated to P$_2$-receptors could occur; by scavenging, the large excess of ATP in the organ bath might prevent the photoincorporation of [^{3}H]ANAPP$_3$ at such sites. If responses to the initial agonist activity of [^{3}H]ANAPP$_3$ were antagonized by prior treatment of the tissues with photolyzed ANAPP$_3$, and if the incorporation of [^{3}H]ANAPP$_3$ were also reduced by this procedure, then additional evidence would be provided for the covalent insertion of radioactivity at specific receptors. Representative results of three experiments designed to test this hypothesis are shown in figs. 8 and 9. In the control vas deferens ('control') which was irradiated in the absence of ANAPP$_3$, the addition of [^{3}H]ANAPP$_3$ (10^{-5} M) elicited a typical contractile response (fig. 8; top panels). In the contralateral tissue, the first addition of nonradiolabeled ANAPP$_3$ (10^{-4} M) elicited a contraction. Following irradiation of the preparation the response to [^{3}H]ANAPP$_3$ (10^{-5} M) was antagonized (fig. 8, bottom panels) in a manner resembling the antagonism of ATP-induced responses (see fig. 1). The electrophoretic profiles of radioactivity obtained from these tissues are shown in fig. 9. In the membrane fraction of the control tissue (no ANAPP$_3$ present during the first irradiation) two regions of radioactivity were seen (ca. 57 and 48 kD); the prior treatment of the contralateral tissue with photolyzed nonradiolabeled ANAPP$_3$ reduced the incorporation of radioactivity in both regions. In the cytosol of the control and ANAPP$_3$-pretreated vasa deferentia a small peak of radioactivity was observed at ca. 45 kD (see also fig. 7); its magnitude was not affected by prior ANAPP$_3$-pretreatment.

3.5. Effect of irradiation of preparations in the presence of various concentrations of ATP on the degree of photoincorporation of [^{3}H]ANAPP$_3$

An estimate of the affinity of ATP for P$_2$-receptors was sought by photolyzing [^{3}H]ANAPP$_3$ in the presence of ATP using concentrations which span the range over which ATP induces contractile responses, e.g., 10^{-7}-10^{-3} M (Fedan et al., 1982c). In general, there was an inverse relationship between the degree of incorporation of radioactivity into the ca. 56 and 48 kD regions of membrane samples and the concentration of ATP that was present in the organ bath during photolysis of [^{3}H]ANAPP$_3$. A reduction in the concentration of ATP to below 10^{-4} M resulted in the gradual heightening of the peaks; at 10^{-7} M ATP there was no appreciable effect. The ability of a competing ligand to protect against the covalent insertion of a photoaffinity label is related to the ligand concentration. However, a competing ligand will reduce the rate, but ultimately not the extent, of photoincorporation. While the results obtained (not shown) from three experiments were not amenable to a kinetic analysis, the binding constant of ATP for the sites was judged to be larger than 10^{-7} M but less than 10^{-5} M, or, perhaps, ca. 10^{-6} M.

