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P_{2U}-Purinergic Receptors on C6–2B Rat Glioma Cells: Modulation of Cytosolic Ca^{2+} and cAMP Levels by Protein Kinase C

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SUMMARY

The activation of P_2-purinergic receptors on C6-2B rat glioma cells caused a transient increase in cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) as detected by Fura 2 fluorescence ratio imaging of single cells. These purinergic receptors are of the P_{2U} subtype because UTP and ATP were equipotent and substantially more potent than the P_{2X}- and P_{2Y}-selective agonists α,β-methylene ATP and 2-methylthio ATP, respectively. There was homologous desensitization of the Ca^{2+} responses between UTP and ATP but no heterologous desensitization between these nucleotides and another Ca^{2+}-mobilizing receptor agonist, α-thrombin. The UTP-induced peak $[Ca^{2+}]_i$ rise was insensitive to chelation of extracellular Ca^{2+} with EGTA. However, the response was abolished after either depletion of intracellular Ca^{2+} stores with the microsomal Ca^{2+}-ATPase inhibitor thapsigargin or blockade of Ca^{2+} release from intracellular stores with the muscle relaxant dantrolene. The activation of P_{2U}-purinergic receptors and thrombin receptors increased the formation of total inositol phosphates (IPs) and inhibited cAMP accumulation elicited with either the β-adrenergic receptor agonist (−)-isoproterenol, or forskolin, a direct activator of adenylyl cyclase. UTP- and α-thrombin-induced changes in the levels of IPs, cytosolic Ca^{2+}, and agonist-elicited cAMP accumulation were dramatically inhibited (>80%) by acute treatment of the cells with the protein kinase C activator 4β-phorbol 12-myristate 13-acetate but not with the inactive ester 4α-phorbol 12,13-didecanoate. We conclude that in C6-2B cells, the increase in $[Ca^{2+}]_i$ after activation of P_{2U}-purinergic receptors is primarily a result of IPs-mediated release of Ca^{2+} from intracellular stores with secondary influx of Ca^{2+} by capacitative mechanisms. Also, the inhibition by UTP and α-thrombin of agonist-elicited cAMP accumulation is mediated through an increase in $[Ca^{2+}]_i$.

There is now a considerable body of evidence to suggest that ATP serves as a neurotransmitter and/or neuromodulator in the central nervous system (1, 2). Recently, miniature and evoked synaptic currents mediated through ATP receptors in the central nervous system have been recorded (3), indicating that ATP can act as a fast excitatory transmitter at synapses between neurons. The various effects of ATP in the central nervous system and peripheral tissues are mediated through cell surface P_2-purinergic receptors (1, 2). Five subtypes of P_2 receptors, namely P_{2X}, P_{2Y}, P_{2T}, P_{2Z} and P_{2U}, have been identified based on the structure activity profile of some nucleotides (4). Of these, P_{2X} and P_{2Y} are the most widely studied subtypes and exhibit distinct structure activity profiles: AMPCPP is the most potent nucleotide at P_{2X} and 2-methylthio ATP is the most potent nucleotide at the P_{2Y} subtype. The P_{2T} and P_{2Z} receptors mediate the proaggregatory effect of ADP in platelets and the plasma membrane permeabilization effect of the unchelated form of ATP^{-4}, respectively, in Swiss 3T6 fibroblasts, mast cells, and macrophages (1, 2). P_{2U} is the most recently described subtype of P_2 receptors that displays equal potency for ATP and the pyrimidine nucleotide UTP. AMPCPP and 2-methylthio ATP are only weakly active at raising $[Ca^{2+}]_i$ (1, 4). P_{2U} receptors have now been identified on a variety of cell types including PC12 cells, HL-60 differentiated leukemia cells, HSG-PA human submandibular duct cells, CF\T43 human airway epithelial cells, and NCB-20 mouse neuroblastoma × Chinese hamster brain explant hybrid cells (5). The first goal of this study was to investigate if P_2-purinergic receptors are present on C6-2B glioma cells.

Activation of P_2-purinergic receptors evokes an increase in $[Ca^{2+}]_i$ through P_{2X} receptor-operated calcium channels in ar-

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ABBREVIATIONS: $[Ca^{2+}]_i$, cytosolic-free calcium ion concentration; IPs, total inositol phosphates; IP_3, 1,4,5-inositoltrisphosphate; PI, phosphatidylinositol; PLC, phospholipase C; PKC, protein kinase C; G_i, inhibitory guanine nucleotide-binding protein; G_q, guanine nucleotide binding protein coupled to phospholipase C; THR, α-thrombin; TG, thapsigargin; AMPCPP, α,β-methylene ATP; SK, substance K; DANT, dantrolene; ISO, (−)-isoproterenol; FO; forskolin; PMA, 4β-phorbol 12-myristate 13-acetate; PDD, 4α-phorbol 12,13-didecanoate; DMSO, dimethylsulphoxide; PTX, pertussis toxin.

terial smooth muscle cells (6) or P_{2Y} or P_{2U} receptor-mediated PI metabolism in several other cell types including HL-60 cells, NCB-20 cells, turkey erythrocytes, and RINm5F insulin-secreting cells (1, 2, 4). ATP-sensitive P_2 receptors have been identified recently on C6 glioma cells by Lin and Chuang (7), although in this study the P_2 receptors were not subtyped. Also, in their paper Lin and Chuang suggest that ATP receptor-gated Ca^{2+} channels are present on C6 glioma cells (7). The second goal of our study was to subtype the P_2 receptor on C6-2B glioma cells and test the hypothesis that nucleotide-gated Ca^{2+} channels are present on these cells.

