0026-895X/98/010022-11$3.00/0

MOLECULAR PHARMACOLOGY, **54**:22–32 (1998).

Isoquinolines as Antagonists of the $P2X_7$ Nucleotide Receptor: High Selectivity for the Human versus Rat Receptor Homologues

BENJAMIN D. HUMPHREYS, CATERINA VIRGINIO, ANNMARIE SURPRENANT, JANET RICE, and GEORGE R. DUBYAK

Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106 (B.D.H., J.R., G.R.D.), and Glaxo Institute for Molecular Biology, Plan-les-Ouates, 1228 Geneva, Switzerland (C.V., A.S.)

Received January 14, 1998; Accepted March 11, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

1-[*N*,*O*-Bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) and *N*-[1-[*N*-methyl-*p*-(5 isoquinolinesulfonyl)benzyl]-2-(4 phenylpiperazine)ethyl]-5-isoquinolinesulfonamide (KN-04) potently inhibit the human lymphocyte P2Z receptor, an ATP-gated cation channel [*Br J Pharmacol* **120**:1483–1490 (1997)]. Although the molecular identity of the lymphocyte P2Z receptor has not been established, it shares many functional characteristics with the cloned $P2X_7$ nucleotide receptor. We have tested whether these isoquinolines inhibit P2X receptor function in human embryonic kidney 293 cells that stably express the human or rat recombinant $P2X_7$ receptors. ATP activation of cation currents and uptake of the organic dye ethidium were potently inhibited by KN-62 and KN-04 in human embryonic kidney cells expressing the human $P2X_7R$ but not the rat $P2X_7R$, even though these species homologues share 80% amino acid identity. Introduction of the first 335 amino acids of the human $P2X_7R$ sequence conferred KN-62 sensitivity to the rat $P2X_7R$; this suggests that isoquinolines interact with residues in the amino-terminal half (containing the large extracellular loop) of the human $P2X_7R$. KN-62 and KN-04 also potently inhibited ATP-gated Ca^{2+} influx and ethidium uptake in several leukocyte cell lines (THP-1, BAC1.2f5, and BW5147) that natively express the human or murine $P2X_7R$ mRNA. The ability of isoquinoline sulfonamides to potently inhibit human and murine $P2X_7R$ signaling will be a useful tool for identifying $P2Z/P2X_7$ functional responses in other cell types. The substantial differences in pharmacological sensitivity between rat and human $P2X_7R$ may also indicate structural domains important in channel/pore activation.

P2 nucleotide receptors mediate the biological responses to extracellular ATP in a wide variety of tissues and cell types (Dubyak and El-Moatassim, 1994). The two major subgroups that compose the P2 receptor family include the metabotropic P2Y receptors and the seven different ionotropic P2X receptors ($P2X_{1-7}$). The similarities and differences among the P2X receptor subtypes have been reviewed (Buell *et al*., 1996; Evans *et al*., 1998). The most recently cloned P2X receptor is the $P2X_7R$ that acts as rapidly gated cation channel in the presence of high concentrations of extracellular ATP. In contrast with the other six P2X receptor subtypes, the $P2X_7R$ also forms a pore permeable to large organic molecules such as ethidium (342 Da) and YoPro-1 (629 Da). The long, cytoplasmic carboxyl-terminal tail of the $P2X_7R$ is required for this permeability to large molecules, but not small cations, and this region is absent from the $P2X_{1-6}$ receptor subtypes (Surprenant *et al*., 1996, Rassendren *et al*., 1997).

The absence of high potency antagonists traditionally has limited the identification and characterization of P2 nucleotide receptors in cells and tissues. Recently, Gargett and Wiley (1997) described a novel class of P2 receptor inhibitors: the isoquinoline derivatives KN-62 [1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine] and KN-04 [*N*-[1-[*N*-methyl-*p*-(5 isoquinolinesulfonyl)benzyl]-2-(4 phenylpiperazine)ethyl]-5-isoquinolinesulfonamide]. Submicromolar levels of these drugs inhibited the ability of ATP to stimulate cation influx, pore formation, phospholipase D activation, and shedding of L-selectin in human chronic lymphocytic leukemia cells. These ATP-dependent responses in lymphocytes are mediated by the P2Z receptor, a functionally defined P2 receptor that shares many properties with the

This work was supported in part by National Institutes of Health Grant GM36387 (G.R.D.).

Portions of this work were presented at a meeting for the American Society of Biology and Molecular Biology (San Francisco, CA) and at Purines '97 (New Orleans, LA).

ABBREVIATIONS: $P2X_7R$, $P2X_7$ receptor; $rP2X_7$, rat $P2X_7$; $hP2X_7$, human $P2X_7$; HEK, human embryonic kidney; CamKII, calcium/calmodulin-dependent protein kinase II; Bz, 3′-*O*-(4-benzoyl)benzoyl; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon-γ; LPS, lipopolysaccharide; IL-1β, interleukin-1β; CLL, chronic lymphocytic leukemia; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

cloned P2X$_7$R (Wiley *et al*., 1990; Gargett *et al*., 1996; Jamieson *et al*., 1996). Although KN-62 is a potent inhibitor of CamKII (Tokumitsu *et al*., 1990), Gargett and Wiley (1997) observed that KN-04, an inactive analog of KN-62, also blocked the P2Z responses in lymphocytes. This indicated that inhibition of the P2Z receptor by KN-62 is not mediated by CamKII. Previous studies have demonstrated that P2Z receptor signaling in nonlymphoid cell types also can be antagonized by isoquinolines. Blanchard *et al*. (1995) reported that KN-62 potently suppressed ATP-induced cytolysis of THP-1 monocytes and monocyte-derived human macrophages. We similarly demonstrated that KN-62 inhibits the ability of P2Z receptors to activate phospholipase D in THP-1 monocytes (Humphreys and Dubyak, 1996).

These various data indicate that isoquinolines are potent inhibitors of P2Z receptor signaling in human hematopoietic cell types. However, the molecular mechanism or mechanisms by which KN-62 inhibits P2Z receptor signaling remain undefined. Although expression of recombinant P2X$_7$R cDNA is sufficient to confer P2Z-like phenotypes to nonhematopoietic cells, it is not known whether signaling by native P2Z receptors also might involve cell-specific modulation of P2X$_7$R structure or function. For example, recombinant P2X$_7$R (human or rat) form pores that readily admit ethidium (342 Da) and the propidium dye Yo-Pro1 (629 Da). In contrast, the P2ZR pore in human leukemic lymphocytes passes ethidium but excludes propidium (414 Da) (Wiley *et al*., 1993). The P2ZR pore in murine thymocytes is even more restrictive given its low permeability to ethidium (Pizzo *et al*., 1991). The factors that underlie these altered permeation characteristics of some P2Z receptors are not known but could indicate the presence of altered subunit interactions between P2X$_7$Rs and other leukocyte proteins or unique posttranslational regulation of the P2X$_7$R when expressed in hematopoietic cells. The multiexon organization of the human P2X$_7$R gene (GenBank accession numbers Y12851, Y12852, Y12853, Y12854, Y12855) raises the additional possibility that some cell types might express variant P2X$_7$ proteins encoded by alternatively spliced transcripts.

The current study was performed to determine the effect of isoquinoline derivatives on P2X$_7$R function in (1) cells expressing only full-length recombinant P2X$_7$Rs in the absence of other P2X family members and (2) various human and murine cell types that natively express both P2X$_7$R mRNA and P2Z-like phenotypes. To establish cell lines expressing homomeric P2X$_7$R, we stably transfected the rat and human P2X$_7$ cDNAs into HEK 293 cells that lack expression of P2X$_{1-7}$. Our results indicate that homomeric human P2X$_7$Rs are potently inhibited by the isoquinoline derivatives. However, we also identified a striking and unexpected difference in drug sensitivity between human and rat P2X$_7$Rs, and chimeric molecules were constructed to explore further the structural requirements for inhibition of huP2X$_7$ by the isoquinolines. Because detailed structure-function studies have not yet been performed on P2X$_7$Rs, little is known about the regions involved in nucleotide binding or channel/pore activation. Our results suggest that the isoquinoline derivatives will be useful pharmacological tools both for identifying THAT$_7$R function in human cell types and for investigating the structural determinants of THAT$_7$R activation.

Experimental Procedures

Materials. Cell culture medium was purchased from Sigma Chemie (Deisenhofen, Germany). KN-62 was from BIOMOL Research Laboratories (Plymouth Meeting, PA), and KN-04 was the generous gift of Dr. R. Ganapathi (Cleveland Clinic Foundation, Cleveland, OH). Lipofectamine was from GIBCO BRL (Gaithersburg, MD), and G418 was from Genentech (South San Francisco, CA). Recombinant human IFN-γ was a generous gift from Genentech. LPS was from List Biochemicals (Campbell, CA). Dr. G. Buell (Glaxo Institute for Molecular Biology, Geneva, Switzerland) kindly provided the human and rat THAT$_7$ cDNAs. BzATP was obtained from Sigma or synthesized as described previously (Williams and Coleman, 1982; Erb *et al*., 1990).

