3.1.7.1. Pick-Up of ES Cell Colonies with Homologous Recombination

1. Prepare 96-well feeder plates.
2. Refeed 10-cm dish 2 h before picking up colonies.
3. Wash one of the dishes with PBS once and add 10 mL of PBS. Leave the others in the incubator until their turn. If necessary, count the number of colonies.
4. Distribute Trypsin (50 µL/well) to fresh 96-well plates (not to feeders).
5. Pick up the 96 colonies (*see* **Note 4**), then incubate the 96-well plate at 37°C for 10 min. It takes 1 h to pick up 96 colonies, but trypsin will not work on ES cells if it is kept at room temperature.
6. During incubation, remove STO medium from 96-well plates with feeders and add 100 µL/well of ES medium.
7. After incubation, add 50 µL/well of ES medium to Trypsin and pipet up and down to make a single-cell suspension. Transfer the suspension to the 96-well feeders (*see* **Note 5**). Move on to the next dish (*see* **step 2**).
8. Refeed each day (*see* **Note 6**).

3.1.7.2. Freeze-Down of ES Cell Colonies and Screening the Positive ES Clones

1. Prepare two sets of gelatinized 96-well plates.
2. Check each well in the plate with picked-up ES cells for confluence.
3. Refeed the 96-well plates 2 h before freezing down.
4. Remove ES medium and wash twice with PBS, then add 50 µL/well of Trypsin.
5. Incubate the 96-well plates at 37°C for 10 min. During incubation, remove gelatin from two sets of gelatinized plates and add 150 µL/well of ES medium.
6. Add 150 µL/well of ES medium to the Trypsinized plate. Add ES medium to all of the wells without changing tips.
7. Set multichannel pipette at 50 µL and mix the medium in the well by pipeting up and down 10 times to make single cells. Then transfer 50 µL of suspension to two of the gelatinized plates (*see* **Note 7**).
8. Add 100 µL/well of 2 × freezing medium to the original plate, and mix by pipeting up and down a couple of times.
9. Seal the original plate with Parafilm. Freeze at –80°C (*see* **Note 8**).
10. Put replica plates in a CO_2 incubator, and refeed everyday until 90–100% confluence is reached (3–4 d).
11. When cells on the replica plates have become confluent, wash the plates twice with PBS and add 50 µL of ES cell lysis buffer per well containing freshly added proteinase K.
12. Put 96-well plates into Tupperware with water and paper towel for humidity, and incubate at 55°C overnight. Make sure the lid is completely secured.
13. The next day, prepare a mix of sodium chloride and ethanol in a solution reservoir. (Per plate: 10 mL of ethanol + 150 µL of 5 *M* sodium chloride.) Add 100 µL/well of salt/ethanol mixture and stand at room temperature for 20–30 min. DNA will precipitate and stick to the bottom.

14. Invert the plate to discard the solution and place on a paper towel to drain. Add 150 µL/well of 70% ethanol to rinse. Discard the solution and place on a paper towel to drain. Repeat this step at least six times. After final rinse, dry the pellet completely at room temperature.
15 Add 30 µL/well of restriction enzyme cocktail. Incubate the plates at 37°C (or proper temperature for the enzymes) overnight.
16. Follow the regular Southern blotting protocol for screening the positive ES clones with homologous recombination (*see* **Note 9**).

3.2. Generating, Analyzing, and Maintaining Knock-Out Mice

ES cells are introduced into developing embryos to generate chimeras, either by blastocyst injection or by aggregation with morula-stage preimplantation embryos (*see* **Fig. 2**). These techniques are described in detail elsewhere ***(10)***. We describe here the blastocyst injection technique that is most commonly used. Blastocysts injected with ES cells are implanted into the uterine of pseudopregnant recipient foster mothers. Chimeras are generally test bred to ascertain the contribution of the ES cells to the germline. Once germline chimeras are identified, the next step will be to obtain homozygous mice for phenotype analysis. Elsewhere, we describe phenotype analysis of opioid receptor knockout mice including our own ***(1–8,11–13)***.

3.2.1. Blastocyst Injection

Blastocysts are very early-stage embryos that can be collected from the uterus of d 3.5 pregnant females. The C57BL/6 inbred strain is often used. The standard procedure is to inject 10–20 ES cells into the blastocoel cavity of the blastocysts. After injection, culture the embryos for a short period (2–3 h) to allow reexpansion of the blastocoel cavity, and then transfer to the uterine horns of the pseudopregnant outbred mice. The CD-1 outbred strain is often used as recipient, because these mice sustain pregnancies well and provide good parental care.

3.2.2. Chimeric Production and Coat Color Strategy

The resulting mouse pup has tissues and organs consisting of a mosaic mixture of cells derived from the original 129 ES cells and the C57BL/6 host blastocysts. The pups are called chimeras because they contain cells from two independent sources. The coat color of chimeric pups is a mosaic of black from the C57 and Agouti from 129 strain. Appearance of the Agouti coat color is a useful early marker of a successful mutation.

Once chimeras are generated, they are bred to the C57BL/6 strain because germline transmission of the ES cell genome can be identified from the coat color of their pups. Half of the Agouti pups should be heterozygous mutants. Heterozygous mating can then be set up to generate wild-type, heterozygous, and homozygous gene-targeted mice.

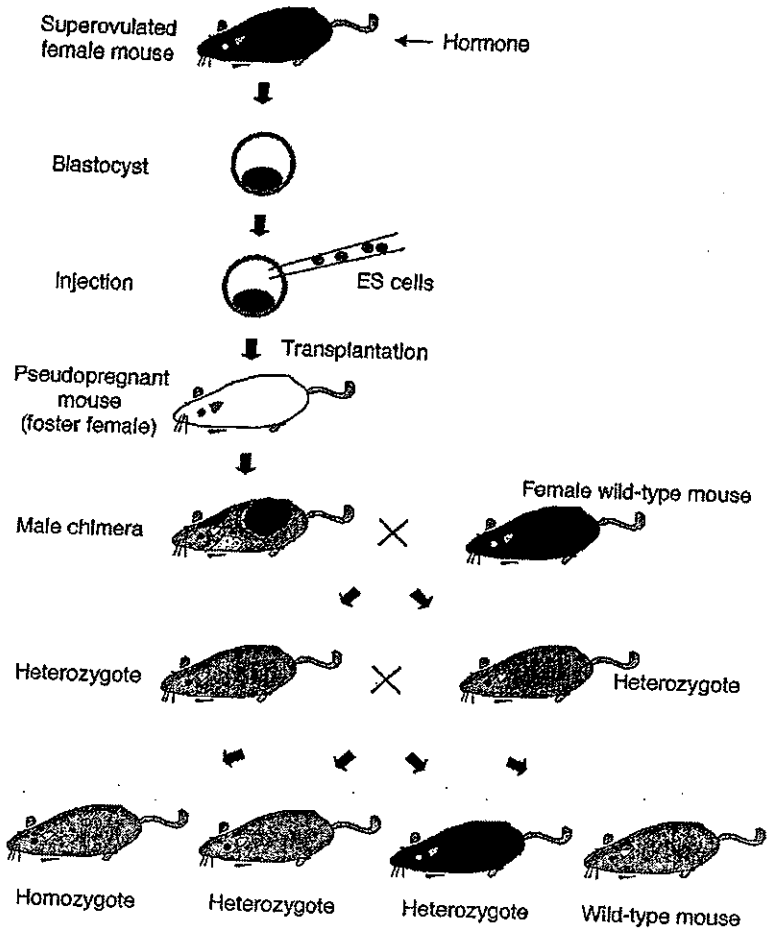

Fig. 2. Schematic illustration of procedure for generation of chimeric mice and derivation of the mouse mutant line. The targeted ES cells are injected into the blastocyst harvested from a superovulated female, then implanted in the uterus of pseudopregnant females. The resulting chimeric male mice are mated with wild-type females to confirm germline transmission. Heterozygous mice born from the wild-type female are mated to produce homozygous mice for phenotypic analyses.

3.2.3. Breeding Scheme

Mice are commonly bred in pairs, in trios (one male with two females), or in harems (one male with more than two females). Weekly rotation of a male mouse through cages containing pairs of females may be used to optimize the yield of offspring from a single male. Mice are weaned at approx 3 wk of age, and should be removed from the breeding cage before the birth of a subsequent litter. Breeding records should be kept to identify those animals to be used as replacement breeders, to maintain pedigree details, and to ensure that unproductive animals can be identified. The mouse database can be kept using any PC spreadsheet software (*see* example in **Table 1**). The breeding nucleus of a congenic strain must be maintained by full-sib mating (brother × sister) whereas outbred strains must be maintained so as to minimize inbreeding.

3.2.4. Analysis of Phenotypes

Phenotypes to be tested for opioid receptor knock-out mice include behaviors such as the analgesia test and reward test. A series of preliminary observations of general health, home cage behavior, sensory abilities, and motor functions is first conducted before analysis of altered responses to opioids, ethanol, and psychostimulant drugs of abuse. If an animal has a major health problem or a gross motor defect, then it will be unable to perform many behavioral tasks for reasons not necessarily specific to the mutation. SHIRPA is a systematic, objective protocol for phenotype analysis that can provide general phenotype assessment *(14)* (http://www.mgu.har.mrc.ac.uk/mutabase/shirpa_summary.html,). The SHIRPA protocol involves three stages, of which the first two give a detailed general phenotype assessment, and the tertiary stage provides a specialized screen tailored primarily to neurological deficits (*see* **Table 2**). The genetic background of knock-out mice influences the phenotype observed to some extent. The issue of genetic background is important, especially in the analysis of complex behavioral phenotypes. Specific protocols for individual behavioral tasks can be found in the original publications, and in several recent reviews *(13,15)*.

Significant numbers of opioid-receptor knock-out mice have now been made. Analyses of μ-opioid receptor (MOR) clearly show that MOR is necessary to mediate morphine action on both pain and reward pathways *(2,3,13)*. MOR knock-out mice show reduced analgesia after administration of morphine, a MOR agonist, but also after administration of δ-opioid receptor agonists *(2,3,11,12)*. κ-opioid receptor (KOR) knock-out mice show no analgesia after administration of KOR agonists, whereas analgesic effects of morphine are intact *(7)*. Conditioning with morphine induced place preference in KOR knock-out mice *(7)*. However, morphine did not induce place preference and self-administration in MOR knock-out mice *(2,13)*.

4. Notes

1. The quality of the FBS is very important for the maintenance of ES cells. Different batches from different suppliers (Hyclone, GIBCO) should be tested for their support growth of pluripotent ES cells. Suitable FBS batches should be purchased in large quantities.
2. One-third or one-quarter of the suspension can be transferred to the same size of fresh dish. Dilution should be no greater than fourfold.
3. Usually 6.5–7.0 ms. If there are extra cells, set up cuvet without DNA to check condition.
4. Set P20 Pipetteman at 2 μL. Under stereomicroscope, circle feeders that surround an ES colony to detach from dish. Suck up the colony and transfer to 96-well plate trypsin. Change tip and move on to the next colony. To avoid confusion, a newly opened tip box should be used.
5. Each well should have 200 μL of suspension. A multichannel pipet is most helpful. Tips must be changed for each row.