Furthermore, the activation of P_{2X}-purinergic receptors on rat hepatocytes (8) and P_{2U} receptors on NCB-20 cells (5) inhibits agonist-elicited cAMP accumulation through G_i and a rise in $[Ca^{2+}]_i$, respectively. In C6-2B cells, increase in $[Ca^{2+}]_i$ either by the activation of stably transfected SK receptors or by the inhibition of a microsomal Ca^{2+}-ATPase pump with TG causes inhibition of agonist-stimulated cAMP accumulation (9, 10). The third goal of this study was to investigate whether activation of P_2-purinergic receptors on C6-2B glioma cells stimulates PI metabolism (thereby increasing IPs formation and $[Ca^{2+}]_i$) and inhibits agonist-elicited cAMP accumulation, and if the latter effect is transduced through G_i or occurs indirectly through the PLC-mediated rise in IPs and $[Ca^{2+}]_i$.

Activation of PLC causes an increase in the levels of IP_3 and the endogenous PKC activator diacylglycerol. An increase in the levels of IP_3 causes the release of Ca^{2+} from IP_3-sensitive intracellular stores. An increase in the levels of diacylglycerol is thought to act as a feedback inhibitor of the Ca^{2+} response by activating PKC (11). To this end, the activation of PKC with phorbol esters has been shown to negatively regulate the agonist-stimulated activity of PLC (11, 12). The fourth goal of this study was to use phorbol esters as a tool to further test the hypothesis that a causal relationship exists between the increase in $[Ca^{2+}]_i$ and inhibition of cAMP accumulation in C6-2B glioma cells.

In this study, we provide evidence for the presence of the P_{2U} subtype of purinergic receptors on C6-2B glioma cells. These receptors are coupled to the IP_3-mediated increase in $[Ca^{2+}]_i$ but not to nucleotide-gated Ca^{2+}-channels. Furthermore, we demonstrate that the changes in the levels of IPs, $[Ca^{2+}]_i$, and agonist-elicited cAMP accumulation after activation of these receptors are insensitive to PTX and modulated by PKC, providing strong support to the hypothesis of a cause-effect relationship between increase in $[Ca^{2+}]_i$ and inhibition of cAMP.

Materials and Methods

2-Methylthio ATP and TG were purchased from Research Biochemicals, Inc. (Natick, MA). ATP, AMPCPP, UTP, THR (bovine plasma), HEPES, EGTA, DANT, PMA, and PDD were purchased from Sigma Chemical Co. (St. Louis, MO). FO was purchased from Calbiochem (San Diego, CA), Ro-20–1724 from BIOMOL Research Labs, Inc. (Plymouth Meeting, PA), PTX from List Biochemicals, Inc. (Campbell, CA), Fura 2-AM from Molecular Probes, Inc. (Eugene, OR), and *myo*-[2-^{3}H]inositol (18.3 Ci/mmol) from Amersham Corporation (Arlington Heights, IL). Suramin was a gift from Dr. Anton Wellstein, (Georgetown University Medical School, Washington, DC).

Cell culture. C6-2B rat glioma cells were grown as monolayers in Ham's F-10 medium and 10% calf serum at 37° in the presence of 95% air and 5% CO_2. The C6-$2B_{A2-3}$ clone (C6-2B cells stably transfected with SK receptor cDNA) (9) was grown as described (10).

Single cell Ca^{2+} imaging. An Attofluor digital microscopy system (Atto Instruments Inc., Rockville, MD) was used for single cell Ca^{2+} imaging with the fluorescent Ca^{2+} indicator Fura 2 (13). The cells were grown on 25-mm-round and 1-mm-thick glass coverslips and loaded at 37° for 30 min with the cell-permeable acetoxy methyl ester of Fura 2 (Fura 2-AM) (5 μM) in Ham's F-10 medium supplemented with 20 mM HEPES, pH 7.4. Cells were then washed and imaged in the same medium at 30 to 32° with a Zeiss Axiovert 35 microscope and a 63× oil immersion objective.

Measurement of cAMP. An Atto-Flo automated radioimmunoassay system (Atto Instruments) was used to measure cAMP content in intact cells. Drug treatments were performed at 37° in serum-free Ham's F-10 medium buffered to pH 7.4 with 10 to 20 mM HEPES in the presence of 100 to 500 μM 3-isobutyl-1-methylxanthine and 100 to 200 μM Ro20-1724 to prevent cAMP breakdown (9, 10).

Measurement of IPs. The accumulation of IPs was measured as described (9). Briefly, after pretreatment with 1 μCi/2 ml of *myo*-[2-^{3}H]inositol (18.3 Ci/mmol) for 40 to 48 hr, C6-2B cells were washed in serum-free medium and treated for 5 min with 10 mM LiCl, which was included in all subsequent drug treatments.