Cell culture. THP-1 cells (American Type Culture Collection, Rockville, MD) were cultured in Iscove's modified Dulbecco's medium plus 10% iron-supplemented newborn calf serum (Hyclone Laboratories, Logan, UT). At the initiation of culture, cell density was 3×10^5/ml, and medium was changed when cells reached 1×10^6/ml (every ~3 days). For experiments involving IFN-γ or LPS, cells were plated onto 100-mm culture dishes (10 ml) at a cell density of 1×10^6/ml in fresh medium. IFN-γ (1000 units/ml) or LPS (1 μg/ml) was added from sterile stocks for 2 or 3 days, as indicated. The BAC1.2f5 macrophage cell line, a clone of the SV40 transformed murine macrophage cell line BAC1, was maintained using protocols described previously (El Moatassim and Dubyak, 1992). Murine BW5147 cells, a thymocyte line, were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% iron-supplemented newborn calf serum (Hyclone). They were split every three days. HEK 293 cells (a generous gift from Dr. Cathy Carlin) were maintained in Dulbecco's minimal essential medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% iron-supplemented newborn calf serum (Hyclone). Stably transfected HEK lines were obtained using Lipofectamine according to the protocol supplied by the manufacturer (GIBCO BRL) or by electroporating 1×10^7 cells with 20 μg of DNA at 300 mV and 500 μF with a time constant of 6–8 msec (Gene Pulser II; BioRad, Hercules, CA). G418 (19 mg/ml) was added 2 days after transfection, and >100 G418-resistant colonies were pooled 2 weeks later. The stably transfected cell lines were maintained in G418-containing medium.

Construction of P2X$_7$R chimeras. A *Hin*dIII/*Bsr*G1 fragment corresponding to the 5′ end of each cDNA was excised and subcloned into the opposite plasmid. This resulted in two chimeras, hu-ratP2X$_7$ (human 1–335, rat 336–595) and rat-huP2X$_7$ (rat 1–335, human 336–595). Sequencing of these recombinant receptor DNAs confirmed the expected chimeric sequences of nucleotides. Stably transfected HEK 293 cell lines were obtained by electroporation of 20 μg of DNA followed by selection in G418.

Intracellular Ca^{2+} concentration measurements. Intracellular Ca^{2+} concentration was measured using the fluorescent indicator Fura-2 in a thermostatically controlled and magnetically stirred fluorimeter exactly as described (El-Moatassim and Dubyak, 1992).

Changes in plasma membrane permeability. ATP or BzATP-activated pore function was measured with the fluorescent dye ethidium bromide. Cells (1.5 ml) of resuspended in divalent cation-free basal salt solution were incubated in the fluorimeter cuvette (37°) at a final concentration of 0.5×10^6 cells/ml. Ethidium bromide was added to a final concentration of 20 μM. Cells were allowed to equilibrate for 10 min and then stimulated with nucleotide agonists, and the fluorescence was monitored at wavelengths of 360 nm excitation and 580 nm emission. In each assay, maximum cell permeabilization/ethidium uptake was defined as the fluorescence value achieved by adding digitonin (20 μg/ml) to the cuvette. Agonist-stimulated fluorescence increases were quantified as the fraction of maximum fluorescence attained 3 min after nucleotide addition. These measurements were expressed as percentages by normalizing

to the maximal changes in fluorescence triggered by 1 mM ATP or 100 μM BzATP.

Electrophysiology. HEK 293 cells stably or transiently expressing the rat, human, or chimeric $P2X_7R$ cDNAs were used in all electrophysiological experiments (Surprenant *et al.*, 1996; Rassendren *et al.*, 1997). Standard whole-cell recordings were obtained using an EPC9 patch-clamp amplifier and Pulse acquisition software (HEKA, Lambrecht, Germany). Patch pipettes (4–7 MΩ) were filled with 165 mM NaCl, 10 mM HEPES, and 11 mM EGTA. Standard extracellular solution was 154 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 12 mM glucose. "Low divalent" solution contained no added $MgCl_2$ and 0.1 mM $CaCl_2$. All solutions were maintained at pH 7.3 and 300–310 mOsmol/liter. Agonists were applied using a fast-flow U-tube delivery system (Fenwick *et al.*, 1982). Antagonists were present in both superfusion and fast-flow solutions. Results are shown as mean ± standard error.

Semiquantitative RT-PCR. Total RNA was isolated from THP-1, BAC1.2f5, and BW5147 cultures by the acid-guanidinium thiocyanate extraction method (Chomczynski and Satchi, 1987). RNA (1.0 μg) was reverse transcribed to cDNA in a 20-μl reaction volume containing 0.5 μg of oligo(dT) primer (Promega, Madison, WI), 40 units of RNasin (Boehringer-Mannheim, Mannheim, Germany), 10 mM $MgCl_2$, and 25 units of avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim) dissolved in reverse transcriptase buffer (Promega). The reactions were incubated for 1 hr at 42°, heated to 90° for 2 min, and diluted to 100 μl with RNase-free water. In each experiment, a parallel aliquot of RNA was subjected to mock reverse transcription as indicated above except avian myeloblastosis virus reverse transcriptase was left out. Diluted aliquots from these reactions were used as templates for PCR. The $P2X_7$ primers were from the rat cDNA sequence (sense 5′-GGCAGTTCAGGGAGGAATCATGG-3′ and antisense 5′-AAAGCGCCAGGTGGCATAGCTC-3′), generating a 939-bp product. Commercial primers to human GAPDH (Stratagene, La Jolla, CA) or murine β actin (Clontech) also were used to generate 600- and 540-bp products, respectively. $P2X_7R$ reactions included 1.0 μM concentration of each primer, 0.8 mM concentration of dNTPs, 60 mM Tris·HCl, pH 8.5, 15 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, and 1.25 units of *Taq* polymerase (Boehringer-Mannheim) preincubated with 275 ng of TaqStart antibody (Clontech) for 5–30 min at room temperature. huGAPDH and murine β actin reactions included 1.0 μM concentration of each primer, 0.8 mM concentration of dNTPs, 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, and *Taq* polymerase pretreated as above. The PCR cycling protocol was as follows: 1 min at 94°, 2 min at 60°, and 2 min at 72°. This protocol was carried out for 35 cycles and included an initial 5-min denaturation at 94° and a final 7-min extension at 72°. Products were electrophoresed on 1% agarose gels containing ethidium bromide and photographed. In control experiments, standard curves were generated using serial dilutions of the RT reactions as templates for PCR with each primer set. The linear range of the assay thus was determined for each primer set, and products from the original 20-μl RT were diluted into the final PCR volume to ensure nonsaturation of the PCR amplification reactions. A 1:50 dilution of the RT reaction was used for $P2X_7R$ analysis; a 1:500 dilution was used for huGAPDH and muβ-actin.

Data presentation. Values are presented as average ± standard error, and each experiment was repeated two to five times with similar results.

Results

Functional analysis of $P2X_7R$ pore activity by ethidium bromide uptake. Activation of the $P2X_7R$ is accompanied by a dramatic increase in plasma membrane permeability to organic solutes up to 900 Da in mass. We used uptake of the fluorescent dye ethidium bromide as an index of flux through this gated pore. On activation of $P2X_7Rs$ by ATP or BzATP, extracellular ethidium rapidly enters the cytoplasm and binds nucleic acids, resulting in increased fluorescence. These experiments were performed in the absence of extracellular divalent cations that inhibit $P2X_7$/P2Z receptor function by mechanisms involving reduction in the concentration of free ATP^{4-} (Steinberg *et al.*, 1987), reduction in receptor affinity for ATP (Virginio *et al.*, 1997), or direct blockade of permeation (Surprenant *et al.*, 1996). Neither ATP nor BzATP activated any ethidium influx in untransfected HEK 293 cells (data not shown). In cells stably transfected with either the human $P2X_7$ cDNA (HEK-h$P2X_7$) or the rat $P2X_7$ cDNA (HEK-r$P2X_7$), ATP elicited a dose-dependent increase in the rate of ethidium influx (Fig. 1, A and B). Although the maximal rate of ATP-induced ethidium influx for HEK-r$P2X_7$ was faster than that in HEK-h$P2X_7$, the ATP dose responses were identical (Fig. 1C).

Effect of KN-62 on ATP-mediated ethidium influx in HEK-hu$P2X_7$ cells versus HEK-rat$P2X_7$ cells. Because HEK 293 cells do not natively express any of the known P2X receptor subtypes (Evans *et al.*, 1998), the $P2X_7R$ will be expressed as a homomeric receptor in this cell type. We tested the ability of KN-62, a CamKII antagonist, to inhibit homomeric $P2X_7R$ function. Fig. 2A shows that increasing concentrations of KN-62 in the nanomolar range inhibited the ethidium influx induced by 1 mM ATP in the HEK-h$P2X_7$cells. Unexpectedly, even a high concentration of KN-62 (3 μM) had no detectable effect on ATP-mediated ethidium uptake by the HEK-r$P2X_7$ cells (Fig. 2B). Stimulating the HEK-r$P2X_7$ cells with submaximally active concentrations of ATP failed to unmask inhibition by KN-62 (data not shown). Because the surface protein expression of HEK-r$P2X_7$ may differ from that of HEK-h$P2X_7$, we reduced the number of functional cell surface rat $P2X_7R$ by partial inhibition with oxidized ATP (oATP), a drug that irreversibly inactivates the $P2X_7R$. A 1-hr incubation of HEK-r$P2X_7$ cells with 100 μM oATP (followed by removal of unreacted oATP) reduced the magnitude of ATP-induced ethidium influx by about half, but these cells remained KN-62 insensitive (data not shown). A longer incubation with oATP resulted in greater inhibition of rat $P2X_7R$ activity but no increase in relative KN-62 sensitivity. Fig. 2C shows the concentration-response relationships that characterize these isoquinoline effects; KN-62 has an IC_{50} value of ~100 nM against the human $P2X_7R$ but no inhibitory activity against the rat $P2X_7R$.

Because KN-04, an analog of KN-62 with no inhibitory activity toward CamKII, also inhibited signaling by the lymphocyte P2ZR, Gargett and Wiley (1997) proposed that isoquinolines directly block the P2z/$P2X_7R$ by CamKII-independent mechanisms. We also observed that 1 μM KN-04 inhibited ATP-stimulated ethidium uptake in the HEK-h$P2X_7$ cells (75.3 ± 1.6% inhibition, four experiments) but had no inhibitory effects on the HEK-r$P2X_7$ cells. These results demonstrate that the isoquinoline derivatives KN-62 and KN-04 inhibit the transmembrane fluxes associated with agonist-activated $P2X_7Rs$. This inhibition is not mediated by CamKII, and there is dramatic species specificity to isoquinoline sensitivity.

