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ACCELERATED COMMUNICATION

Irreversible Activation of Phospholipase C-Coupled P_{2Y}-Purinergic Receptors by 3′-*O*-(4-Benzoyl)benzoyl Adenosine 5′-Triphosphate

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SUMMARY

3′-*O*-(4-Benzoyl)benzoyladenosine 5′-triphosphate (BzATP), a photoaffinity analog of ATP, was used as a ligand for the phospholipase C-linked P_{2Y}-purinergic receptor in turkey erythrocytes. In membranes from [^{3}H]inositol-labeled turkey erythrocytes, BzATP stimulated inositol phosphate formation in a concentration-dependent manner ($K_{0.5}$ = 172 ± 4 nM). The effect of BzATP was strictly dependent on the presence of guanine nucleotides and was not additive with the effect of other full P_{2Y}-purinergic receptor agonists. Photolysis of [^{3}H]inositol-labeled membranes in the presence of BzATP had no effect on the magnitude of the maximally attainable response of phospholipase C to the P_{2Y}-purinergic receptor agonist adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS) plus guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS). However, the effects of submaximally effective concentrations of GTPγS were markedly increased in membranes previously photolyzed in the presence of BzATP. In addition, the rate of activation of phospholipase C by GTPγS in membranes photolyzed in the presence of BzATP was increased 3-fold, as compared with control membranes. BzATP effects on phospholipase C were prevented by photolysis in the presence of ATP or ADP, but not by the presence of the weak P_{2Y}-purinergic receptor agonist β,γ-methyleneadenosine 5′-triphosphate. These results suggest that BzATP is a full P_{2Y}-purinergic receptor agonist, which after photolysis becomes irreversibly associated with turkey erythrocyte membranes and promotes P_{2Y}-purinergic receptor-mediated guanine nucleotide-dependent activation of phospholipase C.

Extracellular effects of ATP have been documented for a broad range of cell types (see Ref. 1 for review). These responses are mediated by at least two different receptor subtypes, i.e., P_{2X}- and P_{2Y}-purinergic receptors, originally classified by Burnstock and Kennedy (2) on the basis of the relative potencies of ATP and ADP analogs. The structure and function of these receptors, as well as those of the other molecular entities involved in P_2-purinergic receptor-regulated second messenger signalling, have yet to be identified.

Using membranes from [^{3}H]inositol-labeled turkey erythrocytes, we recently have characterized a purinergic receptor coupled to the activation of phospholipase C (3). The order of potency of a series of ATP and ADP analogs for the stimulation of inositol phosphate formation in these membranes is consistent with that for the P_{2Y}-purinergic receptor subtype (2). In the present study, we describe the properties of an ATP analog, BzATP, which upon photolysis irreversibly activates the turkey erythrocyte P_{2Y}-purinergic receptor resulting in a guanine nucleotide-dependent stimulation of phospholipase C.

Experimental Procedures

Materials. HEPES, ATP, ADPβS, ADP, β, γ-MeATP, and GTPγS were obtained from Boehringer Mannheim. 2MeSATP was from Research Biochemicals Inc. (Natick, MA). 2-[^{3}H]myo-Inositol (23 Ci/mmol) was from American Radiolabelled Chemicals Inc. Inositol-free Dulbecco's modified Eagle's medium was from GIBCO. BzATP was either synthesized as described by Williams and Coleman (4) or obtained from Sigma Chemical Co. The results were the same irrespective of the source of the BzATP.

Radiolabeling of turkey erythrocyte phosphoinositides. Fresh turkey blood was obtained by venipuncture from female turkeys and collected in a heparinized syringe. Red blood cells were washed and labeled with [^{3}H]inositol as previously described in detail (3).