Results and Discussion

Rat glioma C6-2B cells are a useful model for studying the interaction of the second messengers cAMP and Ca^{2+} because these cells accumulate cAMP when challenged with ISO or FO and are rich in type VI adenylyl cyclase (14), which can be inhibited by concentrations of Ca^{2+} in the submicromolar range (9, 15). However, C6-2B cells, unlike the parent C6 glioma cells (16, 17), do not consistently respond to several receptor agonists (such as endothelin, carbachol, and angiotensin II) with an increase in $[Ca^{2+}]_i$.[1] Therefore, to study the interaction of Ca^{2+} and cAMP, we stably transfected SK receptor cDNA in C6-2B cells and demonstrated SK-induced Ca^{2+} transients in the C6-$2B_{A2-3}$ subclone (9). We now report that C6-2B cells consistently respond to activation of at least two endogenous cell surface receptors with an increase in $[Ca^{2+}]_i$. One of these receptors is activated by ATP and the other by THR.

Identification of the P_2 purinergic receptor subtype on C6-2B glioma cells. Several cell types respond to activation of P_{2X}, P_{2Y} and P_{2U} receptors with an increase in $[Ca^{2+}]_i$ (1, 4). Preliminary experiments revealed that C6-2B cells also respond to ATP with an increase in $[Ca^{2+}]_i$. This effect on $[Ca^{2+}]_i$ was not a result of the breakdown of ATP to adenosine and the subsequent effect of adenosine on P_1 purinergic receptors (18) because the response persisted in the presence of adenosine deaminase (data not shown). Inosine, the product of adenosine deamination, is not active at P_1 receptors (19). Moreover, when the cells were challenged with 5 μM ATP (approximate concentration for half maximal response, EC_{50}, of the nucleotide for $[Ca^{2+}]_i$ rise; see below), the cytosolic Ca^{2+} response was inhibited by the non-selective P_2 purinergic receptor antagonist, suramin (1, 4) (data not shown), indicating that the ATP-evoked response is mediated through P_2-purinergic receptors.

Four different nucleotides were used to identify the P_2-purinergic receptor subtype on C6-2B glioma cells. These cells responded weakly to the P_{2X}- and P_{2Y}-purinergic receptor-specific agonists, AMPCPP and 2-methylthio ATP, respectively ($EC_{50} > 100$ μM; $n = 3$) (Fig. 1). However, the endogenous nucleotides UTP and ATP were much more potent and displayed comparable potencies for elevation of $[Ca^{2+}]_i$ (mean EC_{50}

[1] R. Munshi, M. A. DeBernardi, and G. Brooker, unpublished observations.

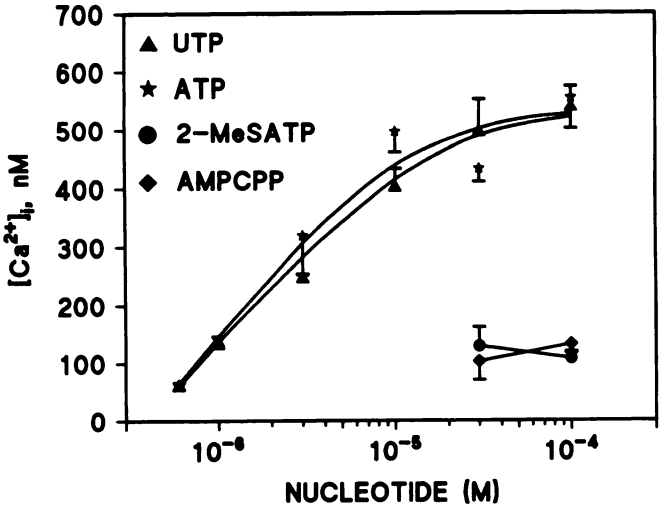

Fig. 1. Concentration-dependent effect of nucleotides on $[Ca^{2+}]_i$ in C6-2B cells. Cells loaded with Fura 2-AM were exposed to various concentrations of nucleotides between 1 and 100 μM as shown. The peak $[Ca^{2+}]_i$ from a total of four to six coverslips (22–51 cells per coverslip in a single field) in three different experiments were averaged and the population mean ± standard error was plotted on a semi-logarithmic scale.

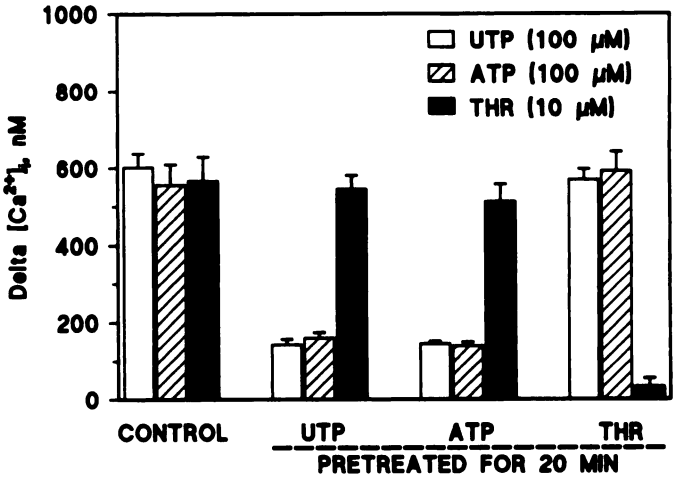

Fig. 2. Homologous desensitization of the Ca^{2+} increase by the nucleotides and THR in C6-2B cells. Cells loaded with Fura 2-AM were exposed to either 100 μM UTP, 100 μM ATP, or 10 μM (0.69 NIH U/ml) THR for 20 min. After washing of the agonists for 2 min, the cells were rechallenged with the same concentrations of nucleotides or THR as above. The data from four to six coverslips (25–48 cells per coverslip in a single field) in two to three experiments was pooled and the population mean ± standard error was plotted. The agonist-evoked delta $[Ca^{2+}]_i$ for each cell was calculated by subtracting the average resting $[Ca^{2+}]_i$ of the cell from the peak $[Ca^{2+}]_i$, measured over 15 to 25 seconds before drug addition.