KN-62 partially antagonizes BzATP-induced ethidium uptake in HEK-h$P2X_7$ cells. Because BzATP is the most potent and selective agonist for $P2X_7$/P2Z receptors,

we examined the ability of KN-62 to inhibit BzATP-mediated ethidium uptake in the HEK-hP2X$_7$ cells. BzATP (100 μM) maximally stimulated ethidium influx (data not shown), and KN-62 markedly inhibited this influx. However, KN-62 was less efficacious as an inhibitor of BzATP-induced ethidium uptake than of ATP-induced ethidium uptake. KN-62 (3 μM) inhibited BzATP-stimulated ethidium influx by 61.6 ± 1.7% (three experiments; Fig. 3, A and B) compared with 87.5 ± 1.8% (three experiments) inhibition for ATP-stimulated influx (Fig. 2, A and C). The inhibitory effect of KN-62 was most apparent during the first 10 sec after BzATP application; the initial rate of ethidium influx was slowed greatly but then increased. Thus, at these earlier time points, inhibition by KN-62 was more dramatic than that after several minutes of BzATP stimulation (Fig. 3A). Gargett *et al*. (1997) also observed that KN-62 is less efficacious as an antagonist of BzATP-activated versus ATP-activated P2Z receptors in human CLL lymphocytes.

Effect of KN-62 and KN-04 on ATP and BzATP-induced ionic currents. The P2X$_7$/P2ZR can exist in two functionally distinct states. The first is a channel that opens rapidly in response to agonist and passes small inorganic cations such as Na^+ and Ca^{2+} but not larger molecules. With prolonged or repetitive nucleotide application, the receptor forms the pore capable of passing the larger organic ions such as *N*-methyl-glucamine, ethidium, and Yo-Pro (Nuttle *et al*., 1993, Surprenant *et al*., 1996). Gargett and Wiley (1997) observed that isoquinoline derivatives inhibited the ability of ATP to activate influx of both Ca^{2+} and ethidium in CLL lymphocytes. This indicated that these compounds should inhibit both functional states of the human but not the rat P2X$_7$R. This was confirmed by electrophysiological analysis of whole-cell ionic currents recorded from HEK 293 cells that stably express either of these recombinant receptors. As reported previously (Surprenant *et al*., 1996; Rassendren *et al*., 1997), BzATP (1–300 μM) and ATP (100–1000 μM) evoked rapid inward currents in HEK 293 cells transfected with either the rP2X$_7$ or hP2X$_7$ cDNAs (Fig. 4, A and B). The decay of inward current after a brief (1–2 sec) application of agonist was markedly slower at the rP2X$_7$R than at the hP2X$_7$R. KN-62 (10–1000 nM) was without effect on the BzATP or the ATP-evoked current recorded from rP2X$_7$ transfected cells (12 experiments). The reduction in the rate of decay of the inward current during repeated agonist pulses is observed in the presence or absence of KN-62 (Fig. 4B). This phenomenon was originally described by Surprenant *et al*.(1996) in their initial characterization of the cloned rP2X$_7$R. In contrast, KN-62 produced a dose-dependent inhibition of agonist-evoked currents in hP2X$_7$ transfected cells (Fig. 4C). The half-maximal concentrations of KN-62 required to inhibit the currents evoked by 100 μM BzATP or 1 mM ATP was 38 ± 1.4 nM (six experiments) and 34 ± 3 nM (four experiments), respectively. These latter currents were recorded from cells incubated in saline containing low concentrations of divalent cations. KN-62 exhibited a similar potency in inhibiting currents evoked by BzATP (300 μM) when the external solution contained normal divalent cations (i.e., 2 mM $CaCl_2$ and 1 mM $MgCl_2$; five experiments). The inhibition by KN-62 was only

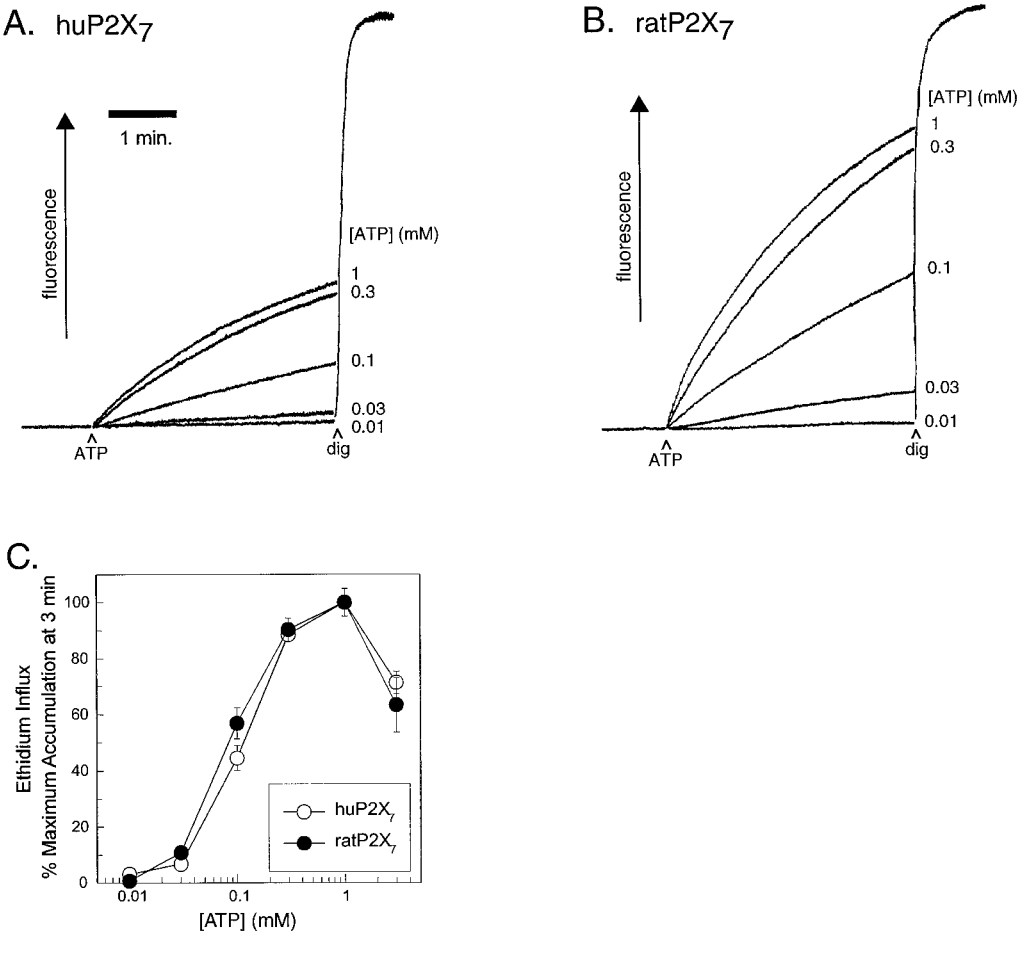

Fig. 1. Functional characterization of HEK 293 cells stably transfected with human or rat P2X$_7$ receptor. A, Effects of increasing ATP concentration on ethidium influx into HEK-huP2X$_7$ cells. Ethidium influx studies were performed in divalent free-basal salt solution as described in Experimental Procedures. Digitonin (20 μg/ml) was added to cell suspensions after 3 min to determine maximal ethidium accumulation as indicated. B, Effects of increasing ATP concentration on ethidium influx into HEK-ratP2X$_7$ cells. C, Concentration-response relationships describing ATP-induced ethidium accumulation in HEK-huP2X$_7$ (○) and HEK-ratP2X$_7$ (●). Ethidium influx at 3 min after the indicated [ATP] was measured and normalized to the maximal ATP-induced accumulation of ethidium. Values represent mean ± standard error of triplicate samples.

partially reversible after 10-min washout of the compound; for example, 300 nM KN-62 inhibited the BzATP-evoked current by 95 ± 2% (five experiments) within 4 min of application, and current had returned to 62 ± 4% of control amplitude 10 min after KN-62 was removed from the bath.

Similar results were obtained in experiments with KN-04 in which 1 μM produced no significant change in the BzATP current at the rP2X$_7$R (four experiments), whereas 30 and 300 nM inhibited the BzATP current by 29 ± 3% and 72 ± 6% (four experiments) at the hP2X$_7$R.

Chimeric receptors localize KN-62 sensitivity to the amino-terminal half of huP2X$_7$. The striking difference in isoquinoline sensitivity between human and rat P2X$_7$Rs despite 80% shared amino acid identity suggested that KN-62 sensitivity might be localized to only a few amino acid residues of the human receptor homologue. Gargett and Wiley (1997) speculated that the inhibitory actions of KN-62 might be mediated by an interaction with the unique intracellular carboxyl-terminal domain of the P2X$_7$R. To address this possibility, we examined the isoquinoline sensitivity of HEK cells stably transfected with chimeric P2X$_7$Rs in which the carboxyl-terminal tails of human and rat receptors were exchanged. Fig. 5A shows that ATP-mediated ethidium uptake in cells expressing the human-rat P2X$_7$R construct (h-rP2X$_7$; human amino terminus, rat carboxyl terminus) was inhibited by 1 μM KN-62 (88.1 ± 1.2% inhibition, four experiments). In contrast, cells stably expressing the rat-human P2X$_7$ construct (r-hP2X$_7$; rat amino terminus, human carboxyl terminus) displayed ATP-stimulated ethidium uptake that was completely insensitive to KN-62 (Fig. 5B) or KN-04 (data not shown). Similar results were obtained when ATP or BzATP-evoked currents were measured in HEK cells expressing these chimeric receptors (Fig. 5C). The IC$_{50}$ value (40 nM) that characterized KN-62 inhibition of BzATP-activated current through the h-rP2X$_7$R was equivalent to the IC$_{50}$ observed in HEK cells expressing the full-length human P2X$_7$R (Fig. 4C). In contrast, even micromolar amounts of KN-62 failed to substantially inhibit current through the rat-human P2X$_7$R chimera. These experiments indicate that the first 335 amino acids of the hP2X$_7$R mediate isoquinoline sensitivity, ruling out a KN-62 binding site in the intracellular carboxyl-terminal domain of the receptor.

