0026-895X/99/061020-08$3.00/0

MOLECULAR PHARMACOLOGY, **55:**1020–1027 (1999).

Bombesin Receptors Inhibit G Protein-Coupled Inwardly Rectifying K^+ Channels Expressed in *Xenopus* Oocytes through a Protein Kinase C-Dependent Pathway

EDWARD B. STEVENS, BHAVAL S. SHAH, ROBERT D. PINNOCK, and KEVIN LEE

Parke-Davis Neuroscience Research Centre, Cambridge University Forvie Site, Cambridge, United Kingdom

Received November 16, 1998; accepted March 9, 1999

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

ABSTRACT

Although activation of G protein-coupled inward rectifying K^+ (GIRK) channels by G_i/G_o-coupled receptors has been shown to be important in postsynaptic inhibition in the central nervous system, there is also evidence to suggest that inhibition of GIRK channels by G_q-coupled receptors is involved in postsynaptic excitation. In the present study we addressed whether the G_q-coupled receptors of the bombesin family can couple to GIRK channels and examined the mechanism by which this process occurs. Different combinations of GIRK channel subunits (Kir3.1, Kir3.2, and Kir3.4) and bombesin receptors (BB_1 and BB_2) were expressed in *Xenopus* oocytes. In all combinations tested GIRK currents were reversibly inhibited upon application of the bombesin-related peptides, neuromedin B or gastrin-releasing peptide in a concentration-dependent manner. Incubation of oocytes in the phospholipase C inhibitor U73122 or the protein kinase C (PKC) inhibitors chelerythrine and staurosporine significantly reduced the inhibition of GIRK currents by neuromedin B, whereas the Ca^{2+} chelator, BAPTA-AM had no effect. The involvement of PKC was further demonstrated by direct inhibition of GIRK currents by the phorbol esters, phorbol-12,13-dibutyrate and phorbol-12-myristate-13-acetate. In contrast, the inactive phorbol ester 4α-phorbol and protein kinase A activators, forskolin and 8-bromo cAMP did not inhibit GIRK currents. At the single-channel level, direct activation of PKC using phorbol ester phorbol-12,13-dibutyrate caused a dramatic reduction in open probability of GIRK channels due to an increase in duration of the interburst interval.

G protein-coupled inwardly rectifying K^+ (GIRK) channels are important in regulating cell excitability in both the heart and the central nervous system (CNS; Dascal, 1997). In the CNS, GIRK channels are coupled to many types of inhibitory neurotransmitter receptors via pertussis toxin (PTX)-sensitive G proteins, e.g., γ-aminobutyric acid type B ($GABA_B$), 5-hydroxytryptamine type 1A (serotonin), δ and μ opioid, somatostatin, and adenosine A_1 receptors (Andrade et al. 1986; Trussell and Jackson, 1987; Penington et al., 1993; Velimirovic et al., 1995). In agreement with this, Lüscher et al. (1997) recently showed that GIRK channels perform an important role in inhibition of hippocampal neurons using Kir3.2 knockout mice. Furthermore, immunocytochemical staining has revealed the presence of Kir3.1 on presynaptic nerve terminals in many areas of the brain, suggesting a role for GIRK channels in presynaptic inhibition (Morishige et al., 1996; Ponce et al. 1996).

The mechanism by which neurotransmitters interact to activate the GIRK channel is now well established. In the presence of agonist, G_i/G_o-coupled receptors catalyze the turnover of trimeric G proteins releasing $\beta\gamma$ subunits, which directly bind to the GIRK channel complex, stabilizing phosphatidyl inositol 4,5-bis-phosphate binding, with consequent channel activation (Huang et al., 1997, 1998).

In addition to their role in neuronal inhibition, there is increasing evidence to suggest that GIRK channels have an important function in neuronal excitation induced by G_q-coupled neurotransmitter receptors (Velimirovic et al., 1995; Farkas et al., 1997). However, in contrast to the wealth of knowledge concerning G_i/G_o-mediated activation of this channel complex, little is known regarding how G_q-coupled receptors inhibit these channels. In view of this, in the present study we have used the *Xenopus* oocyte expression system to examine the mechanism by which G_q-coupled receptors of the bombesin family interact with and inhibit GIRK channel activity. Some of these findings have recently been reported to The Physiological Society (Stevens et al., 1998).

Materials and Methods

Molecular Biology. Rat Kir3.1 and Kir3.2 cDNAs engineered into pBG7.2 were linearized using *Nde*I, whereas rat bombesin re-

ABBREVIATIONS: GIRK, G protein-coupled inwardly rectifying K^+ channel; GRP, gastrin-releasing peptide; IP_3, inositol 1,4,5-triphosphate; NMB, neuromedin B; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; PTX, pertussis toxin.

ceptor (BB_1 and BB_2) cDNAs engineered into pBluescript SK (Invitrogen, Netherlands) were linearized using *Bam*HI and Spe1, respectively. Capped cRNAs were transcribed in vitro from linearized cDNAs using T7 polymerases (Promega, Southampton, UK).

Isolation of *Xenopus* Oocytes. *Xenopus laevis* were anesthetized by immersion in 0.3% (w/v) 3-amino benzoic acid (Sigma, Poole, U.K.) and ovarian lobes were removed. Oocytes were dissociated using 0.3% (w/v) collagenase (Sigma) in Ca^{2+}-free solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6). Prepared oocytes were microinjected with 50 nl of cRNAs dissolved in water. Oocytes were incubated at 18°C in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES).

Two-Electrode Voltage Clamp Recording. Two-electrode voltage clamp recordings were performed 3 to 6 days after microinjection of cRNAs using a GeneClamp 500 amplifier (Axon Instruments, Burlingame, CA) interfaced to a Digidata 1200 A/D board with Clampex software (version 6, Axon Instruments) and recorded on DAT (Sony, Toyoko, Japan). Records were replayed on a Gould TA240 chart recorder. Oocytes were continually perfused with standard recording solution (90 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM HEPES, pH 7.4). Microelectrodes filled with 3 M KCl had resistances between 0.5 and 2 MΩ. Ten-millivolt hyperpolarizing steps were applied every second from a holding potential of −80 mV. Currents were filtered at 1 kHz. Data were analyzed using Clampfit (version 6; Axon Instruments) and Prism (version 2; GraphPad Software, San Diego, CA). Oocytes were preincubated with chelerythrine, staurosporine, U-73122 ({1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione}), U73343 ({1-[6-((17β-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione} or BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester) for 2 h. Statistical analyses were performed using a Student's unpaired *t* test. Data were assumed to be normally distributed and a significant difference was accepted when the two-tailed *P* value was less than 0.01. Averaged data are presented as means ± S.E.M. Dose-response data were fitted with the equation:

$$y = 1/(1 + ([NMB]/EC_{50})^n) \quad (1)$$

where EC_{50} is the half-maximally effective concentration of neuromedin B (NMB), [NMB] is the test concentration of NMB, n is the Hill coefficient, and y is the amount of inhibition of current relative to the control level.

