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European Journal of Pharmacology 431 (2001) 133–141

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Desensitization of endogenously expressed δ-opioid receptors: no evidence for involvement of G protein-coupled receptor kinase 2

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Received 20 June 2001; accepted 27 August 2001

Abstract

The involvement of G protein-coupled receptor kinase 2 (GRK2) in the agonist-induced desensitization of δ-opioid receptor-mediated inhibition of cAMP formation in NG108-15 mouse neuroblastoma × rat glioma hybrid cells was investigated. Pretreatment of wild-type cells with the δ-opioid receptor agonist [D-Pen^{2,5}]-enkephalin (DPDPE; 100 nM) for as little as 5 min produced marked desensitization of subsequent DPDPE-mediated inhibition of iloprost (300 nM)-stimulated cAMP formation. In NG108-15 cells stably overexpressing wild-type GRK2 or dominant negative mutant GRK2 (DNM GRK2), the DPDPE-induced desensitization of cAMP inhibition was the same as in plasmid-transfected control cells. Pretreatment of wild-type cells with the inhibitors of receptor internalization, concanavalin A (0.25 mg ml^{-1}) or hypertonic sucrose (0.4 M), also failed to inhibit DPDPE-mediated desensitization. Finally, in NG108-15 cells stably overexpressing G protein-coupled receptor kinase 6 (GRK6), DPDPE-induced desensitization was significantly increased as compared to plasmid-transfected control cells. These results indicate that GRK2 is unlikely to mediate the desensitization of endogenous δ-opioid receptors in NG108-15 cells, but that other GRKs, such as GRK6, may be more important.

Keywords: Desensitization; G protein-coupled receptor kinase; δ-Opioid receptor; NG108-15 cell

1. Introduction

As with many other G protein-coupled receptors (GPCRs), δ-opioid receptors become less responsive to continuous or repeated activation with agonist (Beaumont and Henderson, 1999; Law et al., 2000b). This process, known as desensitization, can involve a number of mechanisms, which occur at differing time periods after initial agonist stimulation (Krupnick and Benovic, 1998). Rapid desensitization is thought to occur mainly through receptor phosphorylation or receptor internalization (Pitcher et al., 1998). Chronic agonist exposure can lead to decreased receptor synthesis and increased receptor degradation within the cell, processes known as downregulation (Tsao and von Zastrow, 2000). Together all these processes lead to a loss of receptor responsiveness, and in the case of opioid receptors, are thought to be involved in the mechanisms of addiction and drug tolerance (Beaumont and Henderson, 1999).

Opioid receptor desensitization has been studied widely but the exact mechanisms involved remain unclear. However, a role for δ-opioid receptor phosphorylation in desensitization has been indicated from a number of studies. For example, relatively nonselective inhibitors of protein kinases, such as staurosporine, can reduce the agonist-induced desensitization of δ-opioid receptor responsiveness (Cai et al., 1996; Wang et al., 1998; Yoon et al., 1998; Song and Chueh, 1999). Furthermore, phosphorylation by G protein-coupled receptor kinases (GRKs) has been implied both by the use of nonselective GRK inhibitors (Hasbi et al., 1998) and by coexpression of G protein-coupled receptor kinase 2 (GRK2; Guo et al., 2000) or dominant negative mutant GRK2 (DNM GRK2; Pei et al., 1995). GRKs are a family (GRKs 1–6) of serine–threonine kinases that phosphorylate receptors upon agonist binding to initiate receptor desensitization (Pitcher et al., 1998; Krupnick and Benovic, 1998). Perhaps the GRK subtype most extensively studied is GRK2, which is highly expressed in many cell types, including NG108-15 cells (Mundell et al., 1997). These cells also express significant levels of δ-opioid receptors, coupled to inhibition of cAMP

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PII: S0014-2999(01)01360-7

accumulation, a response that is known to undergo homologous agonist-induced desensitization (Law et al., 1982). In the present study, we have examined whether GRK2 mediates the agonist-induced desensitization of δ-opioid receptors endogenously expressed in NG108-15 cells. Our findings indicate that GRK2 manipulation has no effect on δ-opioid receptor function. However, overexpression of G protein-coupled receptor kinase 6 (GRK6) increased the extent of δ-opioid receptor desensitization upon agonist stimulation, suggesting that GRKs other than GRK2 may be more important for δ-opioid receptor desensitization.

2. Materials and methods

2.1. Materials

[5′ 7′-^{3}H]naltrindole (1.5 TBq mmol^{-1}) was purchased from Tocris Cookson (Bristol, UK) and [8-^{3}H]cAMP (925 GBq mmol^{-1}) from Amersham International, UK. Cell culture materials, media and Lipofectamine were from GIBCO Life Sciences, UK. Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes, ECL detection kit, Hyperfilm-ECL luminescence detection film, and sheep anti-mouse and donkey anti-rabbit immunoglobulin (Ig) horseradish peroxidase-linked secondary antibodies were all obtained from Amersham International. All other reagents were from Sigma (UK).