Table 1
Example of Mouse Database

Tag#	Location	Color	ID #	Male parent	Female parent	Tagged Date	DOB	Sex	Genotype	Used	Date	Outcome	Date
60	RS	A	MK1-3-1	MK25	MK21	2/7/01	12/21/00	F	+/–	Breeder	3/2/01	Analgesia	9/12/01
61	LN	A	MK1-3-2	MK25	MK21	2/7/01	12/21/00	F	–/–			Died	3/10/01
62	R	B	MK1-3-3	MK25	MK21	2/7/01	12/21/00	M	+/–	CPP	5/16/01	Euthanasia	5/16/01

Tag#: Metal ear tag ID number. Location: location of ear tag, L-left, R-right, S-slash, N-notch. Color: coat color, A-agouti, B-black. ID #: ID number based on male parent. Male parent: ear tag ID number. Female parent: ear tag ID number. DOB: date of birth.

Table 2
Overview of SHIRPA Protocol Stages

Primary Screen for Behavioral Observation Profile

1. Behavior recorded in the viewing jar:
   Body position, spontaneous activity, respiration rate, tremor.
2. Behavior recorded in the arena:
   Transfer arousal, locomotor activity, palpebral closure, piloerection, startle response, gait, pelvic elevation, tail elevation, touch escape, positional passivity.
3. Behavior recorded on or above the arena:
   Trunk curl, limb grasping, visual placing, grip strength, body tone, pinna reflex, corneal reflex, toe pinch, wire manoeuvre.
4. Behavior recorded during supine restraint:
   Skin color, heart rate, limb tone, abdominal tone, lacrimation, salivation, provoked biting, righting reflex, contact righting reflex, negative geotaxis, fear, irritability, aggression, vocalization, body temperature.

Secondary Screen

1. Locomotor activity.
2. Food and water intake.
3. Balance and coordination.
4. Analgesia.
5. Histology.
6. Biochemistry.

Tertiary Screen

1. Anxiety.
2. Learning and memory.
3. Prepulse inhibition.
4. Electroencephalography.
5. Nerve conduction.
6. Magnetic resonance imaging.

6. Most wells will become confluent within 4 d. When they are 80% confluent, prepare two sets of gelatinized 96-well plates.
7. These plates are replicas (50 µL × 2 of suspension will be removed, leaving 100 µL in the original well). Tips must be changed for each row. To avoid confusion, a newly opened tip box should be used.
8. Put the plates in a plastic bag, then put these in a used Styrofoam box.

Southern screen uses a restriction enzyme that cuts once outside one region of homology and a second time within (or at the other side of) the construct, in combination with a single-copy probe outside the region of homology.

References

1. Kieffer, B. L. (1999) Opioids: first lessons from knockout mice. *Trends Pharmacol. Sci.* **20,** 19–26.
2. Matthes, H. W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., et al. (1996) Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* **383,** 819–823.
3. Sora, I., Takahashi, N., Funada, M., Ujike, H., Revay, R. S., Donovan, D. M., et al. (1997) Opiate receptor knockout mice define mu receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc. Natl. Acad. Sci. USA* **94,** 1544–1549.
4. Tian, M., Broxmeyer, H. E., Fan, Y., Lai, Z., Zhang, S., Aronica, S., et al. (1997) Altered hematopoiesis, behavior, and sexual function in mu opioid receptor-deficient mice. *J. Exp. Med.* **185,** 1517–1522.
5. Loh, H. H., Liu, H. C., Cavalli, A., Yang, W., Chen, Y. F., and Wei, L. N. (1998) mu Opioid receptor knockout in mice: effects on ligand-induced analgesia and morphine lethality. *Brain Res. Mol. Brain Res.* **54,** 321–326.
6. Filliol, D., Ghozland, S., Chluba, J., Martin, M., Matthes, H.W., Simonin, F., et al. (2000) Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat. Genet.* **25,** 195–200.
7. Simonin, F., Valverde, O., Smadja, C., Slowe, S., Kitchen, I., Dierich, A., et al. (1998) Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *Embo. J.* **17,** 886–897.
8. Schuller, A. G., King, M. A., Zhang, J., Bolan, E., Pan, Y. X., Morgan, D. J., et al. (1999) Retention of heroin and morphine-6 beta-glucuronide analgesia in a new line of mice lacking exon 1 of MOR-1. *Nat. Neurosci.* **2,** 151–156.
9. Tymms, M. J. and Ismail, I., eds. (2001) *Gene Knockout Protocols*. Methods in Molecular Biology, vol. 158, Humana, Totowa, NJ.
10. Joyner, A. L., ed. (2000) *Gene Targeting - A Practical Approach* (Second Edition), Oxford University Press, New York.
11. Fuchs, P. N., Roza, C., Sora, I., Uhl, G., and Raja, S. N. (1999) Characterization of mechanical withdrawal responses and effects of mu-, delta- and kappa-opioid agonists in normal and mu-opioid receptor knockout mice. *Brain Res.* **821,** 480–486.
12. Sora, I., Funada, M., and Uhl, G. R. (1997) The mu-opioid receptor is necessary for [D-Pen2,D-Pen5]enkephalin- induced analgesia. *Eur. J. Pharmacol.* **324,** R1–R2.
13. Sora, I., Elmer, G., Funada, M., Pieper, J., Li, X.-F., Hall, S., et al. (2001) Mu opiate receptor gene dose effects on different morphine actions: evidence for differential in vivo mu receptor reserve. *Neuropsychopharmacology* **25,** 41–54.
14. Rogers, D. C., Fisher, E. M., Brown, S. D., Peters, J., Hunter, A. J., and Martin, J. E. (1997) Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm. Genome* **8,** 711–713.
15. Crawley, J. N. (2000) *What's Wrong With My Mouse?: Behavioral Phenotyping of Transgenic and Knockout Mice*. Wiley-Liss, New York.

British Journal of Pharmacology (2003) 138, 1119–1128

www.nature.com/bjp

Inhibition of G protein-activated inwardly rectifying K^+ channels by fluoxetine (Prozac)

*,[1]Toru Kobayashi, [1]Kazuo Washiyama & [2]Kazutaka Ikeda

[1]Department of Molecular Neuropathology, Brain Research Institute, Niigata University, 1-757 Asahimachi, Niigata, Niigata 951-8585, Japan and [2]Department of Molecular Psychiatry, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan

1 The effects of fluoxetine, a commonly used antidepressant drug, on G protein-activated inwardly rectifying K^+ channels (GIRK, Kir3) were investigated using *Xenopus* oocyte expression assays.

2 In oocytes injected with mRNAs for GIRK1/GIRK2, GIRK2 or GIRK1/GIRK4 subunits, fluoxetine reversibly reduced inward currents through the basal GIRK activity. The inhibition by fluoxetine showed a concentration-dependence, a weak voltage-dependence and a slight time-dependence with a predominant effect on the instantaneous current elicited by voltage pulses and followed by slight further inhibition. Furthermore, in oocytes expressing GIRK1/2 channels and the cloned *Xenopus* A_1 adenosine receptor, GIRK current responses activated by the receptor were inhibited by fluoxetine. In contrast, ROMK1 and IRK1 channels in other Kir channel subfamilies were insensitive to fluoxetine.

3 The inhibitory effect on GIRK channels was not obtained by intracellularly applied fluoxetine, and not affected by extracellular pH, which changed the proportion of the uncharged to protonated fluoxetine, suggesting that fluoxetine inhibits GIRK channels from the extracellular side.

4 The GIRK currents induced by ethanol were also attenuated in the presence of fluoxetine.

5 We demonstrate that fluoxetine, at low micromolar concentrations, inhibits GIRK channels that play an important role in the inhibitory regulation of neuronal excitability in most brain regions and the heart rate through activation of various G-protein-coupled receptors. The present results suggest that inhibition of GIRK channels by fluoxetine may contribute to some of its therapeutic effects and adverse side effects, particularly seizures in overdose, observed in clinical practice.

British Journal of Pharmacology (2003) **138**, 1119–1128. doi:10.1038/sj.bjp.705172

Keywords: Fluoxetine; antidepressant; GIRK; Kir channel; ethanol; *Xenopus* oocyte

Abbreviations: Ado, adenosine; DMSO, dimethyl sulfoxide; E_K, K^+ equilibrium potential; EtOH, ethanol; GIRK, G protein-activated inwardly rectifying K^+ channel; hK, high potassium; 5-HT, serotonin; Kir channel, inward-rectifier K^+ channel; ND98, K^+-free high sodium; n_H, Hill coefficient; NMDG, *N*-methyl-D-glucamine; QX-314, lidocaine *N*-ethyl bromide; SSRI, selective serotonin reuptake inhibitor; *wv*, *weaver*; XA1 receptor, *Xenopus* A_1 adenosine receptor

Introduction

Fluoxetine, known by the trade name of Prozac, has been used widely in the treatment of depression and other psychiatric disorders, such as obsessive-compulsive disorder, bulimia nervosa, post-traumatic stress disorder and alcoholism (Wong *et al.*, 1995; Baldessarini, 2001). Inhibition of serotonin (5-hydroxytryptamine; 5-HT) transporters in the brain is generally thought to have important implications in its therapeutic effects, and fluoxetine is classified as a selective serotonin reuptake inhibitor (SSRI) (Wong *et al.*, 1995). Recent studies have also shown that fluoxetine inhibits the functions of several receptors and ion channels, such as 5-HT_{2C} (Ni & Miledi, 1997) and 5-HT_3 receptors (Fan, 1994), nicotinic acetylcholine receptors (García-Colunga *et al.*, 1997; Maggi *et al.*, 1998), voltage-gated Ca^{2+}, Na^+ and K^+ channels (Pancrazio *et al.*, 1998; Choi *et al.*, 1999; 2001; Yeung *et al.*, 1999; Deák *et al.*, 2000; Perchenet *et al.*, 2001; Thomas *et al.*, 2002) and Cl^- channels (Maertens *et al.*, 1999). In addition to inhibition of serotonin transporters, these effects might be involved in the molecular and cellular mechanisms underlying the multiple therapeutic effects and side effects of fluoxetine.

G protein-activated inwardly rectifying K^+ (GIRK) channels (also known as Kir3 channels) are members of a family of inward-rectifier K^+ (Kir) channels that includes seven subfamilies (Doupnik *et al.*, 1995; Reimann & Ashcroft, 1999). Four GIRK channel subunits have been identified in mammals (Kubo *et al.*, 1993b; Lesage *et al.*, 1995; Wickman *et al.*, 1997). Neuronal GIRK channels are predominantly heteromultimers composed of GIRK1 and GIRK2 subunits in most brain regions (Kobayashi *et al.*, 1995; Lesage *et al.*, 1995; Karschin *et al.*, 1996; Liao *et al.*, 1996) or homomultimers composed of GIRK2 subunits in the substantia nigra (Inanobe *et al.*, 1999), and atrial GIRK channels are heteromultimers composed of GIRK1 and GIRK4 subunits (Krapivinsky *et al.*, 1995). Various G-protein-coupled receptors, such as M_2 muscarinic, α_2 adrenergic, D_2 dopamine, 5-HT_{1A}, opioid,

*Author for correspondence; E-mail: torukoba@bri.niigata-u.ac.jp

nociceptin/orphanin FQ receptors and A_1 adenosine (Ado) receptor, activate GIRK channels (North, 1989; Ikeda *et al.*, 1995, 1996, 1997) through direct action of G protein $\beta\gamma$-subunits released from pertussis toxin-sensitive G proteins (Reuveny *et al.*, 1994). In addition, ethanol (EtOH) activates GIRK channels independent of G-protein-coupled signalling pathways (Kobayashi *et al.*, 1999; Lewohl *et al.*, 1999). Activation of GIRK channels causes membrane hyperpolarization, and thus the channels play an important role in the inhibitory regulation of the neuronal excitability and the heart rate (North, 1989; Signorini *et al.*, 1997; Wickman *et al.*, 1998). Therefore, modulators of GIRK channel activity may affect many brain and cardiac functions. Here we show that fluoxetine inhibits brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels expressed in *Xenopus* oocytes, whereas two different SSRIs: fluvoxamine and zimelidine have no significant effect on the channels. In contrast, ROMK1 (Kir1.1) and IRK1 (Kir2.1) channels in other Kir channel subfamilies were insensitive to fluoxetine. Moreover, fluoxetine inhibited EtOH-induced GIRK currents. We propose that some effects of fluoxetine in clinical practice may involve inhibition of GIRK channels in the brain and heart.

