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Purinergic Receptor Regulation of Signal Transduction in NCB-20 Cells

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SUMMARY

In the present paper, P_1 and P_2 purinergic receptors and their control of signal transduction pathways were investigated in NCB-20 cells. ATP elicited an increase in $[Ca^{2+}]_i$. The purinergic receptor subtype involved was identified by comparing the actions of a range of nucleotides. UTP was the most potent agonist in elevating $[Ca^{2+}]_i$, with an EC_{50} value of 6.2 ± 0.5 μM. UTP, ATP (EC_{50}, 17.3 ± 1.5 μM), adenosine-5′-*O*-(3-thio)triphosphate (23 ± 3 μM), and ITP (55 ± 4 μM) exerted similar maximal effects. Other nucleotides tested, including β,γ-methylene-ATP and 2-methylthio-ATP, which are considered prototypic agonists for P_{2x} and P_{2y} receptors, respectively, were ineffective; in general, modifications in the ribose-triphosphate chain and substitution on the 2-position of the purines reduced the efficacy of nucleotides. This pharmacological characterization indicated that a putative P_{2u} receptor mediates the $[Ca^{2+}]_i$ elevation elicited by nucleotides in NCB-20 cells. The increase in $[Ca^{2+}]_i$ originates from intracellular Ca^{2+} stores; blockade of Ca^{2+} entry does not affect the rise in $[Ca^{2+}]_i$. In contrast, pretreatment with the Ca^{2+}-ATPase inhibitor thapsigargin or with bradykinin, a hormone that releases Ca^{2+} from inositol trisphosphate-sensitive stores, does preclude the increase in $[Ca^{2+}]_i$ induced by ATP. ATP and UTP also transiently inhibit cAMP accumulation in the intact cell, presumably via a Ca^{2+}-mediated mechanism. The finding of a P_{2u} receptor in NCB-20 cells adds to a growing perception that P_2 receptors are widely distributed. Besides the P_{2u} receptor, NCB-20 cells express adenosine A_2 receptors, coupled to stimulation of cAMP accumulation. The presence of both P_1 and P_2 purinergic receptors permits a sequential modulation of distinct second messenger levels associated with a common stimulus, ATP.

For some time, adenosine has been recognized to play important roles in regulating a range of physiological processes, including cardiac function and neurotransmitter release (1). More recently, the likely metabolic precursor of adenosine, ATP, has been implicated in a growing number of effects (2). The effects of both of these compounds are mediated by cell surface receptors that can be classified as P_1 receptors, which are sensitive to adenosine and its analogues, and P_2 receptors, which respond to the adenine nucleotides (3).

Although the role of adenosine as a neuromodulator had been suggested as early as 1929 (4), the existence of membrane-bound adenosine receptors was established only in the late 1970s (5, 6). These P_1 receptors were divided into A_1 and A_2 subtypes. The adenosine A_2 receptor couples in a stimulatory manner to adenylyl cyclase, whereas the adenosine A_1 receptor activates the inhibitory G protein G_i, resulting in the inhibition of adenylyl cyclase, activation of outward potassium channels, and inhibition of Ca^{2+} channels (7). Selective agonists and antagonists for both adenosine receptors are available (8), which has facilitated their characterization. Recently, cDNAs encoding both adenosine A_1 and A_2 receptors have been identified (9–11).

Although the existence of P_2 receptors had been proposed as early as 1972 (12), the molecular pharmacology of P_2 receptors has been slow to be elucidated (2). P_2 receptor research is impeded by a shortage of stable and selective agonists and antagonists. To date, five distinct subtypes have been described, based on agonist studies, i.e., P_{2x}, P_{2y}, P_{2t}, P_{2z}, and P_{2u} (13–16). Most information on structure-activity relationships and biochemical properties is available for the P_{2x} receptor, at which AMP-PCP is more potent and 2-CH_3S-ATP is less potent than ATP, and the P_{2y} receptor, at which this agonist potency is reversed (13, 15). The P_{2t} receptor is specific for platelets, with ADP as the natural ligand, and the P_{2z} receptor mediates the ATP-induced permeabilization of the plasma membrane in, for example, mast cells and J774 macrophages (13). The P_{2u} receptor, which is sensitive to UTP as well as ATP, is a recent addition to this family (14, 16). The properties and distribution

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ABBREVIATIONS: AMP-PCP, β,γ-methylene-ATP; AMP-PNP, β,γ-imido-ATP; BK, bradykinin; $[Ca^{2+}]_i$, cytosolic calcium concentration; DMEM, Dulbecco's modified Eagle's medium; GMP-PNP, β,γ-imido-GTP; IBMX, 3-isobutyl-1-methylxanthine; PTX, pertussis toxin; *R*-PIA, N^6-(*R*)-1-phenyl-2-propyladenosine; TLC, thin layer chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; ATPγS, adenosine-5′-*O*-(3-thio)triphosphate; G protein, GTP-binding protein; IP_3, inositol trisphosphate; 2-CH_3-S-ATP, 2-methylthio-ATP.

of this latter receptor subtype are still relatively unknown; indeed, it is possible that, because UTP is not always tested in pharmacological studies on P_2 purinergic receptors, the P_{2u} subtype may be overlooked.

In the present study, we have explored the presence of purinergic receptors, in particular P_2 receptors, and their control of signal-transduction pathways in NCB-20 cells, mouse neuroblastoma × Chinese hamster brain explant hybrid cells that exhibit some properties of cholinergic neurons (17). We demonstrate that, in addition to muscarinic cholinergic, opiate, serotonin, prostaglandin, and BK receptors, these cells express P_{2u} receptors, which are coupled to intracellular Ca^{2+} mobilization, and adenosine A_2 receptors, which elevate cAMP accumulation.