± standard error = 5.0 ± 0.7 and 3.6 ± 0.7 μM, n = 3, respectively), indicating the presence of P_{2U}-purinergic receptors on C6-2B cells. Similar EC_{50} values for UTP and ATP at P_{2U}-purinergic receptors on NCB-20 (5) and CF/T43 (20) cells have been reported. These half-maximal concentrations of the nucleotides are not unphysiologic because much higher concentrations can be achieved during exocytosis and repetitive nerve stimulation (1, 2).

To investigate whether UTP and ATP both activated the same population of P_2-purinergic receptors, the cells were pretreated for 20 min with 100 μM UTP or ATP, washed, and rechallenged with the nucleotides. Each nucleotide homologously desensitized the Ca^{2+} response to the other; the desensitized peak $[Ca^{2+}]_i$ were 24 to 29% that of the control (Fig. 2). Under these conditions, the peak $[Ca^{2+}]_i$ after addition of another Ca^{2+}-mobilizing receptor agonist (THR) did not diminish in that 98 to 100% of the control response was observed (Fig. 2), indicating a lack of heterologous desensitization between the nucleotides and THR. Pretreatment of the cells with THR also caused homologous desensitization of its response (<10% of the control response), but under these conditions, the response to the nucleotides was >90% of their control response (Fig. 2).

Mobilization of intracellular Ca^{2+} by UTP. To investigate if the nucleotide-evoked cytosolic Ca^{2+} increase had intracellular or extracellular origin, C6-2B cells were challenged with 20 μM UTP either in the absence or presence of EGTA. When the cells were pretreated with 2 mM EGTA for 2 min, the peak $[Ca^{2+}]_i$ was minimally affected (from an average of 266 ± 95 nM in the absence to 211 ± 67 nM in the presence of EGTA; n = 3) (Fig. 3A). These data indicate that a UTP-induced rise in $[Ca^{2+}]_i$ primarily involves the mobilization of Ca^{2+} from intracellular stores and is not a result of Ca^{2+} influx. Although the rate of onset of the response was comparable, the rate of decay was about 4 times higher in the presence of EGTA ($t_{1/2}$ of decay approximately 37 sec in the presence and >154 seconds in the absence of EGTA (Fig. 3A). This difference in the two decay slopes is likely a result of the capacitative entry of Ca^{2+} triggered by the depletion of internal stores (10, 11, 21).

Further support for the hypothesis that intracellular Ca^{2+} is the single main source of the cation for the peak response to UTP came from experiments in which the internal stores were depleted of Ca^{2+} with the microsomal Ca^{2+}-ATPase inhibitor TG (10, 11) or the release of Ca^{2+} from the stores was blocked with DANT (10), a drug used clinically to treat malignant hyperthermia (22). When C6-2B cells were challenged with 40 nM TG (concentration that produces submaximal Ca^{2+} response) (10), the $[Ca^{2+}]_i$ peaked between 7 to 8 min after the application of TG and and decayed slowly to levels slightly above the resting $[Ca^{2+}]_i$ within 20 min (Fig. 3B). In these TG-treated cells, the response to 100 μM UTP, added after washing, was blocked, although in the same experiment another group of cells not pretreated with TG responded to UTP as shown in Fig. 3B. When C6-2B cells were exposed to DANT (40 μM), there was a 2- to 3-fold increase in the resting $[Ca^{2+}]_i$ (Fig. 3C) (10). After 8 min, the drug was washed off and the cells were challenged with 20 μM UTP in the absence of DANT. The response to UTP was blocked, whereas in the same experiment, another group of cells pretreated with the vehicle (0.4% DSMO) responded to UTP as shown in Fig. 3C.

Although it is conceivable that blockade of Ca^{2+} release from intracellular pools triggers a slow influx of Ca^{2+} from the extracellular space, the exact mechanism of DANT-induced increase in $[Ca^{2+}]_i$ is currently under investigation. Because the increase in $[Ca^{2+}]_i$ in response to UTP (but not ionomycin) is abolished under conditions when there is no Ca^{2+} release from intracellular stores, there must not be a primary influx of Ca^{2+} after exposure of the cells to UTP. These data are in sharp contrast with those of Lin and Chuang, who suggest that ATP-gated Ca^{2+} channels in C6 glioma cells may account primarily for the Ca^{2+}-transient response to the nucleotide (7). Lin and Chuang's nucleotide gated Ca^{2+} channel hypothesis is based

