Articles

Specific Binding of ATP to Extracellular Sites on *Torpedo* Acetylcholine Receptor†

B. J. Carlson and M. A. Raftery*

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108, and Department of Pharmacology, University of Minnesota, 435 Delaware Street, Minneapolis, Minnesota 55455

Received November 20, 1992; Revised Manuscript Received March 26, 1993

ABSTRACT: The β- and δ-subunits of the nicotinic acetylcholine receptor from *Torpedo californica* were covalently photolabeled at the synaptic surface with the ATP photoaffinity analogue [α-^{32}P]-8-azido-ATP. The specificity of labeling for nucleotide binding sites was demonstrated by the saturation of labeling with increasing concentration of 8-azido-ATP and the inhibition of photolabeling by ATP. Protection studies suggest that the binding sites for the photolabel are unique and are not associated with the cholinergic ligand binding sites.

In addition to the modulating effects of ATP[1] on various ion channels [reviewed by Stanfield (1987) and Bernardi *et al.* (1992)], it has been proposed that ATP released simultaneously with AcCh at cholinergic synapses is a modulator of the nicotinic AcChR (Saji *et al.*, 1975; Ewald, 1976; Akasu *et al.*, 1981, 1983; Akasu & Konetsu, 1985; Eterovic *et al.*, 1989, 1990). This suggests that ATP interacts with the AcChR at a specific binding site, presumably different from those to which AcCh and competitive antagonists bind due to the lack of similarity in structure, with the same being true for noncompetitive antagonists [reviewed in Eterovic *et al.* (1990)]. In this article we describe one approach toward identification of such binding sites for ATP on the AcChR from *Torpedo californica*, using the photoaffinity label [α-^{32}P]-8-azido-adenosine-5′-triphosphate. The results demonstrate that specific binding sites exist on two of the four subunits that constitute this receptor [see Conti-Tronconi and Raftery (1982)]. We also show that such binding sites appear to be located on the synaptic surface of the AcChR, as would be expected if ATP coreleased with AcCh exerts a modulatory physiological effect.

METHODS

Materials. [α-^{32}P]-8-Azido-ATP was purchased from ICN. ADP and ATP-γ-S were obtained from Boehringer Mannheim. Unlabeled 8-azido-ATP, ATP, GTP, and carbamylcholine were acquired from Sigma. [^{125}I]-α-Bungarotoxin was obtained from the Miami Serpentarium and calibrated by the procedures of Blanchard *et al.* (1979). *Torpedo californica* was purchased from Pacific Biomarine (Venice, CA). AcChR-enriched membranes were prepared as described by Duguid and Raftery (1973), and peripheral proteins were removed by alkali extraction (Neubig *et al.*, 1979; Elliott *et al.*, 1980). Sealed membrane vesicles enriched with AcChR were obtained according to Jeng *et al.* (1981). The specific activities of purified membrane preparations, as determined by [^{125}I]-α-bungarotoxin binding (Blanchard *et al.*, 1979) ranged from 2 to 4 nmol of α-bungarotoxin binding sites/mg of protein.

Photolabeling. Standard buffer for [α-^{32}P]-8-azido-ATP photolabeling was 100 mM potassium phosphate, pH 7.5, with concentrations of [α-^{32}P]-8-azido-ATP, AcChR, and other additions as described in the relevant figures. Stock [α-^{32}P]-8-azido-ATP solution in methanol was transferred via pipet into 0.5-mL microcentrifuge tubes and evaporated with a stream of air. A solution of unlabeled 8-azido-ATP in buffer was added and kept at 4 °C. AcChR-rich membranes and other additions were prepared (at 4 °C) in 0.5-mL microcentrifuge tubes. An aliquot of [α-^{32}P]-8-azido-ATP was added to the sample, vortex mixed, and transferred to a glass spotplate. Irradiation was accomplished using a Mineralight UVSL-25. The samples utilizing the Mineralight UVSL-25 (Ultra Violet Products, Inc.) were irradiated at 3 in. for various time intervals at the shortwave setting (180–254 nm). Photolabeling mixtures were quenched immediately following irradiation by addition of equal volumes of 50 mM DTT, 10% glycerol, 3% SDS, and 63 mM Tris. The procedures used were essentially those described previously by Potter and Haley (1982).