Experimental Procedures

Materials. The following chemicals were used in this study: [^{3}H] adenine (New England Nuclear, Boston, MA), DMEM (GIBCO, Grand Island, NY), fetal bovine serum (Irvine, Santa Ana, CA), gentamicin (Elkins-Sinn, Cherry Hill, NJ), fura-2 acetoxymethyl ester and pluronic acid (Molecular Probes, Eugene, OR), thapsigargin (LC Services Corp, Woburn, MA), ADP, AMP, AMP-PCP, ATP (grade I), BK, cAMP, CTP, forskolin, IBMX, *R*-PIA, and UTP (Sigma, St. Louis, MO), PTX (List, Campbell, CA), CGS21680 (Dr. K. A. Jacobson, National Institutes of Health, Bethesda, MD), Ro 20–1724 (Dr. K. Prasad, University of Colorado Health Science Center, Denver, CO), AMP-PNP, ATPγS, GMP-PNP, ITP, UMP, and UDP (Boehringer, Mannheim, FRG), and 2-CH_3S-ATP (Research Biochemicals, Inc., Natick, MA). All other chemicals were obtained from standard commercial sources and were of analytical grade. The concentration of nucleotide stock solutions was established by UV absorbance.

Cell culture. Early passage NCB-20 cells were provided by Dr. G. Dawson (University of Chicago). Cells were grown in 75-cm^2 culture flasks, in DMEM with 5% fetal bovine serum and 6 μg/ml gentamicin, at 37°, in an atmosphere of 10% CO_2. Cells were used 4–6 days after passage.

Measurement of cAMP accumulation. cAMP formation in intact cells was measured according to the method of Evans *et al.* (18). NCB-20 cells were incubated in DMEM (60 min, 30°) with ~2 μCi of [^{3}H] adenine/10^6 cells, to label the ATP pool. The cells were detached with phosphate-buffered saline (12.1 mM Na_2HPO_4, 4 mM KH_2PO_4, 130 mM NaCl, pH 7.4) containing 0.02% EDTA, centrifuged for 4 min at 1000 rpm in an IEC-HN-SII table-top centrifuge, and resuspended in incubation buffer (in mM: NaCl, 110; KCl, 5.4; $MgSO_4$, 0.8; $CaCl_2$, 1.38; NaH_2PO_4, 0.9; D-glucose, 25; with bovine serum albumin, 1 mg/ml). After 10 min of equilibration at 30°, the suspension was divided into 900-μl aliquots, each containing approximately 9 × 10^5 cells. Before incubation with the test compounds, the cells were preincubated for 10 min at 30° with 2 units/ml adenosine deaminase and phosphodiesterase inhibitors (500 μM IBMX and 100 μM Ro 20–1724). When adenosine receptor ligands were tested, IBMX was omitted and the concentration of Ro 20–1724 was increased to 500 μM. cAMP accumulation was initiated by addition of the test compounds (100 μl) to the cell suspension for a period of 2 min, unless otherwise indicated, and terminated by addition of 100% (w/v) trichloroacetic acid (50 μl). Unlabeled cAMP (100 μl, 10 mM) was added, the mixture was centrifuged, and the [^{3}H] ATP and [^{3}H]cAMP content of the supernatant was quantified according to the method of Salomon *et al.* (19). The conversion of [^{3}H]ATP to cAMP was defined as: conversion = [^{3}H]cAMP (cpm)/[[^{3}H]ATP (cpm) + [^{3}H]cAMP (cpm)].

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in an H&L series 300 fluorimeter, essentially as described,[1] using the change in fluorescence ratio at 340 and 380 nm of fura-2. Briefly, cells were detached with phosphate-buffered saline containing 0.02% EDTA and were loaded with 3.5 μM fura-2 acetoxymethyl ester and 0.02% pluronic acid for 20 min at room temperature. After washing, the cells were kept at room temperature until use. An aliquot (~4 × 10^6 cells) was diluted with 3 ml of Krebs buffer (in mM: NaCl, 120; KCl, 4.75; KH_2PO_4, 1; $NaHCO_3$, 5; $MgSO_4$, 1.44; $CaCl_2$, 1.1; EGTA, 0.1; glucose, 11; HEPES, 25; adjusted to pH 7.4 with Tris), centrifuged, resuspended in 3 ml of Krebs buffer, and transferred to a stirred cuvette that was thermostatted at 30°. After equilibration for 1 min, a base line was recorded, after which additions were made by means of a Hamilton syringe that reached into the cuvette. Test compounds were added from a 100-fold concentrated stock. Extracellular fura-2 was estimated by addition of 1 mM $MnCl_2$, which immediately quenches fura-2 outside the cell, followed by a slower quenching of intracellular fura-2.[1] Ratios were converted to $[Ca^{2+}]_i$ values as described,[1] based on the formula of Grynkiewicz *et al.* (20), after correction for autofluorescence of the cells and differences in viscosity between the cytosol and calibration solutions (21).

Assessment of nucleotide purity by TLC. The purity of the nucleotide stock solutions was verified by TLC, using polyethyleneimine-cellulose as the stationary phase and 0.38 M potassium phosphate (pH 3.4) as the mobile phase. The nucleotides were visualized with UV light. In none of the nucleotide stock solutions kept on ice for several hours was a substantial degree of degradation detected.