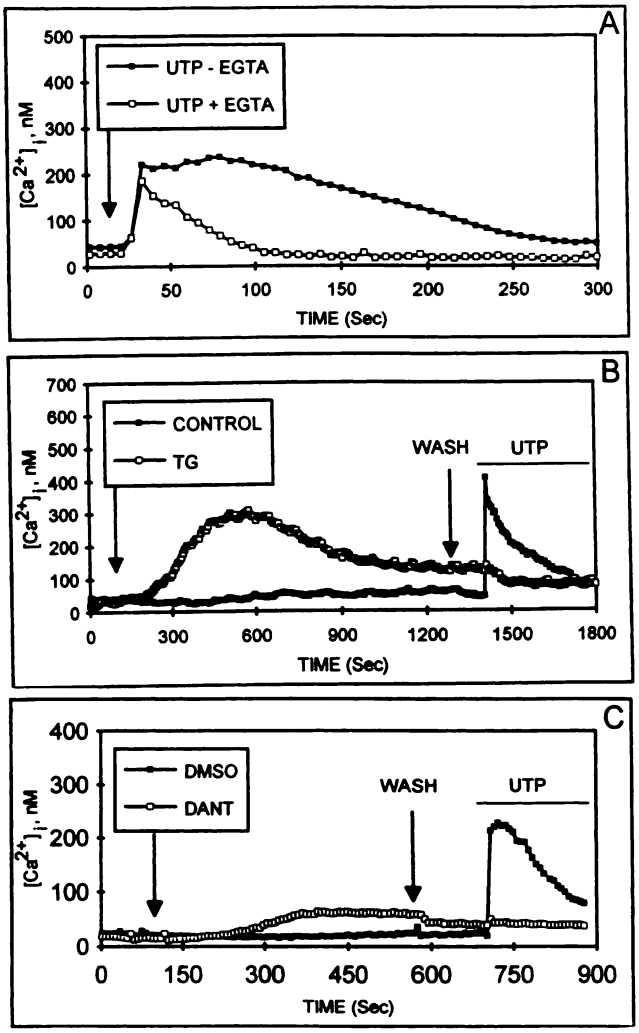

Fig. 3. Mobilization of intracellular Ca^{2+} by UTP in C6-2B cells. A, Cells loaded with Fura 2-AM were challenged with 20 μM UTP in Ham's F-10 medium as shown. In parallel, cells were pretreated for 2 min with Ham's F-10 medium containing 2 mM EGTA to chelate extracellular Ca^{2+}. Then 20 μM UTP was added in EGTA-containing medium. B, The cells were treated with the F-10 medium either in the absence or presence of 40 nM TG for 20 min, washed for 2 min with the medium, and challenged with 100 μM UTP in the absence of TG. C, The cells were treated with either 40 μM DANT or the vehicle (0.4% DSMO) for 8 min, washed for 2 min with F-10 medium, and challenged with 20 μM UTP in the absence of DANT/DSMO. The data represent the population mean from one coverslip and are representative of five to six coverslips (12–35 cells per coverslip in a single field) in two to three different experiments.

upon partial sensitivity of the Ca^{2+}-response (but not the PI response) to 1 mM La^{3+} and a Ca^{2+}-response to ATP in BAPTA-AM-pretreated cells. One wonders if there are differences between the parent C6 and the subclonal C6-2B glioma cells, at least with respect to P_2-purinergic receptor signalling pathways. It is possible that the parent cells have two populations of P_2 receptors (one coupled to PLC and the other to a receptor-gated Ca^{2+} channel (7)) whereas the subclonal cells have a single population of P_2-purinergic receptors coupled to PLC. Because P_{2X} receptors are known to couple to a receptor-gated Ca^{2+} channel, such as in arterial smooth muscle cells (6), it is conceivable that C6 glioma cells have a P_{2X} and either a P_{2Y} or P_{2U} receptor subpopulation. Lin and Chuang did not subtype the ATP-sensitive receptors in C6 glioma cells (7). On the other hand, P_{2X} and P_{2Y} receptor-specific nucleotides are only weakly active in C6-2B cells (Fig. 1), indicating the functional absence of these receptors in the subclonal cells.

Effect of UTP on IPs formation. In several cell systems, DANT-sensitive intracellular Ca^{2+} stores comprise the IP_3-and TG-sensitive stores (10). Because UTP-induced Ca^{2+} increase was sensitive to both DANT and TG, we investigated if UTP released Ca^{2+} through the activation of PLC and consequent formation of IPs. When C6-2B cells prelabeled with *myo*-[2-^{3}H]inositol were challenged with 100 μM UTP, there was an increase in the basal level of IPs formation by over 40% (Fig. 4A). We have shown previously that overnight treatment with 1 μg/ml PTX quantitatively ADP-ribosylates the toxin-sensitive G proteins in C6-2B glioma cells (10). However, there were no detectable changes in either IPs formation (Fig. 4A) or $[Ca^{2+}]_i$ rise (Fig. 4B) stimulated by UTP in cells treated for 20 to 24 hr with 1 μg/ml PTX compared with control, toxin-untreated cells. P_{2U} receptors couple to PLC either partially through PTX-sensitive G proteins in CF\T43 cells (20) and human fibroblasts (23) or exclusively through toxin-insensitive G proteins in HSG-PA (24) and NCB-20 cells (5). The lack of sensitivity to PTX of both IPs formation and the subsequent rise in $[Ca^{2+}]_i$ indicates that, in C6-2B cells, P_{2U} receptors couple to PLC not through G_i but possibly G_q (11, 25). The activation of PLC could be mediated by α- or $\beta\gamma$-subunits of G proteins (26). It has been shown recently that PLC-β2 isozyme is specifically stimulated by G protein $\beta\gamma$-subunits (27, 28). In C6-2B cells, release of $\beta\gamma$-subunits through activation of G_i-coupled adenosine A_1 receptors (18) with 100 nM cyclopentyladenosine (in the presence of 4 U/ml adenosine deaminase) did not affect either the IPs formation or resting $[Ca^{2+}]_i$,[1] indicating that PLC-β2 isozyme may not be present in these cells unless there is compartmentalization of G proteins and

