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Identification and characterization of an endogenous $P2X_7$ (P2Z) receptor in CHO-K1 cells

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1 CHO-K1 cells were examined for their cellular responses to the P2 receptor agonist, 2′- and 3′-O-(4-benzoylbenzoyl)-ATP (DbATP), and for the presence of mRNA for P2X receptors.

2 Reverse transcriptase-polymerase chain reactions, using primers directed against the rat P2X subunits, detected the presence of $P2X_7$ but not $P2X_1$-$P2X_6$ subunits.

3 DbATP ($EC_{50} \sim 100\ \mu M$) evoked non-desensitizing inward currents which reversed at ~ 0mV, suggesting activation of a non-selective cation channel. ATP also evoked inward currents but was less potent than DbATP.

4 DbATP also stimulated the accumulation of ^{45}calcium ($^{45}Ca^{2+}$) and the DNA binding dye, YO-PRO-1, in CHO-K1 cells. Both responses were inhibited by NaCl and $MgCl_2$. In 280 mM sucrose buffer, $^{45}Ca^{2+}$ accumulation was measurable within 10–20 s of agonist addition, whereas YO-PRO-1 accumulation was only detectable after 8 min. ATP and ATPγS were also agonists but were less potent than DbATP, while UTP, 2-methylthio ATP, ADP and $\alpha\beta$methylene ATP were inactive at concentrations up to 100 μM.

5 DbATP increased lactate dehydrogenase release from CHO-K1 cells, suggesting cell lysis, although this effect was only pronounced after 60–90 min.

6 These data suggest that CHO-K1 cells express an endogenous $P2X_7$ receptor which can be activated by DbATP to cause a rapid inward current and accumulation of $^{45}Ca^{2+}$. Prolonged receptor activation results in a delayed, increased permeability to larger molecules such as YO-PRO-1 and ultimately leads to cell lysis. Importantly, the presence of an endogenous $P2X_7$ receptor should be considered when these cells are used to study recombinant P2X receptors.

Keywords: $P2X_7$ receptor; CHO-K1 cell line

Introduction

The ability of ATP to permeabilize a number of cell types to molecules with molecular weights up to 900 daltons has been recognized for many years and has been attributed to the action of ATP on a pore forming P2Z receptor (Steinberg *et al*., 1987; Dubyak & El-moatassim, 1993). Recently, a seventh member of the P2X receptor family of ligand-gated cation channels has been cloned and found to exhibit many of the properties of the P2Z receptor (Surprenant *et al*., 1996). With brief application of ATP, the $P2X_7$ receptor, similar to the other members of the P2X receptor family, functions as a ligand-gated, non-selective cation channel, which is permeable to small inorganic ions of MW <200 daltons. However, with prolonged agonist application, the currents gated by the P2Z/$P2X_7$ receptor increase or become prolonged (Surprenant *et al*., 1996; Chessell *et al*., 1997) and the channel becomes permeable to larger molecules with molecular weights up to 900 daltons (Steinberg *et al*., 1987). It is thought that the increase in current carried by the $P2X_7$ receptor and the increase in size of permeant molecules reflects formation of a large pore form of the $P2X_7$ receptor (Surprenant *et al*., 1996, Chessell *et al*., 1997).

The P2Z/$P2X_7$ receptor is found in many cells of the immune system including macrophages, mast cells and lymphocytes (Dubyak & El-moatassim, 1993). In addition, ATP has been shown to permeabilize a number of transformed cell lines (Weisman *et al*., 1984) suggesting that these cells may also express the $P2X_7$ receptor. The CHO-K1 cell line is a transformed variant of the CHO parent cell line and is used in studies on recombinant receptors due to the fact that it either does not express, or expresses low levels of endogenous receptors for most neurotransmitters. If these cells are to be used for studies on recombinant P2 purinoceptors, it is important that they should not express endogenous P2 purinoceptors. It is already known that these cells express a G-protein coupled P2Y receptor (Iredale & Hill, 1993), thereby limiting their use in studies on recombinant P2Y receptors. It is not known if CHO-K1 cells express endogenous P2X receptors, although metabolically inhibited CHO-K1 cells can be permeabilized by extracellular ATP (Kitagawa & Akamatsu, 1986). In the present study we have therefore examined CHO-K1 cells for their cellular responses to the selective P2Z receptor agonist, 2′- & 3′-O-(4-benzoylbenzoyl)-ATP (DbATP) (Erb *et al*., 1990), and describe the presence of an endogenous P2 purinoceptor which exhibits many of the properties of the recombinant $P2X_7$ receptor.

Methods

Cell culture

The majority of studies were performed on CHO-K1 cells that were obtained from ECACC (Porton Down, U.K.) and were used from passage 8–50 after receipt. There were no obvious differences in the functional properties of the CHO-K1 cells with increasing passage number. In addition, CHO-K1 cells expressing recombinant somatostatin receptors were obtained

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from Affymax (Palo Alto, California, U.S.A.) and Glaxo Wellcome (Stevenage, U.K.) and found to respond in a similar manner to the CHO-K1 cells obtained from ECACC. Cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 nutrient mix containing 10% foetal bovine serum and were maintained as a monolayer culture at 37°C in a humidified atmosphere containing 5% CO_2. The cells were used when confluent.

Reverse transcriptase-polymerase chain reaction (RT–PCR)

Total RNA from CHO-K1 cells was prepared using a micro RNA isolation kit (Stratagene, Cambridge, U.K.) according to the manufacturer's instructions, and treated with RNase-free, DNase I (Pharmacia) for 30 min at 37°C. First strand cDNA was synthesized (first strand cDNA synthesis kit; Clontech, Palo Alto, U.S.A.) from 5 mg of total RNA using an oligo(dT)18 primer and Moloney-murine leukaemia virus reverse transcriptase (60 min, 42°C). Control reactions in the absence of reverse transcriptase were also carried out. Sequence specific primers (45mers) for all seven known rat P2X subunits were used in polymerase chain reactions (PCR). The bases against which the primers were directed were as follows and are described relative to the start methionine ATG codon with the A of this codon defined as base 1:- $P2X_1$ (accession number X80477) forward primer directed to bases 596–641 and reverse primer to 1190–1235; $P2X_2$ (accession number U14414) forward primer directed to bases 581–626 and reverse primer to bases 1361–1406; $P2X_3$ (accession number X91167) forward primer directed to bases 571–616 and reverse primer to bases 1150–1195; $P2X_4$ (accession number X87763) forward primer directed to bases 612–657 and reverse primer to bases 1122–1167; $P2X_5$ (accession number X92069) forward primer directed to bases 622–667 and reverse primer to bases 1320–1365; $P2X_6$ (accession number X92070) forward primer directed to bases 621–666 and reverse primer to bases 1092–1137; $P2X_7$ (accession number X95882) forward primer directed to bases 1–45 and reverse primer to bases 621–666.