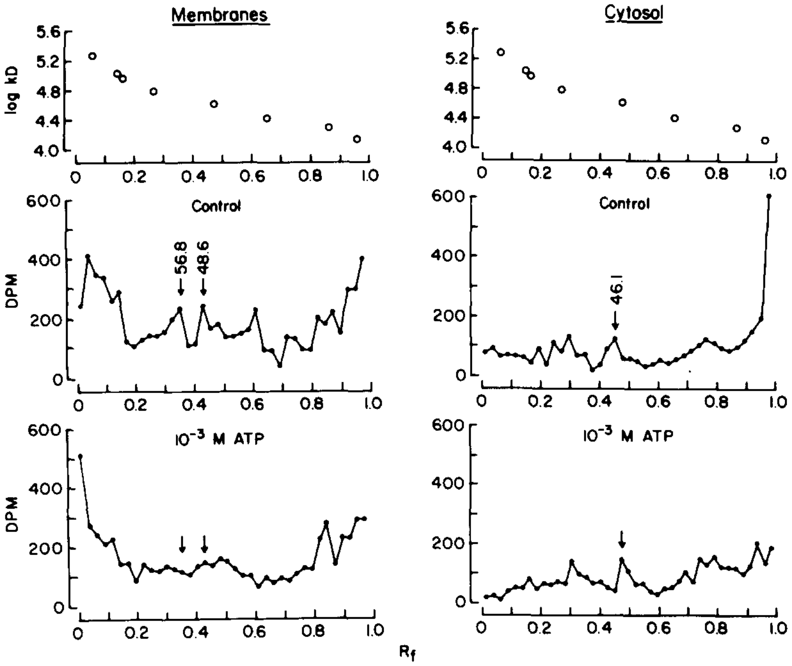

Fig. 6. Comparison of the distributions of ^3H on SDS-polyacrylamide gels of samples of membranes (left panels) and cytosol (right panels) following incubation of vas deferens with 10^{-5} M [^{3}H]ANAPP$_3$ and photolysis. Middle panels: irradiation in the absence of ATP ('Control'). Bottom panels: irradiation of the contralateral tissue in the presence of 10^{-3} M ATP. For each condition (i.e., absence or presence of ATP) the membranes and cytosol were obtained from the same vas deferens.

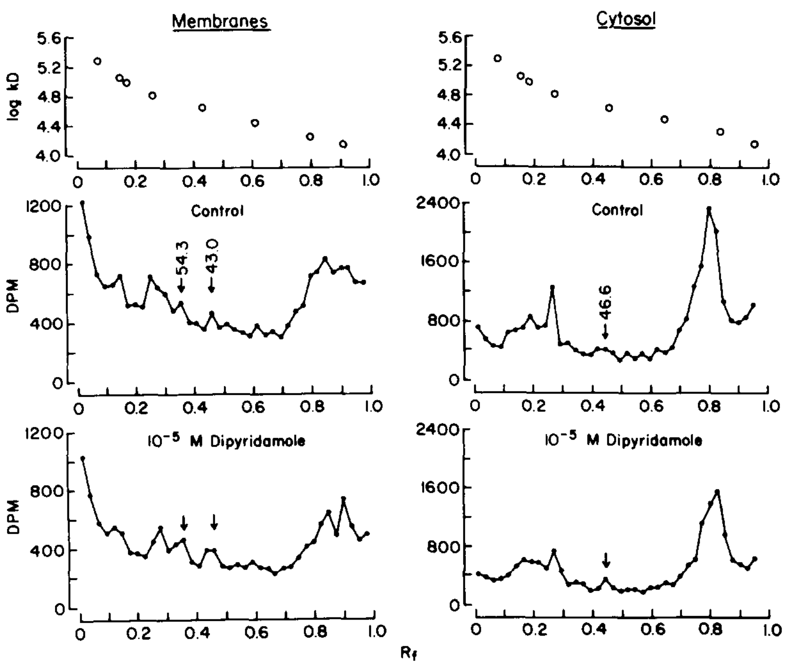

Fig. 7. Effect of dipyridamole (10^{-5} M) on photoaffinity labeling of membranes (left panels) and cytosol (right panels). Middle panels: irradiation of 10^{-5} M [^{3}H]ANAPP$_3$ in the absence of dipyridamole ('Control'); bottom panels: irradiation of the contralateral tissue in the presence of dipyridamole. For each condition the membranes and cytosol were obtained from the same vas deferens.

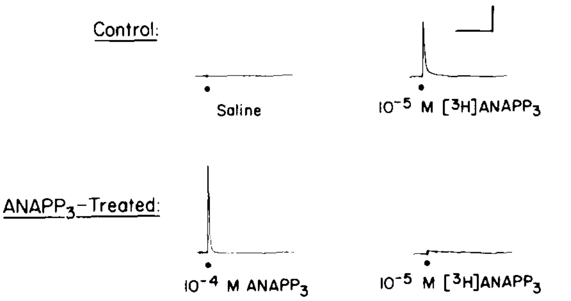

Fig. 8. Effect of treatment of vas deferens with irradiated, nonradiolabeled $ANAPP_3$ (10^{-4} M) on the response obtained following the subsequent addition of 10^{-5} M [^{3}H]$ANAPP_3$. Top panels ('Control'): $ANAPP_3$ was absent during the first irradiation period (irradiation occurred between the responses shown). Bottom panels ('$ANAPP_3$-treated'): following the addition of 10^{-4} M $ANAPP_3$ (left) the tissues were irradiated (not shown) prior to the addition of 10^{-5} M [^{3}H]$ANAPP_3$ (right) and its subsequent photolysis. See section 2.4.3 for additional details.