Methods

Preparation of specific mRNAs

Plasmids containing the entire coding sequences for the mouse GIRK1, GIRK2, *weaver* (*wv*) GIRK2 and GIRK4 channel subunits and the *Xenopus* A_1 (XA1) Ado receptor were obtained using the polymerase chain reaction method as described previously (Kobayashi *et al.*, 1995, 1999, 2000, 2002). In addition, cDNAs for rat ROMK1 in pSPORT and mouse IRK1 in pcDNA1 were provided by Dr Steven C. Hebert and Dr Lily Y. Jan, respectively. These plasmids were linearized by digestion with an appropriate enzyme as described previously (Ho *et al.*, 1993; Kubo *et al.*, 1993a; Kobayashi *et al.*, 2000), and the specific mRNAs were synthesized *in vitro* using the mMESSAGE mMACHINE™ *In Vitro* Transcription Kit (Ambion, Austin, TX, U.S.A.).

Electrophysiological analyses

Adult female *Xenopus laevis* frogs were purchased from Copacetic (Soma, Aomori, Japan) and maintained in the laboratory until use. Frogs were anesthetized by immersion in water including 0.15% tricaine (Sigma Chemical Co., St Louis, MO, U.S.A.). A small incision was made on the abdomen to remove several ovarian lobes from the frogs that were humanely killed after the final collection. Oocytes (Stages V and VI) were isolated manually from the ovary and maintained in Barth's solution (Kobayashi *et al.*, 2002). *Xenopus laevis* oocytes were injected with mRNA(s) for GIRK1/GIRK2 or GIRK1/GIRK4 combinations (each ~0.4 ng), GIRK2 (~5 ng), *wv*GIRK2 (~12.5 ng), ROMK1 (~5 ng) or IRK1 (~0.5 ng) and/or XA1 (~10 ng). The oocytes were incubated at 19°C in Barth's solution, and defolliculated following treatment with 0.8 mg ml^{-1} collagenase as described previously (Kobayashi *et al.*, 2002). Whole-cell currents of the oocytes were recorded from 2 to 10 days after the injection with a conventional two-electrode voltage clamp (Kobayashi *et al.*, 1999). The membrane potential was held at −70 mV, unless otherwise specified. Microelectrodes were filled with 3 M KCl. The oocytes were placed in a 0.05 ml narrow chamber and superfused continuously with a high-potassium (hK) solution (composition in mM: KCl 96, NaCl 2, $MgCl_2$ 1, $CaCl_2$ 1.5 and HEPES 5), a K^+-free high-sodium (ND98) solution (composition in mM: NaCl 98, $MgCl_2$ 1, $CaCl_2$ 1.5 and HEPES 5) or a large organic cation *N*-methyl-D-glucamine (NMDG) solution (composition in mM: NMDG 98, $MgCl_2$ 1 and HEPES 5) at a flow rate of 2.5 ml min^{-1}. The acidic or basic solutions were made by adding HCl or NaOH, respectively. For examining the effect of intracellular fluoxetine, 23 nl of 10 mM fluoxetine or 30 mM lidocaine *N*-ethyl bromide (QX-314) dissolved in distilled water was injected into an oocyte using a Nanoliter injector (World Precision Instruments, Sarasota, FL, U.S.A.) (Kobayashi *et al.*, 1999) and the oocyte currents were continuously recorded for 30–40 min. Owing to a volume of ~1 μl in the oocyte, the intracellular concentrations of fluoxetine or QX-314 were presumed as ~225 or ~674 μM, respectively. In the hK solution, the K^+ equilibrium potential (E_K) was close to 0 mV and inward K^+ current flow through Kir channels was observed at negative holding potentials. Data were fitted to a standard logistic equation using SigmaPlot (SPSS Science, Chicago, IL, U.S.A.) in the analysis of concentration–response relations. The EC_{50} value, which is the concentration of a drug that produces 50% of the maximal current response for that drug, IC_{50} value which is the concentration of a drug that reduces control current responses by 50% and the Hill coefficient (n_H) were obtained from the concentration–response relations.

Statistical analysis of results

The values obtained are expressed as mean±s.e.m., and n is the number of oocytes tested. Statistical analysis of differences between groups was carried out using paired t-test, Student's t-test or one-way ANOVA followed by Tukey–Kramer *post hoc* test. A probability of 0.05 was taken as the level of statistical significance.

Compounds

Fluoxetine hydrochloride and fluvoxamine maleate were purchased from Tocris Cookson Limited (Bristol, U.K.). Zimelidine dihydrochloride and Ado were purchased from Research Biochemical International (Natick, MA, U.S.A.). QX-314 was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Fluoxetine and fluvoxamine were dissolved in dimethyl sulfoxide (DMSO) or distilled water. Other drugs were dissolved in distilled water. The stock solutions of these compounds were stored at −30°C until use. EtOH was purchased from Wako Pure Chemical Industries (Osaka, Japan). Each compound was added to the perfusion solution in appropriate amounts immediately before the experiments.

Results

Inhibition of GIRK channels by fluoxetine

To investigate whether fluoxetine interacts with brain-type GIRK1/2 and cardiac-type GIRK1/4 channels, we used

Xenopus oocyte expression assays. In oocytes coinjected with GIRK1 and GIRK2 mRNAs, basal GIRK currents, which are known to depend on free G protein $\beta\gamma$-subunits present in the oocytes because of the inherent activity of G proteins (Dascal, 1997), were observed under the conditions of a hK solution containing 96 mM K^+ and negative membrane potentials (Kobayashi *et al.*, 2000; Figure 1a). Application of fluoxetine, at 10 μM, immediately and reversibly caused a reduction of the inward currents through basally active channels in the hK solution (Figure 1a). The current responses were abolished in the presence of 3 mM Ba^{2+}, which blocks the Kir channel family including GIRK channels ($n=4$; Figure 1a). Fluoxetine produced no significant response in a K^+-free ND98 solution containing 98 mM Na^+ instead of the hK solution (5±5 nA, $n=3$), suggesting that the fluoxetine-sensitive current components show K^+ selectivity. In uninjected oocytes, fluoxetine and Ba^{2+} caused no significant response (0 nA at 200 μM and 5.5±0.7 nA at 3 mM at a membrane potential of −70 mV, $n=4$ and 29, respectively; Figure 1a), suggesting no effect of fluoxetine on intrinsic oocyte channels. In addition, application of DMSO, the solvent vehicle, at the highest concentration (0.3%) used had no significant effect on the current responses in oocytes coinjected with GIRK1 and GIRK2 mRNAs ($n=4$; data not shown). These results suggest that fluoxetine inhibits GIRK1/2 channels. However, the channels were insensitive to two different SSRIs: fluvoxamine and zimelidine (6.1±2.3% inhibition and 0.7±1.2% inhibition of the 3 mM Ba^{2+}-sensitive current component at 100 μM, $n=11$ and 10, respectively). Furthermore, similar results were obtained in oocytes co-injected with GIRK1 and GIRK4 mRNAs (Figure 1a), suggesting that fluoxetine also inhibits GIRK1/4 channels.

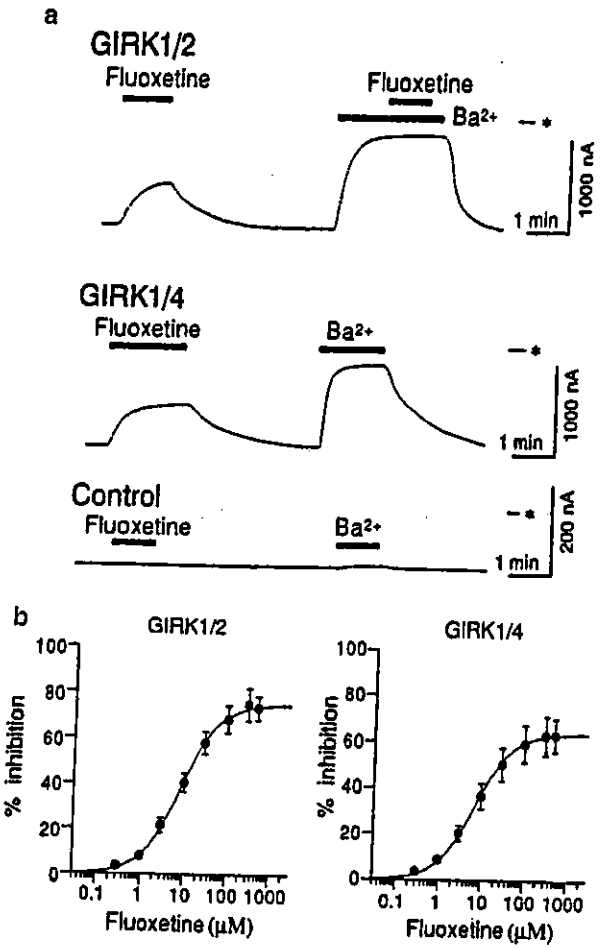

Figure 1 Inhibition by fluoxetine of brain-type GIRK1/2 channels and cardiac-type GIRK1/4 channels expressed in *Xenopus* oocytes. (a) *Top*: In an oocyte coinjected with GIRK1 and GIRK2 mRNAs, current responses to 10 μM fluoxetine and 10 μM fluoxetine in the presence of 3 mM Ba^{2+}. *Middle*: In an oocyte coinjected with GIRK1 and GIRK4 mRNAs, current responses to 10 μM fluoxetine and 3 m*M* Ba^{2+}. *Bottom*: In an uninjected oocyte, no significant current responses to 200 μM fluoxetine and 3 mM Ba^{2+}. Asterisks show the zero current level. Bars show the duration of application. (b) Concentration–response relations for fluoxetine in regard to the effects on GIRK1/2 channels (left) and GIRK1/4 channels (right). The magnitudes of inhibition of GIRK current by fluoxetine were compared with the 3 mM Ba^{2+}-sensitive current components, which were 741.6±118.0 nA ($n=5$) in oocytes expressing GIRK1/2 channels and 600.8±268.0 nA ($n=5$) in oocytes expressing GIRK1/4 channels, respectively. Current responses were measured at a membrane potential of −70 mV in a high-potassium solution containing 96 mM K^+ (pH 7.4). Each point and error bar represents the mean and s.e.m. of the percentage responses obtained from five oocytes. Data points were fitted using a logistic equation.

Characteristics in fluoxetine inhibition of GIRK channels

We investigated the concentration–response relations of the inhibitory effects of fluoxetine on GIRK channels expressed in *Xenopus* oocytes, compared with the current components sensitive to 3 mM Ba^{2+} which blocked basal GIRK currents (Kobayashi *et al.*, 2002). Inhibition of GIRK1/2 and GIRK1/4 channels by fluoxetine was concentration-dependent with similar potency and effectiveness at micromolar concentrations (Figure 1b). Fluoxetine reduced the GIRK currents to a limited extent even at high concentrations. Table 1 shows the EC_{50} and n_H values obtained from the concentration–response relations for fluoxetine and the percentage inhibition of the GIRK currents by the drug at the highest concentrations tested. In addition, to further compare the effects of fluoxetine on GIRK channels, the drug concentrations required to inhibit the GIRK currents by 50% are shown in Table 1.