RT–PCR was performed on 2 μl of the first-strand reaction using the forward and reverse primer sets (200 ng of each primer) designed to amplify each P2X subunit partial cDNA. The reactions also contained 200 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$ and 2.5 units of Dynazyme DNA polymerase (Flowgen, Sittingbourne, U.K.). The conditions were: 1 min at 94°C, 1 min at either 53°C ($P2X_7$) or 58°C ($P2X_{1-6}$), 1 min at 72°C for 35 cycles and, finally, 10 min at 72°C. The obtained PCR products were cloned into the pCR 2.1 vector (TA cloning kit; Invitrogen, Leek, The Netherlands) according to the manufacturer's instructions and sequenced completely using an automated DNA sequencer.

Electrophysiology

Electrophysiological recordings were made from CHO-K1 cells attached to glass coverslips (13 mm; Menzel-Glaser, Fisher, Leicester, U.K.). For each experiment, coverslips were transferred to a perfused recording chamber (volume approximately 400 μl, flow rate 2 ml min^{-1}) mounted on the stage of an inverted microscope (Nikon Diaphot, Nikon U.K.). The whole cell configuration of the patch-clamp technique (Hamill *et al*., 1981) was used to record nucleotide-evoked ionic currents. Cells were perfused with a low-divalent cation containing solution, consisting of (in mM): NaCl 145, KCl 2, $CaCl_2$ 0.5, HEPES 10, D-glucose 10 (pH 7.3, osmolarity 300 mOsm). Patch electrodes were pulled from 1.2 mm borosilicate glass (GC120F-10, Clarke Electromedical Supplies, Pangbourne, U.K.), firepolished, and when filled with (in mM) Cs aspartate 145, EGTA 11, HEPES 5, NaCl 2 (pH 7.3, osmolarity 290 mOsm) had resistances of 2–5 MΩ.

Tight seal (>10 GΩ) whole-cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.). Currents were filtered with a corner frequency of 1–5 kHz (8-pole Bessel filter), digitized at 2–10 kHz using a Digidata 1200A (Axon Instruments) interface, and stored on computer. Data was only recorded from cells with a residual series resistance of less than 18 MΩ, and compensation for series resistance was used (>75%). Cells were voltage-clamped at −90 mV, unless otherwise stated. For determination of reversal potentials, reported voltages were corrected for the junction potential between the internal solution and the extracellular solution in which zero current was obtained before forming a seal. All experiments were performed at room temperature (22–24°C).

In all cases, DbATP or ATP was applied using a computer-controlled fast-flow U-tube system (Fenwick *et al*., 1982), modified to include an extra solenoid valve, which allowed both rapid application and removal of applied drugs (onset and offset latencies of approximately 90 and 50 ms, respectively). Concentration-effect curves to agonists were determined using a 2 s agonist application time with serially increasing agonist concentrations, and a wash period of 2 min between applications. Only one agonist was tested on each cell. In some cells a first concentration-effect curve was followed by determination of a second curve using the same agonist, after an intervening period of 5 min. Current-voltage relationships for DbATP (100 μM) induced responses were determined by stepping the holding voltage to the desired value 500 ms prior to application of agonist. In all electrophysiological studies the data were expressed as a percentage of the initial response to a single application of 1 mM ATP.

^{45}Calcium accumulation

The accumulation of ^{45}calcium ($^{45}Ca^{2+}$) was studied using methods similar to those described previously (Michel *et al*., 1996a). The assay buffer comprised 10 mM HEPES, 5 mM N-methyl-D-glucamine (NMDG), 5 mM KCl, 10 mM glucose and 0.5 mM $CaCl_2$ (pH 7.4.). In the majority of studies this buffer was supplemented with 280 mM sucrose, although in some studies monovalent cations (as their chloride salts) were iso-osmotically substituted for sucrose. Cells were harvested by incubation in 10 ml of ice-cold phosphate buffered saline (PBS) containing 0.1 mM EDTA and no added $CaCl_2$ or $MgCl_2$ (PBS/EDTA) at 4°C for 10 min. Following addition of ice-cold PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ the cells were harvested and centrifuged at $250 \times g$ for 5 min. The supernatant was discarded and the cells were gently resuspended in assay buffer using a 5 ml Gilson pipette and centrifuged at $250 \times g$ for a further 5 min. Finally the cells were gently resuspended in assay buffer to a concentration of approximately 5×10^5 cells ml^{-1}.

Reactions were usually initiated by addition of 200 μl of cells (1×10^5 cells) to 1 ml mintubes (Skatron, Cambridgeshire, U.K.) containing 50 μl of $^{45}Ca^{2+}$ (3.3 Kbq) and test compounds. Assay tube contents were mixed by agitation immediately after addition of the cells and thereafter shaken for 10 s at 1500 cycles min^{-1} every 5 min using a microplate shaker. Reactions were terminated by vacuum filtration over GF/B glass fibre filters using a Canberra Packard Filtermate

cell harvester. In order to reduce filter binding of $^{45}Ca^{2+}$, the filters were pretreated with wash buffer (80 mM NaCl; 80 mM $CaCl_2$) before use and samples were diluted with 0.5 ml of this buffer before filtration. Following filtration, the filters were rinsed for 4 s with wash buffer, dried and, after addition of 50 μl of microscint-0 scintillation fluid, the $^{45}Ca^{2+}$ was measured using liquid scintillation spectrophotometry on a Canberra Packard TopCount scintillation counter. The data are presented as $^{45}Ca^{2+}$ accumulation in d.p.m. rather than as absolute concentrations of calcium. However, if it is assumed that the volume of a CHO-K1 cell is 1×10^{-12} litre then a $^{45}Ca^{2+}$ accumulation of 1000 d.p.m. would represent an approximate cellular $^{45}Ca^{2+}$ concentration of 6 μM.