To assess possible breakdown of the nucleotides during $[Ca^{2+}]_i$ measurements, the stability of ATP (used as a model compound) was studied under conditions similar to those used in $[Ca^{2+}]_i$ measurements, i.e., at 30° in the presence of cells. NCB-20 cells in Krebs buffer (~1.3 × 10^6 cells/ml, the cell density usually present in the cuvette) were incubated at 30° with 300 μM ATP, with a trace amount of [^{32}P]ATP. After 15, 60, and 300 sec, samples (100 μl) were removed and added to 50 μl of potassium phosphate (0.38 M, pH 3.4) at 0°. Samples were centrifuged for 5 min at 13,000 × *g*, and the supernatant was frozen on dry ice. The supernatant (5 μl) was analyzed by TLC, as described above. To quantitate the distribution of ^{32}P between ATP, ADP, AMP, and P_i, either the TLC plates were scanned in an AMBIS scanner or individual lanes were cut into sections (representing ATP, ADP, and AMP), eluted with HCl (1 ml, 0.1 mM), and counted after addition of 15 ml of liquid scintillation cocktail. Both methods for quantitation gave identical results. Under the conditions described above, 300 μM ATP was marginally (<10%) degraded after 15 sec or 1 min; after 5 min ~50% of the nucleotide was converted to ADP. In the absence of Mg^{2+}, ATP breakdown was more pronounced. After 1 min and 5 min approximately 20% and 80%, respectively, of the ATP added was degraded.

Results

P_2 Receptors in NCB-20 Cells and Effects on $[Ca^{2+}]_i$

Pharmacological characterization of the P_2 receptor in NCB-20 cells. ATP evoked a rapid rise in $[Ca^{2+}]_i$ in NCB-20 cells, as characterized in detail below. To identify the P_2 receptor that mediated the effect, a number of mono-, di-, and triphosphate nucleotides were screened, at a concentration of 100 μM. Among the adenine nucleotides, ATP and ATPγS were the most efficacious analogues, ADP, AMP-PNP, 2′-deoxy-ATP, and 2-CH_3S-ATP were less effective, and AMP and AMP-PCP were without effect at a concentration of 100 μM (Fig. 1). This activity profile does not correspond to that of either a P_{2x} or P_{2y} receptor. It has been suggested, however, that in some tissues limited effects of 2-CH_3S-ATP may result from a low intrinsic activity rather than a low affinity for P_{2y} receptors (22). To determine whether 2-CH_3S-ATP might be a partial agonist in this system, the compound (100 μM) was added either before or simultaneously with a submaximally

[1] Garritsen, A., and D. M. F. Cooper. Manipulation of intracellular calcium in NCB-20 cells. *J. Neurochem.* In press.

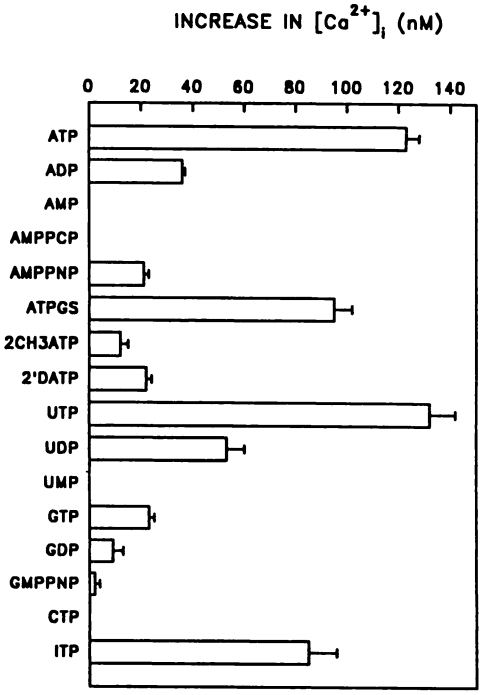

Fig. 1. Effects of various nucleotides on $[Ca^{2+}]_i$. The indicated compounds (100 μM final concentration) were added to fura-2-loaded cells, and $[Ca^{2+}]_i$ was determined as described in Experimental Procedures. The data, expressed as the increase in peak $[Ca^{2+}]_i$ (mean ± standard error), are the average of three to nine independent experiments (basal $[Ca^{2+}]_i$ levels were 97 ± 3 nM, over 11 experiments).

effective concentration of ATP. 2-CH_3S-ATP did not preclude the rise in $[Ca^{2+}]_i$ elicited by ATP (data not shown).

The structural requirements for P_2 receptor activation in NCB-20 cells were further studied by investigating the effect of nucleotides with bases other than adenine. UTP and, to a lesser extent, ITP, UDP, and GTP elevated $[Ca^{2+}]_i$. The following overall order of potency for the nucleotides, tested at a single concentration of 100 μM, was observed: UTP > ATP > ATPγS > ITP > UDP > ADP > GTP, AMP-PNP, 2′-deoxy-ATP > 2-CH_3S-ATP, GDP > GMP-PNP > AMP, AMP-PCP, CTP (inactive) (Fig. 1).

To obtain a more accurate structure-activity profile, concentration-response curves for the most efficacious nucleotides were determined (Fig. 2). UTP was the most potent agonist of this receptor, with an EC_{50} value of 6.2 ± 0.5 μM. Other nucleotides were less potent, with EC_{50} values of 17.3 ± 1.5 μM (ATP), 23 ± 3 μM (ATPγS), and 55 ± 4 μM (ITP). These compounds exerted maximal effects that were not different from that of UTP, and Hill coefficients were close to unity. Because ADP had not produced a maximal effect at the maximal concentration tested (300 μM), an EC_{50} value could not be calculated. UDP elicited a modest rise in $[Ca^{2+}]_i$, even at low concentrations; this elevation gradually increased to 50% of the increase induced by 100 μM UTP at 300 μM UDP.