Effects of KN-62 on BzATP-activated ionic fluxes in leukocyte cell lines that natively express the human or murine P2X$_7$R. Having established that KN-62 inhibits homomeric human P2X$_7$R, we next examined the KN-62 sensi-

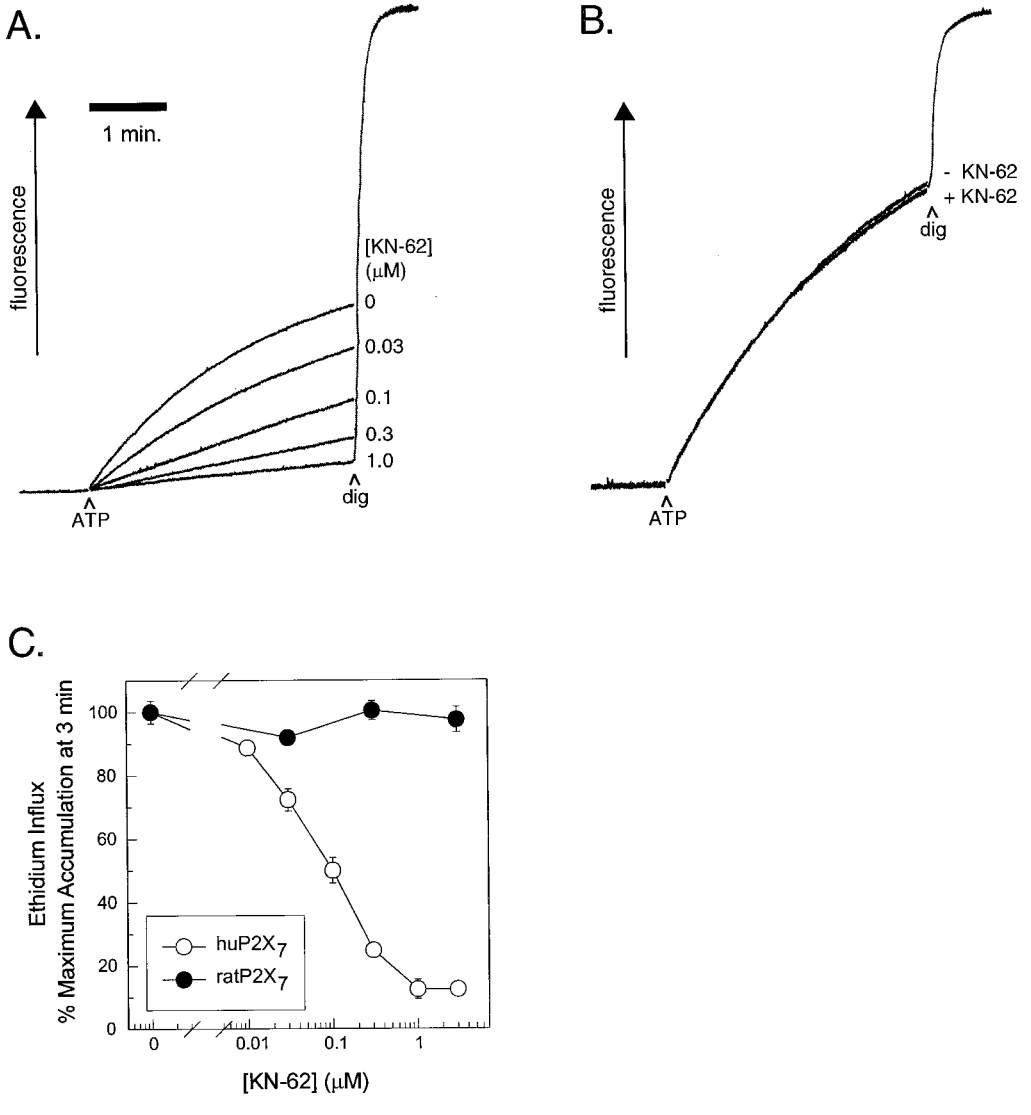

Fig. 2. Effect of the isoquinoline derivative KN-62 on ATP-evoked ethidium influx in HEK-huP2X$_7$ and HEK-ratP2X$_7$. A, HEK-huP2X$_7$ cells were challenged with 1 mM ATP in the absence or presence of the indicated concentrations of KN-62 and ethidium fluorescence measured. KN-62 was added 10 min before the addition of ATP. B, HEK-ratP2X$_7$ cells were challenged with 1 mM ATP in the absence or presence of 3 μM KN-62. C, Ethidium influx at 3 min after ATP was measured and normalized to the maximal ATP-induced accumulation assayed in the absence of isoquinoline. The data points were obtained from HEK-huP2X$_7$ (○) or HEK ratP2X$_7$ (●) over a range of KN-62 concentrations (10–3000 nM). Values represent mean ± standard error of triplicate samples.

tivity of P2X$_7$R as natively expressed in three different cell backgrounds: human macrophages (activated THP-1 monocytic leukemia cells), murine macrophages (the BAC1.2f5 macrophage line), and murine thymocytes (the BW5147 thymoma line). We previously established that human THP-1 monocytes treated with IFN-γ and LPS express both human P2X$_7$R mRNA and P2Z-like functional responses (Humphreys and Dubyak, 1996). Many studies of P2Z receptor function have used murine macrophage cell lines such as the BAC1.2f5 line used in our experiments. However, the sequence of the murine P2X$_7$R has not been reported, and its relative similarity to the human and rat P2X$_7$ homologues remains to be established. Finally, as noted above, the permeability characteristics of the P2Z receptor expressed in murine thymocytes differs from those observed in cells expressing the recombinant P2X$_7$Rs. Fig. 6A verifies that all of these leukocyte cell lines express P2X$_7$R mRNA. The primers used for these RT-PCR analyses were derived from the rat P2X$_7$R cDNA and will amplify a mRNA segment corresponding to the protein domain starting at Val245 and ending at Phe558. This encompasses 84 residues from the extracellular loop, the entire second transmembrane domain, and 85% of the 239 residue intracellular carboxyl-terminal tail. The absence of bands in the mock RT reactions rules out genomic DNA contamination of the RNA preparations, and amplification of human GAPDH or murine β actin confirms the quality of the RTs. We tested the ability of KN-62 to attenuate BzATP-activated calcium influx in each of these cell types. Analysis of P2Z-mediated changes in cytosolic Ca^{2+} in human myeloid cells is complicated by coexpression of Ca^{2+}-mobilizing P2Y$_2$ receptors, which also can be activated by 100 μM BzATP (Humphreys BD and Dubyak GR, unpublished observations). Thus, we routinely activated and desensitized these latter receptors by challenging THP-1 cells with 100 μM UTP for 10 min before the addition of BzATP. We previously confirmed that this protocol results in complete desensitiza-

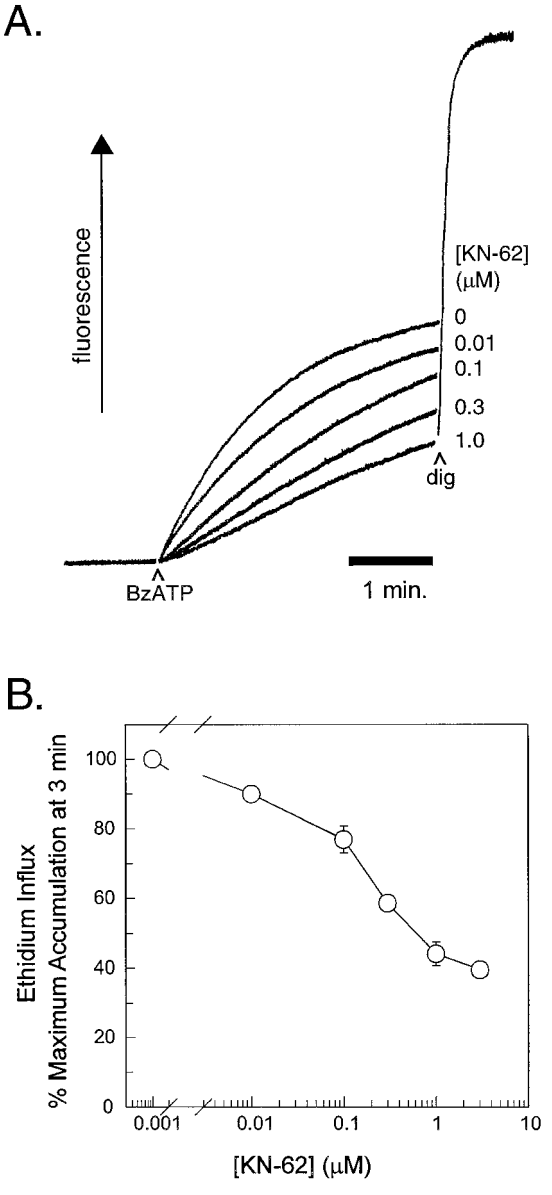

Fig. 3. Inhibition of BzATP-mediated ethidium influx by KN-62 in HEK-huP2X$_7$. A, Ethidium influx in response to 100 μM BzATP recorded from HEK-huP2X$_7$ in the absence or presence of KN-62 (10–3000 nM). Cell suspensions were challenged with BzATP after a 10-min preincubation in the presence of KN-62. B, Concentration-response relationship for inhibition of BzATP-mediated ethidium uptake by KN-62. Ethidium influx at 3 min after BzATP was measured and normalized to the maximal BzATP-induced accumulation assayed in the absence of isoquinoline. Values represent mean ± standard error of triplicate samples.

