Kinetics of Activation of Phospholipase C by P$_{2Y}$ Purinergic Receptor Agonists and Guanine Nucleotides*

José Luis Boyer‡§, C. Peter Downes¶, and T. Kendall Harden‡∥

From the ‡Department of Pharmacology, University of North Carolina, School of Medicine, Chapel Hill, North Carolina 27599 and the ¶Department of Cellular Pharmacology, Smith Kline & French Research Limited, The Frythe, Welwyn, Hertfordshire AL6 9AR, United Kingdom

Membranes prepared from [^{3}H]inositol-labeled turkey erythrocytes express a phospholipase C that is markedly stimulated by stable analogs of GTP (Harden, T. K., Stephens, L., Hawkins, P. T., and Downes, C. P. (1987) *J. Biol. Chem.* 262, 9057–9061). We now report that P$_2$-purinergic receptor-mediated regulation of the enzyme occurs in the membrane preparation. The order of potency of a series of ATP and ADP analogs for stimulation of inositol phosphate formation, *i.e.* 2-methylthioadenosine 5′-triphosphate (2MeSATP) > adenosine 5′-O-(2-thiodiphosphate) > adenosine 5′-O-(3-thiotriphosphate) > ATP > 5′-adenylyl imidodiphosphate ~ ADP > α,β-methyleneadenosine 5′-triphosphate > β,γ-methyleneadenosine 5′-triphosphate, was consistent with that for the P$_{2Y}$-purinergic receptor subtype. Agonist-stimulated effects were completely dependent on the presence of guanine nucleotide. Activation of phospholipase C by guanosine 5′-O-(3-thiotriphosphate) (GTPγS) occurred with a considerable time lag. The rate of activation followed first order kinetics and was markedly increased by increasing concentrations of a P$_{2Y}$ receptor agonist; in contrast, the rate of activation at a fixed agonist concentration was independent of guanine nucleotide concentration. Addition of guanosine 5′-O-(2-thiodiphosphate) (GDPβS) prior to addition of agonist and GTP, 5′-guanylyl imidodiphosphate (Gpp(NH)p), or GTPγS blocked in a concentration-dependent manner the stimulatory effect of guanine nucleotide. GDPβS, added subsequent to preactivation of membranes with 2MeSATP and GTPγS or Gpp(NH)p had only small inhibitory effects on the rate of inositol phosphate production observed over the subsequent 10 min. In contrast, addition of GDPβS to GTP-preactivated membranes resulted in a rapid return of enzyme activity to the basal state within 60 s. Taken together, the data are consistent with the idea that P$_{2Y}$ receptor activation increases the rate of exchange of GTP and GTP analogs for GDP on the relevant guanine nucleotide regulatory protein. Once the active enzymic species is formed, hydrolysis of guanine nucleotide reverts the enzyme to the inactive state.

G-proteins[1] play an obligatory role in the coupling of a variety of extracellular signal-regulated receptors to intracellular effector proteins, *e.g.* adenylate cyclase, cGMP phosphodiesterase, and ion channels (1–5). Evidence also has accumulated recently indicating that receptors for calcium-mobilizing hormones are linked through a G-protein to the stimulation of phospholipase C. Guanine nucleotide-sensitive high affinity binding of agonists to phospholipase C-linked receptors (6–9) is correlated with the capacity of these agonists to stimulate inositol phosphate formation (10), and receptor-stimulated guanine nucleotide-dependent activation of phospholipase C has been observed in cell-free preparations from a variety of tissues (11–18).

The turkey erythrocyte membrane is a useful model to study regulation of phospholipase C by a putative G-protein; stable analogs of GTP markedly stimulate inositol phosphate formation, and the response is relatively long-lived (19, 20). Preliminary data with turkey erythrocyte membranes (20) and intact turkey erythrocytes[2] suggest that the phospholipase C in these cells is activated by a purinergic receptor. In the current report we take advantage of this highly responsive phospholipase C to examine the kinetics of activation of the enzyme by guanine nucleotides and by P$_{2Y}$-purinergic receptor agonists. The data indicate that the overall steps in the regulatory cycle for activation of phospholipase C in turkey erythrocyte membranes are similar in character to those observed previously in this and other tissues for the G-protein-dependent activation of adenylate cyclase.

EXPERIMENTAL PROCEDURES

Materials—Hepes, GTP, GTPγS, GDPβS, Gpp(NH)p, App(NH)p, App(CH$_2$)p, ADP, ATP, ATPγS, and ADPβS were from Boehringer Mannheim; Ap(CH$_2$)pp was from Sigma; 2MeSATP was from Research Biochemicals Inc., Natick, MA, and 2-[^{3}H]*myo*-inositol (15 Ci/mmol) was from American Radiolabeled Chemicals Inc; Inositol-free Dulbecco's modified Eagle's medium and chicken serum were from Gibco.

Radiolabeling of Turkey Erythrocyte Phosphoinositides—Fresh turkey blood was centrifuged at 300 × g for 5 min, plasma and buffy

coat were aspirated and discarded, and packed erythrocytes were resuspended in 5 volumes of cold Dulbecco's modified Eagle's medium and centrifuged at 300 × g for 5 min. This washing procedure was repeated two times. One ml of washed, packed cells was combined with 2.4 ml of inositol-free Dulbecco's modified Eagle's medium, 0.8 ml of chicken serum, and 0.5 mCi of 2-[^{3}H]*myo*-inositol. The cells were incubated in stirred glass scintillation vials at 39 °C for 18–24 h in a humidified 95% O_2, 5% CO_2 atmosphere.