In Krebs buffer, ATP is mainly present in the Mg^{2+}-bound form. In order to assess whether this was the form of the nucleotide responsible for the effect observed in NCB-20 cells, the effect of ATP was determined in Krebs buffer without Mg^{2+}. As shown in Fig. 3, 3, 10, and 30 μM ATP elicited considerably stronger effects in the absence of Mg^{2+} (compare Fig. 3, *upper* and *lower*). The difference between the effect of ATP in Mg^{2+}-containing and Mg^{2+}-free Krebs was virtually

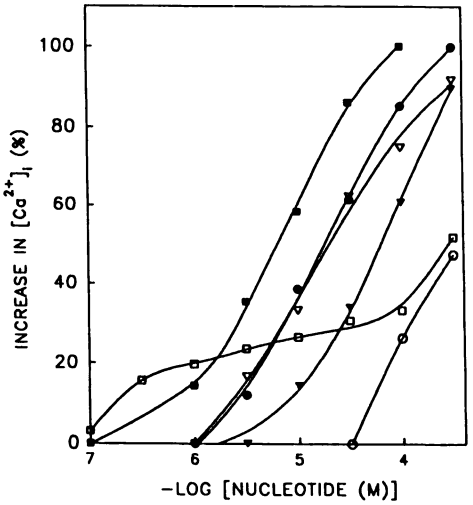

Fig. 2. Concentration-response relationship for the elevation of $[Ca^{2+}]_i$ by various nucleotides. The indicated concentrations of nucleotides were added, and $[Ca^{2+}]_i$ was measured as described in Experimental Procedures. The results were expressed as a percentage of the increase in $[Ca^{2+}]_i$ induced by 100 μM UTP. Data shown are the means of three or four experiments. ■, UTP; ●, ATP; ▽, ATPγS; ▼, ITP; □, UDP; ○, ADP.

absent at 100 μM. Thus, omission of Mg^{2+} resulted in an apparent increase in the affinity of the receptor for ATP.[2]

Source of Ca^{2+} mobilized by nucleotides. In order to identify the source of the Ca^{2+} mobilized by ATP in NCB-20 cells, compounds that reduce the availability of Ca^{2+} from the extracellular space or from intracellular stores were used.

First, the role of Ca^{2+} entry was investigated. Activation of receptors may enhance Ca^{2+} influx through voltage-sensitive or receptor-operated Ca^{2+} channels. To block Ca^{2+} entry, 2.5 mM EGTA was added to the cell suspension 15 sec before ATP. This addition reduced the extracellular Ca^{2+} concentration to ~100 nM. After addition of EGTA, the fluorescence ratio dropped immediately, due to the quenching of extracellular fura-2. When the data were corrected for the presence of extracellular fura-2 (see Experimental Procedures), the increase in $[Ca^{2+}]_i$ was identical in the absence or presence of EGTA (Fig. 4A).

The foregoing evidence, which indicates that Ca^{2+} entry plays no role in the $[Ca^{2+}]_i$ elevation induced by ATP, suggests that the major source of elevated $[Ca^{2+}]_i$ is IP_3-sensitive intracellular stores. The P_{2u} receptor increases phosphoinositide turnover in other cell lines (14, 23, 24). These IP_3-sensitive stores can be depleted by thapsigargin, a Ca^{2+}-ATPase inhibitor that specifically blocks Ca^{2+} uptake into nonmitochondrial stores (25). In NCB-20 cells, the initial effect of thapsigargin was to elevate $[Ca^{2+}]_i$ (Fig. 4B). After 3 min of exposure to thapsigargin, addition of ATP did not elevate $[Ca^{2+}]_i$ further (Fig. 4B), suggesting that the P_{2u} receptor recruits Ca^{2+} from thapsigargin-sensitive stores.

In NCB-20 cells, BK also elevates $[Ca^{2+}]_i$ by release of Ca^{2+}

[2] In Mg^{2+}-free incubation medium the effect of 300 μM ATP became biphasic; after a fast transient increase in $[Ca^{2+}]_i$, a sustained increase in fura-2 fluorescence was observed. In contrast, the increase in $[Ca^{2+}]_i$ induced by UTP in Mg^{2+}-free buffer was monophasic at all concentrations tested (data not shown). The persistent elevation of fura-2 fluorescence might be due to ATP-induced permeabilization or lysis of cells, possibly mediated by a P_{2z} receptor (at which UTP would not be effective). The fact that metabolism of ATP was more rapid in Mg^{2+}-free than in Mg^{2+}-containing buffer (see Experimental Procedures) suggests that cell lysis, which would allow intracellular nucleotidases to contribute to ATP degradation, occurs.

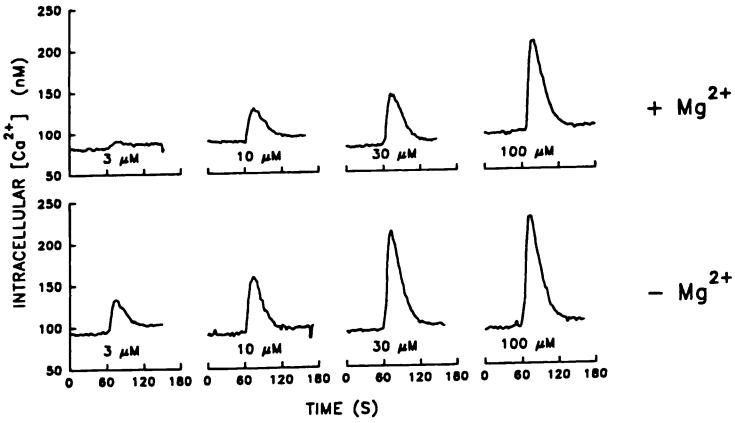

Fig. 3. Ca^{2+} mobilization by ATP in NCB-20 cells, in the absence and presence of Mg^{2+}. The indicated concentrations of ATP were added to fura-2-loaded cells suspended in Mg^{2+}-containing (*upper*) or -free (*lower*) Krebs buffer, and $[Ca^{2+}]_i$ was measured. Results are from a representative experiment that was repeated with virtually identical results.

