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ACCELERATED COMMUNICATION

# Coexpression with Potassium Channel Subunits Used to Clone the $Y_2$ Receptor for Neuropeptide Y

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**SUMMARY**

*Xenopus* oocytes were injected with RNAs for the two inward-rectifier potassium channel subunits Kir3.1 (GIRK1) and Kir3.4 (rcKATP or CIR) in addition to RNA from the neuroblastoma cell line KAN-TS. Potassium currents were evoked by neuropeptide Y in oocytes injected with polyadenylated RNA or with cRNA from pools of a neuroblastoma (KAN-TS) cDNA library, and progressive subdivision of responding pools yielded a single cDNA. The encoded protein contains 381 amino acids, has the seven hydrophobic domains characteristic of G protein-coupled receptors, and is 31% identical to the $Y_1$ receptor: potassium currents were induced by neuropeptide Y ($EC_{50}$ = 60 pM) and $Y_2$-selective analogues. Coexpression with potassium channel subunits will be a generally useful method for the cloning of G protein-coupled receptors.

Expression cloning of cDNAs is a powerful method of obtaining *de novo* information about receptor structure. The isolation of a single cDNA is typically followed by measurement of the binding of a radioactive or fluorescent ligand (for review, see Ref. 1), by observation of the opening of an integral ion channel (e.g., glutamate receptors; Ref. 2), or by measurement of a downstream second messenger response such as the calcium-activated chloride channel in *Xenopus* oocytes (e.g., tachykinin receptors; Ref. 3). The downstream responses of the large subset of G protein-coupled receptors that signal through $G_{\alpha i}$ are typically inhibition of adenylyl cyclase, inhibition of voltage-dependent calcium currents, and activation of inward-rectifying potassium currents (reviewed in Ref. 4). Several of these potassium channel genes have recently been cloned (5–8); their coexpression thus provides the possibility to follow the isolation of the receptor cDNA through direct measurement of potassium currents.

One member of this subset of G protein-coupled receptors is the $Y_2$ receptor for NPY. NPY is a 36-amino acid peptide with important physiological roles in feeding behavior, anxiety, and control of blood pressure (9, 10). $Y_2$ receptors are abundant in brain and peripheral nerves; their activation inhibits adenylyl cyclase through pertussis toxin-sensitive G proteins, inhibits voltage-dependent calcium currents, and inhibits transmitter release (11–13). We therefore hypothesized that activation of the $Y_2$ receptor would lead, through a pertussis toxin-sensitive G protein, to the opening of inwardly rectifying potassium channels. This was tested by coexpressing the potassium channel subunits in *Xenopus* oocytes with RNA from KAN-TS cells, which are derived from a cell line (SMS-KAN) known from ligand-binding and functional studies to be rich in $Y_2$ receptors (14, 15).

## Materials and Methods

Total RNA was isolated according to the guanidinium isothiocyanate method from human neuroblastoma cell line KAN-TS (Amersham, Little Chalfont, UK), and the poly(A)$^+$ RNA was subsequently purified with oligo(dT)-cellulose. First-strand cDNA was primed with 5′-$(GA)_5GCGGCCGC(T)_{15}$-3′ and synthesized with Superscript (Bethesda Research Laboratories, Gaithersburg, MD). After conversion double-strand (16), *Eco*RI linkers containing *Xba*I, *Sfi*I, and *Apa*I sites were ligated to the cDNA, and the product was digested with *Not*I. The *Eco*RI/*Not*I cDNA was fractionated on a 5–20% potassium acetate gradient into seven groups ranging in size from 0.8 to 5 kilobases, and a unidirectional library was constructed through ligation of the cDNA into pBKCMV (Stratagene, San Diego, CA) digested with the same enzymes. The seven library fractions were electroporated into *Escherichia coli* DH10B and spread onto Luria broth plates containing 20 μg/ml kanamycin. The plasmid DNA from the pools was prepared through alkaline lysis maxiprep and purified