Membrane Preparation—[^{3}H]Inositol-labeled erythrocytes were lysed in 15 volumes of cold lysis buffer (5 mM sodium phosphate, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA), and membranes were obtained as described previously in detail (19).

Assay of Phospholipase C Activity—Twenty-five μl of membranes (≈175 μg of protein), containing approximately 200,000 cpm were added to a medium (final volume 200 μl) containing 424 μM $CaCl_2$, 0.91 mM $MgSO_4$, 2 mM EGTA, 115 mM KCl, 5 mM KH_2PO_4, and 10 mM Hepes, pH 7.0. Free Ca^{2+} concentration was approximately 1 μM. Since preliminary experiments indicated that GTP was hydrolyzed by the membrane preparation, all experiments with this nucleotide included a regenerating system consisting of 15 mM creatine phosphate and 4 units of phosphocreatine kinase. All solutions of P_{2Y}-receptor agonists were prepared in equimolar $MgCl_2$. Membranes were incubated at 30 °C for 5 min, unless otherwise stated. The reaction was stopped by the addition of 750 μl of 6.25% perchloric acid, and the samples were centrifuged at 2000 × g for 15 min. Twenty μl of 100 mM EDTA and 1.0 ml of tri-n-octylamine:1,1,2-trichlorotrifluoroethane (1:1 by volume) were added to the supernatants, and the samples were vortexed vigorously for 10 s and centrifuged at 1000 × g at room temperature. Seven hundred and fifty μl of the aqueous upper phase were transferred to Dowex 1-X8 (200–400 mesh) columns (0.6 ml bed volume) with 10 ml of H_2O. Ten ml of 200 mM ammonium formate, 100 mM formic acid were added and the eluate usually discarded. Total inositol phosphates were eluted by the addition of 8.0 ml of 1.2 M ammonium formate, 100 mM formic acid, the eluate was collected in scintillation vials, and [^{3}H]inositol phosphates quantitated by scintillation spectroscopy (25% efficiency).

Data Presentation—All assays were performed in duplicate or triplicate, using a fresh membrane preparation in each experiment. Figures present representative data that were obtained with at least three different membrane preparations in at least three different experiments.

RESULTS

Activation of Turkey Erythrocyte Phospholipase C by P_{2Y}-purinergic Receptor Agonists—As illustrated in Fig. 1, a series of ATP and ADP analogs in the presence of 1 μM GTPγS stimulate inositol phosphate formation in turkey erythrocyte membranes. 2MeSATP was the most potent agonist tested ($K_{0.5}$ = 12 ± 1 nM, n = 8 different preparations); ATP and ADP were 100–1000-fold less potent, and the ATP analog App(CH_2)p had essentially no effect. No additivity between the effects of agonists was observed (data not shown). Based on the potency order observed with the series of agonists, *i.e.* 2MeSATP > ADPβS > ATPγS > ATP > App(NH)p ≈ ADP > Ap(CH_2)pp ≫ App(CH_2)p (Fig. 1), and the fact that adenosine, AMP, phenylisopropyladenosine, and 5′-dideoxyadenosine had no effect at concentrations up to 1 mM (data not shown), the receptor specificity is consistent with that described by Burnstock and Kennedy (21) for a P_{2Y}-purinergic receptor.

All putative P_2 receptor agonists, with the exception of App(CH_2)p and ATPγS, gave similar maximal responses. The response to App(CH_2)p was routinely 5–10% of that observed with 2MeSATP, and the response to ATPγS was 80–95% of that of 2MeSATP. As we have described previously (20), the effect of ATP was not greater than that of 2MeSATP and other P_{2Y} receptor agonists at incubation times less than 5–7 min. At incubation times of 10 min or greater, the effects of high concentrations of ATP, *i.e.* greater than 300 μM, were always greater than that of maximal concentrations of other P_{2Y} agonists. As discussed previously in detail (20), this effect probably represents a contribution of ATP to substrate avail-

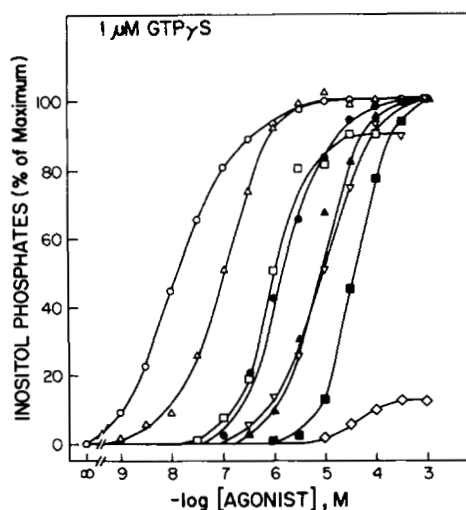FIG. 1. **Concentration-dependent stimulation of inositol phosphate formation by ATP and ADP analogs in turkey erythrocyte membranes.** ^{3}H-Labeled membranes were incubated for 5 min with the indicated concentrations of adenine nucleotides in the presence of 1 μM GTPγS as described under "Experimental Procedures." Individual concentration-effect curves represent the mean of 2–10 different experiments performed in duplicate for each agonist. Data are expressed as a percentage of the maximum stimulation obtained with 2MeSATP in the same experiment. ○, 2MeSATP; △, ADPβS; □, ATPγS; ●, ATP; ▲, App(NH)p; ▽, ADP; ■, Ap(CH_2)pp; ◇, App(CH_2)p.

ability. That is, in the presence of high concentrations of ATP, [^{3}H]PtdIns(4,5)P_2 levels are maintained and inositol phosphate production is linear for up to 20 min, whereas in the absence of ATP (and presence of stable ATP or ADP analogs) inositol phosphate production is linear for only 5–7 min, at which time significant decreases in [^{3}H]PtdIns(4,5)P_2 levels have occurred.