Single-Channel Recording. Single-channel recordings were performed using an Axopatch 200B patch clamp amplifier (Axon Instruments). Currents were recorded at 10 kHz and filtered at 2 kHz. The electrode solution contained: 140 mM KCl, 2 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 0.1 mM $GdCl_3$, pH 7.2, whereas the bathing solution contained: 140 mM KCl, 3 mM $MgCl_2$, 5 mM EGTA, and 5 mM HEPES, pH 7.2. Patch electrodes were coated with dental wax and had resistances between 1 to 2 MΩ. Data was acquired using Fetchex (versin 6; Axon Instruments) and analyzed using TAC and TACfit (version 5; Bruxton Corp., Seattle, WA). All experiments were performed at 22 to 24°C.

All events were detected using the "50% threshold" technique and were visually inspected before being accepted. Events shorter than 100 μs were excluded from the histograms. Open and closed duration histograms were binned logarithmically and the distributions were fitted by a sum of exponential probability density functions using the maximum likelihood method.

The critical closed time (t_{crit}) is the duration below which closed times are considered to occur within a burst rather than between bursts and was used to define burst lengths. t_{crit} was calculated by the method of Colquhoun and Sigworth (1995) using the equation:

$$a_f e^{-t_{crit}/\tau f} = a_s(1 - e^{-t_{crit}/\tau s}) \quad (2)$$

where τ_f and τ_s are the fast and slow time constants of the closed time distribution, whereas a_f and a_s are their respective areas.

Open probability (P_o) was calculated over a 5-min duration before and after application of phorbol-12-myristate-13-acetate (PMA).

Results

Inhibition of Kir3 by Bombesin Receptors. Oocytes coinjected with GIRK channel and bombesin receptor cRNA were recorded using two-electrode voltage clamp. Oocytes expressing heteromeric Kir3.1-Kir3.2 channels and the BB_1 receptor gave rise to Ba^{2+}-sensitive currents at −80 mV in standard recording solution. A 2-min application of the preferred BB_1 agonist, NMB, inhibited the GIRK current in a concentration-dependent manner that was slowly reversible on washout (Fig. 1, a, b, and d). Voltage ramps applied from −120 mV to +40 mV before and after NMB application, suggested that NMB reduced current amplitude, but not rectification properties (Fig. 1b). In initial experiments, the time course of GIRK channel inhibition by NMB was obscured due to the activation of an endogenous Ca^{2+}-dependent Cl^- current (Barish, 1983; Fig. 1a) at NMB concentrations above 50 pM (Fig. 2a–c). However, preincubation of oocytes with the membrane-permeant Ca^{2+} chelator BAPTA-AM abolished this Cl^- conductance at all tested NMB concentrations while having no effect on the inhibition of the GIRK current (Fig. 1c and Fig. 3). Under these conditions, inhibition of the GIRK current was preceded by a small activation, which could be due to either residual Ca^{2+}-activated C^- currents or promiscuous coupling of the bombesin receptor to G proteins of the G_i/G_o family. Using this approach, it was possible to construct a concentration-inhibition curve for NMB. Fitting these data to eq. 1 gave a Hill coefficient of 1.09 ± 0.25 and an IC_{50} of 5.4 ± 0.04 pM (Fig. 2a).

The effects of BB_1 receptor expression on the amplitude of the GIRK current in the absence of agonist was investigated by comparing oocytes expressing Kir3.1-Kir3.2 with and without the BB_1 receptor. GIRK currents in oocytes coexpressing Kir3.1-Kir3.2 and BB_1 receptor were not significantly different from oocytes expressing Kir3.1-Kir3.2 alone (6.9 ± 0.5 μA; $n = 7$, and 6.8 ± 0.8 μA; $n = 6$; at −80 mV), suggesting that the unstimulated BB_1 receptor cannot catalyze the turnover of G proteins in the absence of agonists.

Currents flowing through homomeric Kir3.2 and heteromeric Kir3.1-Kir3.4 channels were also reversibly inhibited by activation of the BB_1 receptor by 10 nM NMB (Fig. 1d). In addition, the related bombesin receptor, BB_2 also inhibited Kir3.1-Kir3.2 currents in the presence of the preferred BB_2 agonist, gastrin-releasing peptide (GRP; 10 nM) (Fig. 1d).

The most common GIRK channel in the CNS is the Kir3.1-Kir3.2 heteromultimer (Lesage et al., 1995; Liao et al., 1996) whose distribution overlaps with BB_1 receptor in many areas of the brain (Wada et al. 1991; Ladenheim et al., 1992; Karschin et al. 1996; Murer et al., 1997). In attempt to investigate the interaction between these bombesin receptors and GIRK channels with relevance to the CNS, the Kir3.1-Kir3.2 and BB_1 receptor combination was chosen for more extensive study.

Inhibition Is Mediated by Protein Kinase C (PKC). Bombesin receptors have previously been shown to couple to phospholipase C (PLC) via G_q in *Xenopus* oocytes, which activates both the inositol 1,4,5-triphosphate (IP_3) and PKC pathways (Shapira et al., 1994). To determine whether PLC performed a role in inhibition of GIRK currents via interaction with the BB_1 receptor, oocytes were preincubated in the

PLC inhibitor U73122. GIRK current inhibition was significantly reduced by U73122, whereas the inactive analog, U73343 had no effect (Fig. 3).

To examine whether IP_3-mediated release of Ca^{2+} from intracellular stores was involved in the present sequence of events, oocytes were preincubated in BAPTA-AM. However, as shown previously, this process had no effect on the ability of NMB to inhibit the GIRK current (Fig. 3), suggesting that Ca^{2+} release stimulated by IP_3 is not involved in this coupling.