In contrast to these results ATP had no effect on the magnitude of the ca. 45 kD peak observed in the cytosolic fraction of the tissues (three experiments; data not shown). These findings are in general agreement with the consistent inability of any procedure to modify the peak of radioactivity in cytosolic samples.

4. Discussion

The results of the present study indicate that the incorporation of [^{3}H]$ANAPP_3$ into membrane constituents of ca. 54-66 (region I) and 43-57 kD (region II) is related directly to blockade of P_2-receptors. This conclusion is supported by an approach in which the functional effects of agents and procedures in organ baths were compared

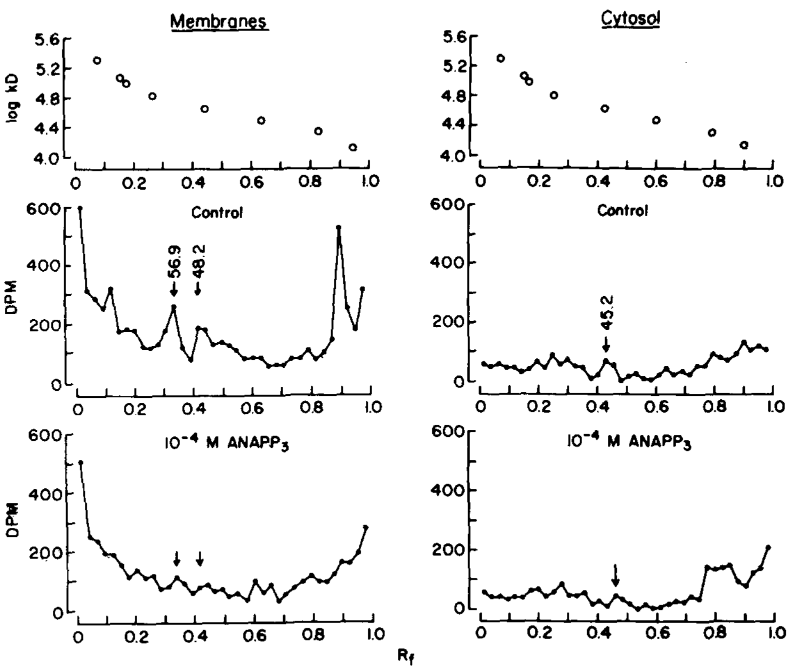

Fig. 9. Effect of prior irradiation of vas deferens with nonradiolabeled $ANAPP_3$ (10^{-4} M) on the subsequent photoincorporation of [^{3}H]$ANAPP_3$ (10^{-5} M) into membranes (left panels) and cytosol (right panels). Middle panels ('Control'): nonradiolabeled $ANAPP_3$ was absent during the first irradiation period; bottom panels: the tissue was first irradiated in the presence of nonradiolabeled $ANAPP_3$ prior to irradiating in the presence of [^{3}H]$ANAPP_3$. For each condition the membranes and cytosol were obtained from the same vas deferens. Tension responses of these tissues are shown in fig. 8.

with those seen in electrophoretic profiles from the same tissues. (1) The presence of ATP in the organ bath antagonized the [^{3}H]ANAPP$_3$-induced contractile response and reduced the incorporation of photolyzed [^{3}H]ANAPP$_3$ into regions I and II of samples of homogenate and membrane fraction. (2) The antagonistic effect of ANAPP$_3$ on ATP-induced responses was not reversible following photolysis (Hogaboom et al., 1980; Fedan et al., 1982a, b; O'Donnell et al., 1983) but could be reversed if the compound was not photolyzed (Hogaboom et al., 1980). Likewise, the incorporation of [^{3}H]ANAPP$_3$ into regions I and II occurred only after photolysis. (3) The prior photolytic treatment of tissues with nonradiolabeled ANAPP$_3$ antagonized contractile responses to [^{3}H]ANAPP$_3$ added subsequently, and reduced the level of incorporation of [^{3}H]ANAPP$_3$ into regions I and II. (4) Dipyridamole had no effect on [^{3}H]ANAPP$_3$-induced contractions or on the incorporation of photolyzed [^{3}H]ANAPP$_3$ into regions I and II.