Guanine Nucleotide Dependence of the Effect of ATP and ADP Analogs—In the absence of a guanine nucleotide, the occupation of the putative P_{2Y}-purinergic receptor by 2MeSATP (Fig. 2) or other agonists (data not shown) did not result in stimulation of inositol phosphate formation. However, P_{2Y} receptor agonists stimulated phospholipase C activity in a concentration-dependent manner in the presence of GTPγS (Fig. 2).

GTP (Fig. 3) and Gpp(NH)p (data not shown) also supported 2MeSATP-stimulated accumulation of inositol phosphates. The maximal effect observed with GTP and 2MeSATP was 15–25% of that observed with GTPγS and 2MeSATP. The $K_{0.5}$ for 2MeSATP in the presence of GTP (170 ± 37 nM) was essentially identical to the $K_{0.5}$ of 2MeSATP for stimulation of inositol phosphate formation in intact erythrocytes.[2]

In the absence of ATP or analogs of ATP or ADP, GTPγS, at concentrations over 300 nM, promoted a concentration-dependent activation of phospholipase C (Fig. 4, *bottom line*). The presence of 2MeSATP (Fig. 4) or other P_{2Y} receptor agonists (data not shown) enhanced in a concentration-dependent manner the effectiveness of GTPγS for stimulation of inositol phosphate accumulation. The concentration effect curve for GTPγS determined in a 5-min assay was shifted at least 50-fold to the left by a maximally effective concentration of 2MeSATP.

Inositol phosphate formation in the presence of GTPγS occurs with a time course exhibiting a considerable lag. The extent of the lag was decreased in a concentration-dependent manner by 2MeSATP (Fig. 5A). Activation in the presence
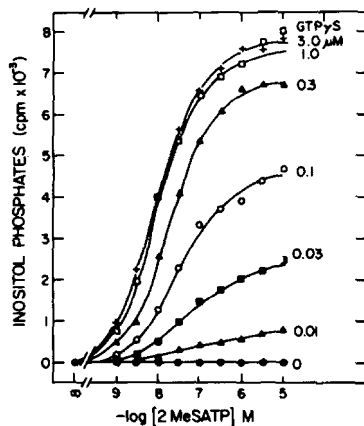
Guanine Nucleotide-regulated Phospholipase C

FIG. 2. **Effect of GTPγS on the P2Y receptor-mediated activation of phospholipase C in turkey erythrocyte membranes.** ^{3}H-Labeled turkey erythrocyte membranes were incubated for 5 min with the indicated concentrations of 2MeSATP in the absence (●) or in the presence of the following concentrations of GTPγS: 0.01 μM (▲), 0.03 μM (■), 0.1 μM (○), 0.3 μM (△), 1 μM (□), or 3 μM (+). The effect of GTPγS alone on [3H]inositol phosphate accumulation was subtracted from the GTPγS plus agonist data, (262 cpm for 0.01 μM, 285 cpm for 0.03 μM, 399 cpm for 0.1 μM, 600 cpm for 0.3 μM, 1077 cpm for 1 μM, and 1839 cpm for 3 μM GTPγS). The data are the mean of duplicate determinations and are representative of results obtained in three different membrane preparations.

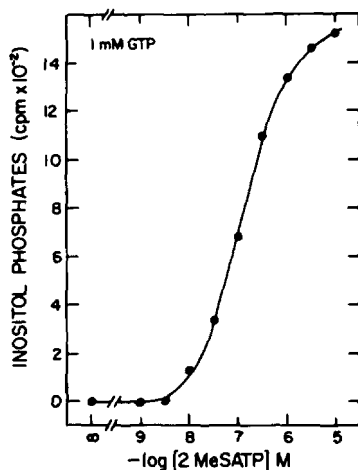FIG. 3. **Concentration-dependent stimulation of inositol phosphate formation by 2MeSATP in the presence of GTP.** ^{3}H-Labeled erythrocyte membranes were incubated in the presence of 1 mM GTP for 5 min with the indicated concentrations of 2MeSATP as described under "Experimental Procedures." The data are from a representative experiment repeated four times with similar results.

of agonist and GTPγS followed pseudo-first order kinetics (Fig. 5B); the k_{obs} increased with increasing concentrations of 2MeSATP in a saturable manner indicating that the rate of activation was increased by receptor stimulation. The value for k_{obs} was 0.03 min⁻¹ with GTPγS alone and increased to 1.06 min⁻¹ in the presence of GTPγS and 1 μM 2MeSATP. The apparent activation constant for 2MeSATP, *i.e.* the concentration of agonist producing one-half of the maximal increase in k_{obs}, was 87 nM.