The instantaneous GIRK1/2 currents elicited by the voltage step to −100 mV from a holding potential of 0 mV diminished in the presence of 10 μM fluoxetine (Figure 2a). The percentage inhibition of the steady-state GIRK current at the end of the voltage step by fluoxetine was slightly higher than that of the instantaneous current (4.6±0.2%, 4.6±0.1% and 5.2±0.1% at −80, −100 and −120 mV; paired *t*-test, $P<0.05$, 0.005 and 0.005; $n=11$, 10 and 12, respectively). These results suggest that the channels were primarily inhibited at the holding potential of 0 mV and in only a slight time-dependent manner by external fluoxetine.

Like 3 mM Ba^{2+}-sensitive currents corresponding to basal GIRK currents, fluoxetine-sensitive currents in oocytes expressing GIRK channels increased with negative membrane potentials and the current–voltage relations showed strong inward rectification (Figure 2b), indicating a characteristic of GIRK currents.

For GIRK1/2 channels, the percentage inhibition of GIRK currents by 10 μM fluoxetine tended to decrease with membrane potentials from −80 to −20 mV, although there was no significant difference in the percentage inhibition at membrane potentials between −120 and −40 mV (Figure 2c). For

Table 1 Inhibitory effects of fluoxetine on GIRK channels

Channel	*EC_{50}*	*IC_{50}*	*% inhibition (μM)*	*n_H*	*(n)*
GIRK1/2	7.89±0.60	16.9±4.0	73.6±6.1 (300)	0.83±0.06	(5)
GIRK1/4	7.05±0.65	18.4±4.3	63.3±8.5 (300)	0.85±0.03	(5)
GIRK2	13.03±1.89	89.5±24.3	57.5±7.5 (300)	0.89±0.02	(7)
*wv*GIRK2	7.85±2.24	9.37±2.81	92.0±3.4 (200)	0.82±0.05	(4)

The mean±s.e.m. of the EC_{50} values and the drug concentrations required to reduce basal GIRK currents by 50% (IC_{50}) are shown in μM. The values of % inhibition indicate the mean±s.e.m.% inhibition of basal GIRK currents by fluoxetine at the highest concentrations tested. The highest concentrations tested (μM) and the number of oocytes tested (*n*) are indicated in parentheses. The n_H values indicate the mean±s.e.m. of the Hill coefficients.

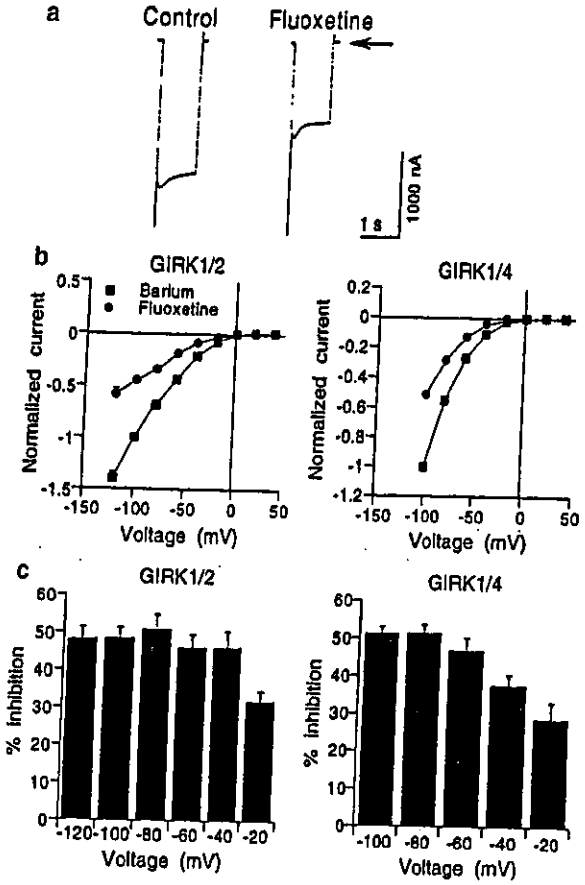

Figure 2 Characteristics in the effects of fluoxetine on GIRK currents. (a) Representative GIRK1/2 currents elicited by voltage step to −100 mV for 1 s from a holding potential of 0 mV in the absence and presence of 10 μM fluoxetine. Current responses were recorded in a high-potassium solution containing 96 mM K^+. Arrow indicates the zero current level. (b) Current–voltage relations of 3 mM Ba^{2+}-sensitive inward currents and 10 μM fluoxetine-sensitive inward currents in oocytes expressing GIRK1/2 channels or GIRK1/4 channels. Current responses were normalized to the 3 mM Ba^{2+}-sensitive current component measured at a membrane potential of −100 mV. The Ba^{2+}-sensitive current components were 2426.9±661.3 nA ($n=7$) in oocytes expressing GIRK1/2 channels and 1203.3±307.0 nA ($n=4$) in oocytes expressing GIRK1/4 channels. (c) The percentage inhibition of GIRK channels by fluoxetine over the voltage range of −120 to −20 mV. There was a significant interaction between the fluoxetine effect and the membrane potential effect ($P<0.05$ for GIRK1/2, $n=5-16$ for the groups, and $P<0.001$ for GIRK1/4, $n=4$ for each group; one-way ANOVA), and then there were significant differences between the effects at −80 or −100 mV and that at −20 mV for GIRK1/2 channels, and between the effects at −60, −80 or −100 mV and that at −20 mV and between the effects at −80 or −100 mV and that at −40 mV for GIRK1/4 channels ($P<0.05$; Tukey–Kramer *post hoc* test). All values are mean and s.e.m.

GIRK1/4 channels, the percentage inhibition of GIRK currents by 10 μM fluoxetine decreased with membrane potentials from −80 to −20 mV (Figure 2c). When membrane potentials were more negative than −100 mV, the percentage inhibitions by 10 μM fluoxetine were almost the same both for GIRK1/2 and GIRK1/4 channels, suggesting that some changes in the interaction between fluoxetine and GIRK channels may occur at very negative membrane potentials.

At physiological pH or below, fluoxetine exists mainly in a protonated form and the proportion of the uncharged form increases by increasing pH, because of a pK_a value of 9.5 (Maertens *et al.*, 1999). We examined whether changes in pH affect fluoxetine inhibition of GIRK channels. The effects of pH on the fluoxetine inhibition were not observed in the concentration–response relations for fluoxetine (Figure 3), suggesting that the inhibition is mediated by both forms of fluoxetine with almost the same effectiveness. It also appears unlikely that the inhibition by fluoxetine is caused by hydrophobic interactions with GIRK channels within the membrane bilayer.

Moreover, we examined the effect of intracellular fluoxetine on GIRK channels. Injection of fluoxetine into oocytes expressing GIRK1/2 channels had no or little effect on the basal currents (3.8±1.8% inhibition of control at the presumed intracellular concentration of ~225 μM, $n=9$). Therefore, the result suggests that fluoxetine directly inhibits GIRK channels from the extracellular side of the cell membrane.

We further investigated the effects of fluoxetine on GIRK channels activated by G-protein-coupled receptors. In oocytes coexpressing GIRK1/2 channels and XA1 receptor (Kobayashi *et al.*, 2002), application of 10 nM Ado induced inward GIRK currents (Figure 4). Effects of fluoxetine were evaluated by measuring the amplitude of the Adoinduced current response during application of fluoxetine at different concentrations. The current responses to 10 nM Ado were reversibly inhibited by fluoxetine with an IC_{50} value of 9.26±2.00 μM and a n_H value of 0.88±0.08 ($n=5$, Figure 4). The extent of inhibition by fluoxetine was similar to that of basally active GIRK1/2 channels ($P>0.05$ at each concentration, Student's *t*-test), suggesting interaction of fluoxetine with GIRK channels. In addition, the Ado-induced GIRK currents were not significantly affected by intracellularly applied fluoxetine (112.2±14.4% of pretreated control current, paired *t*-test, $P>0.1$, $n=5$), whereas the GIRK currents were significantly inhibited by intracellularly applied QX-314 (55.2±11.9% of pretreated control current, paired *t*-test, $P<0.01$, $n=8$) as reported previously (Zhou *et al.*, 2001). The results, therefore, suggest that extracellular fluoxetine can inhibit the effect of GIRK channels activated by G-protein-coupled receptors.

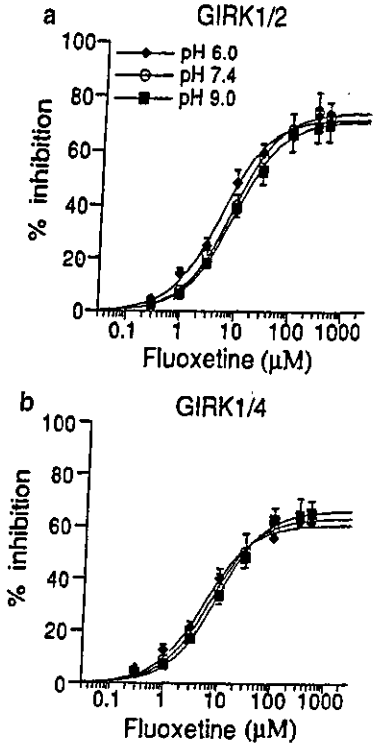

Figure 3 Concentration–response relations for inhibition of GIRK1/2 channels and GIRK1/4 channels by fluoxetine at three different pH values. The magnitudes of inhibition of GIRK current by fluoxetine were compared with the 3 mM Ba^{2+}-sensitive current components, which were 1033.3±184.8 nA ($n=6$, pH 6.0), 741.6±118.0 nA ($n=5$, pH 7.4) and 822.0±166.3 nA ($n=4$, pH 9.0) in oocytes expressing GIRK1/2 channels (a), and 625.5±117.4 nA ($n=6$, pH 6.0), 600.8±268.0 nA ($n=5$, pH 7.4) and 328.8±114.0 nA ($n=4$, pH 9.0) in oocytes expressing GIRK1/4 channels (b), respectively. Current responses were measured at a membrane potential of −70 mV in a high-potassium solution. Each point and error bar represents the mean and s.e.m. of the percentage responses obtained. Data points were fitted using a logistic equation.