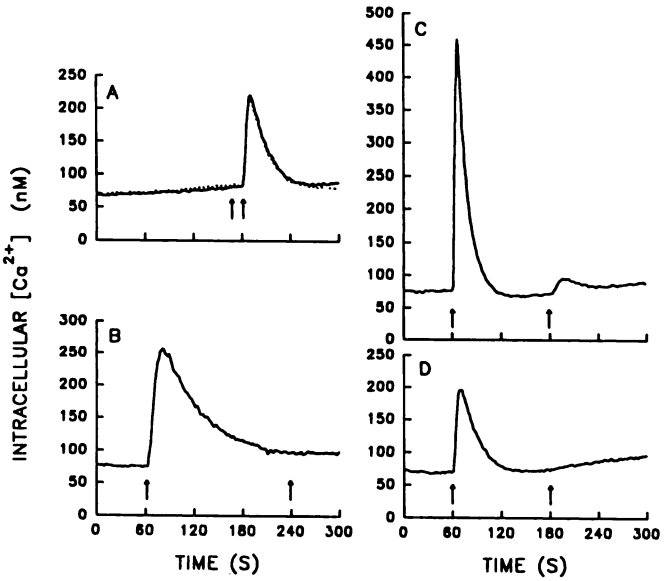

Fig. 4. Effects of EGTA, thapsigargin, BK, and UTP on the response to ATP. Fura-2-loaded cells were exposed (*first arrow*) to 2.5 mM EGTA for 15 sec (A) (*dotted line*), 1 μM thapsigargin for 3 min (B), 1 μM BK for 2 min (C), or 100 μM UTP for 2 min (D), after which 100 μM ATP was added (*second arrow*). *Solid line in A*, response to 100 μM ATP under control conditions. The traces are from a representative experiment that was repeated with identical results. $[Ca^{2+}]_i$ values were corrected for 8% extracellular fura-2, estimated as described in Experimental Procedures.

from IP_3-sensitive stores (26).[1] ATP and UTP were less effective than BK in elevating $[Ca^{2+}]_i$ (see Fig. 4, A and C); the maximal response of UTP was 36 ± 3% of that elicited by BK in the same experiments. BK and ATP, although interacting with distinct receptors, may recruit Ca^{2+} from the same intracellular Ca^{2+} stores. To study the relation between the Ca^{2+} pools that are the targets of BK and ATP, a maximally effective concentration of BK (1 μM) was added 2 min before 100 μM ATP (Fig. 4C). Such pretreatment largely precluded the $[Ca^{2+}]_i$ elevation elicited by ATP, although a small effect of ATP remained. In similar experiments, identical results were obtained with UTP (data not shown). In contrast to the partial inhibition of the ATP effect by BK, addition of a maximally effective concentration of UTP (100 μM) completely blocked a subsequent elevation of $[Ca^{2+}]_i$ by ATP (Fig. 4D). Given that (i) ATP and UTP elicit the same elevation in $[Ca^{2+}]_i$, which is only 36% of that elicited by BK, and (ii) pretreatment with UTP precludes a subsequent response to ATP, these data are consistent with the suggestion that UTP and ATP act on the same receptors and Ca^{2+} pools.

Role of PTX-sensitive G proteins. In HL-60 cells, ATP and UTP increase $[Ca^{2+}]_i$ via a P_{2u} receptor coupled to a PTX-sensitive G protein (27). To investigate the role of PTX substrates in P_2 receptor-mediated Ca^{2+} mobilization in NCB-20 cells, cells were pretreated for 18 hr with 125 ng/ml PTX. This treatment did not affect the response to either ATP or UTP (or BK[1]) at maximal or submaximal concentrations, although it was fully capable of blocking muscarinic receptor-mediated inhibition of cAMP accumulation (data not shown).

Effects of Nucleotides on cAMP Accumulation

Another second messenger system whose activity may be modulated by P_2 receptor activation is adenylyl cyclase. It is difficult to determine the effects of nucleotides on adenylyl cyclase activity in broken cell preparations, because many of these compounds serve not only as receptor ligands but also as substrates for the enzyme. In the intact cell, it may be assumed that effects of nucleotides are mediated by cell surface receptors, because the highly charged compounds cannot enter the cell. Therefore, such studies can indicate whether cAMP generation may be modulated by P_2 receptors.

ATP and UTP significantly inhibited cAMP accumulation stimulated by 10 μM prostaglandin E_1, whereas AMP-PCP and 2-CH_3S-ATP were without effect on cAMP accumulation (Fig. 5). Inhibition by ATP and UTP was slightly less than that induced by 2 μM BK. To avoid possible interference from P_1 receptor activation (see below), the assays were performed in the presence of adenosine deaminase (2 units/ml) and IBMX (500 μM).

From concentration-response curves of the inhibition of cAMP accumulation by nucleotides, IC_{50} values of about 10 and 30 μM for UTP and ATP, respectively, were estimated (data not shown). This agonist potency corresponds to that for the elevation of $[Ca^{2+}]_i$. Recently, we demonstrated that Ca^{2+} can inhibit adenylyl cyclase activity in plasma membranes from NCB-20 cells (26). Such an interaction could be a powerful feedback mechanism between these two second messenger systems in the intact cell. Indeed, BK inhibits cAMP accumulation in intact NCB-20 cells in a transient manner (26), apparently as a consequence of its elevation of $[Ca^{2+}]_i$.[3] To determine

[3] A. Garritsen, Y. Zhang, J. A. Firestone, M. D. Browning, and D. M. F. Cooper. Inhibition of cAMP accumulation in intact NCB-20 cells as a direct result of elevation of $[Ca^{2+}]_i$. Submitted for publication.