**ABBREVIATIONS:** NPY, neuropeptide Y; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

through CsCl equilibrium centrifugation (17). For the progressive subdivision of the pools, the bacteria were split into subpools and grown in 5 ml Luria broth containing 50 $\mu$g/ml kanamycin. Cells were harvested; then, plasmid DNA from the subpools was prepared through alkaline lysis miniprep followed by LiCl precipitation (17). *Not*I linearized cDNA was transcribed *in vitro* with T3 RNA polymerase in the presence of the cap analogue $m^7GpppG$ (17). *In vitro* transcribed RNAs for Kir3.1 (GIRK; Ref. 6) and Kir3.4 (Ref. 7) were synthesized from *Not*I-linearized cDNAs with the use of T7 RNA polymerase. cRNA from the pools (20 nl) was coinjected with potassium channel subunit RNAs (25 ng each) into defolliculated *Xenopus* oocytes. cRNA of the individual clones was quantified on a 1.2% agarose/6% formaldehyde gel.

Oocytes were prepared by standard procedures, and two electrode voltage-clamp recordings were made 36–48 hr after injection of cRNAs. Electrodes contained KCl (3 M). For screening of cDNA pools, oocytes were held at −60 mV in 90 mM KCl with 3 mM $MgCl_2$ and 5 mM HEPES, pH 7.5. For pharmacological characterization of the $Y_2$ receptor, oocytes were injected with cRNA (250 pg) transcribed from a truncated form of the $Y_2$ cDNA (the ApaI/*Eco*RI fragment) that contained the coding region plus 132 bases of 5′ utr and 28 bases of 3′ utr.

Both strands of the cDNA were sequenced with a combination of *Bal*31 digestion, the Erase-a-Base system (Promega, Madison, WI) and oligodeoxynucleotides. Data obtained through fluorescent DNA sequencing (Applied Biosystems, Redwood City, CA) were compiled with Sequencher 2.1 (Gene Codes Corporation, Ann Arbor, MI).

## Results and Discussion

NPY (100 nM) evoked a potassium current in oocytes that had been injected with cRNAs encoding Kir3.1 (GIRK1) and Kir3.4 (rcKATP or CIR) in addition to poly(A)$^+$ RNA from KAN-TS cells (Fig. 1a); this was not mimicked by [$Leu^{31}$,$Pro^{34}$]NPY, which is selective for $Y_1$ receptors. A cDNA library was made from KAN-TS cells; cRNA from four of seven size-fractionated pools was tested. In one pool (80,000 cDNA clones) NPY evoked a potassium current; six additional rounds of screening subpools resulted in the isolation of a single cDNA clone (Fig. 1b). The current activated by NPY increased as the pool size decreased, but with the single cDNA, currents became much smaller; large currents were then restored by 200-fold dilution of the RNA before injection. This may be because overexpression of the receptor results in tonic activation (and down-regulation) of the potassium current in the absence of applied agonist; with 50 ng of $Y_2$ RNA (and 25 ng of each potassium channel RNA), the mean holding current at −60 mV (in 90 mM potassium) was 245 ± 9 nA and NPY (10 nM) evoked an inward current of only 13 ± 2 nA (five oocytes), whereas with 250 of pg $Y_2$ RNA, the holding current was 469 ± 83 nA and NPY evoked a current of 182 ± 38 nA (five oocytes of the same batch). In normal potassium concentrations (2 mM), currents evoked by NPY were outward at −40 mV (Fig. 1c); they reversed polarity at ~−85 mV (Fig. 1d), which is close to the expected value for a potassium-selective current.