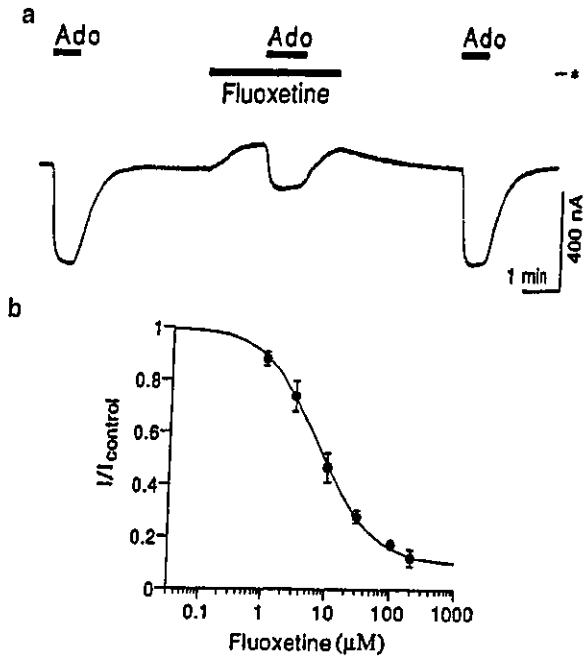

Figure 4 Inhibitory effect of fluoxetine on GIRK channels activated by a G-protein-coupled receptor. (a) In an oocyte coinjected with mRNAs for GIRK1 and GIRK2 channels and XA1 receptor, current responses to adenosine (Ado), Ado in the presence of 10 μM fluoxetine and Ado are shown. The concentration of Ado used was 10 nM. Bars show the duration of application. Asterisk indicates the zero current level. (b) Concentration-dependent inhibition of fluoxetine on Ado-induced GIRK currents. $I_{control}$ is the amplitude of GIRK currents induced by 10 nM Ado (407.2±109.0 nA, $n=5$) and I is the current amplitude in the presence of fluoxetine. Current responses were measured at a membrane potential of −70 mV in a high-potassium solution. Each point and error bar represents the mean and s.e.m. of the relative responses. Data points were fitted using a logistic equation.

Comparison of the fluoxetine effects on members of Kir channels

We examined whether fluoxetine interacts with ROMK1, an ATP-regulated inwardly rectifying K^+ channel, and IRK1, a constitutively active Kir channel, among G-protein-insensitive Kir channels. In oocytes expressing ROMK1 or IRK1 channels, application of 100 μM fluoxetine had no significant effect on the inward currents through the channels in the hK solution (0.3±0.3 inhibition and 0.8±0.3% inhibition of the 3 mM Ba^{2+}-sensitive current components that were 946.0±126.0 and 967.7±418.1 nA at a membrane potential of −70 mV, $n=3$ for ROMK1 and $n=6$ for IRK1, respectively; Figure 5a).

GIRK2 channels form homomultimers (Lesage *et al.*, 1995; Inanobe *et al.*, 1999). The GIRK2 channels of *wv* mutant mice, which have a missense point mutation in the pore-forming region (Patil *et al.*, 1995), show constitutive activation in a G-protein- and EtOH-independent manner and permeability to sodium as well as potassium ions because of the lack of K^+ selectivity (Navarro *et al.*, 1996; Slesinger *et al.*, 1997; Kobayashi *et al.*, 1999). To further address the relation between fluoxetine and GIRK channels, we investigated the effects of fluoxetine on these homomeric channels. In oocytes expressing GIRK2 channels, fluoxetine inhibited the channels to the similar extent when compared with GIRK heteromeric channels ($P>0.05$ at 300 μM, Student's t-test), although the IC_{50} value for GIRK2 channels was ~5 times higher than those for the heteromeric channels (Table 1). On the other hand, in oocytes expressing *wv*GIRK2 channels, large inward currents were observed when the oocytes were perfused with either ND98 or hK solution instead of NMDG solution, which does not contain Na^+ or K^+ (3118.6±190.1 and 3094.6±364.4 nA, $n=7$ and 25, respectively). The addition of 100 μM fluoxetine to ND98 or hK solutions immediately reduced the inward currents (89.9±1.9% of the ND98 current component and 80.9±4.1% of the hK current component, $n=4$ and 7, respectively; Figure 5a). As shown in Figure 5 and Table 1, the inhibitory effect of fluoxetine on *wv*GIRK2 channels was more efficacious than that of GIRK2 channels ($P<0.01$, Student's t-test). Moreover, injection of fluoxetine inhibited only 8.4±2.3% of the hK current component, which was 1329.8±232.9 nA, by the presumed intracellular concentration of ~225 μM ($n=5$). The results suggest the possibilities that extracellular fluoxetine may easily approach its action sites on *wv*GIRK2 channels because of a mutation in the pore-

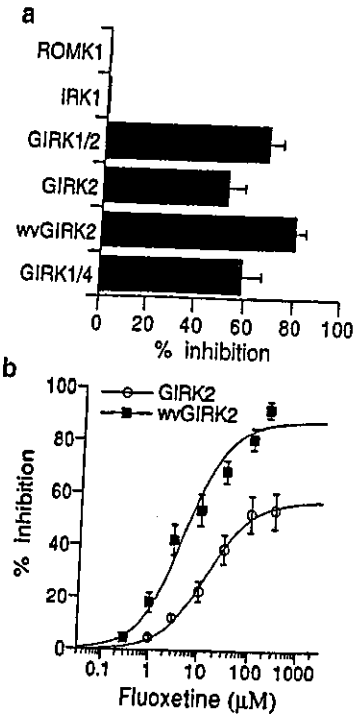

Figure 5 Comparison of the fluoxetine effects on members of inwardly rectifying potassium channels. (a) Action of fluoxetine on GIRK, ROMK1 and IRK1 channels expressed as homomeric or heteromeric channels in *Xenopus* oocytes. The concentration of fluoxetine used was 100 μM. Current responses were measured at a membrane potential of −70 mV in a high-potassium solution. (b) Distinct concentration-dependent responses to fluoxetine for GIRK2 channels and *weaver* (wv) GIRK2 channels. The magnitudes of inhibition of GIRK current by fluoxetine were compared with the 3 mM Ba^{2+}-sensitive current components (685.8 ± 183.6 nA, $n=5$) in oocytes expressing GIRK2 channels, and with the hK current components (2857.8 ± 321.0 nA, $n=4$) in oocytes expressing *wv*GIRK2 channels, respectively. Current responses were measured at a membrane potential of −70 mV in a high-potassium solution or a NMDG solution. Each point and error bar represents the mean and s.e.m. of the percentage responses obtained. Data points were fitted using a logistic equation.

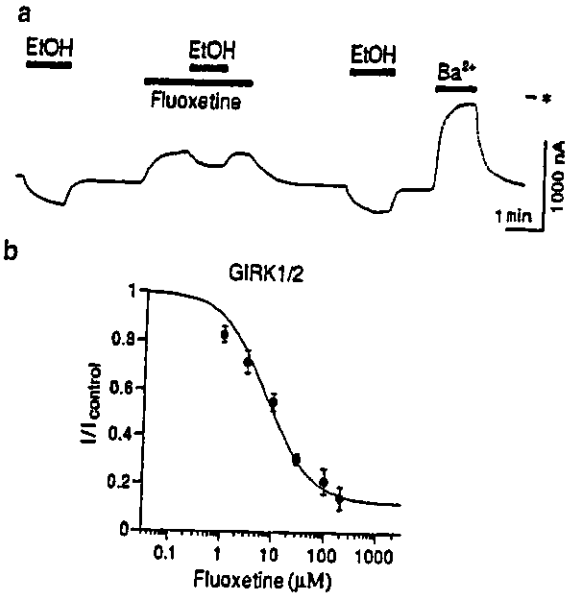

Figure 6 Inhibitory effect of fluoxetine on the ethanol-induced GIRK currents in *Xenopus* oocytes expressing GIRK1/2 channels. (a) In an oocyte coinjected with GIRK1 and GIRK2 mRNAs, current responses to ethanol (EtOH), EtOH in the presence of 10 μM fluoxetine, EtOH and 3 mM Ba^{2+} are shown. The concentration of EtOH used was 100 mM. Asterisk indicates the zero current level. Bars show the duration of application. (b) Concentration-dependent inhibition of fluoxetine on EtOH-induced GIRK currents. $I_{control}$ is the amplitude of GIRK currents induced by 100 mM EtOH (340.8 ± 18.1 nA, $n=5$) and I is the current amplitude in the presence of fluoxetine. Current responses were measured at a membrane potential of −70 mV in a high-potassium solution containing 96 mM K^+. Each point and error bar represents the mean and s.e.m. of the relative responses. Data points were fitted using a logistic equation.

forming region and/or that it may easily cause a distinct conformational change in the channels with the different channel properties.

Fluoxetine inhibits EtOH-induced GIRK currents

GIRK channels are also activated by EtOH independent of G-protein signalling pathways (Kobayashi *et al.*, 1999). Several studies have shown that fluoxetine reduces EtOH consumption (Rockman *et al.*, 1982; Naranjo & Knoke, 2001), suggesting a relation between the fluoxetine and EtOH effects. We next examined the effect of fluoxetine on EtOH-induced GIRK currents. In oocytes expressing GIRK1/2 channels, the EtOH-induced GIRK currents were attenuated in the presence of fluoxetine, with an IC_{50} value of 11.1 ± 2.5 μM and a n_H value of 0.78 ± 0.02, in a reversible manner ($n=5$; Figure 6). Furthermore, we compared the EtOH-induced GIRK currents before and after injecting fluoxetine into the oocytes. The EtOH-induced GIRK currents were not significantly affected by intracellularly applied fluoxetine (105.9 ± 4.6% of pretreated control current, paired t-test, $P>0.1$, $n=5$). The results, therefore, suggest that extracellular fluoxetine can inhibit the effect of GIRK channels induced by EtOH.

Discussion

We have demonstrated that extracellular fluoxetine inhibits both brain-type GIRK1/2 and GIRK2 channels and cardiac-type GIRK1/4 channels to a limited extent at high concentrations. However, two different SSRIs fluvoxamine and zimelidine have no significant effect on the channels. The inhibition by fluoxetine showed a concentration-dependence, a weak voltage-dependence and a slight time-dependence with a predominant effect on the instantaneous current elicited by voltage pulses and followed by slight further inhibition. On the other hand, blockade by extracellular Ba^{2+} and Cs^+, typical of Kir channel blockers that occlude the pore of the open channel, shows a concentration-dependence, a strong voltage-dependence and a time-dependence with a comparatively small effect on the instantaneous current, but a marked inhibition on the steady-state current at the end of voltage pulses (Lesage *et al.*, 1995). These observations suggest that

fluoxetine probably causes a conformational change in the channels, but does not act as typical open channel blockers of Kir channels like Ba^{2+} and Cs^{+}, although all these molecules act at the channels from the extracellular side. The different mechanism from Ba^{2+} for GIRK channels may be involved in the incomplete blockade and slower time course of inhibition, compared with the inhibition by Ba^{2+} as shown in Figure 1. Since *wv*GIRK2 channels were highly sensitive to fluoxetine, but ROMK1 and IRK1 channels among members of the Kir channel family were insensitive to fluoxetine, further studies using chimeric GIRK channels replaced with the homologous regions from ROMK1 and IRK1 channels and mutant GIRK channels may clarify the critical sites mediating the effects of fluoxetine on GIRK channels. In addition, single channel analyses of wild-type GIRK channels and *wv*GIRK2 channels in the presence of fluoxetine may be useful for understanding the mechanisms of the action of fluoxetine on GIRK channels and the greater inhibition by fluoxetine of the mutant channels with different channel properties.

The therapeutic plasma concentrations of fluoxetine range approximately from 0.3 to 1.5 μM (Baldessarini, 2001). The brain concentrations of fluoxetine are approximately 10 or 20 times higher than the corresponding blood levels (Karson *et al.*, 1993; Bolo *et al.*, 2000). Therefore, the present findings suggest that GIRK channels in the brain may be inhibited by fluoxetine at clinically relevant concentrations.