Membrane Preparation. [^{3}H]Inositol-labeled erythrocytes (1 ml

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ABBREVIATIONS: BzATP, 3-*O*-(4-benzoyl)benzoyl adenosine 5′-triphosphate; G protein, guanine nucleotide regulatory protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); 2MeSATP, 2 methylthioadenosine 5′-triphosphate; App(NH)p, 5′-adenylyl imidodiphosphate; EGTA, [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid; ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); β,γ-MeATP, adenylyl (β,γ-methylene)-diphosphonate.

of packed cells) were resuspended in 15 volumes of cold lysis buffer (5 mM sodium phosphate, pH 7.4, 5 mM $MgCl_2$, and 1 mM EGTA) and centrifuged at 10,000 × *g* for 10 min at 4°. The supernatant was aspirated, the membranes were gently resuspended by carefully swirling the centrifuge tube with 1–2 ml of lysis buffer, the tight white pellet was aspirated and discarded, and the membranes were resuspended to a 35-ml final volume. This procedure was repeated two more times and, after a final wash with lysis buffer without EGTA, membranes were resuspended with 20 mM HEPES, pH 7.0, to a final concentration of 3–4 mg/ml.

Photoaffinity labeling of turkey erythrocyte membranes. Two hundred microliters of [^{3}H]inositol-labeled membranes (~700 μg of protein) were transferred to 12-well culture plates and incubated with the indicated concentrations of ligands for 3 min at 30°. The medium (1.0 ml final volume) contained 20 mM HEPES, 5 mM KH_2PO_4, pH 7.0, 1.6 mM $MgCl_2$, 38 mM KCl, and 0.6 mM EGTA. After incubation, the culture plates were transferred to ice and either kept in the dark or irradiated for 2–4 min using a low intensity short-wave UV lamp (Mineralight) placed at a distance of 3 cm above the sample. The membranes were transferred to centrifuge tubes and washed three or four times by sequential resuspension and centrifugation with 10 ml of lysis buffer without EGTA. The final membrane pellet was resuspended in 20 mM HEPES, pH 7.0, to approximately 2 mg of membrane protein/ml. Membranes were immediately used for phospholipase C assays.

Assay of phospholipase C activity. Fifty microliters of membranes containing approximately 200,000 cpm of ^{3}H were added to a medium consisting of (final concentrations) 242 μM $CaCl_2$, 0.91 mM $MgSO_4$, 2 mM EGTA, 115 mM KCl, 5 mM KH_2PO_4, and 20 mM HEPES, pH 7.0. The free concentration of Ca^{2+} was approximately 1 μM. Membranes were incubated for 5 min at 30° and the reaction was stopped by the addition of 0.75 ml of 6.25% perchloric acid and centrifugation at 2000 × *g* for 10 min at 4°. The total inositol phosphate fraction in the perchloric acid supernatant was neutralized and purified by anion exchange chromatography on Dowex 1-X8 (200–400 mesh), as previously described in detail (3, 5).

Results

Effects of BzATP on phospholipase C activity. The capacity of BzATP (structure shown in Fig. 1) to promote receptor-mediated activation of phospholipase C in [^{3}H]inositol-labeled turkey erythrocyte membranes was examined. In order to compare the relative potency of BzATP with that of other P_{2Y}-purinergic receptor agonists, inositol phosphate accumulation was determined in the absence of light as previously described for other P_{2Y}-purinergic receptor agonists (3). BzATP stimulated phospholipase C activity in a concentration-dependent manner (Fig. 2). In the absence of guanine nucleotides, BzATP did not produce activation of phospholipase C and in

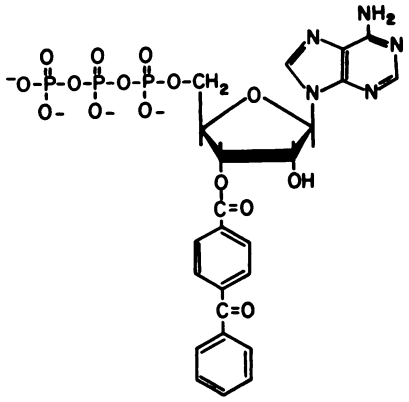

Fig. 1. Structure of BzATP.