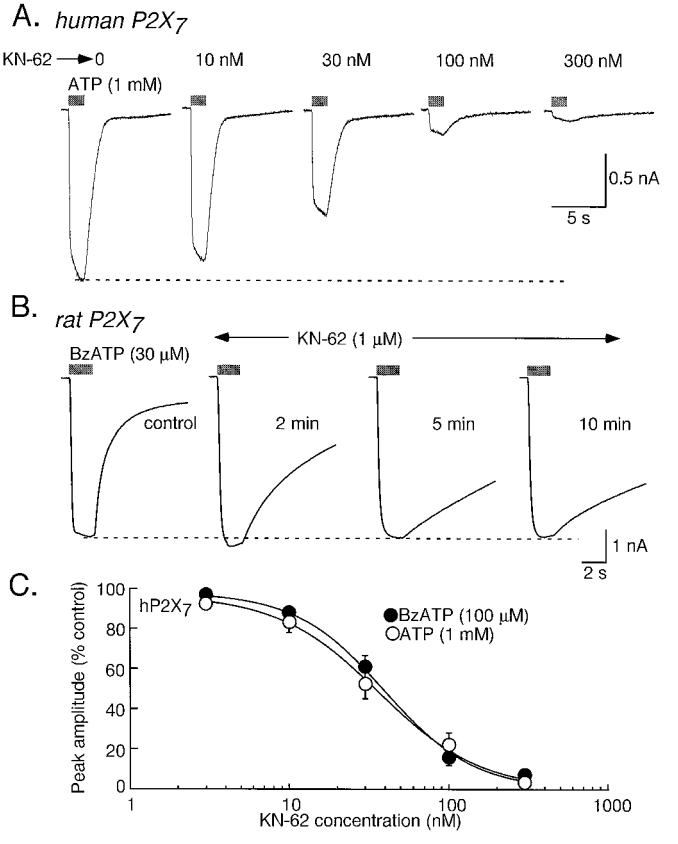

Fig. 4. KN-62 potently inhibits currents through the human P2X$_7$ receptor but is ineffective at the rat P2X$_7$ receptor. Representative currents recorded from HEK 293 cells expressing the human P2X$_7$ (A) or rat P2X$_7$ (B) receptor in response to a brief application of ATP (A) or BzATP (B) as indicated (*bar above trace*). A, Records are shown after 4 min in the presence of KN-62 at the indicated concentrations. B, Records are for before and during the continued presence of 1 μM KN-62 for the indicated durations. C, Concentration-response curve for inhibition of hP2X$_7$-mediated currents induced by ATP (1 mM, ○) or BzATP (100 μM, ●). All results are from experiments in which low divalent cation external solutions were used. Values represent mean ± standard error of four to eight experiments.

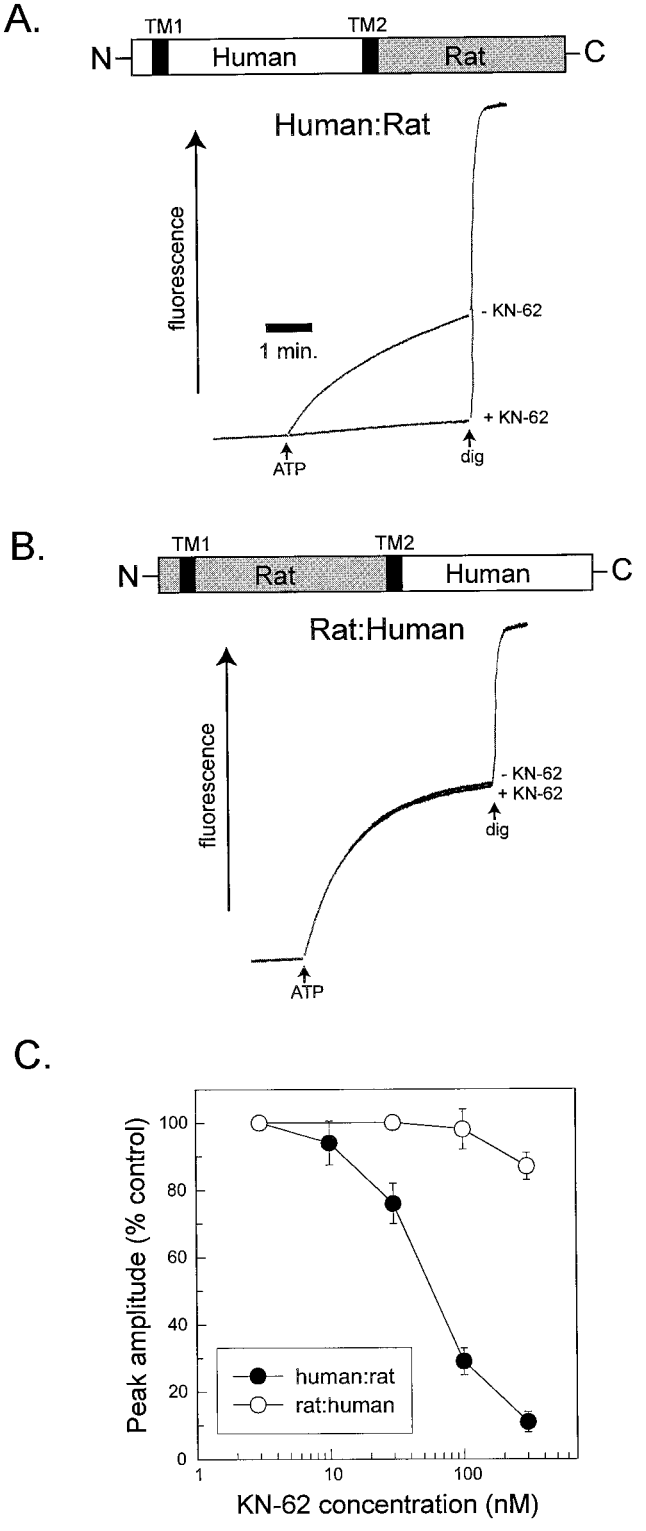

Fig. 5. Chimeric $P2X_7R$ constructs localize KN-62 sensitivity to the amino-terminal 335 amino acids of the human $P2X_7R$. A and B, $P2X_7R$ chimeras were constructed between human and rat $P2X_7$ receptors and stably expressed in HEK 293 cells as described in Experimental Procedures. Ethidium influx was assayed in response to 1 mM ATP after a 10-min preincubation of the cells in absence or presence of 1 μM KN-62. Ethidium influx was inhibited by 88.1 + 1.2% (four experiments) in the human-rat $P2X_7$ chimera (A), whereas the rat/human chimera (B) was insensitive to KN-62. C, KN-62 inhibition of currents evoked by ATP (300 μM) at the chimeric $P2X_7$ receptors. Traces were obtained as described in the legend to Fig. 4. ●, From the rat/human chimera. ○, From the human/rat chimera. Three to five experiments were conducted for each value.

tion of the human $P2Y_2$ receptors (Humphreys and Dubyak, 1996). This desensitization step was not required with the BAC-1.2f5 macrophages because the murine $P2Y_2$ receptors expressed in these cells are not activated by 100 μM BzATP (El-Moatassim and Dubyak, 1992). Likewise, the BW5147 thymocytes were not pretreated with UTP because these murine lymphoid cells lack expression of any Ca^{2+}-mobilizing $P2Y_2$ receptors (Humphreys BD and Dubyak GR, unpublished observations).

Fig. 6B confirms that the BW5147 murine thymocytes express functional $P2X_7R$ as assayed by the ability of BzATP to activate rapid Ca^{2+} influx; no BzATP-induced change in Ca^{2+} was observed when these cells were suspended in Ca^{2+}-free medium. Significantly, the rapid rise in cytosolic Ca^{2+} was completely inhibited when 3 μM KN-62 was added 10 min before stimulation (Fig. 6B). Likewise, KN-62 completely inhibited the BzATP-activated Ca influx observed in IFN/LPS-differentiated THP-1 monocytes (Fig. 6C). Finally, the BzATP-induced influx of Ca^{2+} into the murine BAC1.2f5 macrophages was significantly (but incompletely) attenuated in the presence 3 μM KN-62 (Fig. 6D). These data indicate the isoquinolines can inhibit the ATP-gated ion channel functions of natively human and murine $P2X_7Rs$.

Effect of KN-62 on BzATP-activated pore formation in leukocyte cell lines that natively express the human or murine $P2X_7R$. A similar analysis was used test the effects of KN-62 on the pore-forming function of natively expressed human and murine $P2X_7Rs$. Although KN-62 completely inhibited BzATP-activated ethidium influx in the IFN/LPS-treated THP-1 human monocytes (Fig. 7A), it only partially inhibited the same response in the BAC1.2f5 murine macrophages (Fig. 7B). Characterization of the concentration-response relationships for KN-62 inhibition of ethidium influx in the two cell types suggested that reduced efficacy rather than reduced potency underlies the partial inhibitory effect observed in the murine macrophages (Fig. 7C). We were unable detect any BzATP-induced ethidium influx in the BW5147 thymocytes (data not shown), which is consistent with the findings from previous studies of P2Z receptor function in murine thymocytes (Pizzo *et al.*, 1991).