Neuronal GIRK channels are widely present in various brain regions, such as the frontal cortex, hippocampus, amygdala, thalamus, substantia nigra, ventral tegmental area, brain stem and cerebellum (Kobayashi *et al.*, 1995; Karschin *et al.*, 1996; Liao *et al.*, 1996). GIRK channels are thought to play an important role in inhibiting the neuronal excitability through activation of various G-protein-coupled receptors. Therefore, inhibition of GIRK channels by fluoxetine may affect many brain functions. Recent studies have demonstrated that there are regional abnormalities in the prefrontal cortex, hippocampus, amygdala, thalamus and ventral striatum in depressed individuals (Manji *et al.*, 2001). It is generally thought that the therapeutic effects of fluoxetine and fluvoxamine are primarily because of the inhibition of the reuptake of serotonin in the brain. Although a comparative study of fluoxetine and fluvoxamine showed that both drugs were equally effective in the treatment of patients with major depression (Rapaport *et al.*, 1996), cohort studies showed that fluvoxamine was significantly less effective in the treatment of depression, anxiety and other disorders than fluoxetine (Mackay *et al.*, 1997). In addition, fluvoxamine is approved for the treatment of obsessive-compulsive disorder in the US, but not for the treatment of depression (Barbey & Roose, 1998). The present study demonstrates that in contrast to fluoxetine, fluvoxamine has no significant effect on GIRK channels. GIRK2-deficient mice show an increase in motor activity and less anxiety (Blednov *et al.*, 2001). Taken together, inhibition of neuronal GIRK channels by fluoxetine may contribute to additive therapeutic effects for depression and other related psychiatric disorders.

On the other hand, the incidence of seizures during treatment with SSRIs including fluoxetine and fluvoxamine is a serious side effect in contrast with various benign side effects (Barbey & Roose, 1998; Neely, 1998; Baldessarini, 2001). The molecular mechanisms underlying seizures during treatment with SSRIs remains unclear. The serotonin syndrome, which is a toxic hyperserotonergic state, typically includes not only changes in mental state, myoclonus, tremor and various autonomic responses, but also seizures in severe cases (Sternbach, 1991; Baldessarini, 2001). Seizures associated with SSRIs may be related to a consequence of aberrant responses in serotonergic system. Moreover, overdoses of fluoxetine have been reported to be associated with seizures (Barbey & Roose, 1998; Neely, 1998). The plasma concentrations of fluoxetine in several patients who experienced seizures were reported to be approximately 3.3 – 7.1 μM (Barbey & Roose, 1998; Neely, 1998). Therefore, fluoxetine at the corresponding brain levels may potently inhibit neuronal GIRK channels. Bupivacaine, a local anesthetic, also inhibits GIRK channels but does not affect other Kir channels (Zhou *et al.*, 2001). Overdoses of bupivacaine are associated with seizures, and the blood levels are close to the IC_{50} values for GIRK channels. In addition, fluoxetine inhibits two types of neuronal voltage-gated K^{+} channels, Kv1.1 and Kv3.1 channels (Yeung *et al.*, 1999; Choi *et al.*, 2001). GIRK2-deficient mice show spontaneous seizures (Signorini *et al.*, 1997), and the inhibition of these K^{+} channels could lead to an increase in neuronal excitability. Therefore, potent inhibition of neuronal GIRK channels by fluoxetine together with the Kv channels may also contribute to the cause of seizures and some of other neuropsychiatric toxicity.

Antidepressant drugs including fluoxetine, fluvoxamine and tricyclic antidepressants show analgesic activity (Messing *et al.*, 1975; Lin *et al.*, 1980; Schreiber *et al.*, 1996; Korzeniewska-Rybicka & Plaznik, 2000; Galeotti *et al.*, 2001). Studies using *wv* mutant mice or GIRK2-deficient mice have shown that activation of GIRK channels by opioids or EtOH may be involved in analgesia induced by the drugs, suggesting that the GIRK channel is one of the key molecules in analgesia (Kobayashi *et al.*, 1999; Ikeda *et al.*, 2000; 2002; Mitrovic *et al.*, 2000). However, the present study demonstrates that fluoxetine inhibits GIRK channels, while fluvoxamine has no significant effect on GIRK channels. Therefore, the analgesic effect of fluoxetine and fluvoxamine may be caused by interaction with some of other several targets. Previous studies have shown that the actions of 5-HT may contribute to the analgesic effect (Messing *et al.*, 1975; Lin *et al.*, 1980; Tura & Tura, 1990). Various antidepressant drugs including fluoxetine and tricyclic antidepressants inhibit voltage-gated Na^{+} channels like local anesthetics (Pancrazio *et al.*, 1998). Therefore, the analgesic effect of the antidepressant drugs may share these common mechanisms. In addition, Galeotti *et al.* (2001) suggest that the analgesic effects of tricyclic antidepressants may involve the opening of voltage-gated, ATP-sensitive and Ca^{2+}-activated K^{+} channels. Further studies are required to clarify whether the analgesic effects of SSRIs are also mediated by these channels.

In the heart, acetylcholine released from the stimulated vagus nerve opens atrial GIRK channels via activation of M_2 muscarinic acetylcholine receptors, and ultimately causes slowing of the heart rate (Brown & Birnbaumer, 1990). The binding affinity of fluoxetine for the receptor exhibits low micromolar concentrations (Stanton *et al.*, 1993). In the present study, micromolar concentrations of fluoxetine inhibited cardiac-type GIRK1/4 channels. In clinical practice, fluoxetine overdoses are associated with the incidence of sinus tachycardia (Barbey & Roose, 1998; Neely, 1998). The clinically relevant plasma concentrations become significantly

higher than the micromolar concentrations in the therapeutic use, although the corresponding heart concentrations have not been determined in humans. Sinus tachycardia during fluoxetine treatment may be related to not only antagonism of M_2 muscarinic acetylcholine receptors, but also inhibition of atrial GIRK channels. In addition, 5-HT can elicit tachycardia in humans, although it is known that 5-HT elicits complex changes in the cardiovascular system comprising bradycardia or tachycardia, hypotension or hypertension and vasodilatation or vasoconstriction (Saxena & Villalon, 1990). The 5-HT effect caused by inhibition of 5-HT uptake might also be related to the incidence of tachycardia during treatment with SSRIs. Sinus tachycardia is also observed in fluvoxamine overdose (Barbey & Roose, 1998). Although fluvoxamine has no significant effect on GIRK channels, it has less affinity for muscarinic acetylcholine receptors than fluoxetine (Baldessarini, 2001). Therefore, sinus tachycardia observed in fluvoxamine overdose might be related to the effects of 5-HT and antagonism of muscarinic acetylcholine receptors.

Interestingly, we also demonstrate that extracellar fluoxetine at low micromolar concentrations can inhibit the effect of brain-type GIRK channels induced by ethanol. Fluoxetine reduces EtOH consumption (Rockman *et al.*, 1982; Naranjo & Knoke, 2001). However, GIRK2-deficient mice display no significant change in EtOH consumption (Blednov *et al.*, 2001). So far, there is no evidence to help clarify the *in vivo* effects of fluoxetine on GIRK channel activation by EtOH. Further studies using mice treated with antisense oligodeoxynucleotides to the GIRK mRNAs, mutant mice with GIRK channels insensitive to EtOH alone and a selective unidentified GIRK channel activator or inhibitor may be useful for the understanding of the antagonism of EtOH-induced GIRK effects by fluoxetine *in vivo*. In addition, mutagenesis studies of GIRK channels may clarify the molecular mechanism underlying the inhibitory action of fluoxetine on EtOH activation of GIRK channels.

In conclusion, we demonstrate that fluoxetine, at clinically relevant brain concentrations, acts as an inhibitor at GIRK channels, which play an important role in the inhibitory regulation of neuronal excitability in most brain regions. Moreover, fluoxetine can inhibit the effect of GIRK channels induced by EtOH. Our results suggest that inhibition of GIRK channels by fluoxetine may contribute to some of the therapeutic effects and the adverse side effects, particularly seizures in overdose, observed in clinical practice.

We thank Drs Toshiro Kumanishi and Kansaku Baba for their cooperation, and Tomio Ichikawa and Kazuo Kobayashi for their assistance. We also thank Dr Steven C. Hebert for providing the ROMK1 cDNA, and Dr Lily Y. Jan for providing the IRK1 cDNA. This work was supported by research grants from the Ministry of Education, Science, Sports and Culture of Japan and the RIKEN Brain Science Institute.

References

BALDESSARINI, R.J. (2001). Drugs and the treatment of psychiatric disorders: depression and anxiety disorder. In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*. 10th edn., ed. Hardman, J.G., Limbird, L.E. & Gilman, A.G. pp. 447 – 483. New York: McGraw-Hill.

BARBEY, J.T. & ROOSE, S.P. (1998). SSRI safety in overdose. *J. Clin. Psychiatry*, 59 (Suppl. 15), 42 – 48.

BLEDNOV, Y.A., STOFFEL, M., CHANG, S.R. & HARRIS, R.A. (2001). Potassium channels as targets for ethanol: studies of G-protein-coupled inwardly rectifying potassium channel 2 (GIRK2) null mutant mice. *J. Pharmacol. Exp. Ther.*, 298, 521 – 530.

BOLO, N.R., HODE, Y., NEDELEC, J.-F., LAINE, E., WAGNER, G. & MACHER, J.-P. (2000). Brain pharmacokinetics and tissue distribution *in vivo* of fluvoxamine and fluoxetine by fluorine magnetic resonance spectroscopy. *Neuropsychopharmacology*, 23, 428 – 438.

BROWN, A.M. & BIRNBAUMER, L. (1990). Ionic channels and their regulation by G protein subunits. *Annu. Rev. Physiol.*, 52, 197 – 213.

CHOI, B.H., CHOI, J.-S., YOON, S.H., RHIE, D.-J., MIN, D.S., JO, Y.-H., KIM, M.-S. & HAHN, S.J. (2001). Effects of norfluoxetine, the major metabolite of fluoxetine, on the cloned neuronal potassium channel Kv3.1. *Neuropharmacology*, 41, 443 – 453.

CHOI, J.-S., HAHN, S.J., RHIE, D.-J., YOON, S.-H., JO, Y.-H. & KIM, M.-S. (1999). Mechanism of fluoxetine block of cloned voltage-activated potassium channel Kv1.3. *J. Pharmacol. Exp. Ther.*, 291, 1 – 6.

DASCAL, N. (1997). Signalling via the G protein-activated K^+ channels. *Cell Signal.*, 9, 551 – 573.

DEÁK, F., LASZTOCZI, B., PACHER, P., PETHEO, G.L., KECSKEMETI, V. & SPAT, A. (2000). Inhibition of voltage-gated calcium channels by fluoxetine in rat hippocampal pyramidal cells. *Neuropharmacology*, 39, 1029 – 1036.

DOUPNIK, C.A., DAVIDSON, N. & LESTER, H.A. (1995). The inward rectifier potassium channel family. *Curr. Opin. Neurobiol.*, 5, 268 – 277.

FAN, P. (1994). Effects of antidepressants on the inward current mediated by 5-HT_3 receptors in rat nodese ganglion neurons. *Br. J. Pharmacol.*, 112, 741 – 744.

GALEOTTI, N., GHELARDINI, C. & BARTOLINI, A. (2001). Involvement of potassium channels in amitryptyline and clomipramine analgesia. *Neuropharmacology*, 40, 75 – 84.

GARCÍA-COLUNGA, J., AWAD, J.N. & MILEDI, R. (1997). Blockage of muscule and neuronal nicotinic acetylcholine receptors by fluoxetine (Prozac). *Proc. Natl. Acad. Sci. U.S.A.*, 94, 2041 – 2044.