Analysis of Photolabeling by SDS-PAGE. Quenched mixtures were run on 8.75% polyacrylamide gels and stained with Coomassie Blue (Laemmli, 1970). Autoradiography of dried gels was done at −80 °C using X-Omat RP X-ray film (Kodak) and Cronex Lightning Plus intensifying screens (Du Pont).

Analysis of Photolabeled Mixtures. Immediately following irradiation, photolabeled mixtures were transferred to DEAE–cellulose disks (Whatman, DE-81, 2.4 cm), and the reaction was quenched by placing the mixtures in wash buffer (5 mM sodium pyrophosphate, 50 mM NaCl, 10 mM phosphate, 0.1% Triton X-100, pH 7.4) containing 1 mM DTT. Disks were

† This research was supported by USPHS Grant NS 10294 and by US NIDA program project Grant 5PO1-DA05695.

* To whom correspondence should be addressed.

[1] ATP, adenosine-5′-triphosphate; ATP-γ-S, adenosine-5′-*O*-(3-thiophosphate); AcCh, acetylcholine; AcChR, acetylcholine receptor; 8-azido-ATP, 8-azidoadenosine-5′-triphosphate; DEAE-, (diethylamino)ethyl-; ATPase, adenosinetriphosphatase; SDS, sodium dodecyl sulfate; ADP, adenosine-5′-diphosphate; GTP, guanosine-5′-triphosphate; α-Butx, α-bungarotoxin; Tris, tris(hydroxyethyl)aminomethane; DTT, dithiothreitol; LDH, lactate dehydrogenase; PK, pyruvate kinase; NADH, nicotine adenine dinucleotide; PAGE, polyacrylamide gel electrophoresis.

0006-2960/93/0432-7329$04.00/0

washed over a 30-min period with three changes of wash buffer. Disks were counted by liquid scintillation (Beckman LS 3800).

Analysis of ATP Hydrolysis by AcChR-Rich Membrane Fragments in the Presence of an ATP-Regenerating System. The release of phosphate from ATP was followed in the presence of an enzyme-coupled ATP-regenerating system as described by Pullman *et al.* (1960). The release of phosphate from ATP is measured in the presence of phosphoenolpyruvate and pyruvate kinase (PK) to regenerate ATP. The reaction is followed by spectrophotometrically measuring the formation of pyruvate from phosphoenolpyruvate at 340 nm in the presence of β-NADH and lactate dehydrogenase (LDH). The conditions were 100 mM phosphate, 250 μM β-NADH, 1 mM $MgCl_2$, 2 mM phosphoenolpyruvate, and 2 units/mL of PK. PK/LDH was available from Sigma as a crystalline suspension in ammonium sulfate containing 700 units/mL of PK and 1000 units/mL of LDH. Addition of ATP or ADP was accomplished by direct mixing in the cuvette with a plunger.

RESULTS

Incorporation of [α-^{32}P]-8-azido-ATP into AcChR in enriched membranes was first studied as a function of time of irradiation. The levels of incorporation were determined by centifugation assay as described in Figure 1. As shown in Figure 1A, it was observed that photoinsertion was maximal after about 2 min of irradiation, whereas no insertion was observed without irradiation. This time dependency is similar to that previously observed for a closely related photoaffinity label applied to a different protein system (Rush & Konigsberg, 1990). Therefore, in order to determine the concentration dependency of photoinsertion of the label, all samples were irradiated for this period of time. The results obtained are depicted in Figure 1B. These data show that the photolabeling contains a saturable component with a midpoint at ~330 μM. Since the membrane preparations probably contain a small residual amount of ATPase, even after alkaline extraction, we assayed for ATP hydrolysis using a coupled enzyme assay as described in Methods. No significant hydrolysis of ATP was detectable over the time period of photolysis (data not shown). The stoichiometry of [^{32}P]-8-azido ATP labeling of the AcChR can be determined only as a rough estimate due to the presence of other proteins in the membrane preparations (2–4 nmol of AcChR/mg of protein corresponds to 25–50% AcChR) which could also be labeled specifically (Na, K, ATPase) or nonspecifically (see Figure 2 in the high M_r region). In order to obtain a rough estimate of the extent of AcChR labeling, we assumed that 50% of the label is associated with the receptor. This yields an estimate of the labeling stoichiometry as 1.25 mol of photolabel/mol of AcChR. In order to ascertain subunit localization of the site(s) of photoinsertion, the labeled membranes were analyzed both by SDS-gel electrophoresis followed by staining for proteins using Coomassie Blue and by autoradiography for localization of the photolabel. It was observed that the major photoinsertion of the label was into the β- and δ-subunits of the AcChR, with only trace labeling of the α-subunit (Figure 2, right). This preferential labeling of two of the four subunits attests to the specificity of interaction of the ATP analog with the membrane-bound AcChR. The levels of β- and δ-subunit labeling were roughly equal as determined visually. Unfortunately, it was not possible to determine the stoichiometry for each subunit since this would necessitate a determination of the amounts of each subunit on the SDS gel and reliable methods are not available for such analysis. The presence of high M_r labeled components, some of which may be the result of photoinduced cross-linking, precludes estimation from the amount of AcChR used. In order to ascertain whether the specific labeling we observed is related to extrasynaptic ATP interaction with the AcChR (following vesicular release *in vivo*) or to cytoplasmic ATP, the labeling was repeated using sealed vesicles prepared from the purified membranes as described in Methods. We have previously shown that these enriched membranes containing the AcChR are ~95% outside-out (Hartig & Raftery, 1977) and that a multiple negatively charged species such as aminonaphthalenetrisulfonic acid (Moore & Raftery, 1980) does not leak across closed vesicles over periods of many hours. This was confirmed for the preparations used in the studies reported here. Using this sealed vesicle preparation, we observed no significant difference in the labeling pattern (Figure 2, left) between sealed and unsealed membranes. These reproducible effects ($n = 3$) lead to the conclusion that the specific labeling of the β- and δ-subunits occurs on the extracellular surface of the AcChR.