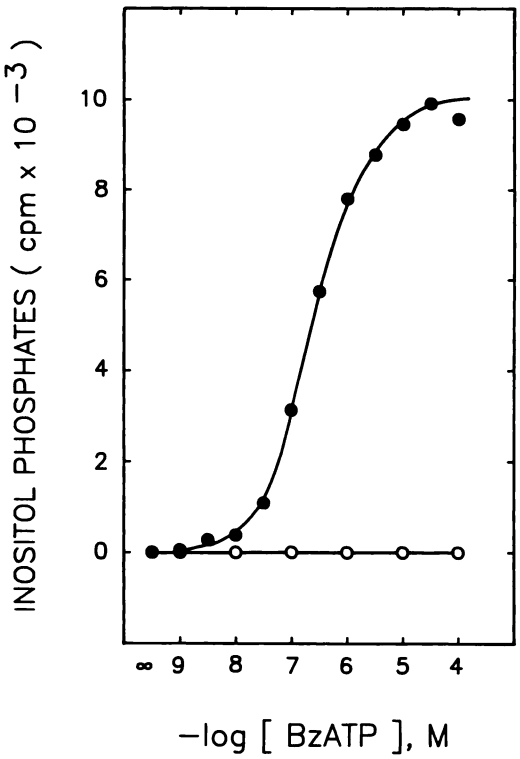

Fig. 2. Concentration-dependent stimulation of inositol phosphate formation by BzATP in turkey erythrocyte membranes. [^{3}H]Inositol-labeled membranes were incubated in the dark for 5 min at 30° with the indicated concentrations of BzATP in the presence (●) or in the absence (○) of 10 μM GTPγS, as described in Experimental Procedures. The data are from a representative experiment repeated four times using different membrane preparations.

the presence of 1–3 μM GTPγS maximal response was attained (Fig. 2). BzATP activation of phospholipase C was also supported by GTP; however, the magnitude of the response was 15–25% of that obtained with GTPγS. The maximal response observed with BzATP was the same as the response obtained with other full agonists (data not shown). BzATP ($K_{0.5}$ = 172 ± 4 nM) was slightly more potent than ATP ($K_{0.5}$ = 828 ± 53 nM) and ADP ($K_{0.5}$ = 7.9 ± 0.6 μM) and somewhat less potent than 2MeSATP ($K_{0.5}$ = 14 ± 2 nM) and ADPβS ($K_{0.5}$ = 110 ± 15 nM) in stimulating inositol phosphate formation.

Photolysis of [^{3}H]inositol-labeled turkey erythrocyte membranes in the presence of BzATP. UV light has deleterious effects on the biological activity of proteins. We, thus, determined the effect of irradiation on the response of turkey erythrocyte phospholipase C under the conditions described in Experimental Procedures. The maximal level of activation of phospholipase C by agonist plus guanine nucleotides was reduced by 20–40% after photolysis under the conditions described. However, the relative magnitude of the effects of guanine nucleotide alone or of guanine nucleotide in the presence of submaximal concentrations of agonist were not modified with irradiation times up to 8 min (data not shown). Moreover, the rate of activation of phospholipase C in the presence of maximal concentrations of agonist plus GTPγS was not changed by photolysis. Qualitatively, the same results were obtained by irradiation with a low intensity long-wave UV light. However, the magnitude of the effect was lower and required longer irradiation times.

BzATP contains a photoreactive carboxybenzophenone

moiety (Fig. 1). Upon irradiation, the unsaturated ketone of BzATP is excited by UV light, forming a diradical triplet intermediary. This diradical induces hydrogen abstraction from a methylene group of the target molecule, resulting in formation of a ketyl radical of the benzophenone plus a target molecule free radical species. A covalent bond then is formed with the target acceptor molecule (6). In [^{3}H]inositol-labeled turkey erythrocyte membranes, the magnitude of the maximal response of phospholipase C to P_{2Y}-receptor agonist plus GTPγS or the response to agonist or GTPγS alone was not modified by preincubation of membranes with BzATP followed by washing, as described in Experimental Procedures (Fig. 3A). Thus, the binding of BzATP is completely reversible under nonphotolyzing conditions, and free ligand is removed from the membranes after several washes. The presence of BzATP during photolysis also did not modify the maximal response subsequently measured in membranes after photolysis (Fig. 3B). However, the response elicited by 10 μM GTPγS was markedly increased compared with nonphotolyzed membranes (Fig. 3B).