The single cDNA fully conferred the properties expected of the $Y_2$ receptor; these were compared with the responses observed when the $Y_1$ receptor was expressed in other oocytes of the same batch (Fig. 2). The dose-response curves for NPY were well-fitted by a logistic function with unit Hill coefficient and midpoint ($EC_{50}$) of 60 ± 30 pM (four oocytes) for the $Y_2$ receptor and 100 ± 20 pM (eight oocytes) for the $Y_1$ receptor. The $Y_2$-selective agonists NPY(13–36) and NPY(2–36) were similar in action to NPY at the cloned receptor [$EC_{50}$ values were 130 ± 20 pM (six oocytes) and 80 ± 40 pM (four oocytes), respectively]. Conversely, the $Y_1$-selective agonist [$Leu^{31}$,$Pro^{34}$]NPY had an $EC_{50}$ value of 3 ± 0.1 nM (four oocytes) in oocytes expressing the $Y_1$ receptor, but in oocytes expressing the cloned receptor 100 nM, [$Leu^{31}$,$Pro^{34}$]NPY evoked only 10 ± 3% (three oocytes) of the current evoked by 100 nM NPY(13–36). The $Y_1$-selective antagonist BIBP3226 (100 nM) (18) had no effect on the resting current; it inhibited the action of NPY (1 nM) in oocytes expressing $Y_1$ receptors by 93 ± 4% (three oocytes), but it had little effect on the current evoked by NPY (1 nM) in oocytes expressing the cloned $Y_2$ cDNA (16 ± 2% inhibition; three oocytes). The coupling from receptor to potassium channel involved a pertussis toxin-sensitive G protein because the mean current induced by NPY (100 nM) was only 8 ± 1 nA in five oocytes that had been injected 2–3 hr previously with pertussis toxin (50 ng); five oocytes from the same batch that were not injected with pertussis toxin gave currents of 188 ± 14 nA. Similar results were obtained in oocytes expressing $Y_1$ receptors (5 ± 1 nA in four pertussis toxin-treated oocytes and 171 ± 18 nA in nine control oocytes). These properties and pharmacological profile demonstrate that the cloned cDNA encodes the human $Y_2$ receptor.

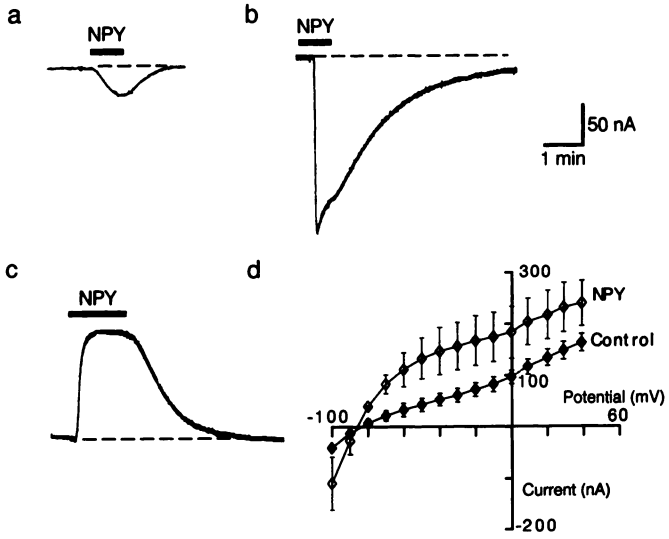


**Fig. 1.** $Y_2$ receptor coupling to heterologously expressed potassium channels in oocytes. a, Inward current evoked by NPY (100 nM) in oocyte coinjected with poly(A)$^+$ RNA from KAN-TS cells as well as cRNA from potassium channel subunits. *Bar*, application time. Holding potential, −60 mV; potassium concentration, 90 mM. Similar responses were observed in 18 of 18 oocytes tested. b, Inward current elicited by 100 nM NPY in oocyte coinjected with cRNA from isolated cDNA clone as well as with cRNA from potassium channel subunits. c, In normal potassium concentration (2 mM), 10 nM NPY(13–36) elicits an outward current at −40 mV in an oocyte expressing $Y_2$ receptor and potassium channel subunits. d, Current-voltage relationship of oocytes expressing $Y_2$ receptor and potassium channel subunits before and after application of 10 nM NPY. Currents were measured during the final 100 msec of a 500-msec step from a holding potential of −40 mV, repeated at 2-sec intervals. Data represent mean ± standard error from four oocytes.

The deduced amino acid sequence of the $Y_2$ receptor has seven hydrophobic domains (Fig. 3). There is a potential *N*-glycosylation site at $Asp^{11}$. There are several potential phosphorylation sites: $Lys^{162}$ and $Arg^{366}$ are possible substrates for cAMP- and cGMP-dependent protein kinases;