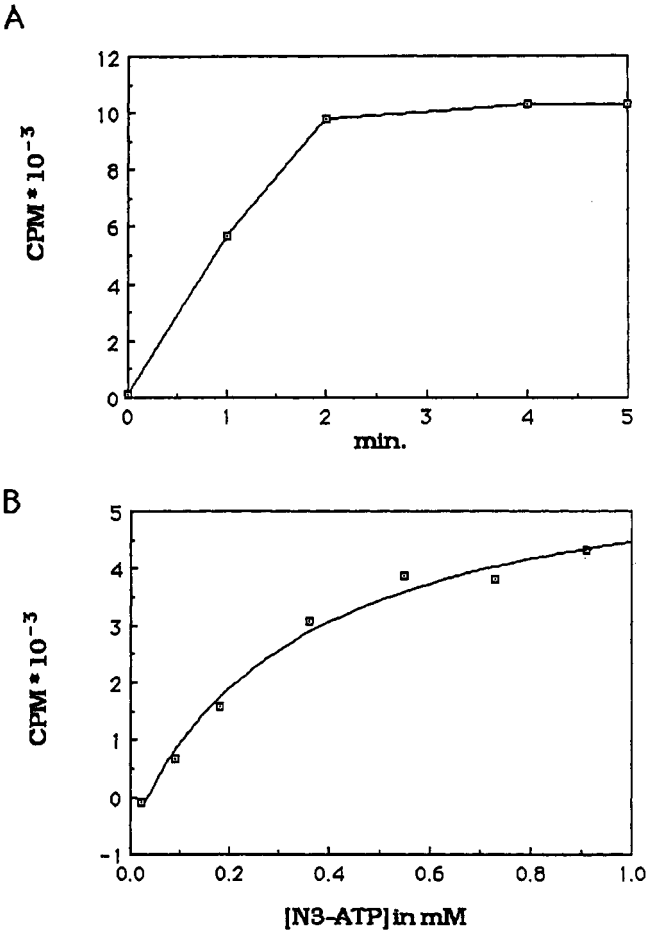

FIGURE 1: (A) Photolabeling as a function of time. Photolysis was done by Mineralight using conditions described in the Methods section. Photolabel mixtures contained 420 μM [α-^{32}P]-8-azido-ATP (5 μCi/μmol), AcChR (1 mg/mL by Lowry assay), and 18 mM $MgCl_2$. Mixtures were transferred to 0.5-mL microcentrifuge tubes containing equal volumes of 10 mM DTT, 10 mM ATP, and 10 mM $MgCl_2$ in buffer and centrifuged for 30 min at 15 000 rpm in a Model 5414 Eppendorf microcentrifuge. Pelleted membranes were counted by liquid scintillation. (B) Concentration dependence of specific labeling of AcChR by 8-azido-ATP. Photolabeling was done by Mineralight, and samples were analyzed on ion-exchange paper disks as outlined in the Methods section. Photolabeled mixtures contained varying concentrations of [α-^{32}P]-8-azido-ATP (3 μCi/μmol) with equal concentration of $MgCl_2$ and 0.7 μM AcChR (α-bungarotoxin sites). Titrations were done in the absence and presence of 10 mM unlabeled 8-azido-ATP. The amount of specific binding is taken as the difference of the two titrations.