Isoquinolines rapidly block ethidium influx through previously activated $P2X_7R$ pores. All of the previous experiments tested inhibitory effects of isoquinolines in cells that were preincubated for 10 min with these drugs before agonist activation of the $P2X_7Rs$. Using IFN/LPS-treated THP-1 monocytes, we tested the effects of KN-62 and KN-04 on ethidium fluxes through $P2X_7R$ pores preactivated by BzATP addition before exposure to the isoquinolines. Fig. 8 shows that isoquinoline addition after nucleotide challenge rapidly attenuated ethidium influx, with maximal inhibition occurring after 20 sec. Similar results were obtained in studies assaying ATP-activated ethidium influx in HEK-$hP2X_7$ cells (data not shown). The rapidity of inhibition is consistent with the notion that KN-62 inhibition may be mediated via its interaction with extracellular domains of the human or murine $P2X_7R$.

Discussion

Activation of $P2X_7Rs$ by extracellular ATP triggers two distinct fluxes. The first reflects a rapid and reversible current through channels permeable to small cations, whereas

the second requires either sustained agonist application or repetitive short exposures and reflects the opening of a nonselective pore through which large organic molecules such as ethidium or YoPro-1 can pass (Nuttle *et al*., 1993, Surprenant *et al*., 1996). We have shown that the isoquinoline derivatives KN-62 and KN-04 block both of these fluxes in HEK 293 cells that express recombinant human P2X$_7$Rs but not recombinant rat P2X$_7$Rs. The extreme species-specific actions of these drugs was surprising given the high degree of amino acid identity (80%) between the human and rat P2X$_7$Rs. Isoquinoline sensitivity is mediated by the first 335 amino acids of the human P2X$_7$R because transfer of the human amino-terminal domain, which included the intracellular amino-terminal tail, the first transmembrane segment, and the entire extracellular loop, to the rat P2X$_7$R resulted in a receptor that exhibited KN-62-sensitive ethidium influx. In contrast, KN-62 had no effect on the reciprocal chimera that transferred the corresponding amino-terminal regions of the rat P2X$_7$R to a segment containing the second transmembrane domain and intracellular carboxyl-terminal tail of the human receptor. The isoquinoline-sensitive span of 335 amino acids from the human receptor contains 64 amino acid differences between human and rat sequences, with one stretch of seven consecutive amino acid differences (153–159). Because P2X$_7$R function in murine cell types (BW5147 and BAC1.2f5) also was isoquinoline sensitive, a sequence comparison among human, rat, and murine (when available) P2X$_7$Rs may help to localize further the KN-62 interaction domain. It is interesting to note that human and rat homologues of the P2X$_4$ receptor also exhibit markedly different sensitivities to the P2 receptor antagonists suramin and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid despite their 89% sequence identity (Garcia-Guzman *et al*., 1996). This suggests that valuable structure-function information may be gained from careful analysis of drug sensitivities across the species homologues of all P2X receptor family proteins.

As first suggested by Gargett and Wiley (1997), the ability of KN-04, an inactive analog of KN-62, to inhibit human P2X$_7$R function suggests that CamKII does not play a role in regulation of either conductance state of this receptor. Also arguing against a role for CamKII is the failure of either isoquinoline derivative to inhibit the rat P2X$_7$R. The cytoplasmic carboxyl-terminal tails of both the human and rat P2X$_7$Rs contain homologous segments (residues 556–562; -RFGSQD- in the human receptor and -RFVSQD- in the rat protein) that comprise the minimal CamKII consensus phosphorylation site (-RXXS/T-) (Braun and Schulman, 1995). However, our chimeric receptors localized KN-62 sensitivity to the amino-terminal half of the human receptor, ruling out CamKII-mediated phosphorylation of the carboxyl-terminal tail as a mechanism for isoquinoline inhibition of P2X$_7$R

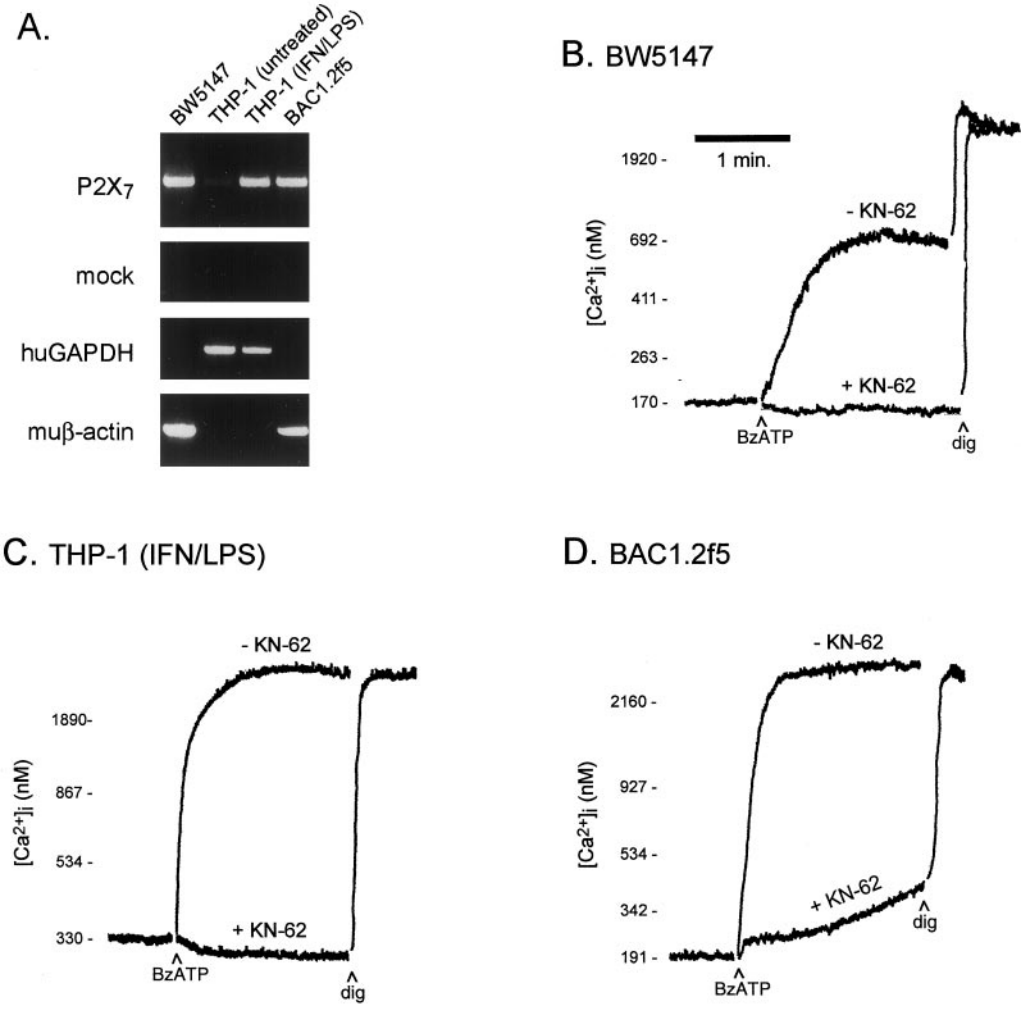

Fig. 6. P2X$_7$R mRNA expression and BzATP-evoked calcium responses in human and murine leukocyte cell lines. A, RNA was isolated from BW5147 murine lymphocytes, undifferentiated THP-1 human monocytes, THP-1 human monocytes differentiated with IFN (1000 units/ml), and LPS (1 μg/ml) for 3 days and BAC1.2f5 murine macrophages. RT-PCR was performed as described in Experimental Procedures using P2X$_7$ primers specific to the unique carboxyl-terminal tail region of the P2X$_7$R; parallel PCRs were performed on mock reverse-transcriptions to test for genomic DNA contamination. All amplified bands were of the expected size. B–D, Cells were loaded with Fura-2, equilibrated for 10 min in the absence or presence of 3 μM KN-62, and then assayed for increases in cytosolic Ca^{2+} in response to 100 μM BzATP. Maximal Ca^{2+}-dependent fluorescence subsequently was determined by permeabilizing the cells with digitonin (20 μg/ml). P2Y$_2$ receptors present on THP-1 cells were desensitized by stimulation with 100 μM UTP for 10 min before the addition of BzATP.

signaling. Gargett and Wiley (1997) speculated that KN-62 might directly bind to the unique carboxyl-terminal tail of the human $P2X_7R$. This is unlikely given our finding that transfer of the intracellular carboxyl-terminal tail of the human protein to the rat receptor resulted in a chimeric $P2X_7R$ that was insensitive to the isoquinolines (Figs. 4C and 5B).

Blanchard *et al.* (1995) proposed that CamKII could act as a specific regulator of P2ZR-mediated cytolysis because both KN-62 and the calmodulin antagonists, trifluoroperazine and calmidazolium, protected human monocyte-derived macrophages from ATP-mediated cytolysis. These authors also suggested that this effect of KN-62 was downstream of the Ca^{2+} influx response to P2ZR activation because the drug failed to block the ATP-induced increase in cytosolic Ca^{2+}. This mechanism seems unlikely because we performed similar studies with human monocyte-derived macrophages and found that KN-04 inhibits ATP-induced release of lactate dehydrogenase to the same degree as KN-62 (Humphreys BD and Dubyak GR, unpublished observations). The analysis of ATP-induced changes in Ca^{2+} in macrophages is complicated by coexpression of the ionotropic $P2X_7Rs$ and the Ca^{2+}-mobilizing $P2Y_2$ receptors (Torre and Trautmann, 1993; Humphreys and Dubyak, 1996). Trifluoroperazine and calmidazolium can block ATP-induced calcium influx by the human $P2X_7R$ (Humphreys BD and Dubyak GR, unpublished observations) whereas calmidazolium additionally blocks the rapid gating of inward current by ATP in HEK cells expressing the rat $P2X_7R$ (Virginio *et al.*, 1997). This suggests that these drugs also are direct inhibitors of $P2X_7R$ function. It is significant to note that KN-62, calmidazolium and trifluoroperazine have been shown to directly block L-type calcium channels (Nakazawa *et al.*, 1993, Li *et al.*, 1992), suggesting that $P2X_7Rs$ and L-type calcium channels share multiple structural determinants that mediate the inhibitory effect of these three drugs.