YO-PRO-1 accumulation

Accumulation of the fluorescent DNA chelating dye, YO-PRO-1 (Hickman *et al*., 1994) was determined in CHO-K1 cells grown on 24-well plates. For these studies the growth medium was removed from confluent monolayers of cells and the wells were filled with assay buffer (as used in studies on $^{45}Ca^{2+}$ accumulation) and incubated for 5 min. The buffer was aspirated and the wells refilled with buffer and incubated for a further 5 min. Finally, the buffer was aspirated and assay buffer and other additions were made in a volume of 250 μl. The final assay concentration of YO-PRO-1 was 1 μM. After the appropriate incubation period the assay buffer was aspirated and the cell monolayer washed by addition of 2 ml of PBS/EDTA which was also aspirated. The cells were harvested by incubation in 0.5 ml of PBS/EDTA for 20 min and transferred to cuvettes. The wells were rinsed with 1.5 ml PBS/EDTA which was also added to the cuvette. YO-PRO-1 fluorescence was measured using an Hitachi spectrofluorimeter using an excitation wavelength of 490 nm and emission wavelength of 509 nm.

Lactate dehydrogenase release

Lactate dehydrogenase (LDH) release was measured in cells grown on 24-well plates. For these studies the cells were processed in a similar manner to that used for the study of YO-PRO-1 accumulation, except that 50 μl aliquots of the assay buffer bathing the cells were removed at various times after initiating reactions. These samples were centrifuged at 250 $\times$ *g* for 5 min prior to determination of LDH in the supernatant using a Promega Cytox96, cytotoxicity kit. Total LDH was determined in separate wells after lysis of the cells in 0.1% Triton X-100.

Data analysis

The data from concentration-effect studies were analysed using a four parameter logistic function by GraphPad Prism to determine the basal and maximal responses and the EC_{50} and slope parameters. Results are presented as the means $\pm$ s.e.mean or the mean and 95% confidence intervals.

Materials

The sources of 2-methyl-thioATP (2-meS-ATP), adenosine 5′-O-(3-thiotriphosphate) (ATPγS), $\alpha\beta$-methylene ATP ($\alpha\beta$ meATP), adenosine, UTP, ADP and ATP were as described previously (Michel *et al*., 1996a). N^6-cyclopentyladenosine (CPA), 5′-N-ethylcarboxamidoadenosine (NECA) and DbATP were from Sigma. $^{45}Ca^{2+}$ (specific activity 0.8 Ci mmol^{-1}) was obtained from Amersham, U.K. YO-PRO-1 was obtained from Molecular Probes (Oregon, U.S.A.). Cytox96 cytotoxicity kits were obtained from Promega.

Results

Amplification of $P2X_7$ partial cDNA by RT–PCR

RT–PCR was applied to a first strand cDNA template synthesized from total RNA extracted from CHO-K1 cells using primer pairs designed to amplify regions of cDNAs encoding the rat $P2X_1$–$P2X_7$ receptors. Only one PCR reaction using the rat $P2X_7$ specific primers resulted in a product (Figure 1 and data not shown). The size of this fragment was approximately 600–700 base pairs on an agarose gel, consistent with the expected size (666 base pairs) for the rat $P2X_7$ partial cDNA at the same region (Surprenant *et al*., 1996 and Figure 1). The amplified partial cDNA was subcloned, sequenced and found to encode the N-terminal one third of the Chinese hamster orthologue of the $P2X_7$ subunit. In contrast, the other P2X subunit specific primers designed from the rat sequences failed to give any products in a PCR reaction with the CHO-K1 template DNA (Figure 1 data for $P2X_4$ and data not shown).

Electrophysiological studies

In low divalent cation containing solution, DbATP and ATP evoked whole-cell inward currents at a holding potential of -90 mV in all CHO-K1 cells tested, with DbATP being more potent than ATP. At the highest concentrations of the agonists

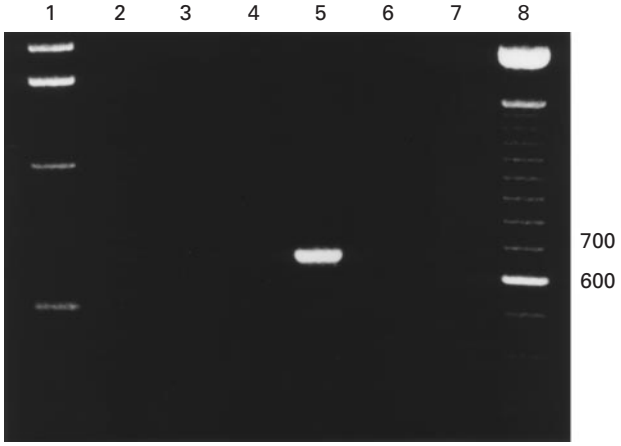

Figure 1 Agarose gel electrophoresis of PCR amplifications from cultured CHO-K1 cells. The gel was stained by ethidium bromide. Lane 1, 1 kb and lane 8, 100 bp DNA ladder (GIBCO/BRL). Lane 2, PCR amplification from the CHO-K1 template DNA using the rat $P2X_4$ specific forward and reverse primers. No product was obtained with these primers. Lane 5, PCR amplification product from CHO-K1 template DNA with the rat $P2X_7$ specific forward and reverse primers. The band corresponds to the predicted size (666 bp) of the rat $P2X_7$ cDNA. Lanes 3, 4, 6 and 7 are control reactions performed either in the absence of template DNA (lanes 3 and 6) or without reverse transcriptase in the first strand reaction (lanes 4 and 7). Lanes 3 and 4 are control reactions with the $P2X_4$ forward and reverse primers and lanes 6, 7 are with the $P2X_7$ forward and reverse primers. PCR amplifications with the rat $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$ and $P2X_6$ specific primer pairs also resulted in no products (data not shown). The PCR amplifications with each of the primer sets were repeated at least three times from two different cDNA syntheses. The 600 and 700 base pair MW markers in lane 8 are indicated.

tested (300 μM for DbATP and 3 mM for ATP), inward currents were 144.9±31.2 and 69.2±9.4 pA, respectively (n=16 and 9). Concentration-effect curves to these agonists did not reach a clearly defined maximum, and thus only estimated EC_{50} values (mean and 95% confidence intervals) of 92.6 (6.5–131.8) μM and 1252.3 (94.6–1656.1) μM, respectively, could be determined (Figure 2a). Determination of a second concentration-effect curve in the same cell for either agonist produced similar EC_{50} and maximal values (data not shown). Current-voltage relationships generated using 100 μM DbATP were approximately linear between −60 and +50 mV, reversing at −2.03±3.1 mV (n=4). The data were well described by linear regression, and thus showed no significant rectification (Figure 2c).