Because Kir3 subunits possess multiple consensus PKC phosphorylation sites, the role of PKC in channel inhibition was tested using PKC activators and inhibitors. Preincubation of oocytes in the PKC inhibitors staurosporine or chelerythrine before electrophysiological recording significantly reduced the ability of NMB to inhibit GIRK currents compared with untreated oocytes (Fig. 3). To verify that PKC is capable of GIRK current inhibition, the effects of phorbol esters on GIRK current amplitude was examined. Continuous application of 1 μM phorbol-12,13-dibutyrate (PDBu) or 1 μM PMA inhibited the GIRK current, which was irreversible over a 30-min period ($n = 5$) (Fig. 4, a, b, and d). As noted for PKC-mediated modulation of ion channels in other native cells and heterologous expression systems (Walsh and Kass, 1988; Henry et al., 1996; Shuba et al., 1996), the inhibitory action of PMA was maximal at 100 nM, because an increase in PMA concentration to 1 μM caused no further increase in inhibition (Fig. 4d). In contrast, the inactive phorbol ester

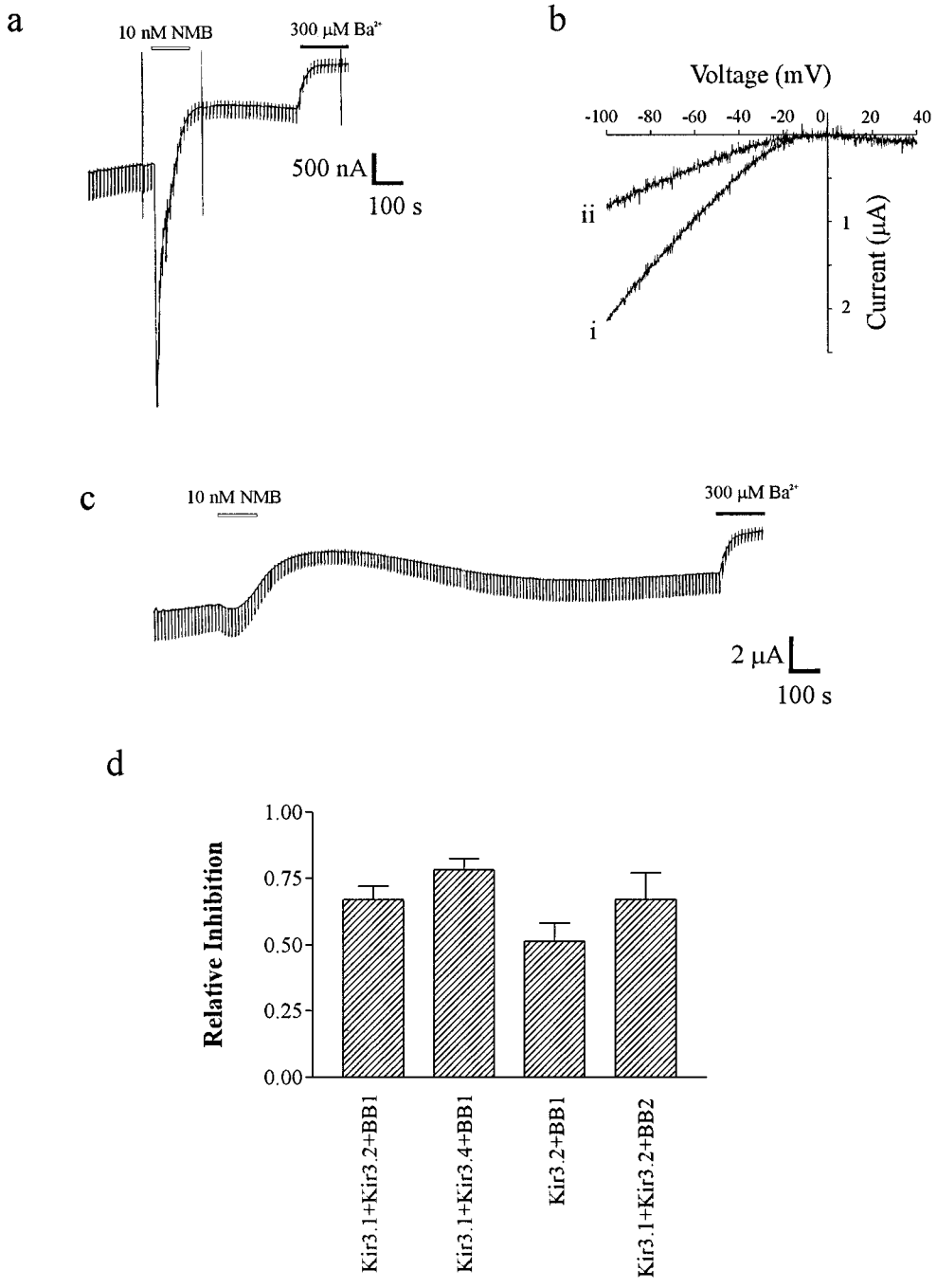

Fig. 1. Inhibition of GIRK channels by coupling to bombesin receptors. a, inward GIRK currents were recorded from oocytes injected with cRNAs for Kir3.1, Kir3.2, and BB_1 receptor. Application of 10 nM NMB both evoked an endogenous inward Ca^{2+}-activated Cl^- current and inhibited the GIRK current, which was also selectively blocked by 300 μM Ba^{2+}. b, current-voltage relationships were obtained using voltage ramps between -120 and $+40$ mV. Voltage ramps were applied before and after application of NMB and in the presence of Ba^{2+}. Current responses to ramps in the presence of Ba^{2+} were subtracted from those before (i) and after (ii) NMB application. c, GIRK currents recorded from oocytes incubated in BAPTA-AM. Endogenous Ca^{2+}-activated Cl^- current is completely abolished by the Ca^{2+} chelator. GIRK current was reversibly inhibited by a 2-min application of NMB. d, histogram of inhibitory effects of bombesin-like peptides on different bombesin receptor-coupled Kir3 subunit combinations ($n = 3$–6). BB_1 receptors were activated with 10 nM NMB, whereas BB_2 receptors were activated with 10 nM GRP. Relative inhibition is current amplitude after drug application relative to current amplitude before drug application. All Ba^{2+}-insensitive currents were subtracted.