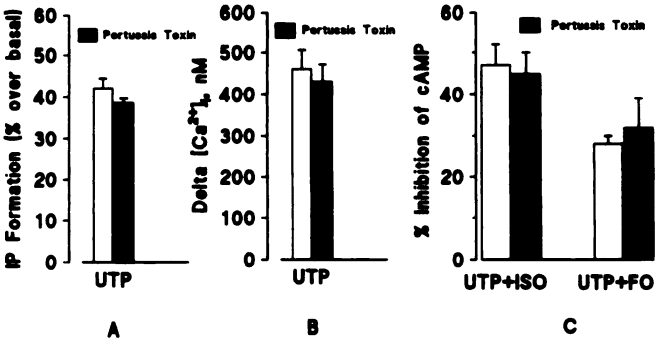

Fig. 4. Effect of UTP on IPs formation, $[Ca^{2+}]_i$, and cAMP accumulation in C6-2B cells. A, The formation of IPs was measured as described in Materials and Methods. The cells were incubated for 20 to 24 hr with 1 μg/ml PTX and then challenged with UTP for 5 min. The basal level of conversion of cell-incorporated *myo*[2-^{3}H]inositol, at 37°, in the presence of 10 mM LiCl was 2.13 ± 0.07% and 2.23 ± 0.03% in control and PTX-treated cells, respectively. The data from a single experiment in triplicate are shown. B, The cells were treated with PTX, loaded with Fura 2-AM, and imaged. The resting $[Ca^{2+}]_i$ was 47 ± 18 and 58 ± 13 nM in control and PTX-treated cells, respectively. The mean ± standard error of delta peak $[Ca^{2+}]_i$ after addition of 100 μM UTP from nine coverslips (25–45 cells per coverslip in a single field) in three experiments are shown. C, Control and PTX-treated cells were challenged for 5 min with ISO (10 μM) or FO (10 μM) in the absence or presence of 20 μM UTP and cAMP accumulation was measured as described in Materials and Methods. The basal level of cAMP accumulation was 42.6 ± 1.5 and 56 ± 9.5 pmol/mg protein in the control and PTX-treated cells, respectively. The data are mean ± standard error of two to three experiments each in triplicate.

PLC. The UTP receptor in C6-2B cells may, therefore, couple to other isozymes of PLC through $G_q\alpha$-subunit.

Effect of UTP on cAMP accumulation. In C6-2B cells, agents that elevate $[Ca^{2+}]_i$, such as the PLC-coupled receptor agonist SK or the microsomal Ca^{2+}-ATPase inhibitor TG, inhibit the accumulation of cAMP elicited by ISO or FO (9, 10). Also, agents that prevent the rise in $[Ca^{2+}]_i$, such as the cell-permeable Ca^{2+} chelator EGTA-AM and the intracellular Ca^{2+} release blocker DANT, inhibit the effect of SK and TG on ISO- and FO-elicited cAMP accumulation (9, 10). In C6-2B cells, UTP inhibited ISO- and FO-elicited accumulation of cAMP by approximately 40 and 30%, respectively, without affecting the basal cAMP accumulation (Fig. 4C). Furthermore, pretreatment of C6-2B cells overnight with 1 μg/ml PTX (9) had no appreciable effect on UTP-induced inhibition of ISO- and FO-elicited cAMP levels (Fig. 4C). These data rule out a direct inhibition of adenylyl cyclase by UTP transduced through G_i and indicate that the effect is mediated through a PTX-insensitive rise in $[Ca^{2+}]_i$. The lack of sensitivity of both the UTP-induced increase in IPs formation and $[Ca^{2+}]_i$ to PTX, as well as UTP-induced inhibition of ISO- and FO-elicited cAMP accumulation, are consistent with the notion that there is a causal relationship between the activation of PLC and inhibition of agonist-elicited cAMP accumulation, as previously reported for C6-2B (9, 10) and NCB-20 cells (5).

Molecular cloning studies have indicated structural diversity in the adenylyl cyclase family, and cDNAs for eight isoforms of the enzyme have been isolated (29). Although all adenylyl cyclases can be inhibited by millimolar concentrations of Ca^{2+} (presumably by competing at the Mg^{2+} binding site) (30), the type VI cyclase is inhibited by concentrations of the cation in the submicromolar range (14, 15) that are achieved during activation of P_2-purinergic and other cell surface Ca^{2+}-mobilizing receptors on C6-2B cells (Figs. 1 and 2). Because type VI is the predominant adenylyl cyclase expressed in C6-2B cells (14), it is conceivable that a UTP-induced rise in $[Ca^{2+}]_i$ inhibits ISO- and FO-elicited cAMP accumulation by negatively regulating type VI adenylyl cyclase.