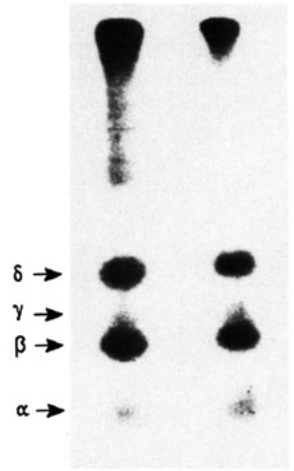

FIGURE 2: Photolabeling of AcChR in sealed vesicles by 8-azido-ATP. Photolabeling was conducted using the Mineralight, and the samples were analyzed by SDS-PAGE and autoradiography as described in the Methods section. Photolabeled mixtures contained 150 μM [α-^{32}P]-8-azido-ATP (42 μCi/mmol), AcChR (1.2 mg/mL by Lowry assay), 10 mM $MgCl_2$, and 100 μM ouabain. Labeling of a sealed vesicle preparation (left lane) and a preparation containing both sealed and open vesicles (right lane) is compared.

These results are consistent with the electrophysiological studies of Eterovic *et al.* (1990), who showed modulation of acetylcholine effects by ATP since they added ATP extracellulary.

As a confirmation of this result using [α-^{32}P]-8-azido-ATP, it was of interest to determine whether ATP could selectively protect against insertion of the photolabel. In Figure 3 (left), the results of such experiments are presented. Using a constant concentration of the photolabel (700 μM) and increasing concentrations of ATP, protection against photoinsertion was observed, with a midpoint of ~200 μM. This protective effect became almost total at submillimolar concentrations of ATP, suggesting that ATP interacts with both the β- and the δ-subunits. This was confirmed by experiments involving SDS-gel electrophoresis, as shown in Figure 3 (right). As the concentration of ATP was increased up to 600 μM, a progressive decline in the labeling of both subunits was observed. The protective effect of ATP was more effective on δ-subunit labeling, presumably due to more effective binding of this nucleotide to this subunit.

Since nucleotides related to ATP have been observed to affect the function of the AcChR (see Introduction and Discussion), we tested some ATP analogues in experiments similar to that depicted in Figure 3 (right). The data depicted in Figure 4 show the protective effects of ATP-γ-S on photoinsertion of the affinity label at concentrations of 0, 75, 150, 300, and 600 μM, as shown in lanes 1–5, respectively. As noted above for ATP protection, ATP-γ-S appeared to protect the δ-subunit from photolabeling more efficiently than the β-subunit, again presumably due to higher affinity for the δ-subunit. Similarly, concentration-dependent protection was evident using GTP (Figure 5, right) and ADP (Figure 5, left) at the same concentrations utilized with ATP-γ-S. It should be noted that in the membrane preparations used for these latter experiments a protein of molecular weight ~90 kDa was present and was photolabeled. This is probably residual Na,K–ATPase, which should also incorporate the photolabel. This notion is bolstered by the observation that both nucleotides also afforded protection against labeling of this entity. In addition, some labeling of the γ-subunit appeared in Figure 5 (right). This was evident only for overexpressed gels and was not significant in comparison with β- and δ-subunit labeling under exposure conditions for optimization of specific photolabeling.

In order to further test whether the binding sites for the photolabel or ATP are unique sites rather than associated with or part of a well-documented cholinergic ligand binding