4α-phorbol (1 μM) had no effect on the magnitude of this current (Fig. 4, c and d), suggesting that the effect of PMA is mediated via PKC and not via some direct interaction with the channel complex. Activation of PKC by PMA was further demonstrated by incubation of oocytes in 10 μM staurosporine, which significantly blocked the inhibitory action of 100 nM PMA on GIRK currents (relative inhibition was 0.11 $\pm$0.05 at -80 mV, $n = 6$). The protein kinase A (PKA) activators forskolin (1 μM) and 8-bromo cAMP (1 μM) had no significant inhibitory effect on the GIRK current (Fig. 4d).

Single-channel Properties in Presence of PMA. Single-channel recordings were performed in the cell-attached recording configuration. Inhibition of the GIRK current by the BB_1 agonist NMB was of insufficient duration (<10 min) for complete single-channel analysis due to desensitization of the receptor (Corjay et al., 1991). Therefore, PKC-mediated inhibition of the GIRK current with PMA was used to examine the effect of PKC activation on GIRK channel activity. The single-channel properties of the Kir3.1-Kir3.2 complex were compared before and after treatment with PMA. Under control conditions, GIRK currents were found to exhibit a unitary amplitude of 2.71 $\pm$ 0.08 pA at -80 mV ($n = 3$). As previously reported in both native and heterologous expression systems, GIRK channel activity was characterized by bursting behavior (Grigg et al., 1996; Luchian et al., 1997), i.e., clusters of openings separated by brief closures (Fig. 5, a and b).

Following bath application of 100 nM PMA, there was a rapid reduction in channel open probability, P_o, from $2.7 \times 10^{-2} \pm 8.1 \times 10^{-3}$ to $8.6 \times 10^{-4} \pm 9.4 \times 10^{-5}$ ($n = 3$). This reduction in channel activity was not associated with any change in single-channel current amplitude (2.63 $\pm$ 0.05 pA at -80 mV). Further kinetic analysis of channel activity revealed that PMA had no significant effect on either the open times (control, $\tau_f = 0.28 \pm 0.03$ ms, $\tau_s = 1.39 \pm 0.05$ ms; PMA, $\tau_f = 0.32 \pm 0.04$ ms, $\tau_s = 1.27 \pm 0.33$ ms, $n = 3$) or burst lengths (control, $\tau_f = 0.32 \pm 0.04$ ms, $\tau_s = 2.20 \pm 0.05$ ms; PMA, $\tau_f = 0.33 \pm 0.03$ ms, $\tau_s = 1.83 \pm 0.27$ ms, $n = 3$) of the GIRK channel complex (Fig. 5, a, b, and c). In contrast, PMA was found to dramatically alter channel closed times (Fig. 5d). In the absence of PMA, closed times were described by two exponential components with time constants of 0.42 $\pm$ 0.01 ms and 57.43 $\pm$ 21 ms ($n = 3$). The first component represents channel closing within a burst, whereas the second component represents channel closing between bursts. In the presence of PMA, closed times were again described by two exponentials with time constants of 0.43 $\pm$ 0.11 ms and 1499 $\pm$ 464 ms ($n = 3$). Thus, PMA-induced inhibition of

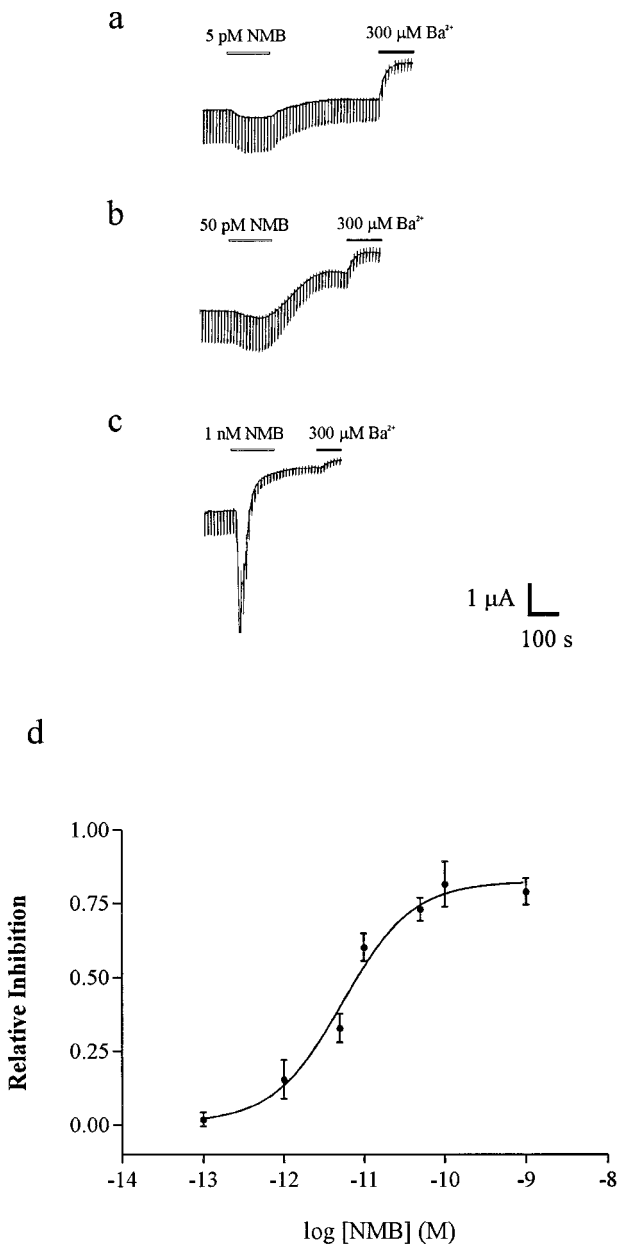

Fig. 2. Dose-dependent inhibition of Kir3.1-Kir3.2 currents by BB_1 receptors in response to NMB. Large transient inward current in c is due to activation of endogenous Ca^{2+}-activated Cl^- current. d, dose-response relationship for NMB. Inhibited currents were normalized relative to current amplitude before application of NMB. Each data point represents $n = 5$. Line is best least-squares fit to eq. 1, where $EC_{50} = 5.4$ pM.