The molecular mechanism or mechanisms that underlie the inhibition of human $P2X_7R$ function by KN-62 remain uncertain. The observed inhibitory effects of these com-

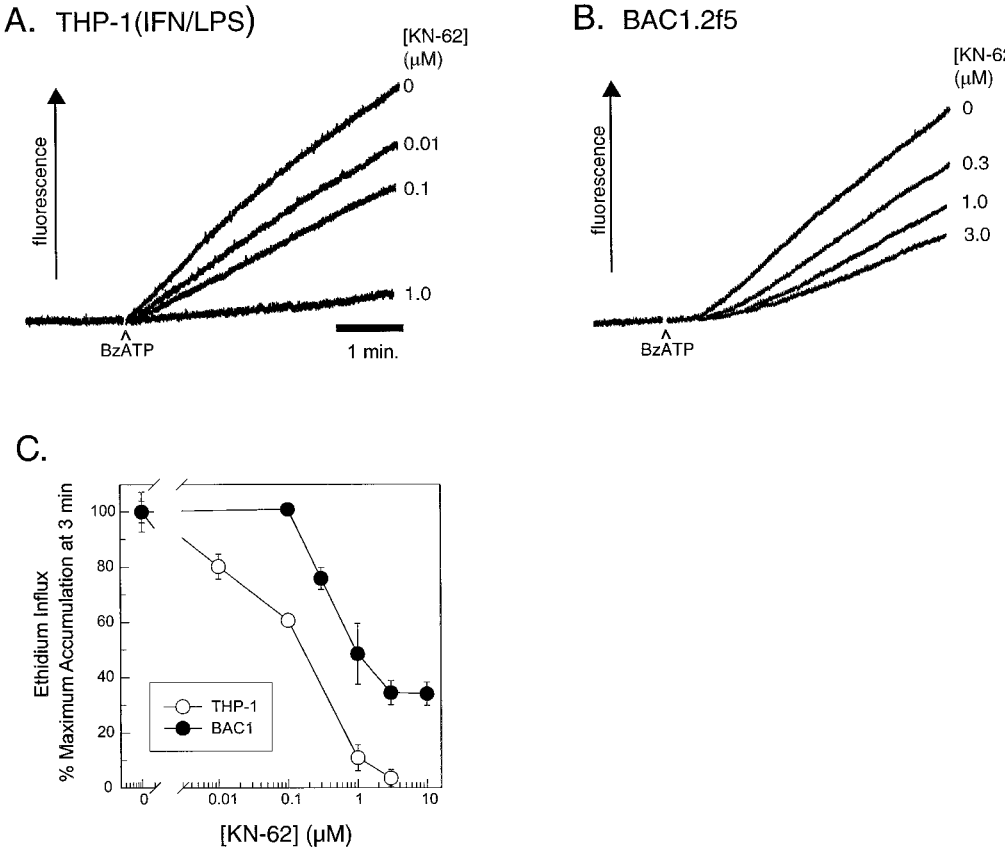

Fig. 7. KN-62 concentration response on BzATP-induced ethidium uptake in THP-1 human monocytes and BAC1.2f5 murine macrophages. A, THP-1 cells that had been incubated with IFN (1000 units/ml) and LPS (1 μg/ml) for 3 days were challenged with 100 μM BzATP in the absence or presence of KN-62 and ethidium influx was measured. B, BAC1.2f5 macrophages were subjected to the same procedure using 100 μM BzATP and different concentrations of KN-62. C, Concentration-response relationships characterizing the effects of KN-62 on ethidium influx activated by 100 μM BzATP in THP-1 monocytes (○) and BAC1.2f5 (●). Ethidium influx at 3 min after BzATP was measured and normalized to the maximal BzATP-induced accumulation assayed in the absence of isoquinoline. Values represent the mean ± standard error of three samples.

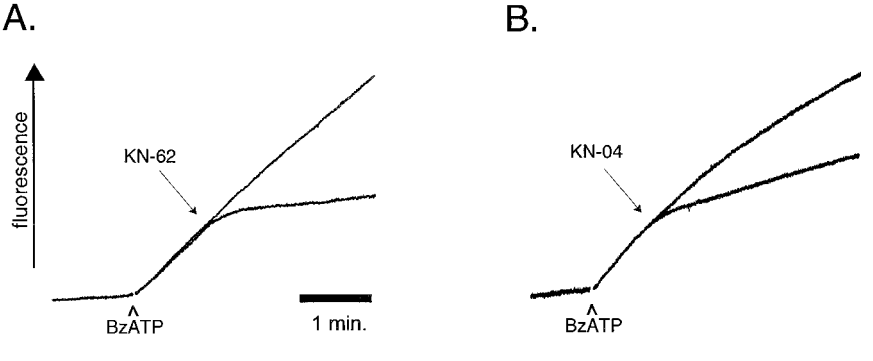

Fig. 8. KN-62 blocks ethidium influx through previously opened $P2X_7$ pores. A and B, To test whether the isoquinoline derivatives could inhibit ethidium influx after $P2X_7R$ activation, THP-1 cells that had been incubated for 3 days in IFN (1000 units/ml) and LPS (1 μg/ml) were challenged with 100 μM BzATP, and ethidium uptake was measured. One minute after BzATP addition, either 1 μM KN-62 (A) or 1 μM KN-04 was added (*arrow*), and ethidium influx compared with cells incubated without drug.

pounds (as described in this study and by Gargett and Wiley, 1997) cannot be ascribed to action as a simple competitive or noncompetitive antagonist. Different IC_{50} values and efficacies were observed depending on (1) the species of nucleotide agonist used to activate the $P2X_7R$, (2) the type of $P2X_7R$ function being assayed, and (3) the cellular background of human $P2X_7R$ expression. The IC_{50} values for KN-62 in our experiments ranged from 30 to 100 nM (Figs. 2–4 and 7). This is significantly higher than the IC_{50} value of 13 nM that characterized the effects of KN-62 on various ATP-induced responses in human CLL leukocytes (Gargett and Wiley, 1997). However, it should be noted that these latter investigators used cells suspended in high KCl/low NaCl medium, whereas our assays were performed using medium with standard extracellular NaCl concentrations. Wiley *et al*. (1992) reported previously that extracellular Na^+ attenuates P2Z receptor function in CLL lymphocytes. The affinity of KN-62 for its binding site or sites on the human $P2X_7R$ may show similar modulation by different monovalent cations or other components of the extracellular medium (e.g., pH, divalent cations).

In contrast, no simple hypothesis can explain the observed variations in KN-62 efficacy among the different experimental systems. KN-62 was a full antagonist of human $P2X_7R$ when ATP was the agonist but was a partial antagonist when BzATP was the agonist (Figs. 2–4 and 7). The ability of KN-62 to fully antagonize ATP-activated human $P2X_7R$ was independent of cellular background (recombinant receptor in HEK cells, native receptor in THP-1 cells) or type of assayed $P2X_7R$ function (rapidly gated cation conductance, ethidium influx pore, phospholipase D, cytolysis). In contrast, the partial antagonism of KN-62 at BzATP-activated human $P2X_7R$ varied with both cell background and assayed function. KN-62 fully antagonized BzATP-activated ethidium influx in THP-1 monocytes but partially inhibited the same phenomenon in HEK-hP2X7 cells. Although KN-62 only partially antagonized the ability of BzATP to induce ethidium influx (indicative of the nonselective pore) in these latter cells, it fully inhibited the ability of BzATP to the trigger the rapidly gated cation current. These findings are consistent with previous speculations that the rapidly gated ion channel and the more slowly induced pore represent functionally and structurally distinct conformations of the $P2X_7R$ (Nuttle and Dubyak, 1994; Surprenant *et al*., 1996, Rassendren *et al*., 1997). This hypothesis has been further supported by the observation of Virginio *et al*. (1997) that calmidazolium can fully antagonize the rapid gating of cation channels by BzATP in HEK cells expressing recombinant rat $P2X_7R$ while having no inhibitory effect on BzATP-activation of Yo-Pro dye uptake. Although our results do not identify the mechanism by which the isoquinolines inhibit $P2X_7R$-mediated transmembrane fluxes, the ability of these drugs to block previously opened $P2X_7$ pores (Fig. 8) suggests that these drugs may directly block pore permeation.

Although expression of recombinant $P2X_7Rs$ confers the ATP-induced ethidium influx phenotype to HEK 293 cells, this type of membrane permeability change was absent in the BW5147 lymphocytes. Our experiments represent the first verification that murine thymocytes do indeed express $P2X_7R$ mRNA despite their lack of the pore-forming $P2X_7R$ phenotype. These cells also displayed a BzATP-activated and KN-62-sensitive Ca^{2+} influx response indicative of the cation-conducting conformation of the $P2X_7Rs$. The failure of this lymphocyte cell line to accumulate ethidium in response to extracellular ATP is consistent with previous studies of P2Z receptor function in rodent lymphocytes; organic dyes, such as ethidium, that readily permeate the macrophage P2ZR failed to pass through the P2ZR of mouse thymocytes (Pizzo *et al*., 1991). It remains unclear why the lymphocyte $P2X_7R$ have a smaller size exclusion limit than $P2X_7R$ expressed in other cell types. Lymphocytes may express another P2X subtype capable of modulating $P2X_7R$ function because some combinations of P2XR subtypes can form heteromeric channels (Lewis *et al*., 1995). Alternatively, efficient pore formation may depend on the local concentration of $P2X_7R$ subunits at the cell surface. In this regard, lymphocytes might have a lower $P2X_7R$ surface density than either macrophages or cells engineered to overexpress recombinant $P2X_7R$.