P2 receptor-mediated $^{45}Ca^{2+}$ accumulation in CHO-K1 cells

In a 280 mM sucrose buffer containing 0.5 mM $CaCl_2$ and no added $MgCl_2$, DbATP (100 μM) produced a time-dependent increase in $^{45}Ca^{2+}$ accumulation in CHO-K1 cell suspensions (Figure 3a). Significant accumulation could be detected within 10 s and was maximal after 32–64 min at 22°C. Accumulation was more rapid at 37 than at 22°C and was maximal after 8–16 min (Figure 3a). 2MeSATP, UTP, $\alpha\beta$meATP, ADP, adenosine, NECA and CPA, at concentrations up to 100 μM did not cause $^{45}Ca^{2+}$ accumulation in CHO-K1 cells at either 22 or 37°C (Figure 4a data for 2meSATP and data not shown). In contrast DbATP (pEC_{50}=5.44±0.06; EC_{50}=3.6 μM), ATP

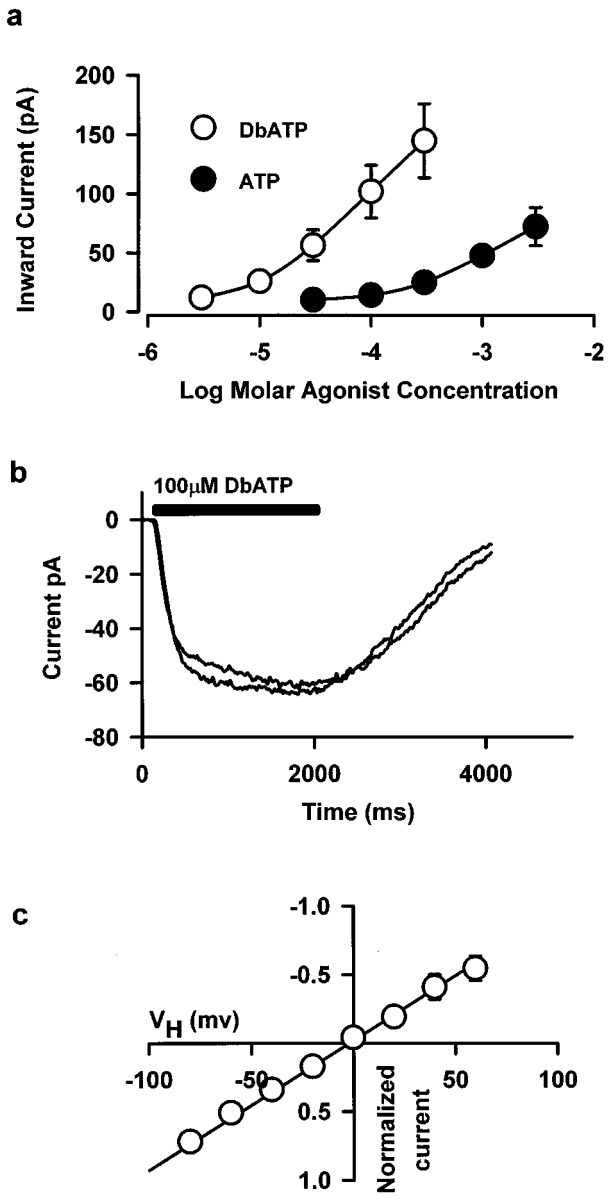

Figure 2 (a) Concentration-effect curves for the ability of ATP and DbATP to activate inward currents in CHO-K1 cells voltage-clamped at −90 mV. Agonists were applied using a fast flow U-tube system for 2 s every 2 min. The data are the means±s.e.mean of six experiments. (b) Representative traces of responses to a 2 s application of 100 μM DbATP obtained in CHO-K1 cells. (c) Current-voltage relationship for 100 μM DbATP-activated responses in CHO-K1 cells. The holding potential of the cells was adjusted to the indicated values 50 ms prior to agonist application. The data are the means±s.e.mean of four experiments.