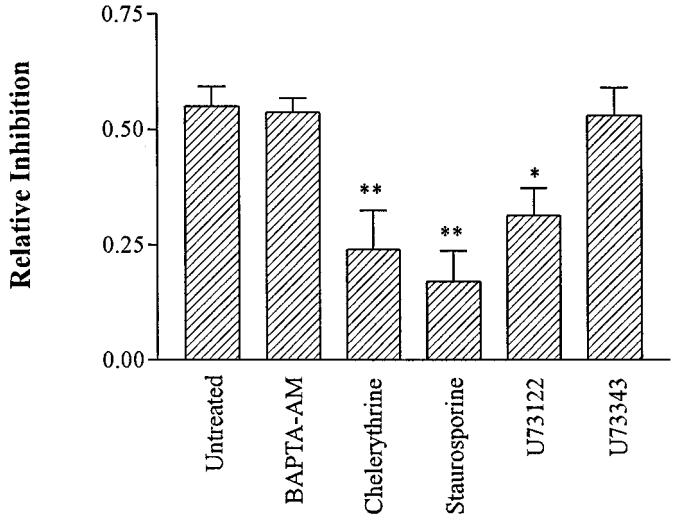

Fig. 3. Effect of PKC inhibitors and BAPTA-AM on Kir3.1-Kir3.2 currents. Histogram shows effect of preincubation of oocytes with the PLC inhibitor U73122 (10 μM) and its inactive analog U73343 (10 μM), PKC inhibitors chelerythrine (10 μM) and staurosporine (10 μM), and Ca^{2+} chelator BAPTA-AM (200 μM) on amount of inhibition of GIRK currents in response to 10 nM NMB ($n = 4$–7). Chelerythrine, staurosporine, and U73122 significantly reduced amount of current inhibition in response to NMB, whereas BAPTA-AM and U73343 had no effect relative to untreated oocytes (*$p < .05$, **$p < .01$).

the channel results in an increase in duration of the burst interval.

Discussion

This study demonstrates that activation of G_q-coupled bombesin receptors using the bombesin-related peptides NMB or GRP reversibly inhibits GIRK currents in *Xenopus* oocytes. The BB_1 receptor has previously been shown to activate phospholipase C via a G_q-mediated pathway in oocytes (Shapira et al., 1994; Woodruff et al., 1996). This results in production of diacylglycerol and IP_3. IP_3 releases Ca^{2+} from intracellular organelles, whereas diacylglycerol activates PKC (Sternweis et al., 1992). The involvement of PLC in coupling between BB_1 receptor and GIRK currents was demonstrated using the specific PLC inhibitor U73122, which decreased the degree of inhibition of GIRK currents in response to NMB. The increase in intracellular Ca^{2+} after binding of NMB to BB_1 receptor is clearly seen in the present study by the activation of the endogenous oocyte Ca^{2+}-dependent Cl^- channel (Barish, 1983). Treatment of oocytes with the Ca^{2+} chelator, BAPTA-AM, abolishes this Cl^- current, but has no effect on inhibition of GIRK current, suggesting that an increase in intracellular Ca^{2+} concentration is not involved in modulation of GIRK channel activity. In contrast, we demonstrate that PKC performs an important role in the modulation of GIRK channel activity using both PKC inhibitors and activators. The PKC inhibitors staurosporine and chelerythrine reduce the amount of GIRK current inhibition in response to NMB, whereas the PKC activators, the phorbol esters PMA and PDBu, induce inhibition of GIRK channel activity in the absence of receptor activation. The specificity of PDBu and PMA for PKC has been demonstrated using the inactive phorbol ester 4α-phorbol, which does not inhibit the GIRK current.

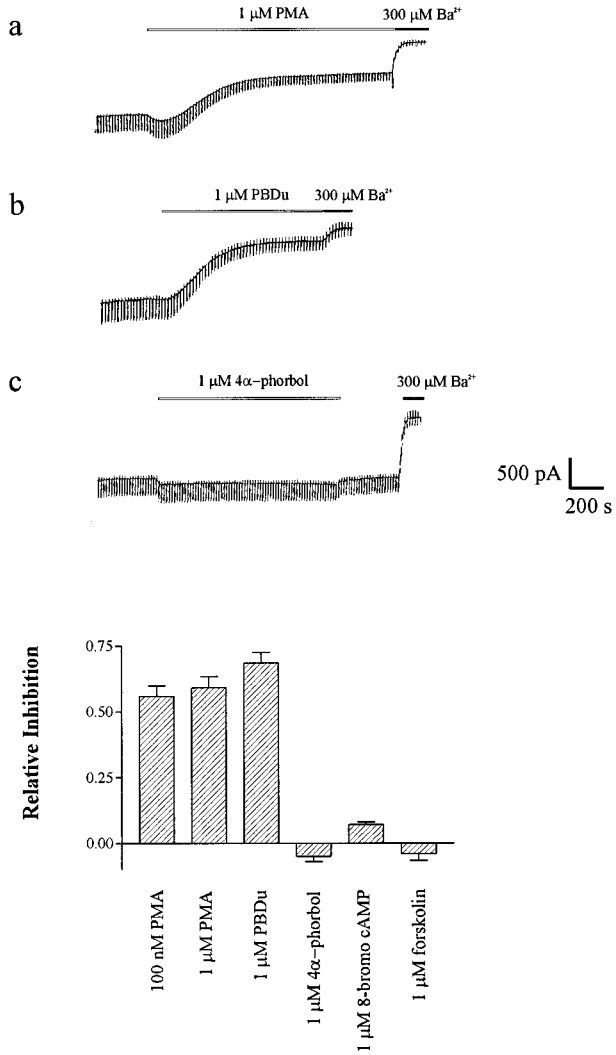

Fig. 4. Effect of PKC and PKA activators on Kir3.1-Kir3.2 currents. a, inhibitory effect of 1 μM PMA on GIRK currents. b, inhibitory effect of 1 μM PBDu on GIRK currents. c, lack of inhibition of GIRK currents by 4α-phorbol. d, histogram showing effects of phorbol esters PMA, PBDu, and 4α-phorbol, and PKA activators 8-bromo cAMP and forskolin. Each bar represents $n = 5$.