The eventual level of activation of phospholipase C attained with GTPγS (300 μM) alone was approximately one-half of that observed with guanine nucleotide and a maximally effective concentration of the P2Y receptor agonist, ADPβS (data not shown). The reason(s) for this difference in the final rates is not known, but apparently the data are not explained by
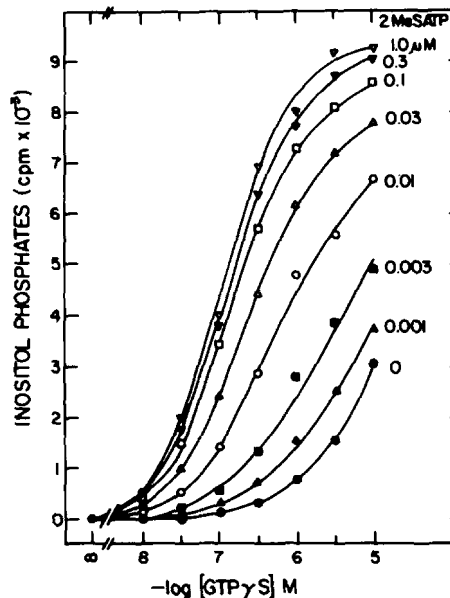

FIG. 4. **Effect of 2MeSATP on the guanine nucleotide-dependent activation of phospholipase C in turkey erythrocyte membranes.** ^{3}H-Labeled membranes were incubated for 5 min with the indicated concentrations of GTPγS in the absence (●) or in the presence of different concentrations of 2MeSATP: 0.001 μM (▲), 0.003 μM (■), 0.01 μM (○), 0.03 μM (△), 0.1 μM (□), 0.3 μM (▼), and 1 μM (∇). The data are from a single experiment carried out in duplicate and are representative of results from three experiments using different membrane preparations.

breakdown of guanine nucleotide in that addition of fresh GTPγS after 3 min of GTPγS incubation produced no further effect, while addition of ADPβS to membranes preincubated with GTPγS resulted in increases in activity to the level observed with membranes initially incubated with ADPβS plus GTPγS.

In the presence of a fixed concentration of 2MeSATP, GTPγS increased inositol phosphate accumulation in a concentration-dependent manner (Fig. 6A). However, the rate of activation was not changed with increasing concentrations of GTPγS, *i.e.*, k_{obs} = 0.85–0.93 min⁻¹ (Fig. 6B). Furthermore, the rate of activation observed with GTPγS and a maximally effective concentration of 2MeSATP was similar to the rate of activation with Gpp(NH)p and 2MeSATP (data not shown). Interestingly, although the final activity attained with GTP is much less, the rate of activation observed in the presence of maximally effective concentrations of GTP and 2MeSATP or ATP was much greater than the rate of activation observed with maximally effective concentrations of GTPγS (Fig. 7) or Gpp(NH)p (data not shown) and P2Y receptor agonists. Steady-state phospholipase C activity was established almost immediately (no detectable lag period) with P2Y receptor agonists and GTP.

Addition of GDPβS prior to or concurrent with addition of GTPγS (Fig. 8A), Gpp(NH)p (Fig. 8B), or GTP (Fig. 8C) blocked activation of phospholipase C by the guanine nucleotides. K_i values for GDPβS calculated by a Schild-type analysis (not shown) were similar for antagonism of stimulation of enzyme activity by all three guanine nucleotides (K_i ≈ 0.6–1.4 μM). The effects of GDPβS on phospholipase C activity in membranes preactivated with 2MeSATP and GTPγS, Gpp(NH)p, or GTP were compared. In contrast to its effects when added prior to or with GTPγS, GDPβS had only negligible effects on observed enzyme activity when added after 4 min of preactivation of membranes with GTPγS and

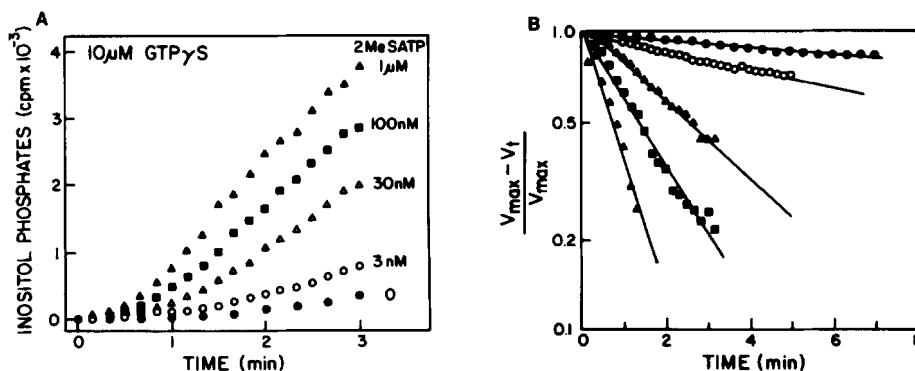FIG. 5. **Time course of GTPγS-mediated activation of phospholipase C as a function of the concentration of P_{2Y} receptor agonist.** ^{3}H-Labeled turkey erythrocyte membranes were incubated for the indicated times at 30 °C in the presence of 10 μM GTPγS and the indicated concentrations of 2MeSATP. *A*, GTPγS-induced accumulation of inositol phosphates in the absence (●) or in the presence of 3 nM (○), 30 nM (△), 100 nM (■), and 1 μM 2MeSATP (▲). *B*, which also includes data from longer incubation times, shows a first order representation of data from *A*. V_{max} is the maximal activity obtained in the presence of a maximal agonist concentration, and V_t is the activity measured at time *t*. The data are representative of results obtained in eight experiments.
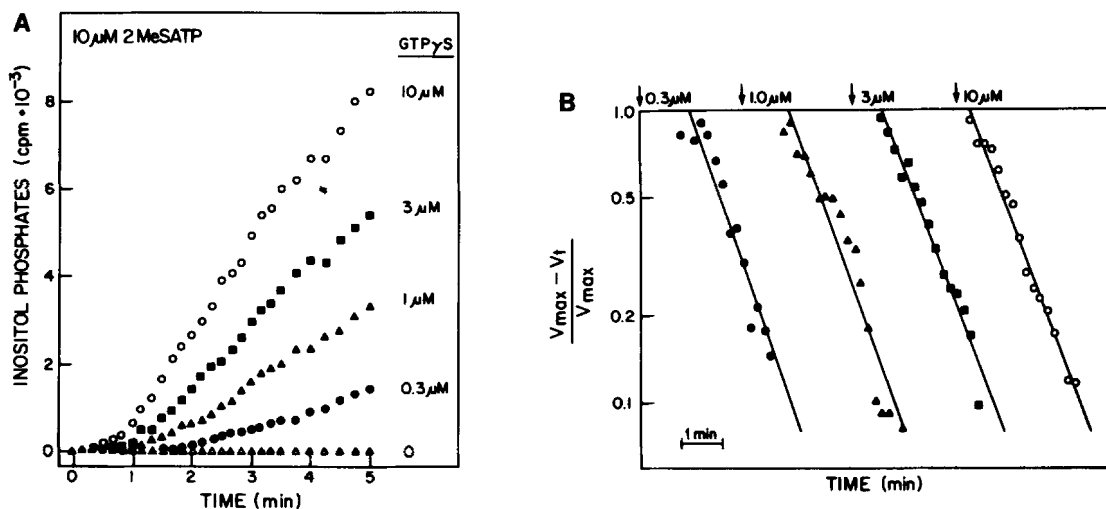