A key step in PI turnover is the reaction whereby phosphatidylinositol 4,5-bisphosphate is hydrolyzed by PLC isoforms to produce IP_3 and diacylglycerol. Both of these reaction products are second messengers involved in the control of intracellular Ca^{2+} fluxes and the activation of PKC, respectively (11). The IP_3-induced release of intracellular Ca^{2+} is then negatively regulated by the diacylglycerol-mediated stimulation of PKC (11). Therefore, if the UTP-evoked cytosolic Ca^{2+} increase in C6-2B cells is a result of enhanced IPs formation, then activation of PKC should inhibit this response. Pretreatment of C6-2B cells for 15 to 20 min with the PKC-activator PMA (1 μM) dramatically reduced (by >80%) the Ca^{2+} response to 30 μM UTP added 2 min after washing the cells with PMA-free medium (Fig. 5). To ascertain that the effect of PMA on UTP-induced increase in $[Ca^{2+}]_i$ is a result of activation of PKC, we used the inactive phorbol ester PDD (1 μM) (31, 32) as a control and found that the magnitude of Ca^{2+} response in PDD-pretreated cells was comparable to that in cells treated with the vehicle (0.01% DMSO) (compare peak $[Ca^{2+}]_i$ in Fig. 5 with that in Fig. 3, A and C). We have reported previously that in C6-2B glioma cells, a 30-min pretreatment with 1 μM PMA (but not with 1 μM PDD) increased the total cellular PKC activity (10), demonstrating specificity of the effect of PMA in these cells.

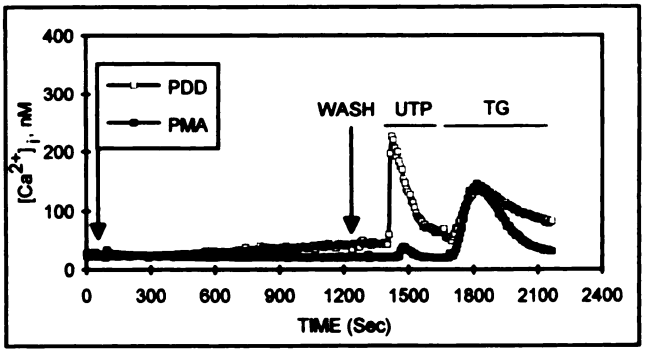

Fig. 5. Sensitivity of UTP-induced Ca^{2+} response to inhibition by PKC in C6-2B cells. Cells loaded with Fura 2-AM were treated with either PDD or PMA, 1 μM each, for 20 min, washed with phorbol ester-free F-10 medium for 2 min, and challenged with 30 μM UTP, followed by 100 nM TG. The data are the population mean from one coverslip (12–42 cells per coverslip in a single field) and is representative of a total of five coverslips from three experiments. The mean ± standard error of delta peak $[Ca^{2+}]_i$ is shown in Fig. 6B.

We exploited this regulatory property of PKC (regulation of the agonist-stimulated transient Ca^{2+} response) to investigate if, in PMA pretreated cells, the inhibition of the UTP-stimulated PLC activity and the subsequent rise in $[Ca^{2+}]_i$ would result in a reversal of the UTP-induced inhibition of the agonist-elicited accumulation of cAMP. Pretreatment with PMA not only inhibited the IPs formation and the $[Ca^{2+}]_i$ rise evoked by UTP, but also reversed the UTP-induced inhibition of agonist-stimulated cAMP accumulation (Fig. 6, A–C). Activation of THR receptors on C6-2B cells and SK receptors on $C6\text{-}2B_{A2\text{-}3}$ subclonal cells also increased the levels of IPs and cytosolic Ca^{2+} and decreased the agonist-elicited cAMP accumulation. The changes in all three parameters were sensitive to inhibition by PMA, but not by PDD (Fig. 6, A–C). However, increase in $[Ca^{2+}]_i$ and inhibition of cAMP accumulation by TG, which acts at a step distal to PLC, remained unchanged after pretreatment with PMA (Fig. 6, B and C). These data provide strong support for our hypothesis that there is a causal relationship between the rise in $[Ca^{2+}]_i$ and the inhibition of cAMP accumulation (9, 10, 14).

The inhibition of the responses to all three agonists also indicates that regulation by PKC occurs at a site common to all three agonists (UTP, THR, and SK). This common site of regulation could be $G_{q\alpha}$, PLC, or distal to PLC. Lounsbury *et al.* (33) recently showed that in permeabilized platelets, under conditions promoting PMA-stimulated phosphorylation of $G_{z\alpha}$, $G_{q\alpha}$ is not phosphorylated, indicating that the G_q subunit is probably not the target for regulation by PKC. Two lines of evidence indicated that in C6-2B cells the effect of PKC is not distal to PLC. First, the rise in $[Ca^{2+}]_i$ in response to TG, which does not affect IPs formation in C6-2B cells (10), was not sensitive to PMA (Figs. 5 and 6B). Second, the activation of PKC inhibited not only the agonist-evoked $[Ca^{2+}]_i$ changes but also the formation of IPs (Fig. 6A).