No agent or procedure had any effect on the incorporation of radioactivity in the cytosol fraction. The photolabeling observed in this fraction is unrelated to its interaction with P$_2$-receptors. The molecular weight of the peak, which was different from the lower molecular weight ones derived from samples of bathing medium and after photolyzing [^{3}H]ANAPP$_3$ in Krebs-Henseleit solution or in water (figs. 3-5), suggests that it originates from the tissue. A number of possibilities could explain its presence: (1) It may reflect the nonspecific incorporation of [^{3}H]ANAPP$_3$ into soluble, extracellular proteins in the cytosolic fraction. (2) There may be entry of [^{3}H]ANAPP$_3$ or its dephosphorylated nucleotide products into the smooth muscle cells. A carrier-mediated transport of *intact* ATP in rat skeletal muscle has been proposed by Chaudry and Baue (1980). Such a mechanism, if it operates in the guinea-pig vas deferens, might transport [^{3}H]ANAPP$_3$ and result in the photolabeling of intracellular proteins. However, the transport of ATP during photolysis and pretreating the tissues with photolyzed, nonradiolabeled ANAPP$_3$ would be expected to reduce the photolabeling of intracellular proteins either by competing with the nucleotide carrier for [^{3}H]ANAPP$_3$, by a blockade of the carrier by photolyzed nonradiolabeled ANAPP$_3$, or by a dilution of the intracellular ATP pool containing [^{3}H]ANAPP$_3$. (3) The degradation of ANAPP$_3$ into arylazido aminopropionyl adenosine by tissue ectophosphohydrolase enzymes, and its subsequent entry into the smooth muscle cells via the nucleoside transporter, could allow the photolabeling of enzymes involved in the intermediary metabolism of adenosine. For example, 8-azidoadenosine, a photoaffinity label analog of adenosine, is a substrate for the nucleoside transporter (Rosenblit and Levy, 1980). Two points argue against this hypothesis. First, dipyridamole, a nucleoside transport inhibitor (Young and Jarvis, 1983), did not reduce the cytosolic peak of radioactivity. Second, in experiments involving the photolytic pretreatment of tissues with nonradiolabeled ANAPP$_3$, in which arylazido aminopropionyl adenosine also would have been generated, the photoincorporation of nonradiolabeled arylazido aminopropionyl adenosine into enzymes related to adenosine metabolism would have reduced the photoincorporation of [^{3}H]arylazido aminopropionyl adenosine produced enzymatically.

Both P$_1$-purinergic receptors and the nucleoside transporter are localized in the plasma membrane of cells, and the possibility that regions I and II are associated with these entities must be considered. This is unlikely, however, for the following reasons. (1) While the existence of prejunctional adenosine autoreceptors, which modulate adrenergic neurotransmitter release, are well-known (Vanhoutte et al., 1981), postjunctional adenosine receptors may not be present to a great extent in the guinea-pig vas deferens. Adenosine does not cause contraction of a resting tissue. In preparations contracted with 60 mM KCl to allow the development of a sustained tension response, adenosine in very high concentrations (10^{-3} M) evokes a very small contraction; relaxation does not occur (unpublished observations). In addition, ANAPP$_3$ has no effect on responses of guinea-pig stomach muscle to ATP; such responses are mediated by adenosine formed in the tissue since they are antagonized by 8-phenyl theophylline (Frew and Lundy, 1982a, b). Thus, there is neither evidence at present for the existence of functional postjunctional adenosine receptors in the guinea-pig vas deferens nor for an action of $ANAPP_3$ on adenosine receptors. (2) Recently, Young et al. (1983) have evaluated some characteristics of the nucleoside transporter from human erythrocyte membranes. Two inhibitory compounds. N^6-(p-azidobenzyl)adenosine, an arylazido adenosine analog, and nitrobenzylthioinosine, which is naturally photolabile, were found to photoincorporate into a membrane constituent with a molecular weight of 45-65 kD. Similarly, 8-azidoadenosine photolabels a 56 kD protein in adipocyte plasma membranes (Rosenblit and Levy, 1980). The overlap in the molecular weights of regions I and II in guinea-pig vas deferens membranes and of the photolabeled nucleoside transporter in erythrocytes and adipocytes might suggest that one or both of regions I and II represent the insertion of [^{3}H]arylazido aminopropionyl adenosine into the nucleoside carrier system. The smooth muscle of the guinea-pig vas deferens is capable of taking up adenosine, which then enters the nucleotide synthetic pathway (Rowe et al., 1976). The strongest evidence which indicates that the nucleoside transporter in the vas deferens was not among the proteins in regions I and II which were labeled by [^{3}H]$ANAPP_3$ or its nucleoside analog is the finding that dipyridamole had no effect on the electrophoretic profiles of regions I and II. Dipyridamole is a competitive inhibitor of nitrobenzylthioinosine binding (Young and Jarvis, 1983 and references inter alia). At present we view the similar relative molecular weights of regions I and II and that of the photolabeled moiety of the nucleoside transporter to be a coincidence.