Recent studies point to important physiological roles for $P2X_7Rs$ in immune cell biology. Expression of the murine T-lymphocyte $P2Z/P2X_7$ is modulated during differentiation, and the receptor is proposed to play a role in thymocyte selection (Chused *et al*., 1996, Ross *et al*., 1997). Extracellular ATP is mitogenic to human T lymphocytes and pharmacological data implicates $P2Z/P2X_7$ in mediating the mitogenic effects of phytohemaglutinin and CD3 stimulation (Baricordi *et al*., 1996). In human macrophages and microglial cells, $P2Z/P2X_7$ activation has clearly been shown to trigger activation of the cysteine protease caspase-1 (interleukin-converting enzyme) with subsequent cleavage and release of pro–IL-1β to bioactive IL-1β (Hogquist *et al*., 1991; Perregaux and Gabel, 1994; Ferrari *et al*., 1997). Two groups have shown that $P2X_7R$ activation by a brief ATP pulses triggers apoptosis of Bacillus-Clamette-Guerin (BCG)-infected monocytes with killing of the intracellular bacilli (Molloy *et al*., 1994, Lammas *et al*., 1997). Moreover, other apoptotic stimuli (CD95 or FAS ligation) directed at the infected macrophages induce apoptosis but do not kill the mycobacteria (Lammas *et al*., 1997). The high potency antagonism of the $P2X_7R$ by isoquinoline derivatives presents a useful tool to study these effects of $P2z/P2X_7$ receptor activation *in vivo*.

Acknowledgments

We are grateful to Dr. R. Ganapathi for the KN-04 used in these studies, Sylvia Kertesy for excellent technical assistance, and Reza Beigi and Erin Clifford for helpful discussions.

References

Baricordi OR, Ferrari D, Melchiorri L, Chiozzi P, Hanau S, Chiari E, Rubini M, and Di Virgilio F (1996) An ATP-activated channel is involved in mitogenic stimulation of human T lymphocytes. *Blood* **87:**682–690.

Blanchard DK, Hoffman SL, and Djeu JY (1995) Inhibition of extracellular ATP-mediated lysis of human macrophages by calmodulin antagonists. *J Cell Biochem* **57:**452–461.

Braun AP and Schulman H (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu Rev Physiol* **57:**417–445.

Buell G, Collo G, and Rassendren F (1996) P2X receptors: an emerging channel family. *Eur J Neurosci* **8:**2221–2228.

Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:**156–161.

Chused TM, Apasoz S, and Sitkovsky M (1996) Murine T lymphocytes modulate activity of an ATP-activated P2Z-type purinoceptor during differentiation. *J Immunol* **157:**1371–1380.

Di Virgilio F (1995) The P2z purinoreceptor: an intriguing role in immunity, inflammation and cell death. *Immunol Today* **16:**524–528.

Dubyak GR and El-Moatassim C (1993) Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol* **265:**C577–C606.

El-Moatassim C and Dubyak GR (1992) A novel pathway for the activation of

phospholipase D by P2z purinergic receptors in BAC1.2f5 macrophages. *J Biol Chem* **267:**23664–23674.
Erb L, Lustig KD, Ahmed AH, Gonzalez FA, and Weisman GA (1990) Covalent incorporation of 3′-O-(4-benzoyl)benzoyl-ATP into a P_2 purinoceptor in transformed mouse fibroblasts. *J Biol Chem* **265:**7424–7432.
Evans RJ, Surprenant A, and North RA (1998) P2X receptors: cloned and expressed, in *The P2 Nucleotide Receptors* (Turner JT, Weisman GA, and Fedan JS, eds) pp 43–61, Humana Press, Totawa, NJ.
Fenwick EM, Marty A, and Neher E (1982) A patch-clamp study of bovine chromaffin cells and of their sensitivity to acetylcholine. *J Physiol (Lond)* **331:**577–597.
Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Melchiorri L, Baricordi OR and Di Virgilio F (1997) Extracellular ATP triggers IL-1β release by activating the purinergic P2z receptor of human macrophages. *J Immunol* **159:**1451–1458.
Ferrari D, Munerati M, Melchiorri L, Hanau S, Di Virgilio F, and Baricordi OR (1994) Responses to extracellular ATP of lymphoblastoid cell lines from Duchenne muscular dystrophy patients. *Am J Physiol* **267:**C886–C892.
Garcia-Guzman M, Soto F, Gomez-Hernandez JM, Lund P, and Stuhmer W (1997) Characterization of recombinant human $P2X_4$ receptor reveals pharmacological differences to the rat homologue. *Mol Pharmacol* **51:**109–118.
Gargett CE, Cornish JE, and Wiley JS (1997) ATP, a partial agonist for the P2Z receptor of human lymphocytes. *Br J Pharmacol* **122**:911–917.
Gargett CE and Wiley JS (1997) The isoquinoline derivative KN-62: a potent antagonist of the P2z-receptor of human lymphocytes. *Br J Pharmacol* **120**:1483–1490.
Hogquist KA, Nett MA, Unanue ER, and Chaplin DD (1991) Interleukin 1 is processed and released during apoptosis. *Proc Natl Acad Sci USA* **88:**8485–8491.
Humphreys BD and Dubyak GR (1996) Induction of the P2z/$P2X_7$ nucleotide receptor and associated phospholipase D activity by lipopolysaccharide and IFN-g in the human THP-1 monocytic cell line. *J Immunol* **157:**5627–5637.
Jamieson GP, Snook MB, Thurlow PJ, and Wiley JS (1996) Extracellular ATP causes loss of L-selectin from human lymphocytes via occupancy of P2z purinoceptors. *J Cell Physiol* **166:**637–642.
Lammas DA, Stober C, Harvey CJ, Kendrick N, Panchalingam S, and Kumararatne DS (1997) ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2z ($P2X_7$) receptors. *Immunity* **7:**433–444.
Lewis C, Neldhart S, Holy C, North RA, Buell G, and Surprenant A (1995) Coexpression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. *Nature (Lond)* **377:**432–435.
Molloy A, Laochumroonvorapong P, and Kaplan G (1994) Apoptosis, but not necrosis of infected monocytes is coupled with killing of intracellular BCG. *J Exp Med* **180:**1499–1509.
Nuttle LC, El-Moatassim C, and Dubyak GR (1993) Expression of the pore-forming P2z purinoceptor in *Xenopus* oocytes injected with poly(A)$^+$ RNA from murine macrophages. *Mol Pharmacol* **44:**93–101.
Perregaux D and Gabel CA (1994) Interleukin-1β maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J Biol Chem* **269**: 15195–15203.
Pizzo P, Zanovello P, Bronte V, and Di Virgilio F (1991) Extracellular ATP causes lysis of mouse thymocytes and activates a plasma membrane ion channel. *Biochem J* **274:**139–144.
Rassendren F, Buell GN, Virginio C, Collo G, North A, and Surprenant A (1997) The permeabilizing ATP receptor, $P2X_7$: cloning and expression of a human cDNA. *J Biol Chem* **272:**5482–5486.
Ross PE, Ehring GR, and Cahalan MD (1997) Dynamics of ATP-induced calcium signaling in single mouse thymocytes. *J Cell Biol* **138**:987–9998.
Steinberg TH, Newman AS, Swanson JA, and Silverstein SC (1987) ATP^{4-} permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J Biol Chem* **262:**8884–8892.
Surprenant A, Rassendren F, Kawashima E, North A, and Buell G (1996) The cytolytic P2z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). *Science (Washington DC)* **272:**735–738.
Tokumitsu H, Chijiwa T, Hagiwara M, Mizutani A, Terasawa M, and Hidaka H (1990) KN-62, 1-[*N*,*O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. *J Biol Chem* **265:**4315–4320.
Torre SR and Trautmann A (1993) Calcium responses elicited by nucleotides in macrophages: interaction between two receptor subtypes. *J Biol Chem* **268:**18640–18647.
Virginio C, Church D, North RA, and Surprenant A (1997) Effects of divalent cations, protons, and calmidazolium at the rat $P2X_7$ receptor. *Neuropharmacology* **36**: 1285–1294.
Wiley JS, Chen R, and Jamieson GP (1993) The ATP^{4-} receptor-operated channel (P2z class) of human lymphocytes allows Ba^{2+} and ethidium$^+$ uptake: inhibition of fluxes by suramin. *Arch Biochem Biophys* **305:**54–60.
Wiley JS, Chen R, Wiley MJ, and Jamieson GP (1992) The ATP^{4-} receptor-operated channel (P2z class) of human lymphocytes: inhibition of fluxes by amiloride analogs and by extracellular sodium ions. *Arch Biochem Biophys* **292:**411–418.
Wiley JS, Jamieson GP, Mayger M, Cragoe EJ Jr, and Jopson M (1990) Extracellular ATP stimulates an amiloride-sensitive sodium influx in human lymphocytes. *Arch Biochem Biophys* **280:**263–268.
Wiley JS, Chen JR, Snook MB, and Jamieson GP (1994) The P2Z-purinoceptor of human lymphocytes: actions of nucleotide agonists and irreversible inhibition by oxidised ATP. *Br J Pharmacol* **112:**946–950.
Williams N and Coleman PS (1982) Exploring the adenine nucleotide binding sites on mitochondrial F_1-ATPase with a new photoaffinity probe, 3′-O-(4-benzoyl)benzoyl adenosine 5′-triphosphate. *J Biol Chem* **257:**2834–2843.

Send reprint requests to: Dr. George R. Dubyak, Department of Physiology & Biophysics, School of Medicine E565, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4970. E-mail: gxd3@po.cwru.edu