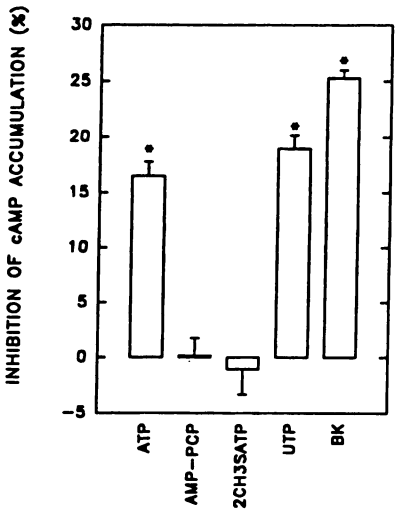

Fig. 5. Effect of nucleotides on cAMP accumulation in NCB-20 cells. Conversion of ATP to cAMP was measured as described in Experimental Procedures. Cells were stimulated for 1 min with 10 μM prostaglandin E_1 and the indicated nucleotides (100 μM) or 2 μM BK, in the presence of 100 μM Ro 20–1724, 500 μM IBMX, and 2 units/ml adenosine deaminase.

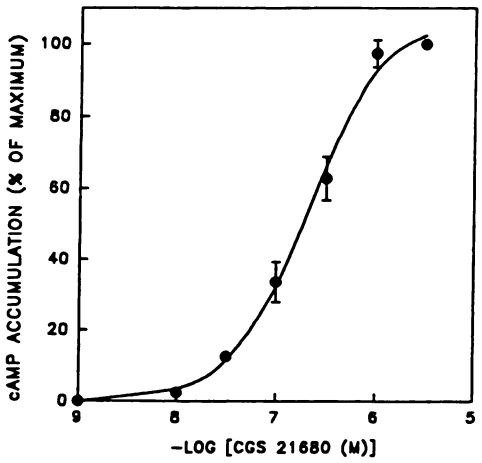

Fig. 6. Concentration-response curve for stimulation of cAMP accumulation by CGS21680 in NCB-20 cells. Conversion of [^{3}H]ATP to cAMP was measured over 3 min, as described in Experimental Procedures, in the presence of 20 μM forskolin, 500 μM Ro 20–1724, 2 units/ml adenosine deaminase, and the indicated concentrations of CGS21680. Data were expressed as a percentage of the maximal stimulation; the average ± standard error of three experiments performed in triplicate is shown. One hundred percent corresponds to an increase in conversion of ATP to cAMP from 2.0 ± 0.4% to 7.9 ± 0.4%.

whether this interaction may also underlie the inhibition of cAMP accumulation by ATP and UTP, cells were pretreated for 3 min with 1 μM thapsigargin, a treatment that precludes a subsequent elevation of $[Ca^{2+}]_i$ by ATP (Fig. 4B). Such pretreatment eliminated the inhibitory effect of ATP and UTP on cAMP accumulation (data not shown).

P_1 Receptors in NCB-20 Cells

Because adenosine is a degradation product of ATP, it was important to evaluate the potential contribution of P_1 receptor stimulation to the observed P_2 effects on cAMP and $[Ca^{2+}]_i$. NCB-20 cells were stimulated with *R*-PIA, a prototypic A_1 agonist (28). No inhibition of cAMP accumulation, as would be expected from activation of adenosine A_1 receptors, was observed at any concentration between 1 nM and 10 μM (data not shown). Moreover, in membrane preparations of NCB-20 cells, neither inhibition of adenylyl cyclase activity by *R*-PIA nor binding of the selective A_1 antagonist 8-[^{3}H]cyclopentyl-1,3-dipropylxanthine was detectable (data not shown). Instead, at concentrations of >1 μM, cAMP accumulation was enhanced, suggesting the activation of A_2 receptors. The presence of A_2 receptors was confirmed by studies with CGS21680, a selective A_2 receptor agonist (29) that stimulated conversion of ATP to cAMP approximately 4-fold in intact cells, with an EC_{50} value of 190 nM (Fig. 6).

To probe the involvement of P_1 receptors in Ca^{2+} mobilization, *R*-PIA and CGS21680 were added to fura-2-loaded NCB-20 cells and $[Ca^{2+}]_i$ was measured. Both compounds were without effect on $[Ca^{2+}]_i$ (data not shown).

Discussion

Characterization of P_2 receptors in NCB-20 cells. ATP elicits a rapid increase in $[Ca^{2+}]_i$ in NCB-20 cells. Of the ATP analogues tested, AMP-PCP and 2-CH_3S-ATP (compounds that are potent at P_{2x} and P_{2y} receptors, respectively) (15) were virtually inactive at elevating $[Ca^{2+}]_i$; this observation suggests that the purinergic receptor involved is neither of the P_{2x} nor of the P_{2y} subtype.

Recently, a distinct subtype of P_2 receptors has been recognized, which is sensitive to uracil nucleotides (16). Because in our studies UTP was more potent than ATP, the characteristics of the P_2 receptor in NCB-20 cells correspond most closely to those of this P_{2u} (14) or 'nucleotide' receptor (16), as has been encountered in pituitary cells (23), human fibroblasts (24), and a variety of leukocytes (27). It is not clear whether in other systems this P_{2u} receptor mediates the effects of ATP, because the effects of UTP have not been studied in most cases.

The following structure-activity profile of the receptor emerges from the compounds examined. (i) Modifications in the triphosphate chain almost always result in a decrease in activity. Replacement of the β-γ oxygen by an imido (AMP-PNP) or methylene (AMP-PCP) group largely reduces activity, although the imido group is better tolerated. Removal of phosphate groups progressively reduces the efficacy of the compounds (ATP > ADP > AMP and UTP > UDP > UMP). The only modification tolerated is the replacement of an ionized phosphate oxygen with sulfur (ATPγS). (ii) The only analogue tested with a modification in the ribose ring (2′-deoxy-ATP) is a very weak agonist. (iii) Substitution of the amino group at C6 with a hydroxyl group (compare ATP with ITP) results in a slightly lower affinity in the case of purines. However, an apparently similar modification in the pyrimidines (compare CTP with UTP) changes a completely inactive compound into the most active in the series of nucleotides tested. This suggests that the six-membered rings of the pyrimidines and purines interact with different domains of the receptor. (iv) Substitution on the 2-position in the purines reduces activity (compare ATP with 2-CH_3S-ATP and ITP with GTP).