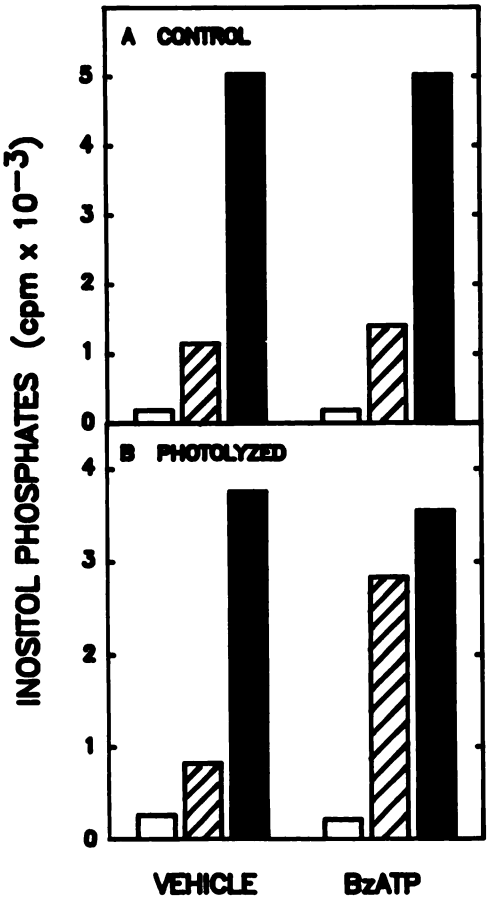

Fig. 3. Irreversible activation of P_{2Y}-purinergic receptors by photolysis in the presence of BzATP. [^{3}H]Inositol-labeled turkey erythrocyte membranes were preincubated for 3 min at 30° in the absence or in the presence of 300 μM BzATP. Following incubation, membranes were transferred to ice and kept in the dark (control) or irradiated with UV light for 3 min (photolyzed), as described in Experimental Procedures. The membranes were washed as described and [^{3}H]inositol phosphate accumulation was measured in response to vehicle (□), 10 μM GTPγS (▨) and 10 μM ADPβS plus 10 μM GTPγS (■). Data presented are the mean of duplicate determinations. The net increase in the response of phospholipase C in the presence of GTPγS in photolyzed BzATP-pretreated membranes varied from 10 to 73% of the response of membranes without photolysis in 17 experiments.

In 17 experiments using different membrane preparations, the response of phospholipase C to 10 μM GTPγS after preincubation of the membranes with 300 μM BzATP and washing *without photolysis* was 15.2 ± 1.9% of the maximal activity obtainable with 100 μM ADPβS plus 10 μM GTPγS added to the membranes. In contrast, in the same membranes photolyzed in the presence of 300 μM BzATP and then washed, the response to GTPγS was increased to 45.7 ± 4.2% of the maximal stimulation obtainable. As shown in Fig. 4, preincubation of membranes with BzATP in the absence of UV irradiation had no effect on the subsequent response to GTPγS measured in the membrane preparation. In contrast, irradiation in the presence of increasing concentrations of BzATP produced membranes that expressed an increased response to GTPγS in a BzATP concentration-dependent manner (Fig. 4).