FIG. 6. **Time course of activation of phospholipase C as a function of GTPγS concentration.** ^{3}H-Labeled turkey erythrocyte membranes were incubated for the indicated times at 30 °C in the presence of 10 μM 2MeSATP and the following final GTPγS concentrations: none, (△); 0.3 μM, (●); 1 μM, (▲); 3 μM (■); 10 μM (○). *A*, 2MeSATP-induced [3H]inositol phosphate accumulation in the presence of various concentrations of GTPγS. First order replots of data from *A* are presented in *B*. For clarity, data for each GTPγS concentration are presented as individual plots with each zero time represented on abscissa by an *arrow*. V_{max} is the maximal activity obtained for each GTPγS concentration, and V_t is the activity measured at time *t*.

2MeSATP (Fig. 8*D*). Addition of GDPβS to membranes preactivated with GppNHp also only resulted in a very slow decrease in the rate of inositol phosphate accumulation and reversal of guanine nucleotide-enhanced activity was not reached within 10 min (Fig. 8*E*). In contrast to the results obtained with GTPγS- and Gpp(NH)p-preactivated membranes, addition of GDPβS to GTP-preincubated membranes resulted in a return to basal levels within 1 min (Fig. 8*F*). A semilogarithmic plot of inositol phosphate accumulation after addition of GDPβS indicated that decay of enzyme activity followed first order kinetics. The calculated rate constants for the GDPβS-induced first order decay of inositol phosphate accumulation were 0.19 ± 0.01 min^{-1} for GTPγS, 0.25 ± 0.01 min^{-1} for Gpp(NH)p, and 1.1 ± 0.15 min^{-1} for GTP. These differences in off-rate observed in the presence of GDPβS apparently account at least in part for the relative differences in maximal accumulation of inositol phosphates observed with the three guanine nucleotides in the presence of 2MeSATP. That is, in the experiment summarized in Fig. 8, the observed

rate of accumulation of inositol phosphates in the presence of GTPγS or Gpp(NH)p was approximately 8-fold greater than that observed in the presence of GTP. The effects of GDPβS on guanine nucleotide plus 2MeSATP-preactivated enzyme activity does not involve competition by GDPβS for the P_{2Y}-purinergic receptor. That is, when inositol phosphate accumulation was measured as a function of 2MeSATP concentration, GDPβS inhibited accumulation in a noncompetitive manner (data not shown).

DISCUSSION

Burnstock (22) proposed in 1978 that purinergic receptors could be classified as P_1- and P_2-purinergic receptors with P_1 receptors exhibiting the potency order of adenosine > AMP > ADP > ATP and P_2-purinergic receptors exhibiting the agonist potency order of ATP > ADP > AMP > adenosine. Burnstock and Kennedy (21) subsequently suggested that P_2 receptors could be subclassified into two subtypes based on the selectivity of ATP and ADP analogs in different tissues.
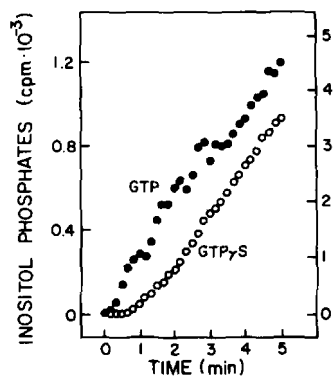
FIG. 7. Time course of activation of turkey erythrocyte phospholipase C by GTP and GTPγS. ^{3}H-Labeled erythrocyte membranes were preincubated for 2 min at 30 °C in the presence of 3 mM ATP. At $t = 0$, 500 μM GTP (●, left ordinate) or 1 μM GTPγS (O, right ordinate) was added. The reaction was stopped at the indicated times, and [3H]inositol phosphates formed were determined as described under "Experimental Procedures." Data shown are the mean of duplicate determinations and are representative of experiments performed at least five times using different membrane preparations.