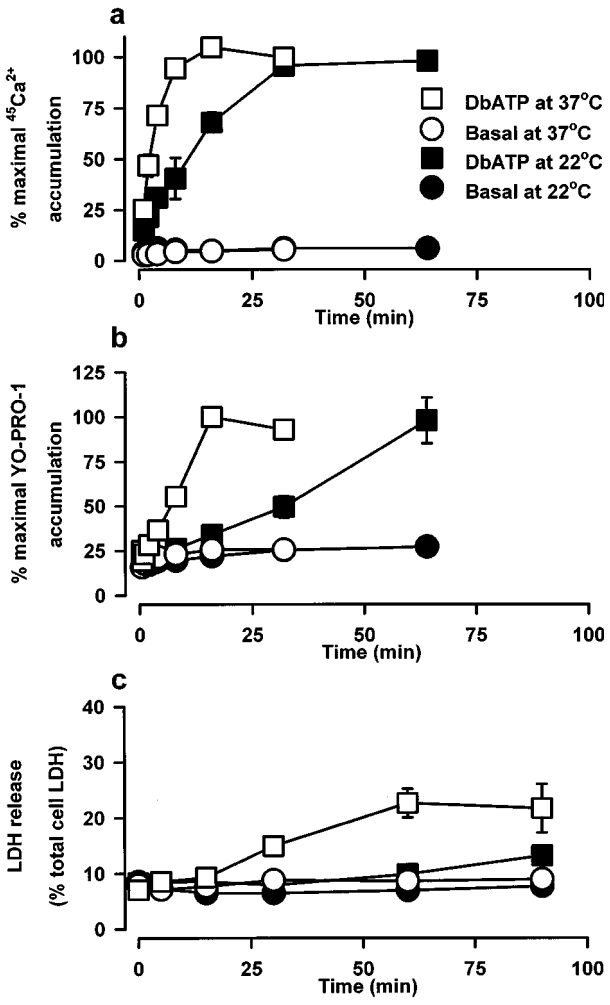

Figure 3 Kinetics of DbATP-stimulated responses in CHO-K1 cells. Cells were incubated in the absence or presence of 100 μM DbATP for varying periods of time at either 22 or 37°C. (a) DbATP-stimulated $^{45}Ca^{2+}$ accumulation was measured in cell suspensions. Reactions were terminated by vacuum filtration. (b) DbATP-stimulated YO-PRO-1 accumulation was measured in cell monolayers grown on 24-well plates. Reactions were terminated after varying times and accumulation measured after washing and harvesting the cells. (c) DbATP-stimulated LDH release was measured in cell monolayers grown on 24-well plates. At the time indicated, aliquots of the cell supernatant were removed and LDH release was measured enzymatically. In all panels the data are the means±s.e.mean of four experiments in which responses were measured at both 22 and 37°C. In a and b the data have been expressed as a percentage of the maximal response obtained at 37°C in each experiment. In c, LDH release was expressed as a percentage of the total cell LDH content.

and ATPγS elicited $^{45}Ca^{2+}$ accumulation which could be detected using a 4 min agonist incubation at 22°C (Figure 4a). Due to the low concentration of $CaCl_2$ employed in these studies, ATP and ATPγS were not assayed at concentrations in excess of 300 μM in order to prevent excessive depletion of calcium through chelation. Consequently it was not possible to estimate their maximal effect or EC_{50} value, although it is clear that ATP and ATPγS were less potent than DbATP (Figure 4a).

When the sucrose in the assay buffer was replaced with the chloride salts of a number of monovalent cations, DbATP-stimulated $^{45}Ca^{2+}$ accumulation measured over 4 min at 22°C was inhibited. Thus, compared to results obtained in 280 mM sucrose, NaCl, LiCl, KCl, guanidinium chloride, N-methyl-D-glucamine chloride and choline chloride inhibited 30 μM DbATP-stimulated $^{45}Ca^{2+}$ accumulation with IC_{50} values (mM with 95% confidence interval in parenthesis) of 8 (7.3–9.4), 12.3 (11.8–12.9), 12.6 (11.7–13.4), 18 (16.2–20.2), 63 (50–79) and 69 (59.3–81), respectively (Figure 5 and data not shown for choline chloride). The reduction in $^{45}Ca^{2+}$ accumulation produced by the monovalent cations was primarily due to a decrease in maximal accumulation with no change in agonist potency. Thus, in buffers containing 280 mM sucrose, 35 mM NaCl/210 mM sucrose, 35 mM KCl/210 mM sucrose or 70 mM choline chloride/140 mM sucrose, pEC_{50} values for DbATP were 5.57 ± 0.10, 5.44 ± 0.02, 5.61 ± 0.07 and 6.03 ± 0.03, respectively ($n = 4$; means ± s.e.mean).

DbATP-stimulated $^{45}Ca^{2+}$ accumulation was also affected by divalent cations (Figure 6 and Table 1). Thus, increasing the concentration of $CaCl_2$ from 0.1–3 mM resulted in a progressive reduction in the absolute levels of $^{45}Ca^{2+}$ accumulation (Figure 6a). However, in these experiments, the concentration of radioactive $^{45}Ca^{2+}$ was kept constant and when the effect of diluting the specific activity of the $^{45}Ca^{2+}$ was taken into account it can be seen that total calcium accumulation increased with $CaCl_2$ concentration (Figure 6b). There was little effect of $CaCl_2$ on agonist potency (Table 1). When the concentration of $MgCl_2$ was increased from 0.3–3 mM in the presence of 0.5 mM $CaCl_2$, the maximal accumulation of $^{45}Ca^{2+}$ and potency of DbATP were reduced (Table 1).

DbATP-stimulated YO-PRO-1 accumulation into CHO-K1 cells

In 280 mM sucrose buffer containing 0.5 mM $CaCl_2$ with no added $MgCl_2$, DbATP (100 μM) elicited a time-dependent (Figure 3b) and concentration-dependent (Figure 4b) accumulation of YO-PRO-1 in CHO-K1 cells grown on 24-well plates. The kinetics for this response were slower than for the accumulation of $^{45}Ca^{2+}$. In particular, at 22°C YO-PRO-1 accumulation was not significant until 8 min and maximal accumulation was only achieved after 64 min. Extending the incubation period did not result in a further increase in YO-PRO-1 accumulation. DbATP-stimulated YO-PRO-1 accumulation was temperature-dependent with a faster rate at 37°C than at 22°C. At 37°C, significant accumulation was evident within 1 min and maximal accumulation was achieved after 16 min and was not significantly different to the maximal level achieved at 22°C. When studied over a 30 min incubation period at 22°C, UTP, αβmeATP, ADP, adenosine, NECA and CPA did not cause YO-PRO-1 accumulation, at concentrations up to 100 μM (data not shown). In contrast, DbATP ($pEC_{50} = 5.11$; $EC_{50} = 7.7$ μM), ATP and ATPγS were able to elicit significant accumulation (Figure 4b).