Activation of phospholipase C by GTPγS alone occurs at relatively high concentrations (>1.0 μM) and after a considerable lag time (5). The rate of activation of phospholipase C is markedly increased by receptor agonists in a concentration-dependent and saturable manner (3). After photolysis of membranes in the presence of BzATP, the rate of activation of phospholipase C by GTPγS was markedly increased (Fig. 5). The k_{obs} for activation of phospholipase C by GTPγS, obtained from first-order plots, was 0.029 ± 0.001 min^{-1} in membranes photolyzed in the absence of BzATP and 0.082 ± 0.007 min^{-1} in membranes photolyzed in the presence of BzATP. No effect

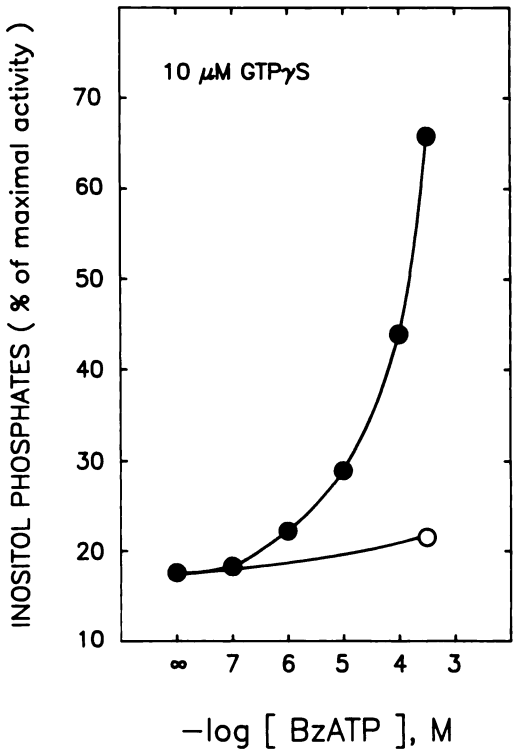

Fig. 4. Concentration-dependent effects of photolyzed BzATP on receptor activation of phospholipase C. [^{3}H]Inositol-labeled turkey erythrocyte membranes were incubated for 3 min at 30° in the presence of the indicated concentrations of BzATP. After incubation the membranes were photolyzed for 3 min (●) or kept in the dark (○) and then washed as indicated in Experimental Procedures. Inositol phosphate formation in response to 10 μM GTPγS was determined. The data are presented as the percentage of the maximal accumulation of inositol phosphates obtained in the presence of maximally effective concentrations of ADPβS and GTPγS. Data shown are from one experiment, which was repeated four times with similar results.

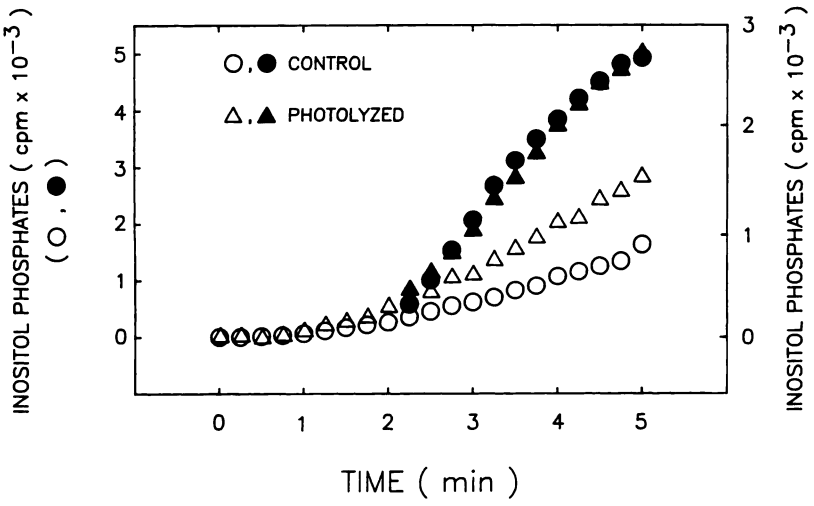

Fig. 5. Effect of BzATP photolysis on the time course of activation of phospholipase C. [^{3}H]Inositol-labeled turkey erythrocyte membranes were incubated for 3 min at 30° in the presence of 100 μM BzATP. After incubation the membranes were transferred to ice, kept in the dark (*circles*) or photolyzed for 3 min (*triangles*), and then washed. Control and photolyzed membranes were incubated for the indicated times in the presence of 10 μM GTPγS. At t = 2 min, buffer (*open symbols*) or 10 μM 2MeSATP (*filled symbols*) was added and the reaction continued for the indicated times. [^{3}H]Inositol phosphate accumulation was determined as described in Experimental Procedures.