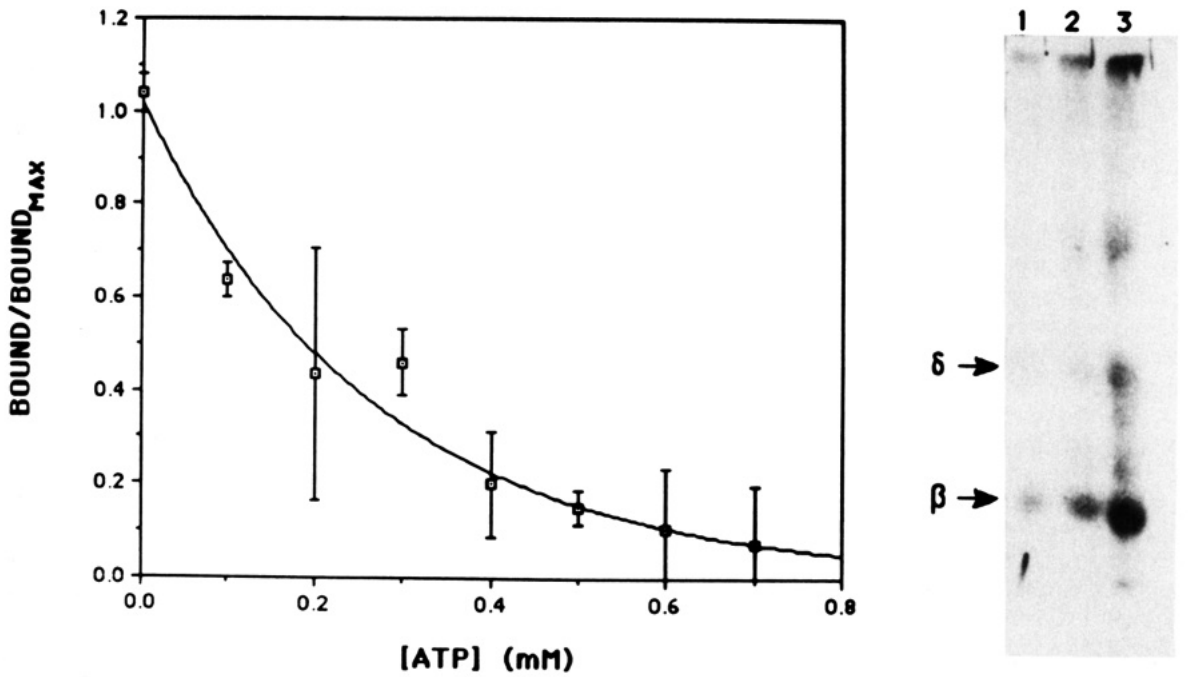

FIGURE 3: Inhibition of photolabeling by ATP. AcChR (0.7 μM) (α-bungarotoxin sites) was photolabeled by 700 μM [α-^{32}P]-8-azido-ATP and 10 mM $MgCl_2$ in the presence of increasing concentrations of ATP. Photolabeling was done using the Mineralight, and samples were analyzed on ion-exchange disks as described in the Methods section. Data from several experiments (n=3) were graphed as fraction of maximum insertion and fitted to an exponential decay curve by a nonlinear graphing program (Graph Pad). Error bars represent the standard deviation of the data at each concentration of ATP. (inset) Photolabeling was performed under standard conditions with 150 μM [α-^{32}P]-8-azido-ATP (22 μCi/μmol), AcChR (1 mg/mL, by Lowry assay), 600 μM $MgCl_2$, and [ATP] = 600 μM (lane 1), 300 μM (lane 2), and 0 μM (lane 3). Photolabeling mixtures were analyzed by SDS-PAGE and autoradiography as described in the Methods section.

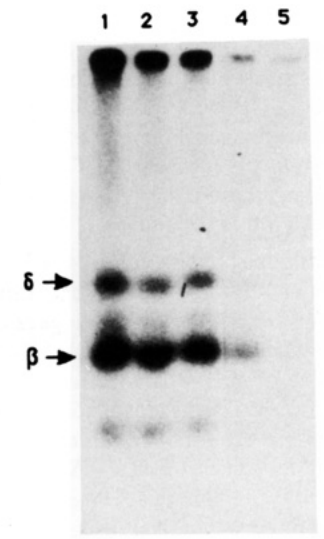

FIGURE 4: Inhibition of photolabeling by ATP-γ-S. Photolabeling was performed under standard conditions with 150 μM [α-^{32}P]-8-azido-ATP (22 μCi/μmol), AcChR (1 mg/mL by Lowry assay), 600 μM $MgCl_2$, and [ATP-γ-S] = 0 μM (lane 1), 75 μM (lane 2), 150 μM (lane 3), 300 μM (lane 4), and 600 μM (lane 5). Photolabeling mixtures were analyzed by SDS-PAGE and autoradiography as described in the Methods section.