Although the agonist-induced increase in IPs formation was inhibited by activation of PKC (Fig. 6A), the basal level of IPs formation was not inhibited. Percent conversion of *myo*[2-^{3}H] inositol into total [^{3}H]IPs in the presence of PDD and PMA was 2.3 ± 0.1 and 2.4 ± 0.2, respectively (n = 3, each in triplicate). This shows that activation of PKC did not affect

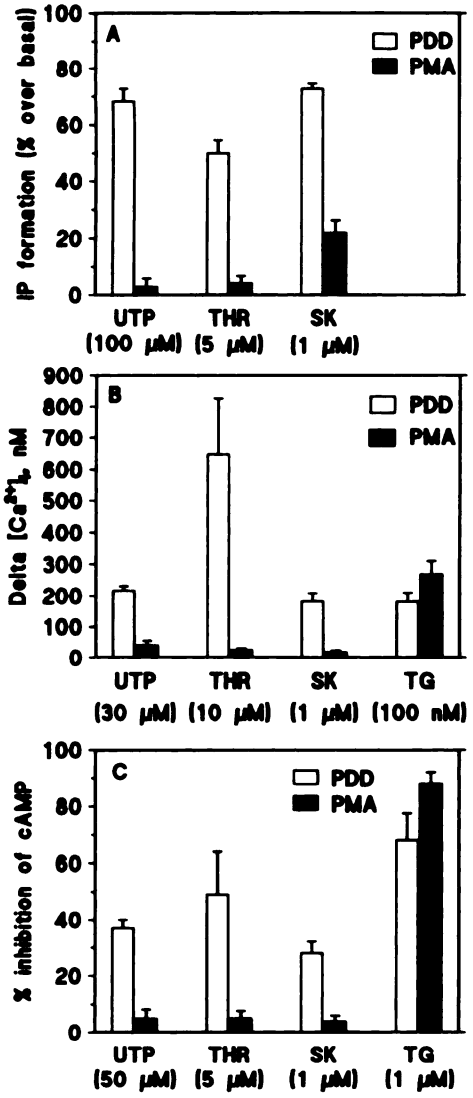

Fig. 6. Effect of activation of PKC on UTP-, THR-, SK-, and TG-induced responses. The cells were treated with either PDD or PMA, 1 μM each, for 15 to 20 min, washed, and challenged with either UTP, THR, SK, or TG. A, IPs formation in response to UTP, THR, and SK was measured in C6-2B$_{A2-3}$ clonal cells. The data are mean ± standard error of three experiments each in triplicate. B, Cells loaded with Fura 2-AM were challenged with UTP, THR, SK, and TG. The data are mean ± standard error of four to five coverslips (12–42 cells per coverslip in a single field) in two to three different experiments. C, The ISO-elicited cAMP accumulation was measured in C6-2B$_{A2-3}$ clonal cells as described in Materials and Methods. The data are mean ± standard error of three experiments each in triplicate.

the intrinsic activity of the effector enzyme and indicates that PKC phosphorylates a site on PLC such that its interaction with the activated $G_{q\alpha}$ is compromised. In permeabilized cells, IPs formation can be stimulated either indirectly through G proteins with GTPγS or directly by activating PLC with exogenously added Ca^{2+} (26). Pachter *et al.* (34) recently reported that in permeabilized rat 6 fibroblasts stably overexpressing the cDNA for PKC-β1, GTPγS-stimulated IP_3 accumulation was greatly reduced compared with mock transfected cells, whereas the IP_3 accumulation elicited by exogenously added Ca^{2+} was similar to the mock transfected cells. In the same study, desensitization of THR-stimulated PLC activity was greater in PKC-β1 overexpressing cells. These data indicate that PKC may modulate G protein-PLC coupling in other cell systems as well. Furthermore, Ryu *et al.* (35) showed that treatment of C6Bu1 glioma, PC12, and NIH 3T3 cells with 1 μM PMA caused phosphorylation of serine residues in PLC-β but not PLC-γ and PLC-δ. In the same study, *in vitro* phosphorylation of PLC-β resulted in stoichiometric incorporation of phosphate at serine 887 without any effect on the activity of PLC-β isoform, strongly indicating that serine phosphorylation by PKC alters the interaction of PLC-β with a putative G protein. The serine residue phosphorylated by PKC probably lies in the carboxyl-terminal domain of PLC because removal of this region in PLC-β1, by either the Ca^{2+}-dependent protease calpain (36) or a series of truncations and deletions of PLC-β1 cDNA (37), abolished the activation of PLC-β by $G_{q\alpha}$ without affecting the catalytic activity measured in the absence of the G protein subunit (36, 37).

In conclusion, we have demonstrated that C6-2B rat glioma cells possess cell surface receptors for nucleotides and THR. The activation of these receptors stimulated PLC enzyme causing accumulation of total IPs, mobilized Ca^{2+} from TG- and DANT-sensitive intracellular Ca^{2+} pools, and inhibited ISO- and FO-elicited cAMP accumulation. There was no direct evidence for an extracellular nucleotide-gated Ca^{2+} channel. The UTP-induced changes in the levels of IPs, $[Ca^{2+}]_i$, and agonist-elicited cAMP were mediated through PTX-insensitive G proteins (possibly G_q), mimicked by another Ca^{2+} mobilizing agonist THR, and sensitive to inhibition by PMA but not PDD. The lack of sensitivity to PMA of the basal levels of both IPs and cytosolic Ca^{2+} indicates a negative regulation by PKC of the coupling of PLC with $G_{q\alpha}$. Furthermore, our data strongly support the hypothesis that rise in $[Ca^{2+}]_i$ through activation of receptors coupled to PLC is a prerequisite for the inhibition of cAMP accumulation in C6-2B glioma cells .

ATP has been shown to be released from stimulated hippocampal slices (38), and the brain is an especially rich source of UTP (39). These nucleotides may mediate neuron-glial interaction through glial cell P_{2U}-purinergic receptors.

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