P_{2X} receptors exhibit the agonist potency order of $Ap(CH_2)pp$ = $App(CH_2)p$ > ATP > 2MeSATP, and P_{2Y} receptors the agonist potency order of 2MeSATP ≫ ATP > $Ap(CH_2)pp$ > $App(CH_2)p$. The second messenger signaling systems associated with P_2 receptor subtypes have not been established unambiguously. However, ATP- and/or ADP-mediated increases in phosphoinositide hydrolysis and/or calcium mobilization have been documented in platelets (24), rat hepatocytes (23, 25), Erlich ascites tumor cells (26), adrenal medullary endothelial cells (27), and bovine endothelial cells (28). Detailed pharmacological analyses of the purinergic receptor regulating phospholipase C have not been reported. However, in several cases (27, 28) the limited studies of agonist specificity agreed with that for a P_{2Y}-purinergic receptor. We recently have observed that ATP and ADP analogs stimulate phosphoinositide hydrolysis in intact turkey erythrocytes with pharmacological properties similar to that expected for a P_{2Y} receptor.[2] Data in the current study with erythrocyte membranes confirm that efficient coupling of this putative P_{2Y} receptor to phospholipase C is maintained in a simple erythrocyte membrane preparation. Thus, we have been able to characterize in detail a P_{2Y}-purinergic receptor-mediated response in a cell-free preparation.

The initial report (10) in this series of studies of turkey erythrocyte phospholipase C involved assays that routinely included high concentrations of ATP and an ATP regenerating system. Subsequently, the influence of ATP on inositol phosphate formation was shown to involve more than provision of substrate via support of conversion of [3H]phosphatidylinositol to [3H]PtdIns(4)P and [3H]PtdIns(4)P to [3H]PtdIns(4,5)P₂ (20). The effect of ATP involving contribution of substrate is only important during incubation times greater than 7 min at 30 °C. That is, a number of analogs of ATP and ADP are just as effective as ATP in stimulating phospholipase C in the presence of guanine nucleotides during the first 5–7 min of stimulation (Fig. 1). After this time a significant amount of PtdIns(4,5)P₂ is apparently hydrolyzed and without ATP-dependent resynthesis from phosphatidylinositol and PtdIns(4)P, substrate becomes limiting (see Ref. 20). This argument, of course, assumes that the majority of the inositol phosphates that accumulate originate from phospholipase C-stimulated hydrolysis of [3H]PtdIns(4,5)P₂, a point
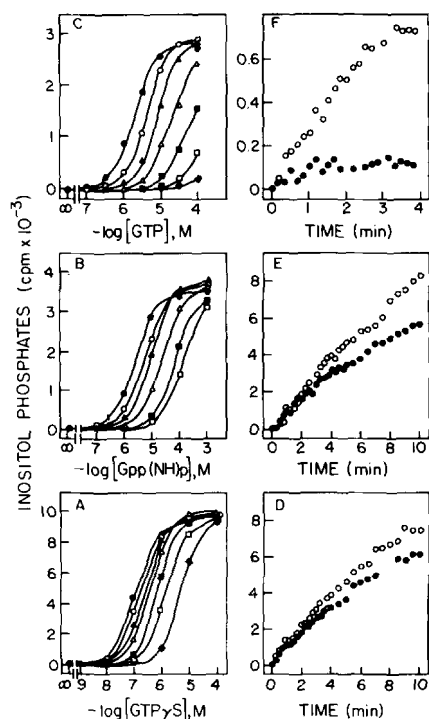

FIG. 8. GDPβS-induced blockade of GTPγS-, GppNHp-, and GTP-stimulated phospholipase C activity. A, B, and C, concentration-dependent inhibition of guanine nucleotide-stimulated inositol phosphate formation by GDPβS. Membranes were incubated for 5 min in the presence of 100 μM 2MeSATP and the indicated concentrations of GTPγS (A), Gpp(NH)p (B), or GTP (C) and the following concentrations of GDPβS: A, none (●), 0.3 μM (O), 1 μM (▲), 3 μM (△), 10 μM (■), 30 μM (□), and 100 μM (♦); B and C, none (●), 1 μM (O), 3 μM (▲), 10 μM (△), 30 μM (■), 100 μM (□), and 300 μM (♦). D, E, and F, GDPβS-induced reversal of GTPγS-, Gpp(NH)p- and GTP-preactivated phospholipase C activity. Turkey erythrocyte membranes were preactivated with 100 μM 2MeSATP and 1 μM GTPγS or 10 μM Gpp(NH)p for 4 min (D and E, respectively) or were preactivated for 2 min in the presence of 100 μM 2MeSATP and 10 μM GTP (F). Subsequent to preactivation, buffer (O) or 300 μM GDPβS (●) was added (time = 0 in D, E, and F) and samples taken at the indicated times. Inositol phosphates formed during the preactivation period were subtracted from data presented in D, E, and F.

we have shown to be the case under these assay conditions (20).