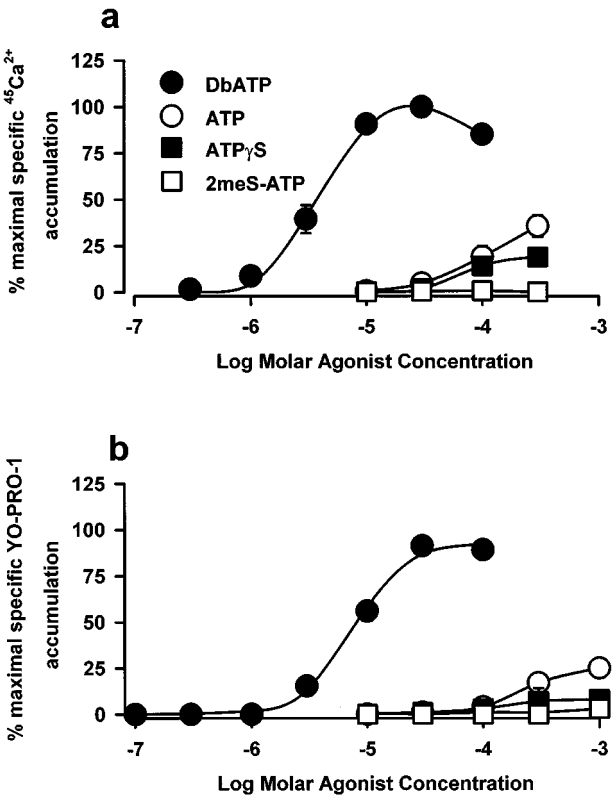

Figure 4 Concentration-effect curves for the ability of DbATP, ATP, ATPγS or 2meS-ATP to stimulate $^{45}Ca^{2+}$ or YO-PRO-1 accumulation in CHO-K1 cells. (a) DbATP-stimulated $^{45}Ca^{2+}$ accumulation was measured in cell suspensions incubated in the absence or presence of the agonists for 4 min at 22°C. Reactions were terminated by vacuum filtration. (b) DbATP-stimulated YO-PRO-1 accumulation was measured in cell monolayers grown on 24-well plates. Reactions were terminated after varying times and accumulation measured after washing and harvesting the cells. In both panels the data are the means ± s.e.mean of four separate experiments in which the four agonists were studied together. The data represent specific accumulation, obtained by subtracting basal accumulation, and have been expressed as a percentage of the maximum response to DbATP in each experiment.

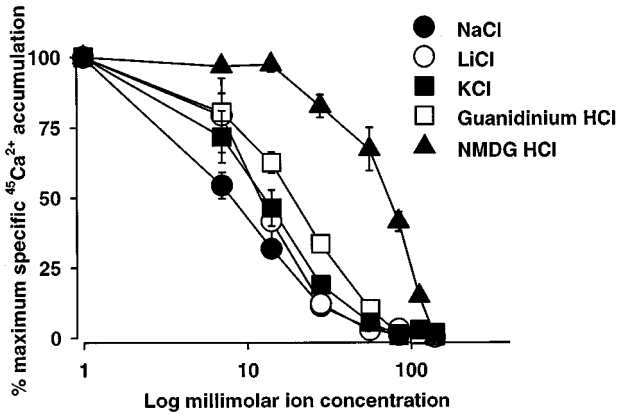

Figure 5 DbATP stimulated $^{45}Ca^{2+}$ accumulation in CHO-K1 cells measured in the presence of increasing concentrations of monovalent cation chloride salts. The ability of DbATP (30 μM) to stimulate $^{45}Ca^{2+}$ accumulation in CHO-K1 cells was studied in assay buffers containing either 280 mM sucrose or buffer in which the indicated concentrations of NaCl, LiCl, KCl, guanidinium chloride or N-methyl-D-glucamine were iso-osmotically substituted for sucrose. Incubations were for 4 min at 22°C and were terminated by vacuum filtration. The data are the means ± s.e.mean of four experiments. In each experiment the data were expressed as a percentage of the maximum specific accumulation stimulated by DbATP in the 280 mM sucrose buffer.

Table 1 Effect of $CaCl_2$ and $MgCl_2$ on DbATP-stimulated $^{45}Ca^{2+}$ accumulation in CHO-K1 cells

Addition (mM)		*DbATP*	*Basal*		
$CaCl_2$	*$MgCl_2$*	*Maximum* (d.p.m.)	*Response* (d.p.m.)	*pEC_{50}*	*n_H*
0.1	0	5731±164	363±10	5.57±0.09	1.6±0.1
0.3	0	3260±83[a]	170±40[c]	5.50±0.09	1.7±0.1
1	0	1436±326[a]	112±28[c]	5.56±0.05	2.1±0.2
3	0	523±8[a]	69±2[c]	5.44±0.02	2.3±0.7
0.5	0	2390±42	134±27	5.50±0.09	1.7±0.1
0.5	0.3	1836±35[b]	149±35	5.25±0.07	1.9±0.1
0.5	1	1132±30[b]	139±33	5.07±0.06[d]	2.0±0.2
0.5	3	386±14[b]	119±28	4.97±0.08[d]	1.7±0.1

CHO-K1 cells were incubated for 4 min at 22°C with DbATP in a 280 mM sucrose buffer (pH 7.4) containing the indicated concentrations of $CaCl_2$ and $MgCl_2$. Incubations were terminated by vacuum filtration and $^{45}Ca^{2+}$ accumulation was determined by scintillation counting. The data were analysed to determine basal and maximal DbATP stimulated $^{45}Ca^{2+}$ accumulation (d.p.m.), the pEC_{50} and the Hill slope (n_H). The data are the means±s.e.mean from four experiments. [a]Significant difference ($P<0.05$) from maximum obtained in 0.1 mM $CaCl_2$ and 0 mM $MgCl_2$. [b]Significant difference ($P<0.05$) from maximum obtained in 0.5 mM $CaCl_2$ and 0 mM $MgCl_2$. [c]Significant difference ($P<0.05$) from basal value obtained in 0.1 mM $CaCl_2$ and 0 mM $MgCl_2$. [d]Significant difference ($P<0.05$) from pEC_{50} obtained in 0.5 mM $CaCl_2$ and 0 mM $MgCl_2$. Statistical test was a one-way ANOVA followed by Duncan's test.