We have proposed earlier (Fedan et al., 1982a, b) that two P_2-receptor types may exist in the smooth muscle of the guinea-pig vas deferens. Evidence was also obtained which suggested that $ANAPP_3$ interacts with both P_2-receptor types (Fedan et al., 1982b). The present results support these hypotheses. It is relevant to consider alternative interpretations, however, of which there are several.

One of the regions of interest may represent the photoaffinity labeling of a receptor while the other the interaction of $ANAPP_3$ with ectophosphohydrolase enzymes, possibly ATPase, nucleotide pyrophosphohydrolase, ADPase or 5′-nucleotidase, during the progressive hydrolysis of $ANAPP_3$ to di- and mono-nucleotide products ($ANAPP_3$ is a substrate for, and photoaffinity labels F_1-mitochondrial and myosin ATPases; Jeng and Guillory, 1975; Russell et al., 1976). This would appear unlikely for two reasons: (1) compared to ATP, contractions elicited by nonphotolyzed $ANAPP_3$ are prolonged, suggesting that $ANAPP_3$ is not readily catabolized by the tissue (O'Donnell et al., 1983). (2) $ANAPP_3$ does not reduce the maximum response of nucleotides which contain normal anhydride oxygen phosphate chains, while it does decrease the maxima of nucleotide analogs with anhydride oxygen substitutions or additions (Fedan et al., 1982a, b).

A second alternative interpretation is that only one receptor is present, i.e., region I, and that region II activity originates from the action of protease enzymes on the region I constituent. Recently, the degree of apparent heterogeneity of β-adrenoceptors following SDS-PAGE, assessed after photolabeling isolated lung membranes with p-azido-m-[^{125}I]iodobenzylcarazolol, was reduced if precautions were taken to inhibit protease activity during homogenization of the tissues and preparation of the membranes (Benovic et al., 1983). As we were only recently aware of this report no precautions were taken in the present investigation to prevent proteolysis. Since intact vasa deferentia were used it would seem very unlikely that proteolysis occurred in the organ bath. We cannot eliminate, however, the possibility that proteases were active during homogenization and membrane preparation that could convert some of the region I · [^{3}H]$ANAPP_3$ complex into region II · [^{3}H]-$ANAPP_3$ complex.

A third alternative interpretation of the presence of two regions of radioactivity in the gels is that one of them is related to the presence of a prejunctional P_2-receptor which has different molecular characteristics. These sites would be silent in the present study in which only postjunctional responses were monitored. There is little reason to suspect this possibility because $ANAPP_3$ treatment had no effect on the neurogenically evoked release of norepinephrine (Fedan et al., 1981), which is evidence which argues against the existence of prejunctional P_2-autoreceptors in this preparation. In addition, the possibility that the molecular characteristics of prejunctional P_2-receptors are different from postjunctional ones is no more likely than the possibility that they are the same. We conclude tentatively, subject to comparisons utilizing chemically or surgically denervated vas deferentia, that regions I and II are associated with postjunctional P_2-receptors.

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