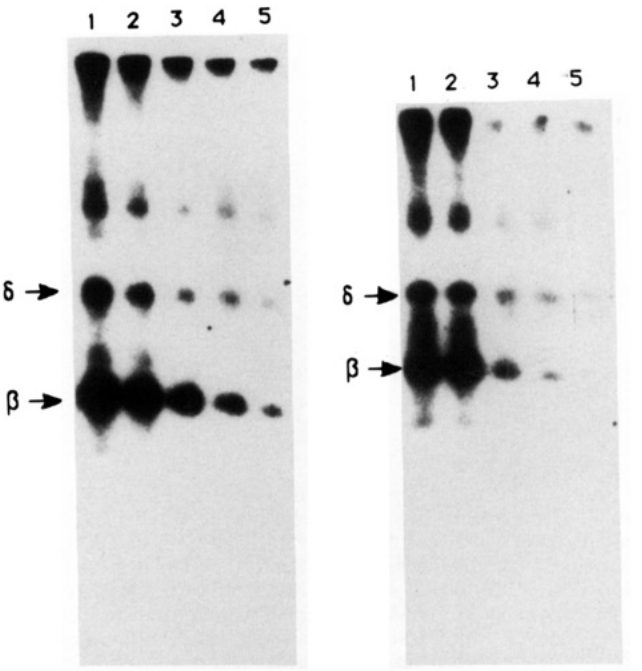

FIGURE 5: (left) Inhibition of photolabeling by GTP. Photolabeling was performed under standard conditions with 150 μM [α-^{32}P]-8-azido-ATP (22 μCi/μmol), AcChR (1 mg/mL by Lowry assay), 600 μM $MgCl_2$, and [GTP] = 0 μM (lane 1), 75 μM (lane 2), 150 μM (lane 3), 300 μM (lane 4), and 600 μM (lane 5). Photolabeling mixtures were analyzed by SDS-PAGE and autoradiography as described in the Methods section. (right) Inhibition of photolabeling by ADP. Photolabeling was performed under standard conditions with 150 μM [α-^{32}P]-8-azido-ATP (22 μCi/μmol), AcChR (1 mg/mL by Lowry assay), 600 μM $MgCl_2$, and [ADP] = 0 μM (lane 2), 150 μM (lane 3), 300 μM (lane 4), and 600 μM (lane 5). Photolabeling mixtures were analyzed by SDS-PAGE and autoradiography as described in the Methods section.

sites, protection studies were conducted using the agonist carbamoylcholine and the antagonist α-Butx. Neither of these compounds had any protective effects on photoinsertion into the β- and δ-subunits (data no shown).

DISCUSSION

The results described above demonstrate that binding sites for nucleotides such as ATP, ATP-γ-S, GTP, and ADP appear to exist on the nicotinic AcChR from *Torpedo californica*. The sites are associated with two of the four constituent subunits, namely, the β- and δ-subunits, with little discernible labeling of the two α-subunits and the single γ-subunit. Furthermore, these binding sites are associated with the extrasympatic surface of the AcChR since no difference was observed in the labeling of sealed vesicle preparations as compared with membrane preparations composed of a mixture of sealed and unsealed vesicles. This extracellular location of direct ATP interaction is reminiscent of other effects of ATP on cation transport, specifically ^{86}Rb flux into cells (Haggblad *et al.*, 1985) where such effects were considered due to interaction with P_2-type receptors (Akasu & Koketsu). Coupling between such nucleotide receptors and nicotinic receptors was not considered likely since α-Butx did not block cation transport. The results presented here also show that such coupling is unlikely, since there is direct interaction of ATP and nucleotide analogues with the AcChR β- and δ-subunits. The results of the experiments described here are also in agreement with the suggestion of Eterovic *et al.* (1990) that the effects of ATP on potentiation of AcCh-induced currents are not due to coupling between purinergic and cholinergic receptors. Those studies and the results presented here strongly indicate a direct action of ATP on the AcChR. The close similiarities between the ATP concentration dependencies of AcCh current potentiation observed by Eterovic *et al.* (1990) and the photoinsertion shown in Figure 1 (midpoint 350 μM) as well as its inhibition by ATP (midpoint ~200 μM) suggest that the ATP binding sites on the β- and δ-subunits we observed here are responsible for the observed electrophysiological effects.

These interactions of ATP and its analogues are distinct from the well-documented kinase-mediated phosphorylation of the AcChR, using [^{32}P]-γ-ATP as substrate (reviewed in Safran *et al.*, 1990). Apart from the difference in location of the ^{32}P label ([^{32}P]-α in the photolabel which precludes kinase-mediated effects), the extracellular rather than intracellular labeling confirms the distinction between these nucleotide-mediated phenomena.