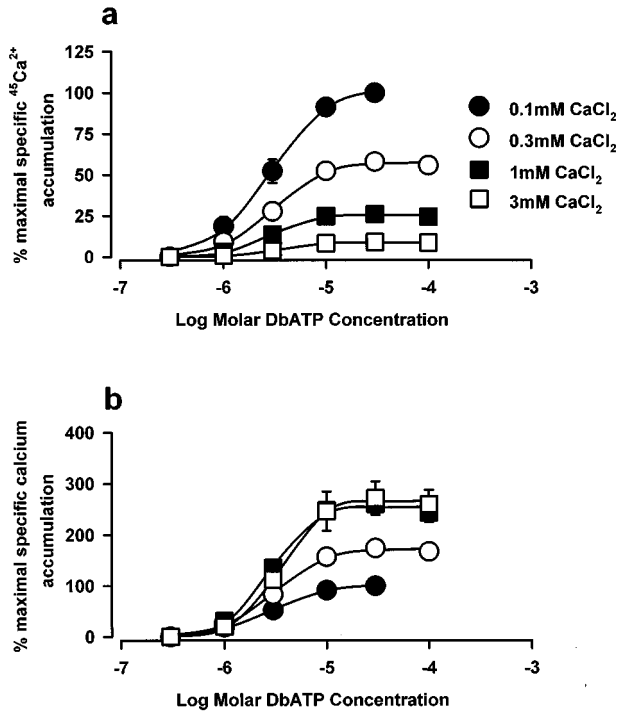

Figure 6 Concentration-effect curves for the ability of DbATP to stimulate $^{45}Ca^{2+}$ accumulation in CHO-K1 cells in the presence of increasing concentrations of $CaCl_2$. DbATP-stimulated $^{45}Ca^{2+}$ accumulation in CHO-K1 cells was studied in 280 mM sucrose assay buffer containing 0.1 mM, 0.3 mM, 1 mM or 3 mM $CaCl_2$. Incubations were for 4 min at 22°C and were terminated by vacuum filtration. The data are the means±s.e.mean of three experiments in which $^{45}Ca^{2+}$ accumulation was measured at all four $CaCl_2$ concentrations. In both panels the data are expressed as a percentage of the maximal specific accumulation stimulated by DbATP in the presence of 0.1 mM $CaCl_2$. (a) The raw data were expressed as $^{45}Ca^{2+}$ accumulation. (b) Total calcium accumulation was calculated based upon the specific activity of the $^{45}Ca^{2+}$.

DbATP-stimulated LDH release from CHO-K1 cells

DbATP increased the release of LDH from CHO-K1 cell monolayers in 24-well plates (Figure 3c). At 22°C this effect was significant after 16 min but was not particularly marked until 60–90 min. At 37°C, a pronounced DbATP-stimulated LDH release was observed after 32 min (Figure 3c). When studied after 30 min, DbATP produced a concentration-dependent increase in the release of LDH at 37°C with a pEC_{50} of 5.34±0.13 ($EC_{50}=4.4$ μM).

Discussion

The main findings of this paper were that CHO-K1 cells express mRNA for the $P2X_7$ receptor and that expression of this receptor can be demonstrated functionally using both electrophysiological and biochemical means.

Among the primer pairs specific for each of the rat P2X subunits used in the RT–PCR studies only the $P2X_7$ primer pair resulted in a product. The identity of this cDNA was confirmed by sequencing and it was found to correspond to the N-terminal one third of the Chinese hamster orthologue of the $P2X_7$ subunit. In addition, RT–PCR confirmed the absence of any other P2X subunits in these cells. The P2X channel present on these cells is therefore probably a homo-oligomeric channel formed by $P2X_7$ subunits. The failure to amplify cDNA for the $P2X_1$-$P2X_6$ receptors may reflect limitations in the use of primers based upon the rat P2X receptors. However, when these primer pairs were used in RT–PCR reactions performed with either rat or murine first strand cDNA templates, products with the expected size for each P2X subunit were obtained (Simon, unpublished observation), suggesting that the primers can identify both rat and mouse P2X receptors.

In addition to demonstrating transcripts for the $P2X_7$ receptor in CHO-K1 cells, functional evidence was also obtained for its expression. Thus, ATP was found to elicit inward currents in the CHO-K1 cells, in agreement with a previous study on this cell line (Michel *et al.*, 1996b). These currents were relatively small but appeared to be due to activation of a non-selective cation channel since they reversed at approximately 0 mV. The potency of DbATP to elicit inward currents (EC_{50} ~93 μM) was considerably less than on the rat recombinant $P2X_7$ receptors (EC_{50} ~2 μM; Surprenant *et al.*, 1996) and slightly lower than determined at the human recombinant $P2X_7$ receptor (50 μM; Rassendren *et al.*, 1997). The demonstration that DbATP also activated similar currents and was considerably more potent than ATP suggests that they were mediated by activation of a P2Z ($P2X_7$) receptor since that is the only ATP-gated cation channel receptor with such operational characteristics (Surprenant *et al.*, 1996).

It was also possible to demonstrate the presence of a P2Z ($P2X_7$) receptor using a number of biochemical methods including measurement of $^{45}Ca^{2+}$ and YO-PRO-1 accumulation and release of LDH. The P2Z receptor has previously been characterized using $^{45}Ca^{2+}$ accumulation (Sela *et al*., 1991; Soltoff *et al*., 1993) and in CHO-K1 cells, DbATP stimulated a marked $^{45}Ca^{2+}$ accumulation. This accumulation could be detected as early as 10 s after agonist exposure and was maximal after 15–60 min. DbATP-stimulated $^{45}Ca^{2+}$ accumulation was sensitive to temperature being more rapid at 37 than at 22°C, although similar maximal accumulation of $^{45}Ca^{2+}$ was observed at the two temperatures. Previous studies have shown a marked temperature sensitivity of the P2Z receptor with more rapid kinetics at 37 than at 22°C (Steinberg *et al*., 1987; Nuttle & Dubyak, 1994). The operational characteristics of the receptor mediating this response were similar to those of the receptor mediating the electrophysiological responses, since DbATP was a more potent agonist than ATP and so it seems probable that $^{45}Ca^{2+}$ accumulation was mediated through activation of a P2Z ($P2X_7$) receptor. There were some differences in absolute agonist potency between the electrophysiological and $^{45}Ca^{2+}$ accumulation studies but this may reflect the use of different buffers for the two techniques.