HO, K., NICHOLS, C.G., LEDERER, W.J., LYTTON, J., VASSILEV, P.M., KANAZIRSKA, M.V. & HEBERT, S.C. (1993). Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature*, 362, 31 – 38.

IKEDA, K., KOBAYASHI, T., ICHIKAWA, T., USUI, H., ABE, S. & KUMANISHI, T. (1996). Comparison of the three mouse G-protein-activated K^+ (GIRK) channels and functional couplings of the opioid receptors with the GIRK1 channel. *Ann. NY Acad. Sci.*, 801, 95 – 109.

IKEDA, K., KOBAYASHI, T., ICHIKAWA, T., USUI, H. & KUMANISHI, T. (1995). Functional couplings of the δ- and the κ-opioid receptors with the G-protein-activated K^+ channel. *Biochem. Biophys. Res. Commun.*, 208, 302 – 308.

IKEDA, K., KOBAYASHI, K., KOBAYASHI, T., ICHIKAWA, T., KUMANISHI, T., KISHIDA, H., YANO, R. & MANABE, T. (1997). Functional coupling of the nociceptin/orphanin FQ receptor with the G-protein-activated K^+ (GIRK) channel. *Mol. Brain Res.*, 45, 117 – 126.

IKEDA, K., KOBAYASHI, T., KUMANISHI, T., NIKI, H. & YANO, R. (2000). Involvement of G-protein-activated inwardly rectifying K^+ (GIRK) channels in opioid-induced analgesia. *Neurosci. Res.*, 38, 113 – 116.

IKEDA, K., KOBAYASHI, T., KUMANISHI, T., YANO, R., SORA, I. & NIKI, H. (2002). Molecular mechanisms of analgesia induced by opioids and ethanol: is the GIRK channel one of the keys? *Neurosci. Res.*, 44, 121 – 131.

INANOBE, A., YOSHIMOTA, Y., HORIO, Y., MORISHIGE, K.-I., HIBINO, H., MATSUMOTO, S., TOKUNAGA, Y., MAEDA, T., HATA, Y., TAKAI, Y. & KURACHI, Y. (1999). Characterization of G-protein-gated K^+ channels composed of Kir3.2 subunits in dopaminergic neurons of the substantia nigra. *J. Neurosci.*, **19**, 1006 – 1017.

KARSCHIN, C., DIßMANN, E., STUHMER, W. & KARSCHIN, A. (1996). IRK(1-3) and GIRK(1-4) inwardly rectifying K^+ channel mRNAs are differentially expressed in the adult rat brain. *J. Neurosci.*, **16**, 3559 – 3570.

KARSON, C.N., NEWTON, J.E.O., LIVINGSTON, R., JOLLY, J.B., COOPER, T.B., SPRIGG, J. & KOMOROSKI, R.A. (1993). Human brain fluoxetine concentrations. *J. Neuropsychiatry Clin. Neurosci.*, **5**, 322 – 329.

KOBAYASHI, T., IKEDA, K., ICHIKAWA, T., ABE, S., TOGASHI, S. & KUMANISHI, T. (1995). Molecular cloning of a mouse G-protein-activated K^+ channel (mGIRK1) and distinct distributions of three GIRK (GIRK1, 2 and 3) mRNAs in mouse brain. *Biochem. Biophys. Res. Commun.*, **208**, 1166 – 1173.

KOBAYASHI, T., IKEDA, K., KOJIMA, H., NIKI, H., YANO, R., YOSHIOKA, T. & KUMANISHI, T. (1999). Ethanol opens G-protein-activated inwardly rectifying K^+ channels. *Nat. Neurosci.*, **2**, 1091 – 1097.

KOBAYASHI, T., IKEDA, K. & KUMANISHI, T. (2000). Inhibition of various antipsychotic drugs on the G-protein-activated inwardly rectifying K^+ (GIRK) channels expressed in *Xenopus* oocytes. *Br. J. Pharmacol.*, **129**, 1716 – 1722.

KOBAYASHI, T., IKEDA, K. & KUMANISHI, T. (2002). Functional characterization of an endogenous *Xenopus* oocyte adenosine receptor. *Br. J. Pharmacol.*, **135**, 313 – 322.

KORZENIEWSKA-RYBICKA, I. & PLAZNIK, A. (2000). Supraspinally mediated analgesic effect of antidepressant drugs. *Pol. J. Pharmacol.*, **52**, 93 – 99.

KRAPIVINSKY, G., GORDON, E.A., WICKMAN, K., VELIMIROVIC, B., KRAPIVINSKY, L. & CLAPHAM, D.E. (1995). The G-protein-gated atrial K^+ channel I_{KACh} is a heteromultimer of two inwardly rectifying K^+-channel proteins. *Nature*, **374**, 135 – 141.

KUBO, Y., BALDWIN, T.J., JAN, Y.N. & JAN, L.Y. (1993a). Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature*, **362**, 127 – 133.

KUBO, Y., REUVENY, E., SLESINGER, P.A., JAN, Y.N. & JAN, L.Y. (1993b). Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. *Nature*, **364**, 802 – 806.

LESAGE, F., GUILLEMARE, E., FINK, M., DUPRAT, F., HEURTEAUX, C., FOSSET, M., ROMEY, G., BARHANIN, J. & LAZDUNSKI, M. (1995). Molecular properties of neuronal G-protein-activated inwardly rectifying K^+ channels. *J. Biol. Chem.*, **270**, 28660 – 28667.

LEWOHL, J.M., WILSON, W.R., MAYFIELD, R.D., BROZOWSKI, S.J., MORRISETT, R.A. & HARRIS, R.A. (1999). G-protein-coupled inwardly rectifying potassium channels are targets of alcohol action. *Nat. Neurosci.*, **2**, 1084 – 1090.

LIAO, Y.J., JAN, Y.N. & JAN, L.Y. (1996). Heteromultimerization of G-protein-gated inwardly rectifying K^+ channel proteins GIRK1 and GIRK2 and their altered expression in *weaver* brain. *J. Neurosci.*, **16**, 7137 – 7150.

LIN, M.-T., CHANDRA, A., CHI, M.-L. & KAU, C.-L. (1980). Effects of increasing serotonergic receptor activity in brain on analgesic activity in rats. *Exp. Neurol.*, **68**, 548 – 554.

MACKAY, F.J., DUNN, N.R., WILTON, L.V., PEARCE, G.L., FREEMANTLE, S.N. & MANN, R.D. (1997). A comparison of fluvoxamine, fluoxetine, sertraline and paroxetine examined by observational cohort studies. *Pharmacoepidem. Dr. S.*, **6**, 235 – 246.

MAERTENS, C., WEI, L., VOETS, T., DROOGMANS, G. & NILIUS, B. (1999). Block by fluoxetine of volume-regulated anion channels. *Br. J. Pharmacol.*, **126**, 508 – 514.

MAGGI, L., PALMA, E., MILEDI, R. & EUSEBI, F. (1998). Effects of fluoxetine on wild and mutant neuronal α_7 nicotinic receptors. *Mol. Psychiatry*, **3**, 350 – 355.

MANJI, H.K., DREVETS, W.C. & CHARNEY, D.S. (2001). The cellular neurobiology of depression. *Nat. Med.*, **7**, 541 – 547.

MESSING, R.B., PHEBUS, L., FISHER, L.A. & LYTLE, L.D. (1975). Analgesic effects of fluoxetine hydrochloride (Lilly 110140) a specific inhibitor of serotonin uptake. *Psychopharmacol. Commun.*, **1**, 511 – 521.

MITROVIC, I., YI, E., MARGETA-MITROVIC, M., STOFFEL, M. & BASBAUM, A.I. (2000). Contribution of GIRK2 channels to morphine and clonidine antinociception: a possible mediator of sex differences. *Soc. Neurosci. Abstr.*, **26**, 2188.

NARANJO, C.A. & KNOKE, D.M. (2001). The role of selective serotonin reuptake inhibitors in reducing alcohol consumption. *J. Clin. Psychiatry*, **62** (Suppl. 20), 18 – 25.

NAVARRO, B., KENNEDY, M.E., VELIMIROVIC, B., BHAT, D., PETERSON, A.S. & CLAPHAM, D.E. (1996). Nonselective and $G_{\beta\gamma}$-insensitive *weaver* K^+ channels. *Science*, **272**, 1950 – 1953.

NEELY, J.L. (1998). Tonic clonic seizures and tachycardia induced by fluoxetine (Prozac^R) overdose. *W. Vir. Med. J.*, **94**, 283 – 285.

NI, Y.G. & MILEDI, R. (1997). Blockage of $5HT_{2C}$ serotonin receptors by fluoxetine (Prozac). *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 2036 – 2049.

NORTH, R.A. (1989). Drug receptors and the inhibition of nerve cells. *Br. J. Pharmacol.*, **98**, 13 – 28.

PANCRAZIO, J.J., KAMATCHI, G.L., ROSCOE, A.K. & LYNCH, CIII. (1998). Inhibition of neuronal Na^+ channels by antidepressant drugs. *J. Pharmacol. Exp. Ther.*, **284**, 208 – 214.

PATIL, N., COX, D.R., BHAT, D., FAHAM, M., MYERS, R.M. & PETERSON, A.S. (1995). A potassium channel mutation in weaver mice implicates membrane excitability in granule cell differentiation. *Nat. Genet.*, **11**, 126 – 129.

PERCHENET, L., HILFIGER, L., MIZRAHI, J. & CLEMENT-CHOMIENNE, O. (2001). Effects of anorexinogen agents on cloned voltage-gated K^+ channel hKv1.5. *J. Pharmacol. Exp. Ther.*, **298**, 1108 – 1119.

RAPAPORT, M., COCCARO, E., SHELINE, Y., PERSE, T., HOLLAND, P., FABRE, L. & BRADFORD, D. (1996). A comparison of fluvoxamine and fluoxetine in the treatment of major depression. *J. Clin. Psychopharmacol.*, **16**, 373 – 378.

REIMANN, F. & ASHCROFT, F.M. (1999). Inwardly rectifying potassium channels. *Curr. Opin. Cell Biol.*, **11**, 503 – 508.

REUVENY, E., SLESINGER, P.A., INGLESE, J., MORALES, J.M., INIGUEZ-LLUHI, J.A., LEFKOWITZ, R.J., BOURNE, H.R., JAN, Y.N. & JAN, L.Y. (1994). Activation of the cloned muscarinic potassium channel by G protein $\beta\gamma$ subunits. *Nature*, **370**, 143 – 146.

ROCKMAN, G.E., AMIT, Z., BROWN, Z.W., BOURQUE, C. & OGREN, S.-O. (1982). An investigation of the mechanisms of action of 5-hydroxytryptamine in the suppression of ethanol intake. *Neuropharmacology*, **21**, 341 – 347.

SAXENA, P.R. & VILLALON, C.M. (1990). Cardiovascular effects of serotonin agonists and antagonists. *J. Cardiovasc. Pharmacol.*, **15** (Suppl. 7), S17 – S34.

SCHREIBER, S., BACKER, M.M., YANAI, J. & PICK, C.G. (1996). The antinociceptive effect of fluvoxamine. *Eur. Neuropsychopharmacol.*, **6**, 281 – 284.

SIGNORINI, S., LIAO, Y.J., DUNCAN, S.A., JAN, L.Y. & STOFFEL, M. (1997). Normal cerebellar development but susceptibility to seizures in mice lacking G protein-coupled, inwardly rectifying K^+ channel GIRK2. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 923 – 927.