A number of hormone receptors that activate phospholipase C also have been shown to stimulate a GTPase activity in the same membrane preparation (29–31). Although such an association only can be considered circumstantial, in analogy to the receptor-regulated adenylate cyclase, these data have been taken to be indicative of a function for a G-protein associated GTPase activity in the catalytic cycle of receptor-activated phospholipase C. The low effectiveness of GTP relative to the effects of hydrolysis resistant analogs of GTP for stimulation of phospholipase C in turkey erythrocyte (Figs. 7 and 8; Ref. 19) and other (12–18) membranes also is consistent with involvement of a GTPase activity. That is, the steady-state level of an enzymatically active GTP-liganded G-protein-phospholipase C complex predictably would be lower than that for a GTPγS-liganded active complex due to the hydrolysis of GTP by an associated GTPase. The experiments with GDPβS reported here (Fig. 8) add a third indirect support for potential importance of a GTPase activity in the catalytic cycle of phospholipase C. Thus, the rate of GDPβS-initiated inactivation of the guanine nucleotide and hormone preactivated enzyme was fastest with enzyme under the influence of GTP

and slowest in the presence of GTPγS. Obviously, this experimental protocol would not distinguish between an active enzymic species that returned to the inactive state by GTPase-catalyzed hydrolysis of guanine nucleotide and one that returned to the inactive state by simple dissociation of guanine nucleotide from the active species. Nevertheless, if the assumption is made that GTPγS and GppNHp are resistant to hydrolysis, then the normal influence of GTPase on the GTP-stimulated enzymic species would explain the rapid decrease in activity observed when GDPβS is added to GTP-pretreated membranes.

It is generally accepted that the interaction of agonists with receptors coupled to the adenylate cyclase effector system promotes the overall "exchange" of GTP for GDP on G_s or G_i (32, 33). It also has been shown in turkey erythrocyte membranes that the lag in onset of the stimulatory effect of guanine nucleotides on the adenylate cyclase is significantly reduced by the presence of β-adrenergic receptor agonists (34–36). The lag time observed in the stimulation of phospholipase C activity by GTPγS in our experiments was greatly reduced (although not abolished) by the P_{2Y} agonist 2MeSATP in a concentration dependent manner (Fig. 5). This result suggests that the interaction of 2MeSATP with the P_{2Y} receptor promotes guanine nucleotide exchange at the G-protein, which in turn, transforms the G-protein into its active GTP (or GTPγS)-liganded state. Preliminary data indicate that the same series of P_{2Y} receptor agonists studied here also increases the rate of binding of [35S]GTPγS to turkey erythrocyte membranes.[3] The fact that activity observed in the presence of GTPγS alone never reaches that observed in the presence of agonist plus GTPγS suggests that receptor stimulation may produce an effect(s) in addition to simple promotion of guanine nucleotide exchange.

It is significant that the rate of activation observed with a given concentration of agonist was independent of concentration of guanine nucleotide. On the other hand, the rate of activation was markedly increased in a concentration-dependent and saturable fashion by P_{2Y} receptor agonists. These results are reminiscent of data reported by Tolkovsky, Levitzki, and colleagues (35–37) and Ross *et al.* (38) for β-receptor-mediated activation of adenylate cyclase in membranes from turkey erythrocytes and S49 lymphoma cells, respectively.

The $K_{0.5}$ for 2MeSATP for stimulation of inositol phosphate production in intact turkey erythrocytes is approximately 100 nM.[2] Thus, in membranes the $K_{0.5}$ (170 nM) for activation of phospholipase C by 2MeSATP in the presence of GTP (Fig. 3) and the $K_{0.5}$ (87 nM) for 2MeSATP-stimulated increases in the first order rate constant for activation in the presence of GTPγS (Fig. 5 and data not shown) are similar to the apparent affinity of agonist observed in intact cells. Since activation of phospholipase C by GTPγS occurs with a lag and with only very slow reversibility, it is not surprising that the $K_{0.5}$ for 2MeSATP determined in the presence of GTPγS becomes smaller with increasing concentrations of stable GTP analog (Fig. 2) and with increasing time (data not shown). Finally, assuming that the ground state of the phospholipase C regulatory cycle involves a GDP-associated state of the involved G-protein, the rate limiting step must not solely involve the simple dissociation of GDP. That is, there was a clear difference in the rate of activation of the enzyme in the presence of GTP plus maximally effective concentration of agonist and GTPγS plus maximally effective concentration of agonist. A similar phenomenon has been observed with adenylate cyclase (39).

Some reservations regarding the turkey erythrocyte phospholipase C need to be voiced. First, this tentative kinetic analysis of G-protein-regulated phospholipase C should be interpreted in light of the realization that radioactive substrate is provided by metabolic labeling of endogenous pools. Thus, the relationship of substrate concentration to K_m of enzyme for substrate is not known. However, it is true that substrate must not be limiting in the sense that accumulation of product is linear for 5–7 min and is independent of potential ATP-dependent provision of more substrate by phosphorylation of phosphatidylinositol and PtdIns(4)P. With high concentrations of ATP included, product formation can be maintained at a linear rate for up to 20 min (20), but due to the added complication of P_{2Y} receptor stimulation by ATP, analysis of inositol phosphate production at times greater than 5–7 min is more complicated than initially (19) envisioned.

Finally, identity of the involved G-protein has not been established; determination of whether dissociation/association of a heterotrimeric G-protein is crucial in the activation/deactivation of phospholipase C must await successful G-protein identification/purification strategies. Nothing in the data presented here proves that hormonal activation of phospholipase C occurs by steps that are completely analogous to those necessary for activation of adenylate cyclase. However, based on these results, knowledge of the structure/function of receptor-regulated adenylate cyclase and cyclic GMP phosphodiesterase should continue to prove heuristic in establishing the molecular details of hormonal regulation of phospholipase C.