Previous studies on interactions between GIRK channels and the G_q/G_{11}-coupled metabotropic glutamate receptors have given conflicting results. Saugstad et al. (1996) reported that type 1a metabotropic glutamate receptors (mGluR1a) activate Kir3.1-Kir3.4 heteromeric channels by coupling to PTX-sensitive G proteins, whereas Sharon et al. (1997) reported that mGluR1a receptors inhibit Kir3.1-Kir3.2 heteromeric channels by coupling to PTX-insensitive G proteins. Sharon et al. (1997) also showed that Kir3.1-Kir3.2 channels display an initial weak activation before the strong inhibition due to promiscuous coupling of the mGluR1a receptor to PTX-sensitive G proteins. In agreement with the present study, Sharon et al. (1997) showed that inhibition of the GIRK channel complex occurs through a PKC-dependent mechanism.

In the present study, single-channel recordings in the presence of PMA provide an insight into the mechanism of channel inhibition. Single-channel conductance, open-time distributions, and burst lengths remain unaffected, whereas the interburst interval is altered. Ivanova-Nikolova and Breitweiser (1997) reported that $\beta\gamma$ binding changes the proportion of channels within three bursting modes, but does not change the burst duration. Termination of bursting occurs independently of $\beta\gamma$ binding and has been shown to be caused by a C-terminal inactivation particle blocking the pore (Luchian et al., 1997). Because intraburst kinetics were unaffected by PKC activation in the present study, inhibition is unlikely to occur through a change in $\beta\gamma$ binding. However, an increased interburst duration suggests that PKC activation stabilizes the inactivated state.

Although there is no direct evidence that phosphorylation by PKC acts directly on the GIRK channel rather than an accessory protein, Kir3.1, Kir3.2, and Kir3.4 contain consensus PKC sites on both the N and C termini of the channel subunits. Another inward rectifier, Kir2.3, has been shown to couple to the G_q-linked muscarinic M_1 receptor and is inhibited by the phorbol esters PMA and PDBu (Henry et al., 1996), suggesting the involvement of PKC. Because Kir3 subunits and Kir2.3 possess a consensus PKC site at identical positions in the N terminus, it is possible to speculate that phosphorylation of this site may be involved in the channel inhibition process.

In order for GIRK channels to be a target for inhibition by coupling to G_q-linked receptors, a proportion of the channels must be activated. GIRK channels have been shown to display tonic activity in hippocampal neurons. Using patch clamp recording from brain slices, the membrane potentials

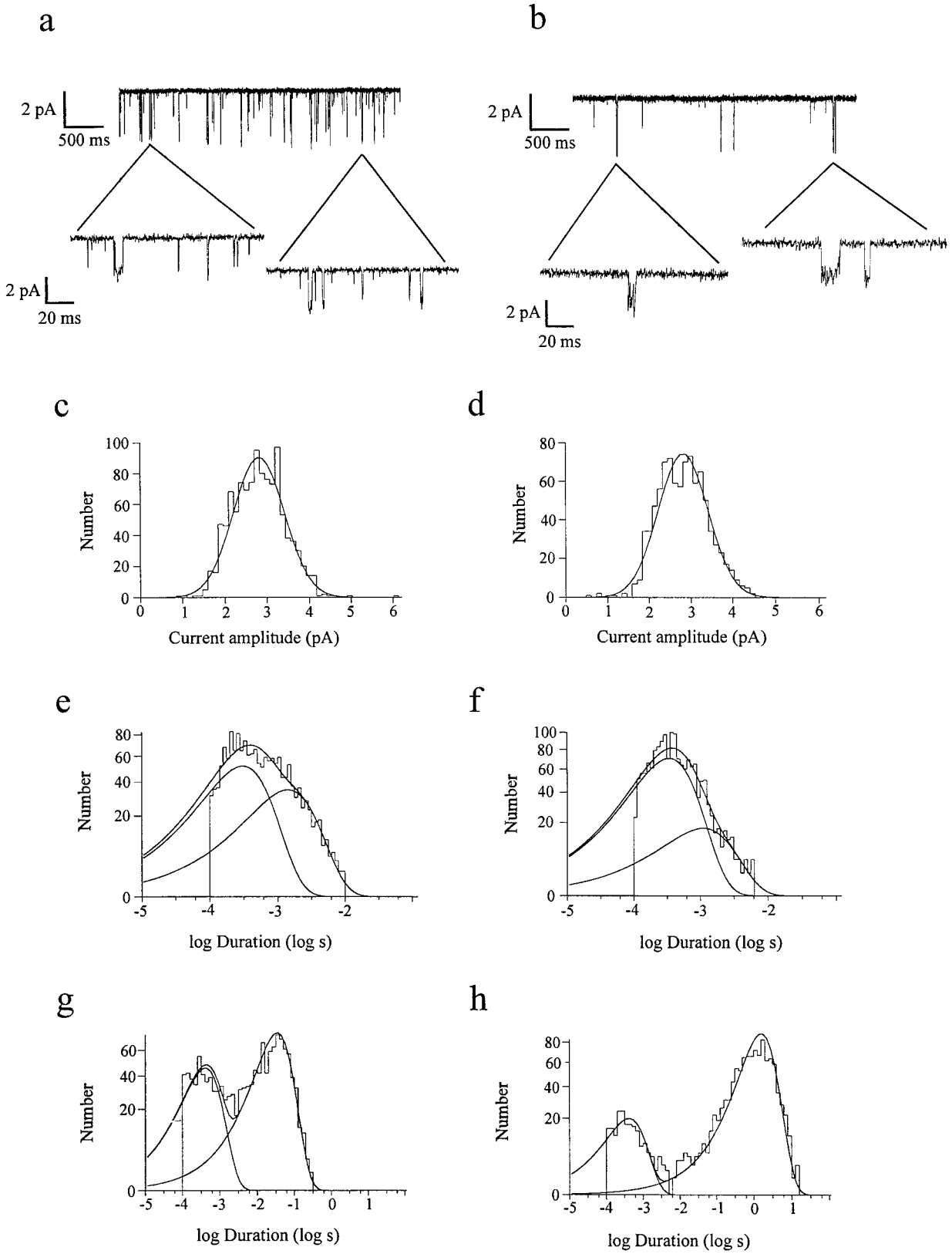

Fig. 5. Change in single-channel properties of Kir3.1-Kir3.2 in response to PMA. Typical single channel records before (a) and after (b) application of 100 nM PMA showing a reduction in open probability. Single-channel amplitude histograms were fitted with a Gaussian function. Single-channel amplitudes before (c) and after (d) application of PMA were identical (2.7 pA). Open-time distributions were fitted with two exponential components. Before application of PMA (e), open times were described with time constants of 0.3 and 1.5 ms, whereas after application of PMA (f), open times were described by time constants of 0.3 and 1.1 ms. Closed-time distributions were fitted with two exponential components. Before application of PMA (g), closed times were described with time constants of 0.4 and 35 ms, whereas after application of PMA (h), closed times were described by time constants of 0.4 and 1480 ms.