Mg-ATP^{2-} is the substrate for enzymatic reactions utilizing ATP, whereas ATP^{4-} is the active form for permeabilization of the plasma membrane in mast cells (30). It has not yet been firmly established which form mediates P_{2x}, P_{2y}, or P_{2u} receptor effects. At increasing $^{45}Ca^{2+}$ efflux in human fibroblasts (24), elevating inositol phosphates in pituitary cells (23), and elevating $[Ca^{2+}]_i$ in NCB-20 cells (the present study), the unchelated

form of ATP is somewhat more potent, and this could thus be the preferred ligand for the P_{2u} receptor. However, it would be anticipated that Mg-ATP^{2-} is the major receptor ligand *in vivo*.

Characterization of Ca^{2+} mobilization by P_{2u} receptor activation. ATP rapidly elevates $[Ca^{2+}]_i$. Chelation of extracellular Ca^{2+} with EGTA did not alter the response, suggesting that Ca^{2+} entry does not play a significant role. Because the response to the nucleotides can be eliminated by pretreatment with the Ca^{2+}-ATPase inhibitor thapsigargin, thapsigargin-sensitive intracellular Ca^{2+} pools appear to be the source of Ca^{2+} mobilized by nucleotides.

Recently, we characterized Ca^{2+} mobilization in NCB-20 cells by BK,[1] which also releases Ca^{2+} from intracellular stores. BK is more effective in elevating $[Ca^{2+}]_i$ than are the nucleotides examined presently. Although prior addition of BK largely reduced the response to ATP, it did not eliminate it, suggesting that the Ca^{2+} stores accessed by ATP and UTP are functionally related to those triggered by BK, but not identical. In contrast, ATP and UTP elicited identical increases in $[Ca^{2+}]_i$, and prior addition of UTP eliminated the effect of ATP, suggesting that these compounds mobilize Ca^{2+} from identical stores. Although it is difficult to establish unequivocally in functional studies, it is likely that ATP and UTP act at the same receptor in NCB-20 cells.

Further characterization of the P_{2u} receptor-effector coupling showed that the G protein involved in NCB-20 cells is insensitive to PTX, whereas in HL-60 (27) and human fibroblasts (24) responses to ATP or UTP are partially reduced by PTX pretreatment. This diversity indicates that, in different cell types, various G proteins, both PTX sensitive and insensitive, mediate the signal-transduction pathway of the P_{2u} receptor.

Effect of P_2 and P_1 receptor activation on cAMP accumulation in intact NCB-20 cells. Because adenosine is a metabolite of ATP, it was critical to evaluate whether P_1 purinergic receptors are present in NCB-20 cells and whether such receptors might confound P_2 receptor-mediated effects. Indeed, NCB-20 cells express not only P_{2u} receptors but also adenosine A_2 receptors, which are coupled to stimulation of cAMP accumulation. CGS21680, a selective adenosine A_2 agonist (29), elevates cAMP levels ~4-fold, with an EC_{50} value around 200 nM. This value is rather high, compared with the IC_{50} value of 22 nM for inhibition of [^{3}H]5′-*N*-ethylcarboxamide adenosine binding in rat striatal membrane preparations (29), but the different conditions under which our experiments were performed (intact cells, physiological buffers) could account for such discrepancies.

In order to observe the effects of ATP on cAMP accumulation, it was crucial to block adenosine A_2 receptors. In the presence of IBMX and adenosine deaminase, both ATP and UTP inhibited cAMP accumulation, whereas AMP-PCP and 2-CH_3S-ATP were without effect. The effects of ATP and UTP occurred in similar concentration ranges as the elevation of $[Ca^{2+}]_i$, with UTP being slightly more potent than ATP. This suggests that the same receptor subtype is involved in Ca^{2+} mobilization and inhibition of cAMP accumulation. Recently, we demonstrated that in the intact cell BK can inhibit cAMP accumulation in a transient, intracellular Ca^{2+}-dependent manner (26).[3] ATP and UTP also inhibited cAMP accumulation transiently.[3] The linkage between Ca^{2+} mobilization by the nucleotides and their inhibition of cAMP accumulation is supported by the fact that blockade of the Ca^{2+} signal by pretreatment with thapsigargin eliminated the inhibition of cAMP accumulation. Therefore, this inhibitory effect probably reflects a direct effect of Ca^{2+} on the catalytic unit of adenylyl cyclase, a novel mechanism for inhibition of adenylyl cyclase on which we have reported in detail elsewhere (26).[3]

In conclusion, NCB-20 cells express both P_1 and P_2 purinergic receptors. Adenosine A_2 receptors increase cAMP accumulation, whereas the P_2 receptor elevates $[Ca^{2+}]_i$ in a PTX-insensitive manner and inhibits cAMP accumulation in an indirect, Ca^{2+}-mediated manner. Pharmacological characterization of the P_2 receptor demonstrates that a receptor is involved that is sensitive not only to adenine nucleotides but also to uracil and guanine nucleotides, i.e., the putative P_{2u} receptor. These studies extend the identification of P_{2u} receptors to NCB-20 cells and add to the perception that P_2 receptors have a widespread distribution. It is possible that P_{2u} and A_2 receptors are spatially separated on neurons, providing, for example, distinct pre- and postsynaptic mechanisms. On some cells, however, ATP and its metabolite adenosine may act sequentially. The elevation of $[Ca^{2+}]_i$ induced by ATP may be followed by a subsequent increase in cAMP induced by adenosine, which is formed from ATP with time. The precise mechanisms whereby intracellular signal transduction is controlled by purinergic receptors must be determined in individual situations.