REFERENCES

1. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649
2. Stryer, L. (1986) *Annu. Rev. Neurosci.* **9**, 87–119
3. Yatani, A., Codina, J., Brown, A. M., and Birnbaumer, L. (1987) *Science* **253**, 207–211
4. Logothetis, D. E., Kurachi, Y., Galper, J., Neer, E. J., and Clapham, D. E. (1987) *Nature* **325**, 321–326
5. Hescheler, J., Rosenthal, W., Trautwein, W., and Schultz, G. (1987) *Nature* **325**, 445–447
6. Glossman, H., Baukal, A., and Catt, K. J. (1974) *J. Biol. Chem.* **249**, 664–666
7. Goodhardt, M., Ferry, N., Geynet, P., and Hanoune, J. (1982) *J. Biol. Chem.* **257**, 11577–11583
8. Boyer, J. L., Garcia, A., Posadas, C., and Garcia-Sáinz, J. A. (1984) *J. Biol. Chem.* **259**, 8076–8079
9. Evans, T., Martin, M. W., Hughes, A. R., and Harden, T. K. (1985) *Mol. Pharmacol.* **27**, 32–37
10. Evans, T., Hepler, J. R., Masters, S. B., Brown, J. H., and Harden, T. K. (1985) *Biochem. J.* **232**, 751–757
11. Cockcroft, S., and Gomperts, B. D. (1985) *Nature* **314**, 534–536
12. Litosch, I., Wallis, C., and Fain, J. N. (1985) *J. Biol. Chem.* **260**, 5464–5471
13. Smith, C. D., Lane, B. C., Kusaka, I., Verghese, M. W., and Snyderman, R. (1985) *J. Biol. Chem.* **260**, 5875–5878
14. Gonzales, R. A., and Crews, R. T. (1985) *Biochem. J.* **232**, 799–804
15. Straub, R. E., and Gershengorn, M. C. (1986) *J. Biol. Chem.* **261**, 2712–2717
16. Martin, T. F. J., Bajjalieh, S. M., Lucas, D. O., and Kowalchyk, J. A. (1986) *J. Biol. Chem.* **261**, 10141–10149
17. Hepler, J. R., and Harden, T. K. (1986) *Biochem. J.* **239**, 141–146
18. Uhing, R. J., Prpic, V., Jiang, H., and Exton, J. H. (1986) *J. Biol. Chem.* **261**, 2140–2146
19. Harden, T. K., Stephens, L., Hawkins, P. T., and Downes, C. P. (1987) *J. Biol. Chem.* **262**, 9057–9061

---

[3] C. L. Cooper and T. K. Harden, unpublished observations.

20. Harden, T. K., Hawkins, P. T., Stephens, L., Boyer, J. L., and Downes, C. P. (1988) *Biochem. J.* **252**, 583–593
21. Burnstock, G., and Kennedy, C. (1985) *Gen. Pharmacol.* **16**, 433–440
22. Burnstock, G. (1978) in *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach* (Straub, R. W., and Bolis, L., eds) pp. 107–118, Raven Press, New York
23. Okajima, F., Tokumitsu, Y., Kondo, Y., and Ui, M. (1987) *J. Biol. Chem.* **262**, 13483–13490
24. Leung, N. L., Vickers, J. D., Kinlough-Rathbone, R. L., Reimers, H.-J., and Mustard, J. F. (1983) *Biochem. Biophys. Res. Commun.* **113**, 483–490
25. Charest, R., Blackmore, P. F., and Exton, J. H. (1985) *J. Biol. Chem.* **260**, 15789–15794
26. Dubyak, G. R. (1986) *Arch. Biochem. Biophys.* **245**, 84–95
27. Forsberg, E. J., Feverstein, G., Shohami, E., and Pollard, H. B. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 5630–5634
28. Pirotton, S., Raspe, E., Demolle, D., Erneux, C., and Boeynaems, J.-M. (1987) *J. Biol. Chem.* **262**, 17461–17466
29. Grandt, R., Greiner, C., Zubin, P., and Jakobs, K. H. (1986) *FEBS Lett.* **196**, 279–283
30. Houslay, M. D., Bojanic, D., and Wilson, A. (1986) *Biochem. J.* **234**, 737–740
31. Wojcikiewicz, R. J. H., Kent, P. A., and Fain, J. N. (1986) *Biochem. Biophys. Res. Commun.* **138**, 1383–1389
32. Cassel, D., and Selinger, Z. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 4155–4159
33. Casey, P. J., and Gilman, A. G. (1988) *J. Biol. Chem.* **263**, 2577–2580
34. Schramm, M., and Rodbell, M. (1975) *J. Biol. Chem.* **250**, 2232–2237
35. Sevilla, N., Steer, M. L., and Levitzki, A. (1976) *Biochemistry* **15**, 3494–3499
36. Tolkovsky, A. M., and Levitzki, A. (1978) *Biochemistry* **17**, 3795–3810
37. Tolkovsky, A. M., Braun, S., and Levitzki, A. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 213–217
38. Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L., and Gilman, A. G. (1977) *J. Biol. Chem.* **252**, 5761–5775
39. Birnbaumer, L., Swartz, T. L., Abramowitz, J., Mintz, P. W., and Iyengar, R. (1980) *J. Biol. Chem.* **255**, 3542–3551