of Kir3.2 knockout mouse CA1 neurons is 8 mV more depolarized than wild-type mouse CA1 neurons (Lüscher et al., 1997). Sodickson and Bean (1996) showed that dissociated rat hippocampal CA3 neurons give rise to $GABA_B$-activated GIRK currents (EC_{50} = 1.6 μM) at extracellular GABA concentrations present basally in the brain (0.2–0.8 μM), suggesting that tonic activity of GIRK channels could arise from tonic activation of receptors. However, because $GABA_B$ and A_1 antagonists do not affect resting potentials of CA1 neurons in slices, Lüscher et al. (1997) suggest that tonic activity of GIRK channels is independent of receptor activation. Tonic activity of GIRK channels in the absence of agonist has been shown to operate through constitutive turnover of G proteins, releasing $\beta\gamma$, in *Xenopus* oocytes (Stevens et al., 1997) and through a Na^+-gating mechanism in both myocytes and oocytes (Lesage et al. 1995; Sui et al. 1996).

Negative coupling of GIRK channels to G_q-linked receptors has been demonstrated in several neuronal preparations. For example, in cultured rat locus coereleus neurons a GIRK channel activated by somatostatin and [Met]enkephalin has been identified by its single-channel conductance (30 picosiemen) and membrane-delimited activation (Grigg et al., 1996). The same GIRK channel is also inhibited by Substance P via a PTX-insensitive G protein (Velimirovic et al., 1995). Similarly, in GTPγS-loaded locus ceruleus neurons application of somatostatin induced a persistent increase in K^+ conductance that was reversed by application of Substance P (Velimirovic et al., 1995). In cultured rat dopaminergic neurons of the midbrain ventral tegmental area a putative GIRK current is activated by dopamine (D_2 receptors) via PTX-sensitive G proteins and inhibited by neurotensin via PTX insensitive G proteins (Farkas et al., 1997)

The distribution of GIRK channels shares a high degree of overlap with BB_1 and BB_2 receptors throughout the CNS (Wada et al. 1991; Ladenheim et al., 1992; Murer et al., 1997; Karschin et al. 1996). Furthermore, inhibition of a resting K^+ conductance by bombesin and related peptides has been reported in neurons of the rat dorsal raphe and suprachiasmatic nucleus (Pinnock and Woodruff, 1991; Reynolds and Pinnock, 1997). Because GIRK channels have been reported to be coupled to 5-hydroxytryptamine type 1A receptors in dorsal raphe neurons (Penington et al., 1993), it is possible that GIRK channels are the target for inhibition induced by bombesin.

In summary, in the present study we demonstrated unequivocally that G_q-coupled neuropeptides are able to inhibit GIRK channels in a nonmembrane-delimited manner involving PKC. Furthermore, we showed at the single-channel level this inhibition by PKC is mediated by a change in interburst interval of the channel complex. In future studies it will be important to examine the interaction between GIRK channels and G_q-coupled receptors in native neurons to test whether a similar mechanism is involved in postsynaptic excitation.

Acknowledgments

We are grateful to Dr. R. Murrell-Lagnado (Department of Pharmacology, University of Cambridge, United Kingdom) for generously supplying the vectors pBG7.2Kir3.1, pBG7.2Kir3.2, and pBG7.2Kir3.4. We are also grateful to J. F. Battey (Laboratory of Molecular Biology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland) for providing us with clones of rat BB_1 receptors, and to M. D. Hall for subcloning the receptor cDNA into Bluescript.

References

Andrade R, Malenka RC, and Nicoll RA (1986) A G protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. *Science* **234**:1261–1265.

Barish ME (1983) A transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *J Physiol (Lond)* **342:**309–325.

Colquhoun D and Sigworth FJ (1995) Fitting and statistical analysis of single-channel records, in *Single-Channel Recording* (Sakmann B and Neyer E eds) pp 483–587, Plenum, New York.

Corjay MH, Dobrzanski DJ, Way JM, Viallet J, Shapira H, Worland P, Sausville EA and Battey JF (1991) Two distinct bombesin receptor subtypes are expressed and functional in human lung carcinoma cells. *J Biol Chem* **266:**18771–18779.

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

Farkas RH, Chien P-Y, Nakajima S and Nakajima Y (1997) Neurotensin and dopamine D2 activation oppositely regulate the same K^+ conductance in rat midbrain dopaminergic neurones. *Neurosci Lett* **231:**21–24.

Grigg JJ, Kozasa T, Nakajima Y and Nakajima S (1996) Single-channel properties of a G-protein-coupled inward rectifier potassium channel in brain neurons. *J Neurophysiol* **75:**318–328.

Henry P, Pearson WL and Nichols CG (1996) Protein kinase C inhibition of cloned inward rectifier (HRK1/Kir2.3) K^+ channels expressed in Xenopus oocytes. *J Physiol (Lond)* **495:**681–688.

Huang CL, Feng, S and Hilgemann, DW (1998) Direct activation of inward rectifier potassium channels by PIP_2 and its stabilization of $G_{\beta\gamma}$. *Nature* **391:**803–806.

Huang CL, Jan YN and Jan LY (1997) Binding of the G protein $\beta\gamma$ subunit to multiple regions of G protein-gated inward-rectifying K^+ channels. *FEBS Lett* **405:**291–298.

Ivanova-Nikolova TT and Breitweiser GE (1997) Effector contributions to G $\beta\gamma$-mediated signalling as revealed by muscarinic potassium channel gating. *J Gen Physiol* **109:**245–253.