on the rate of activation by GTPγS was observed after preincubation of membranes with BzATP and washing without photolysis. The maximal rate of activation of phospholipase C, i.e., the rate of activation by GTPγS in the presence of a maximal concentration of ADPβS, was not different between control and photolyzed samples (data not shown).

The activation of guanine nucleotide-dependent phospholipase C occurring after photolysis of membranes with BzATP was prevented by preincubation with ATP and ATP analogs before photolysis. The order of potency observed was ATP > ADP > App(NH)p ≫ β,γ-MeATP (Fig. 6), which is consistent with a P_{2Y}-purinergic receptor subtype. Apparently due to the low rate constant for dissociation of agonist from high affinity receptor-agonist complexes, protection experiments using other more potent P_{2Y}-purinergic receptor agonists, e.g., ADPβS and 2MeSATP, could not be performed under the conditions described, i.e., preincubation with high concentrations of agonist followed by extensive washing of the membranes resulted in an increased stimulation by GTPγS compared with control.

Discussion

BzATP was initially introduced by Williams and Coleman (4) as a ligand to study the nucleotide binding site in F_1-ATPase. Subsequently, BzATP has been used successfully as a site-specific photoaffinity probe for the study of a number of adenine nucleotide-binding proteins involved in ATP metabolism (4, 6–11). The unique characteristics of benzophenones as photochemically active groups provide a number of advantages over other available photoreactive groups such as azido derivatives of adenine nucleotides (6). For example, the triplet diradical intermediate is generated upon UV irradiation at nearly 100% efficiency. The activated state possesses a relatively long lifetime and once the intermediary activated state is formed it does not undergo intramolecular structural rearrangement and does not react with H_2O, as is the case with arylazido derivatives. Instead, it preferentially abstracts a hydrogen from carbon-hydrogen bonds. In the absence of reaction with a protein, the activated triplet diradical goes back to the ground state (4, 6). Based on these characteristics, BzATP was predicted to be an effective photoaffinity labeling probe for ATP binding sites (4). Recently, Erb *et al.* (12) have used BzATP as a photoaffinity probe for the study of the ATP-induced permeabilization in transformed mouse fibroblasts.