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References

1. Gerlach, E., and B. F. Becker. *Topics and Perspectives in Adenosine Research.* Springer, Heidelberg (1987).
2. Dubyak, G. R., and J. S. Fedan. Biological actions of extracellular ATP. *Ann. N. Y. Acad. Sci.* **603**:1–542 (1990).
3. Burnstock, G. Purinergic nerves and receptors. *Prog. Biochem. Pharmacol.* **16**:141–154 (1980).
4. Drury, A. N., and A. Szent-Györgyi. The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J. Physiol. (Lond.)* **68**:213–237 (1929).
5. Van Calker, D., M. Müller, and B. Hamprecht. Adenosine regulates, via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.* **33**:999–1005 (1979).
6. Londos, C., D. M. F. Cooper, and J. Wolff. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA* **77**:2551–2554 (1980).
7. Cooper, D. M. F., and K. K. Caldwell. Signal transduction mechanisms for adenosine, in *Adenosine and Adenosine Receptors* (M. Williams, ed.). Humana Press Inc., Clifton, NJ, 105–141 (1990).
8. Jacobson, K. A. Adenosine (P1) and ATP (P2) receptors, in *Comprehensive Medicinal Chemistry* (C. Hansch, P. G. Sammes, J. E. Taylor, and J. C. Emmet, eds.), Vol. 3. Pergamon, Oxford, UK, 601–642 (1990).
9. Libert, F., M. Parmentier, A. Lefort, C. Dinsart, J. Van Sande, C. Maenhaut, M.-J. Simons, J. E. Dumont, and G. Vassart. Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science (Washington D. C.)* **244**:569–572 (1989).
10. Maenhaut, C., J. Van Sande, F. Libert, M. Abramowicz, M. Parmentier, J. J. Vanderhaegen, J. E. Dumont, G. Vassart, and S. Schiffman. RDC8 codes for an A_2 adenosine receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* **173**:1169–1178 (1990).
11. Mahan, L. C., L. D. McVittie, E. M. Smyk-Randall, H. Nakata, F. J. Monsma, C. R. Gerfen, and D. R. Sibley. Cloning and expression of an A1 adenosine receptor from rat brain. *Mol. Pharmacol.* **40**:1–7 (1991).
12. Burnstock, G. Purinergic nerves. *Pharmacol. Rev.* **24**:509–581 (1972).
13. Cusack, N. J., and S. M. O. Hourani. Subtypes of P_2-purinoceptors: studies using analogues of ATP. *Ann. N. Y. Acad. Sci.* **603**:172–181 (1990).
14. Dubyak, G. R. Signal transduction by P_2-purinergic receptors for extracellular ATP. *Am. J. Respir. Cell Mol. Biol.* **4**:295–300 (1991).
15. Harden, T. K., J. L. Boyer, H. A. Brown, C. L. Cooper, R. A. Jeffs, and M. W. Martin. Biochemical properties of a P_{2y}-purinergic receptor. *Ann. N. Y. Acad. Sci.* **603**:256–266 (1990).
16. O'Connor, S. E., I. A. Dainty, and P. Leff. Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.* **12**:137–141 (1991).
17. Nirenberg, M., S. Wilson, H. Higashida, A. Rotter, K. Krueger, N. Busis, R.

Ray, J. G. Kenimer, and M. Adler. Modulation of synapse formation by cyclic adenosine monophosphate. *Science (Washington D. C.)* **222**:794–799 (1983).
18. Evans, T., M. M. Smith, L. I. Tanner, and T. K. Harden. Muscarinic cholinergic receptors of two cell lines that regulate cyclic AMP metabolism by different molecular mechanisms. *Mol. Pharmacol.* **26**:395–404 (1984).
19. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
20. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
21. Poenie, M. Alteration of intracellular fura-2 fluorescence by viscosity: a simple correction. *Cell Calcium* **11**:85–91 (1990).
22. Needham, L., N. J. Cusack, J. D. Pearson, and J. L. Gordon. Characteristics of the P_2 purinoceptor that mediates prostacyclin production by pig aortic endothelial cells. *Eur. J. Pharmacol.* **134**:199–209 (1987).
23. Davidson, J. S., I. K. Wakefield, U. Sohnius, P. A. van der Merwe, and R. P. Millar. A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. *Endocrinology* **126**:80–87 (1990).
24. Fine, J., P. Cole, and J. S. Davidson. Extracellular nucleotides stimulate receptor-mediated calcium mobilization and inositol phosphate production in human fibroblasts. *Biochem. J.* **263**:371–376 (1989).
25. Thastrup, O. Role of Ca^{2+}-ATPases in regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal Ca^{2+}-ATPase inhibitor thapsigargin. *Agents Actions* **29**:8–15 (1990).
26. Boyajian, C., A. Garritsen, and D. M. F. Cooper. Bradykinin stimulates Ca^{2+} mobilization in NCB-20 cells, leading to direct inhibition of adenylyl cyclase. *J. Biol. Chem.* **266**:4995–5005 (1991).
27. Dubyak, G. R., and D. S. Cowen. Activation of inositol phospholipid-specific phospholipase C by P_2-purinergic receptors in human phagocytic leukocytes. *Ann. N. Y. Acad. Sci.* **603**:227–245 (1990).
28. Bruns, R. F., G. H. Lu, and T. A. Pugsley. Molecular characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol. Pharmacol.* **29**:331–346 (1986).
29. Hutchison, A. J., R. L. Webb, H. H. Oei, G. R. Ghai, M. B. Zimmerman, and M. Williams. CGS 21690C, an A_2 selective adenosine receptor agonist with preferential hypotensive activity. *J. Pharmacol. Exp. Ther.* **251**:47–55 (1989).
30. Cockcroft, S., and B. D. Gomperts. The ATP^{4-} receptor of rat mast cells. *Biochem. J.* **188**:789–798 (1980).

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