Given the concentration dependency of the inhibition of photoinsertion of the affinity label by ATP ($K_{app} \approx 200$ μM), it seems likely that the ATP binding sites on the β- and δ-subunits are occupied by ATP to a significant extent following release of AcCh and ATP from synaptic vesicles (Morel & Meunier, 1981), estimated to be ~300 μM for AcChR (Kuffler & Yoshikami, 1975) and ~50–80 μM for ATP (Hume & Honig, 1986; Silinsky, 1975) so that the modulation observed in electrophysical studies is likely to parallel similar effects *in vivo*. Whether such effects operate mechanistically by affecting agonist binding, by modulation of agonist coupling to channel activation, or by direct modulation of channel properties remains to be determined. It is also possible that ATP binding mediates yet-to-be-discovered physiological effects.

ACKNOWLEDGMENT

We thank David Okita and Larry Waddell for preparation of the membranes used throughout.

REFERENCES

Akasu, T., & Koketus, K. (1985) *Br. J. Pharmacol. 84*, 525–531.
Akasu, T.; Hirai, K., & Koketsu, K. (1981) *Br. J. Pharmacol. 74*, 505–507.

Akasu, T., Hirai, K., & Koketsu, K. (1983) *Neurosci. Lett. 26*, 673–676.
Bernardi, H., Fosset, M., & Lazdunski, M. (1992) *Biochemistry 31*, 6329–6332.
Blanchard, S. G., Quast, U., Reed, K., Lee, T. Schimerlik, M. I., Vandlen, R., Claudio, T., Strader, C., Moore, H.-P. H., & Raftery, M. A. (1979) *Biochemistry 18*, 1875.
Conti-Tronconi, B. M., & Raftery, M. A. (1982) *Annu. Rev. Biochem. 51*, 491.
Duguid, J. R., & Raftery, M. A. (1973) *Biochemistry 12*, 3593.
Elliott, J., Blanchard, S. G., Wu, W., Miller, J., Strader, L. D., Hartig, P., Moore, H.-P. H., Racs, J., & Raftery, M. A. (1980) *Biochem. J. 185*, 667.
Eterovic, V. A., Escalona de Motta, G., Hann, R. M., Lasalde, J. A., Prieto, J. A., & Ferchmin, P. A. (1989) *J. Receptor Res. 9*, 107–125.
Eterovic, V. A., Li, L., Palma, A., & McNamee, M. G. (1990) *Cell Mol. Neurobiol. 10*, 1990.
Ewald, D. A. (1976) *J. Membr. Biol. 29*, 47–65.
Haggblad, J., Eriksson, H., & Heilbronn, E. (1985) *Acta Physiol. Scand. 125*, 389–393.
Hartig, P. R., & Raftery, M. A. (1977) *Biochem. Biophys. Res. Commun. 77*, 4.
Hume, R. I., & Honig, M. G. (1986) *J. Neurosci. 6*, 681–690.
Jeng, A. Y., St. John, P. A., & Cohen, J. B. (1981) *Biochim. Biophys. Acta 646*, 411–421.
Kuffler, S. W., & Yoshikami, D. (1975) *J. of Physiol. 251*, 462–482.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lowry, O. H., Rosenbrough, N. J., Farr, A., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Moore, H.-P. H., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4509–4513.
Morel, N., & Meunier, F. M. (1981) *J. Neurochem. 36*, 1766–1773.
Neubig, R. R., Krodel, E. K., Boyd, N. D., & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690–694.
Potter, R., & Haley, B. E. (1982) *Methods Enzymol. 91*, 613–633.
Pullman, M. E., Penefsky, H. S., Datta, A., & Raker, E. (1960) *J. Biol. Chem. 235*, 3322–3329.
Rush, J., & Konigsberg, W. H. (1990) *J. Biol. Chem. 265*, 4821–4827.
Safran, A., Provenzano, C., Sagi-Eisenberg, R., & Fuchs, A. (1990) *Biochem. J. 29*, 6730–6734.
Saji, Y., Escalona de Motta, G., & del Castillo, J. (1975) *Life Sci. 16*, 945–954.
Silinsky, E. M. (1975) *J. Pharmacol. 71*, 145–162.
Stanfield, P. R. (1987) *Trends Neurosci. 10*, 335–339.