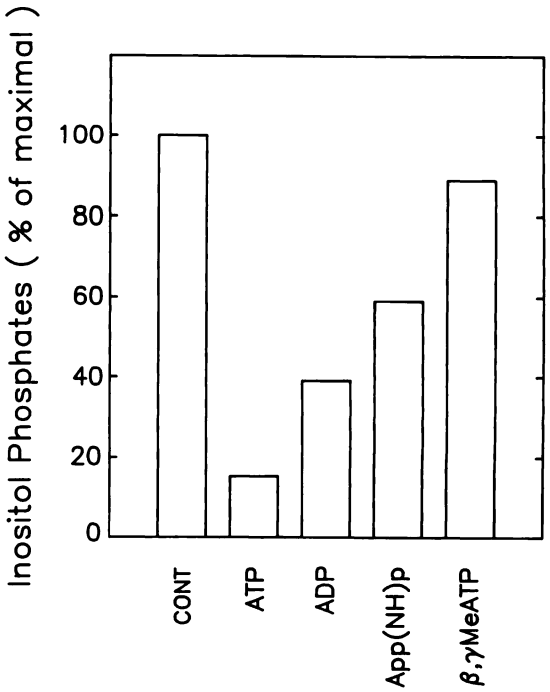

Fig. 6. Effect of ATP and ATP analogs on the photoincorporation of BzATP into P_{2Y}-purinergic receptors coupled to phospholipase C. [^{3}H] Inositol-labeled turkey erythrocyte membranes were preincubated for 3 min in the presence of 10 μM BzATP in the absence (*CONT*) or in the presence of 300 μM ATP, ADP, App(NH)p, or β,γ-methyl-ATP. The membranes were then photolyzed for 2.5 min and washed. Results are presented as percentage of the response to 10 μM GTPγS obtained in membranes photolyzed in the presence of BzATP alone (the responses to 10 μM GTPγS in control and photolyzed membranes were 12 and 36%, respectively, of the maximal attainable response produced by 10 μM ADPβS plus 10 μM GTPγS). The data are representative of results obtained with three different membrane preparations.

The current study suggests that BzATP is also a useful probe for the study of P_{2Y}-purinergic receptors. Under nonphotolyzing conditions, nanomolar concentrations of BzATP stimulated inositol phosphate formation in a guanine nucleotide-dependent manner in turkey erythrocyte membranes. The guanine nucleotide dependence of the effects of BzATP suggests that, as with other ATP and ADP analogs, activation of phospholipase C involves a G protein-dependent process. Based on the fact that the maximal effect of BzATP on phospholipase C was

the same as that of other full P_{2Y}-receptor agonists and that there was no additivity between a maximally effective concentration of BzATP and other full P_{2Y}-receptor agonists, the activity of BzATP apparently is completely accounted for by P_{2Y}-purinergic receptor stimulation.

The experiments reported here suggest that after photolysis BzATP becomes covalently bound to the P_{2Y}-purinergic receptor with the resultant ligand-receptor complex remaining in a functional conformation such that, in the presence of guanine nucleotides, activation of phospholipase C occurs. Several lines of evidence support this idea; (a) phospholipase C activity in photolyzed BzATP-pretreated membranes is not increased in the absence of guanine nucleotides; (b) the rate of activation of phospholipase C by guanine nucleotides is increased after photolysis in the presence of BzATP; (c) the enhanced response to GTPγS after photolysis of membranes in the presence of BzATP was not additive with the effect of maximal concentrations of receptor agonists; and (d) photoincorporation of BzATP in turkey erythrocyte membranes was prevented by ATP and ATP analogs with a potency order consistent with that previously suggested for a P_{2Y}-purinergic receptor.

Although the maximal rate of inositol phosphate production by a P_{2Y} receptor agonist and GTPγS was the same in control membranes and in membranes photolyzed in the presence of BzATP, the number of receptors actually irreversibly occupied by the covalent adduct may be low. That is, the effect of agonist receptor occupation on the activity of phospholipase C is catalytic in nature and, thus, in the presence of nonhydrolyzable guanine nucleotides the agonist occupation of a relatively small percentage of receptors theoretically can maximally activate the effector protein. A small but statistically significant (12 ± 4% of maximum) increase in phospholipase C activity occurred in the presence of GTP with BzATP-photolyzed membranes (data not shown). Based on mass action principles, these data would suggest that 12% or less of the total receptors were covalently modified by BzATP under the conditions employed.

Turkey erythrocytes have proven to be a useful model system for the study of receptor- and guanine nucleotide-mediated activation of adenylate cyclase (13–18). Recently, studies from our research group have shown that turkey erythrocyte membranes are also an advantageous model for the study of receptor- and G protein-regulated phospholipase C (3, 5, 19, 20). Moreover, the possibility of obtaining turkey erythrocytes in large quantities provides a potential source for the identification and isolation of the individual components involved in the phosphoinositide signalling system. Essentially no information is available on the biochemical signalling mechanisms associated with the responses to extracellular ATP. The presence of a P_{2Y} receptor coupled to phospholipase C activation in turkey erythrocytes together with the availability of BzATP as a site-specific probe represent exploitable approaches for advancing information on the structure and function of P_2-purinergic receptors. For example, we are currently attempting to label BzATP with ^{32}P and use this material as a photoaffinity probe to label the P_{2Y}-purinergic receptor.

Acknowledgments

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