SLESINGER, P.A., STOFFEL, M., JAN, Y.N. & JAN, L.Y. (1997). Defective γ-aminobutyric acid type B receptor-activated inwardly rectifying K^+ currents in cerebellar granule cells isolated from *weaver* and *Girk2* null mutant mice. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 12210 – 12217.

STANTON, T., BOLDEN-WATSON, C., CUSACK, B. & RICHELSON, E. (1993). Antagonism of the five cloned human muscarinic cholinergic receptors expressed in CHO-K1 cells by antidepressants and antihistamines. *Biochem. Pharmacol.*, **45**, 2352 – 2354.

STERNBACH, H. (1991). The serotonin syndrome. *Am. J. Psychiatry*, **148**, 705 – 713.

THOMAS, D., GUT, B., WENDT-NORDAHL, G. & KIEHN, J. (2002). The antidepressant drug fluoxetine is an inhibitor of human ether-a-go-go-related gene (HERG) potassium channels. *J. Pharmacol. Exp. Ther.*, **300**, 543 – 548.

TURA, B. & TURA, S.M. (1990). The analgesic effect of tricyclic antidepressants. *Brain Res.*, **518**, 19 – 22.

WICKMAN, K., NEMEC, J., GENDLER, S.J. & CLAPHAM, D.E. (1998). Abnormal heart rate regulation in *GIRK4* knockout mice. *Neuron*, **20**, 103 – 114.

WICKMAN, K., SELDIN, M.F., GENDLER, S.J. & CLAPHAM, D.E. (1997). Partial structure, chromosome localization, and expression of the mouse *Girk4* gene. *Genomics*, 40, 395 – 401.

WONG, D.T., BYMASTER, F.P. & ENGLEMAN, E.A. (1995). Prozac (Fluoxetine, Lilly 110140), the first selective serotonin uptake inhibitor and an antidepressant drug: twenty years since its first publication. *Life Sci.*, 57, 411 – 441.

YEUNG, S.Y., MILLAR, J.A. & MATHIE, A. (1999). Inhibition of neuronal K_v potassium currents by the antidepressant drug, fluoxetine. *Br. J. Pharmacol.*, 128, 1609 – 1615.

ZHOU, W., ARRABIT, C., CHOE, S. & SLESINGER, P.A. (2001). Mechanism underlying bupivacaine inhibition of G protein-gated inwardly rectifying K^+ channels. *Proc. Natl. Acad. Sci. U.S.A.*, 98, 6482 – 6287.

(Received August 26, 2002
Revised November 4, 2002
Accepted January 3, 2003)

Available online at www.sciencedirect.com

SCIENCE DIRECT

Life Sciences 76 (2004) 407–415

Life Sciences

www.elsevier.com/locate/lifescie

Effects of interferon-α on cloned opioid receptors expressed in *Xenopus* oocytes

Toru Kobayashi[a,*], Kazuo Washiyama[a], Kazutaka Ikeda[b]

[a]*Department of Molecular Neuropathology, Brain Research Institute, Niigata University, 1-757 Asahimachi, Niigata, Niigata 951-8585, Japan*

[b]*Department of Molecular Psychiatry, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya-ku, Tokyo 156-8585, Japan*

Received 17 May 2004; accepted 17 July 2004

Abstract

Interferon-α (IFNα) affects the opioid system. However, the direct action of IFNα on cloned opioid receptors remains unknown. Taking advantage of the functional coupling of cloned opioid receptors to G protein-activated inwardly rectifying K^+ (GIRK) channels in a *Xenopus* oocyte expression system, we investigated the effects of recombinant IFNα on cloned μ-, δ- and κ-opioid receptors. In oocytes co-injected with mRNAs for either the δ- or κ-opioid receptor and for GIRK channel subunits, IFNα at high concentrations induced small GIRK currents that were abolished by naloxone, an opioid-receptor antagonist, compared with the control responses to each selective opioid agonist. Additionally, IFNα induced no significant current response in oocytes injected with mRNA(s) for either opioid receptor alone or GIRK channels. In oocytes expressing the μ-opioid receptor and GIRK channels, IFNα had little or no effect. Moreover, in oocytes expressing each opioid receptor and GIRK channels, GIRK current responses to each selective opioid agonist were not affected by the presence of IFNα, indicating no significant antagonism of IFNα toward the opioid receptors. Furthermore, IFNα had little or no effect on the μ/δ-, δ/κ- or μ/κ-opioid receptors expressed together with GIRK channels in oocytes. Our results suggest that IFNα weakly activates the δ-and κ-opioid receptors. The direct activation of the δ- and κ-opioid receptors by IFNα may partly contribute to some of the IFNα effects under its high-dose medication.

Keywords: Interferon α; Opioid receptor; GIRK channel; *Xenopus* oocyte

* Corresponding author. Tel.: +81 25 227 0646; fax: +81 25 227 0818.
E-mail address: torukoba@bri.niigata-u.ac.jp (T. Kobayashi).

0024-3205/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2004.09.007

Introduction

Interferons (IFNs) are multifunctional cytokines that possess antiviral, antitumor, antiproliferative and immunomodulatory actions mediated by specific cellular receptors (Maeyer and Maeyer-Guignard, 1998; Soos and Szente, 2003). Three major classes of IFNs are known; α, β and γ (Maeyer and Maeyer-Guignard, 1998). IFNα has been used for the treatment of chronic viral hepatitis, malignant tumors and several other diseases (Soos and Szente, 2003). However, treatment with IFNα also causes various side effects associated with the central nervous system (CNS), such as somnolence, confusion, depression, fever and sensory disturbance (Dafny, 1998). In animal studies, human IFNα was shown to bind to brain membranes (Janicki, 1992), and IFNα inhibited the binding of [^{3}H]naloxone, [^{3}H][D-Ala^2, D-Leu^5]enkephalin (DADLE) or [^{3}H]dihydromorphine to brain tissues (Blalock and Smith, 1981; Panchenko et al., 1987; Menzies et al., 1992). In addition, previous studies demonstrated immunological cross-reactivity between IFNα and γ-endorphin (Smith and Blalock, 1981), but not between it and β-endorphin (Epstein et al., 1982). Moreover, human IFNα, but not IFNβ or IFNγ, induced endorphin-like CNS effects, including analgesia and catalepsy (Blalock and Smith, 1981; Jiang et al., 2000). It also modulated immobility in a forced swimming test (Makino et al., 2000), wet-dog shakes induced by a $5HT_2$ receptor agonist (Kugaya et al., 1996), neuronal activity in several brain regions (Nakashima et al., 1987, 1988), electroencephalogram (EEG) activity (Birmanns et al., 1990; De Sarro et al., 1990) and hypothalamo-pituitary-adrenocortical secretory activity (Saphier et al., 1993, 1994). These effects were prevented or reversed by naloxone, an opioid receptor antagonist. Therefore, these observations suggest that the IFNα effects may be mediated by opioid receptors, which are implicated in many CNS functions as well as in immune and endocrine functions (Gutstein and Akil, 2001). However, the direct action of IFNα on cloned μ-, δ- and κ-opioid receptors remains unknown. In the present study, taking advantage of the functional coupling of cloned opioid receptors to G protein-activated inwardly rectifying K^+ (GIRK) channels in a *Xenopus* oocyte expression system (Ikeda et al., 1996; Ikeda et al., 2003), we investigated the effects of IFNα on the expressed opioid receptors.

Materials and methods

Preparation of specific mRNAs

Plasmids containing the entire coding sequences for the mouse μ-, δ and κ-opioid receptors and for the mouse GIRK1 and GIRK2 channel subunits were obtained previously (Ikeda et al., 1996; Kobayashi et al., 1995, 1999). The plasmids were linearized by digestion with an appropriate restriction enzyme. The specific mRNAs were synthesized in vitro from the linearized plasmids by using the mMESSAGE mMACHINE™ In Vitro Transcription Kit (Ambion, Austin, TX, USA).

Electrophysiological analyses

Adult female *Xenopus laevis* frogs were purchased from Copacetic (Soma, Aomori, Japan) and maintained in the laboratory until used. Frogs were anesthetized by immersion in water containing 0.15% tricaine (Sigma Chemical Co., St. Louis, MO, USA). A small incision was made on the abdomen to remove several ovarian lobes from the frogs that were humanely sacrificed after the final collection.

For *Xenopus* oocyte expression experiments (Kobayashi et al., 2002; Ikeda et al., 2003), *Xenopus laevis* oocytes (Stages V and VI) were isolated at 19 °C in Barth's solution (composition in mM: NaCl 88, KCl 1, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, $NaHCO_3$ 2.4, Tris-HCl 7.5 mM (pH 7.4), and 0.1 mg ml^{-1} gentamicin sulfate; Wako Pure Chemical Industries, Osaka, Japan), and injected with opioid-receptor mRNA(s) (~10 ng) and/or mRNAs for GIRK1 and GIRK2 subunits (each ~0.3 ng). The oocytes were defolliculated by manual dissection after treatment with 0.8 mg/ml collagenase (Wako Pure Chemical Industries, Osaka, Japan). Whole-cell currents were recorded from 2 to 5 days after the injection with a conventional two-electrode voltage clamp from oocytes which were superfused continuously with a high-potassium solution (composition in mM: KCl 96, NaCl 2, $MgCl_2$ 1, $CaCl_2$ 1.5 and HEPES 5, pH 7.4 with KOH) at ~20 °C. The membrane potential was held at −70 mV. Microelectrodes were filled with 3 M KCl.

Drugs

The following opioid receptor ligands, purchased from Sigma Chemical Co. (St. Louis, MO, USA), were used: [D-Ala^2,N-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO), a selective μ-opioid-receptor agonist; [D-$Pen^{2,5}$]enkephalin (DPDPE), a selective δ-opioid-receptor agonist; *trans*-(±)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide (U50488H), a selective κ-opioid-receptor agonist; and naloxone, an opioid-receptor antagonist. Recombinant human IFNα-2b was purchased from Schering-Plough (Osaka, Japan). All drugs were dissolved in distilled water and added to the high-potassium solution in appropriate amounts before the experiment.

Statistical analysis of results

The values obtained are expressed as the mean ± S.E.M., and n is the number of oocytes tested. Statistical analysis of differences between groups was carried out by using paired t-test or Student's t-test. A probability of 0.05 was taken as the level of statistical significance.

Results

To investigate the effects of IFNα on the cloned μ-, δ- and κ-opioid receptors, we conducted *Xenopus* oocyte expression assays. In oocytes co-injected with either the δ- or κ-opioid receptor mRNA and GIRK1/GIRK2 mRNAs, application of 3000 IU/ml IFNα induced small inward currents, compared with the control responses to each selective opioid agonist (Fig. 1A). The current responses to IFNα were almost completely abolished by the simultaneous presence of 1 μM naloxone, an opioid-receptor antagonist, or fully abolished during application of 3 mM Ba^{2+} which blocks GIRK1/2 channels expressed in oocytes (Kobayashi et al., 2003) (n = 3 for each, data not shown). Furthermore, substitution of the perfusion solution containing additive distilled water used as the solvent vehicle, at the highest content (0.05%), caused no significant current response (n = 4; data not shown). In addition, in oocytes injected with either opioid receptor mRNA alone or GIRK1/GIRK2 mRNAs, neither IFNα nor selective opioid agonists induced any significant current response (n = 3, data not shown). These results suggest that IFNα weakly activated the δ- and κ-opioid receptors and that the current responses were mediated by GIRK channels. Furthermore, the magnitudes of the inward current responses induced by IFNα were