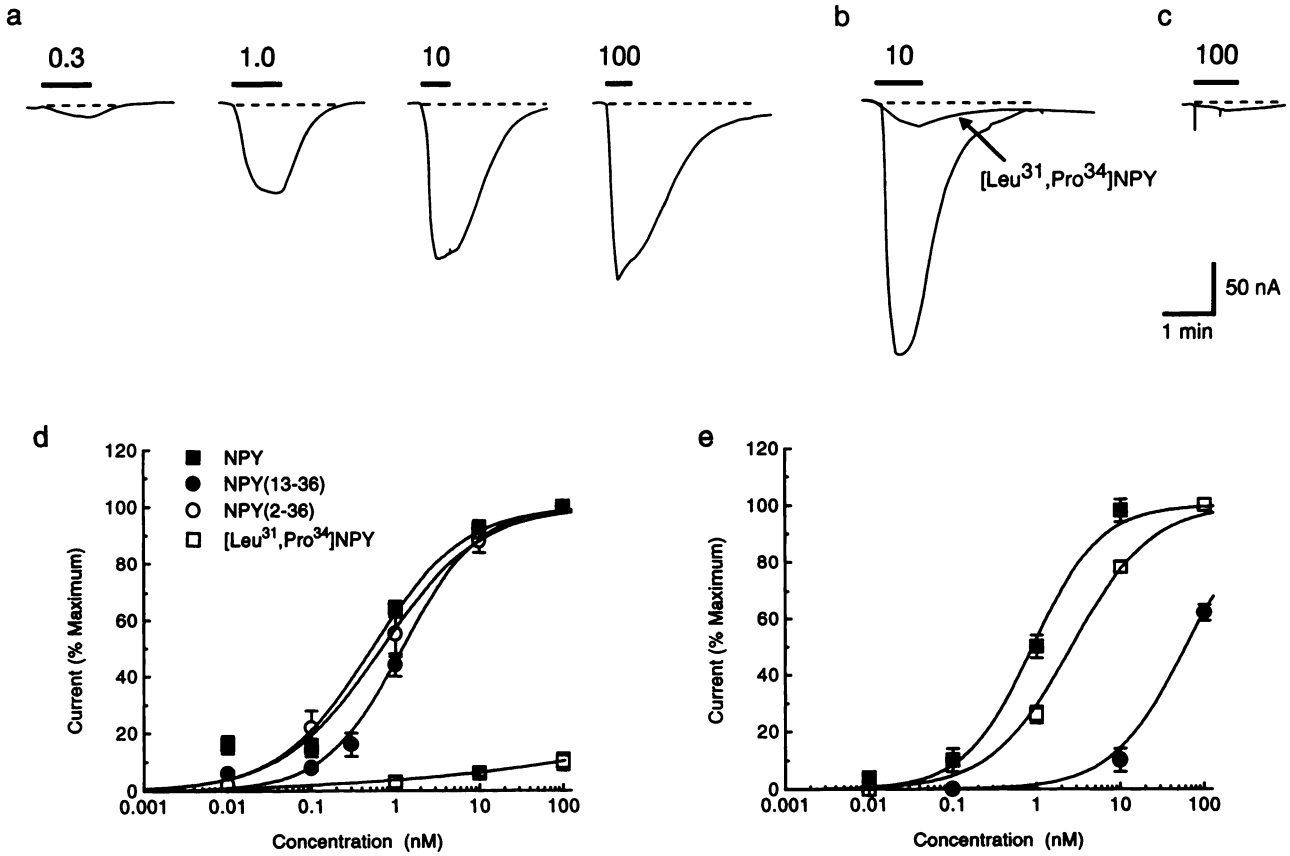


**Fig. 2.** Properties of $Y_2$ and $Y_1$ receptors coexpressed with potassium channel subunits. a–d, Oocytes expressing $Y_2$ receptors. a, Inward currents evoked by different concentrations (indicated in nM) of NPY(13–36) in a single oocyte. Holding potential, −60 mV; potassium concentration, 90 mM. b, Superimposed records of currents evoked by 10 nM [$Leu^{31}Pro^{34}$]NPY and 10 nM NPY(2–36) in the same oocyte. c, Lack of effect of 100 nM NPY in an oocyte injected 2 hr previously with 50 ng of pertussis toxin. d, Concentration-response relations for NPY receptor agonists in oocytes expressing $Y_2$ receptors. e, Concentration-response curves for oocytes expressing $Y_1$ receptors. Each value is the mean ± standard error from three to five experiments of the type illustrated in a. Curves are best fit to hyperbolic functions. In $Y_2$ receptor-expressing oocytes, agonist responses are shown as a percentage of the maximum response evoked by 100 nM NPY(13–36). In $Y_1$ receptor-expressing oocytes, the agonist responses are relative to the maximum induced by 100 nM NPY.