Karschin C, Diβmann E, Stühmer W and 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.

Ladenheim EE, Jensen RT, Mantey SA and Moran TH (1992) Distinct distributions of two bombesin receptor subtypes in the rat central nervous system. *Brain Res* **593:**168–178.

Lesage F, Guillemare E, Fink M, Duprat F, Heurteaux C, Fosset M, Romey G, Barhanin J and Lazdunski M (1995) Molecular properties of neuronal G-protein-activated inwardly rectifying K^+ channels. *J Biol Chem* **270:**28660–28667.

Liao YJ, Jan YN and Jan LY (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.

Luchian T, Dascal N, Dessauer C, Platzer D, Davidson N, Lester HA and Schreibmayer W (1997) A C-terminal peptide of the GIRK1 subunit directly blocks the G protein-activated K^+ channel (GIRK) expressed in *Xenopus* oocytes. *J Physiol (Lond)* **505:**13–22.

Lüscher C, Jan LY, Stoffel M, Malenka RC and Nicoll RA (1997) G-protein coupled inwardly rectifying K^+ (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurones. *Neuron* **19:**687–695.

Morishige KI, Inanobe A, Takahashi N, Yoshimoto Y, Kurachi H, Miyake A, Tokunaga-Y, Maeda T and Kurachi Y (1996) G protein-gated K^+ channel (GIRK1) protein is expressed presynaptically in the paraventricular nucleus of the hypothalamus. *Biochem Biophys Res Commun* **220:**300–305.

Murer G, Adelbrecht C, Lauritzen I, Lesage F, Lazdunski M, Agid Y and Raisman-Vozari R (1997) An immunocytochemical study on the distribution of two G-protein-gated inward rectifier potassium channels (GIRK2 and GIRK4) in the adult rat brain. *Neuroscience* **80:**345–357.

Penington NJ, Kelly JS and Fox AP (1993) Whole-cell recordings of inwardly rectifying K^+ currents activated by $5\text{-}HT_{1A}$ receptors on dorsal raphe neurones of the adult rat. *J Physiol (Lond)* **469:**387–405.

Pinnock RD and Woodruff GN (1991) Bombesin excites a subpopulation of 5-hydroxytryptamine-sensitive neurones in the dorsal raphe nucleus in vitro. *J Physiol (Lond)* **440:**55–65.

Ponce A, Bueno E, Kentros C, Vega-Saenz-de-Miera E, Chow A, Hillman D, Chen S, Zhu L, Wu MB, Wu X, Rudy B and Thornhill WB (1996) G-protein-gated inward rectifier K^+ channel proteins (GIRK1) are present in the soma and dendrites as well as in nerve terminals of specific neurons in the brain. *J Neurosci* **16:**1990–2001.

Reynolds T and Pinnock RD (1997) Neuromedin C decreases potassium conductance and increases a non-specific conductance in rat suprachiasmatic neurones in brain slices in vitro. *Brain Res* **750:**67–80.

Saugstad JA, Segerson TP and Westbrook GL (1996) Metabotropic glutamate receptors activate G-protein-coupled inwardly rectifying potassium channels in *Xenopus* oocytes. *J Neurosci* **16:**5979–5985.

Shapira H, Way J, Lipinsky D, Ooron Y and Battey JF (1994) Neuromedin B receptor, expressed in *Xenopus* oocytes, selectively couples to $G_{\alpha q}$ and not $G_{\alpha 11}$. *FEBS Lett* **348:**89–92.

Sharon D, Vrorobiov D and Dascal N (1997) Positive and negative coupling of the metabotropic glutamate receptors to a G-protein-activated K^+ channel, GIRK, in *Xenopus* oocytes. *J Gen Physiol* **109:**477–490.

Shuba LM, Asai T and McDonald TF (1996) Phorbol ester activation of chloride current in guinea-pig ventricular myocytes. *Br J Pharmacol* **117:**1395–1404.

Sodickson DL and Bean BP (1996) $GABA_B$ receptor-activated inwardly rectifying potassium current in dissociated hippocampal CA3 neurones. *J Neurosci* **16:**6374–6385.

Sternweis PC, Smrcka AV and Gutowski S (1992) Hormone signalling via G-protein: Regulation of phosphatidylinositol 4,5-bisphosphate hydrolysis by G_q. *Philos Trans R Soc Lond B Biol Sci* **336:**35–41.

Stevens EB, Shah BS, Lee K and Pinnock RD (1998) Bombesin receptors inhibit G protein-coupled inwardly rectifying K^+ channels expressed in *Xenopus oocytes* through a PKC-dependent pathway. *J Physiol (Lond)* **513:**P:138P

Stevens EB, Woodward R, Ho IH and Murrell-Lagnado R (1997) Identification of regions that regulate the expression and activity of G protein-gated inward rectifier K^+ channels in *Xenopus* oocytes. *J Physiol (Lond)* **503:**547–562.

Sui JL, Chan KW and Logothetis DE (1996) Na^+ activation of the muscarinic K^+ channel by a G-protein-independent mechanism. *J Gen Physiol* **108:**381–391.

Trussell LO and Jackson MB (1987) Dependence of an adenosine-activated potassium current on a GTP-binding protein in mammalian central neurons. *J Neurosci* **7:**3306–3316.

Velimirovic BM, Koyano K and Nakajima Y (1995) Opposing mechanisms of regulation of a G-protein-coupled inward rectifier K^+ channel in rat brain neurones. *Proc Natl Acad Sci USA* **92:**1590–1594.

Wada E, Way J, Shapira H, Kusano K, Lebacq-Verheyden AM, Coy D, Jensen R and Battery J (1991) cDNA cloning, characterization, and brain region-specific expression of a neuromedin-B-preferring bombesin receptor. *Neuron* **6:**421–430.

Walsh KB and Kass RS (1988) Regulation of a heart potassium channel by protein kinase A and C. *Science* **242:**67–69.

Woodruff GN, Hall MD, Reynolds T and Pinnock RD (1996) Bombesin receptors in the brain. *Ann NY Acad Sci* **780:**223–243.

Send reprint requests to: Dr. Kevin Lee, Parke Davis Neuroscience Research Centre, Cambridge University Forvie Site, Robinson Way, Cambridge CB2 2QB, United Kingdom. E-mail: Kevin.Lee@wl.com