It is unlikely that DbATP-stimulated $^{45}Ca^{2+}$ accumulation occurred through activation of other P2X receptors since mRNA for the $P2X_1$ - $P2X_6$ receptors was not found in CHO-K1 cells. Furthermore, $\alpha\beta$meATP did not elicit $^{45}Ca^{2+}$ accumulation, suggesting that $P2X_1$ and $P2X_3$ receptors are unlikely to be involved. In studies on the recombinant $P2X_1$-$P2X_4$ receptors we have found that it is possible to measure $^{45}Ca^{2+}$ accumulation in cells expressing recombinant $P2X_2$ or $P2X_4$ receptors, however the EC_{50} for ATP activation of $P2X_2$ and $P2X_4$ receptors, under the assay conditions used in this study, of 3–10 μM (Michel, unpublished observations) is much lower than the EC_{50} for ATP (>100 μM) seen in this study. It is also unlikely that $^{45}Ca^{2+}$ accumulation resulted from activation of P2Y receptors given the low potency of 2-meS-ATP and ATP and the lack of effect of UTP and ADP. Similarly, activation of adenosine receptors can be excluded given the lack of effect of adenosine, CPA and NECA.

The DbATP-stimulated accumulation of $^{45}Ca^{2+}$ in CHO-K1 cells was sensitive to the ionic composition of the buffer and was markedly inhibited when sucrose buffer was replaced by NaCl. An inhibitory effect of NaCl on P2Z function has been described previously (Wiley *et al*., 1992). Similar inhibitory effects were also seen with other monovalent cation chloride salts suggesting that such actions are not unique to NaCl.

In addition to the monovalent cation chloride salts, divalent cation chloride salts also affected DbATP-stimulated $^{45}Ca^{2+}$ accumulation in CHO-K1 cells. This is consistent with previous studies which have demonstrated an inhibitory effect of $MgCl_2$ on P2Z receptor function (Steinberg *et al*., 1987). Indeed it is on the basis of this inhibitory effect of divalent cations on nucleotide potency (Dahlquist & Diamont, 1974; Cockcroft & Gomperts, 1979) that the P2Z receptor has also been termed an ATP^{4-} receptor (Cockcroft & Gomperts, 1980). If one assumes that the affinities of DbATP and ATP for magnesium ions are similar then the approximately 3 fold reductions in DbATP potency produced by $MgCl_2$ would be predictable if only the tetrabasic form of DbATP was an agonist. However, it must be noted that calcium ions, which also chelate ATP, did not change the potency of DbATP. This suggests that either DbATP has lower affinity for calcium than ATP or that the effects of magnesium are not solely attributable to a reduction in ATP^{4-} or $DbATP^{4-}$ concentration.

DbATP also stimulated the accumulation of the fluorescent DNA binding dye, YO-PRO-1, into CHO-K1 cells. The ionized form of this dye has a MW of approximately 375 daltons and has been used to monitor P2Z ($P2X_7$) receptor function in macrophages and on the recombinant $P2X_7$ receptor (Hickman *et al*., 1994; Surprenant *et al*., 1996). The potency of DbATP to stimulate YO-PRO-1 accumulation ($pEC_{50} = 5.11$) was similar to that for stimulating $^{45}Ca^{2}$ accumulation ($pEC_{50} = 5.39$), the only differences between the responses being in the relatively slower kinetics of YO-PRO-1 accumulation. This was particularly evident at 22°C where there was a delay of at least 8 min before significant accumulation of YO-PRO-1 could be detected, yet at the same temperature significant $^{45}Ca^{2}$ accumulation could be detected within 10 s. The delayed YO-PRO-1 accumulation could reflect the slow formation of the putative pore form of the $P2X_7$ receptor and may suggest that the initial $^{45}Ca^{2+}$ influx is through the channel form of the $P2X_7$ receptor. DbATP-stimulated YO-PRO-1 accumulation into CHO-K1 cells was temperature-dependent, being much more rapid at 37°C which is consistent with published data concerning the temperature-sensitivity for formation of the larger pore form of the P2Z ($P2X_7$) receptor (Nuttle & Dubyak, 1994).

YO-PRO-1 can also enter dead or dying cells, although it is unlikely that the accumulation measured in this study reflects this. Thus, entry of YO-PRO-1 preceded overt cell death and lysis as measured by release of LDH. Furthermore, at times when maximal YO-PRO-1 accumulation occurred >95% of cells were able to exclude Trypan blue (Michel, unpublished observation). Even if YO-PRO-1 fluorescence was due to entry into dead cells it is still important to note that the response was increased by DbATP and presumably arose as a consequence of P2Z ($P2X_7$) receptor activation.

The P2Z ($P2X_7$) receptor is often referred to as a cytolytic receptor and it was possible to measure a lytic effect of DbATP in CHO-K1 cells. Lysis of cells was assessed on the basis of LDH release and it was shown that DbATP produced a concentration-dependent release of LDH. The potency of DbATP for producing this action ($pEC_{50} = 5.44$) was similar to that for stimulating $^{45}Ca^{2+}$ and YO-PRO-1 accumulation ($pEC_{50} \sim 5.1-5.4$), however, the kinetics were slower. Furthermore, there was a more marked temperature-dependence for DbATP-stimulated LDH release with appreciable release only evident at 37°C. The finding that the potency of DbATP to activate $^{45}Ca^{2+}$ accumulation, YO-PRO-1 accumulation and LDH release were similar suggests that all three responses were mediated *via* the same receptor and were temporally related. Presumably membrane depolarization and $^{45}Ca^{2+}$ accumulation are the primary responses. With prolonged receptor activation the delayed pore formation occurs which allows entry of YO-PRO-1 and other large ions. As a consequence of the considerable permeability changes due to pore formation, it is likely that cell lysis occurs.

Overall, these data provide strong evidence for the presence of a P2Z ($P2X_7$) receptor in CHO-K1 cells. Although this receptor type is usually studied in cells of immune origin it is interesting to note that the P2Z receptor, or a similar permeabilizing receptor for ATP has been described in a number of transformed cell lines (Weisman *et al*., 1984). Indeed a previous study has demonstrated the ability of ATP to permeabilize CHO-K1 cells (Kitagawa & Akamatsu, 1986), although only in the presence of metabolic inhibitors. Finally, since CHO-K1 cells express an endogenous $P2X_7$ receptor this presents a complication if these cells are used to express recombinant P2X receptors.

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