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Y2  MGPIGAEADENQTVEEMKVEQYGPQTTPRGELVPDPEPELIDSTKLIEVQ 050
Y1  MNSTLFSQVENHSVH----SNFSEKNAQLLAFEND------DCHLPLAMI 040
        I                                   II
Y2  VVLILAYCSIILLGVIGNSLVIHVVIKFKSMRTVTNFFIANLAVADLLVN 100
Y1  FTLALAYGAVIILGVSGNLALIIIILKQKEMRNVTNILIVNLSFSDLLVA 090
                        III
Y2  TLCLPFTLTYTLMGEWKMGPVLCHLVPYAQGLAVQVSTITLTVIALDRHR 150
Y1  IMCLPLTFVYTLMDHWVFGEAMCKLNPFVQCVSITVSIFSLVLIAVERHQ 140
                IV
Y2  CIVYHLESKISKRISFLIIGLAWGISALLASPLAIFREYSLIEIIPDFEI 200
Y1  LIINPRGWRPNNRHAYVGIAVIWVLAVASSLPFLIYQVMT-DEPFQNVTL 189
                                        V
Y2  VACTEKW-------PGEEKSIYGTVYSLSSLLILYVLPLGIISFSYTRIW 243
Y1  DAYKDKYVCFDQFPSDSHRLSYTTLL----LVLQYFGPLCFIFICYFKIY 235
                                    VI
Y2  SKLK--NHVSPGAANDHYHQRRQKTTK-MLVCVVVVFAVSWLPLHAFQLA 290
Y1  IRLKRRNNMMDKMRDNKYRSSETKRINIMLLSIVVAFAVCWLPLTIFNTV 285
                VII
Y2  VDIDSQVLDLKEYKLIFTVFHIIAMCSTFANPLLYGWMNSNYRKAF---- 336
Y1  FDWNHQIIATCNHNLLFLLCHLTAMISTCVNPIFYGFLNKNFQRDLQFF 335

Y2  -LSAFRCEQR------LDAIHSEVSVT-FKAKKNLEVRK--NSGPNDSFT 376
Y1  NFCDFRSRDDDYETIAMSTMHTDVSKTSLKQASPVAFKKINNNDDNEKI  384

Y2  EATNV 381
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**Fig. 3.** Sequence alignment of human $Y_2$ and $Y_1$ receptors. Identical residues are *boxed*. *Overlines*, positions of the seven hydrophobic regions. The sequence is the same as that described by Rose *et al.* (20) except that a single nucleotide difference leads to valine at position 134 instead of alanine. The sequence of Rose *et al.* has GenBank accession No. U32500.

$Ser^{374}$ can potentially be phosphorylated by casein kinase II; and protein kinase C might phosphorylate $Ser^{80}$, $Ser^{161}$, $Thr^{266}$, and $Thr^{356}$. The human $Y_2$ receptor is ~31% identical to the human $Y_1$ receptor (Fig. 3), with the highest conservation in transmembrane domains II and VI. Notably absent from the $Y_2$ receptor are two of the three residues ($Tyr^{100}$, $Phe^{286}$, $His^{298}$ in human $Y_1$; Ref. 19) thought to be critical for NPY binding to the $Y_1$ receptor; this is consistent with the prevailing view that NPY binds in fundamentally different ways to the two receptors (9, 10). This relation between $Y_1$

and $Y_2$ receptors is little more than that shown by the receptor with the tachykinin receptors (31%, 27%, and 31% identical to NK1, NK2, and NK3, respectively) or the somatostatin receptors (28%, 26%, 26%, 29%, and 29% identical to SSTR1–5). Very recently, the same $Y_2$ receptor cDNA has been cloned by direct expression in transfected COS-7 cells with assay by binding of $^{125}$I-peptide YY (20).

In conclusion, results of the experiments indicate that the robust coupling between G protein-coupled receptors and inwardly rectifying potassium channels seen in *Xenopus* oocytes can be used to isolate novel cDNA clones. The method is sensitive and relatively fast and does not require high affinity radioactive ligands.

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