2.2. Cell culture

NG108-15 mouse neuroblastoma × rat glioma hybrid cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 6% fetal calf serum, 1 μM aminopterin, 100 μM hypoxanthine, 16 μM thymidine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin under humidified conditions, at 37 °C and 6% CO_2. NG108-15 cells stably transfected with wild-type bovine GRK2 in pMEP4 vector (B7), or pMEP4 vector alone (P1), were used as described previously (Mundell et al., 1998). Both B7 and P1 cells were maintained in the above media supplemented with 200 μg ml^{-1} hygromycin. NG108-15 cells stably transfected with DNM GRK2 (D19), or pcDNA3 vector alone (P10) were used as described previously (Mundell et al., 1997). D19 or P10 cells were maintained in the above media supplemented with 300 μg ml^{-1} geneticin. Furthermore, wild-type NG108-15 cells were transfected with either vector alone (pcDNA3) or human GRK6 (in pcDNA3) using Lipofectamine according to the manufacturer's instructions. After transfection, cells were cultured in medium containing 300 μg ml^{-1} geneticin and surviving cells expanded to cell lines. Two clones that overexpressed GRK6 (GRK6/7 and GRK6/14), along with the plasmid-only controls, P3 and P2, were selected for further study.

2.3. Western blotting

Cellular expression of either wild-type GRK2 or DNM GRK2 was assessed by Western blotting with a GRK2-specific monoclonal antibody that recognises an epitope within residues 500–531 of the carboxyl terminus of bovine GRK2 (Loudon et al., 1996). GRK6 expression was detected with a rabbit polyclonal antibody that recognises epitope 98–136 of human GRK6 (Loudon et al., 1996). Confluent cell monolayers were lysed by the addition of 200–500 μl of ice-cold lysis buffer (20 mM HEPES, pH 7.4, 200 mM NaCl, 10 mM EDTA, 1% Triton-X 100, 0.2 mg ml^{-1} benzamidine, 0.1 mg ml^{-1} leupeptin and 0.5 mM phenylmethylsulphonylfluoride). Insoluble cell fractions were then pelleted by centrifugation at 13,000 rpm and 4 °C for 3 min. Aliquots of the supernatant were then snap-frozen in liquid nitrogen and stored at −80 °C. When required, 40 μg of cell lysate was added to sodium dodecyl sulphate (SDS) sample buffer (final concentration 63 mM Tris, pH 6.5, 100 mM dithiothreitol, 1% SDS, 11.6% glycerol and 0.02% bromophenol blue) and resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Recombinant GRK2 or GRK6 (20 ng) were used as standard. Protein was transferred to nitrocellulose and incubated first with the monoclonal antibodies described above. A sheep anti-mouse Ig horseradish peroxidase-linked secondary antibody was then used for GRK2 or DNM GRK2, while a donkey anti-rabbit Ig horseradish peroxidase-linked secondary antibody was used for GRK6. Finally, ECL was used with Hyperfilm according to the manufacturer's instructions to visualise GRK expression.

2.4. Desensitization experiments

Desensitization of the δ-opioid receptor responsiveness was determined by comparison of the ability of [D-Pen^{2,5}]-enkephalin (DPDPE; a δ-opioid receptor-selective agonist) to induce inhibition of the prostanoid IP receptor agonist iloprost-stimulated cAMP formation in DPDPE-pretreated or nonpretreated cells. Cells were seeded into 24-well culture plates. When confluent, medium was removed and replaced with 0.5 ml of fresh medium. After 1 h, cells (with the exception of nonpretreated controls) were pretreated with DPDPE for various time periods. Cell monolayers were then washed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4. Inhibition of cAMP production by DPDPE was assessed by the concurrent addition of iloprost (300 nM) and DPDPE (100 nM) for 10 min in 0.5 ml of fresh medium at 37 °C, containing the phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one (Ro 201724; 250 μM). The reaction was terminated by addition of 20 μl of trichloroacetic acid (100% v/v). Fifty microliter-aliqouts of each sample were removed and added to 50 μl of NaOH (1 M) and 200 μl

of TE buffer (50 mM Tris, pH 7.5, 4 mM EDTA). cAMP concentrations were determined using [^{3}H]cAMP in a protein binding assay exactly as described previously (Mundell et al., 1998). Protein content was determined by the method of Bradford (1976), and cAMP accumulation was expressed as picomoles per milligram protein. The percentage inhibition of iloprost-stimulated cAMP formation was calculated as follows: {[(cAMP in presence of iloprost) − (cAMP basal)] − [(cAMP in presence of iloprost + DPDPE) − (cAMP basal)]/(cAMP in presence of iloprost) − (cAMP basal)} × 100. For some experiments, receptor internalization was inhibited by the addition of either hypertonic sucrose (0.4 M) or the plant lectin concanavalin A (0.25 mg ml^{-1}) for 5 and 15 min, respectively, before and during pretreatment with DPDPE (100 nM; 5 min). Furthermore, in some experiments, zinc chloride (250 μM) was added to cells for 15 min before and during pretreatment with DPDPE (100 nM; 5 min).

2.5. Determination of δ-opioid receptor number

Cell membranes were prepared by homogenization in Tris (20 mM), EDTA (10 mM) buffer, pH 7.5. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatant was removed and centrifuged at 20,000 × *g* for 20 min at 4 °C to pellet the membrane fraction, which was then resuspended in HEPES (20 mM), $MgCl_2$ (1 mM) buffer, pH 7.5. Saturation binding studies were performed using the specific δ-opioid receptor antagonist [^{3}H]naltrindole. For determination of nonspecific binding, the opioid receptor antagonist naloxone (10 μM) was included. Increasing concentrations of [^{3}H]naltrindole (0.01–30 nM) were added to appropriate samples and incubated for 30 min at 37 °C. Bound and free ligand were separated via rapid filtration using a Brandell cell harvester. Binding was determined by liquid scintillation spectroscopy, and protein concentration of individual samples was determined by the method of Bradford (1976). Specific binding was determined as: [(total binding) − (nonspecific binding in the presence of 10 μM naloxone)]. The resulting data were fitted to a single site hyperbolic

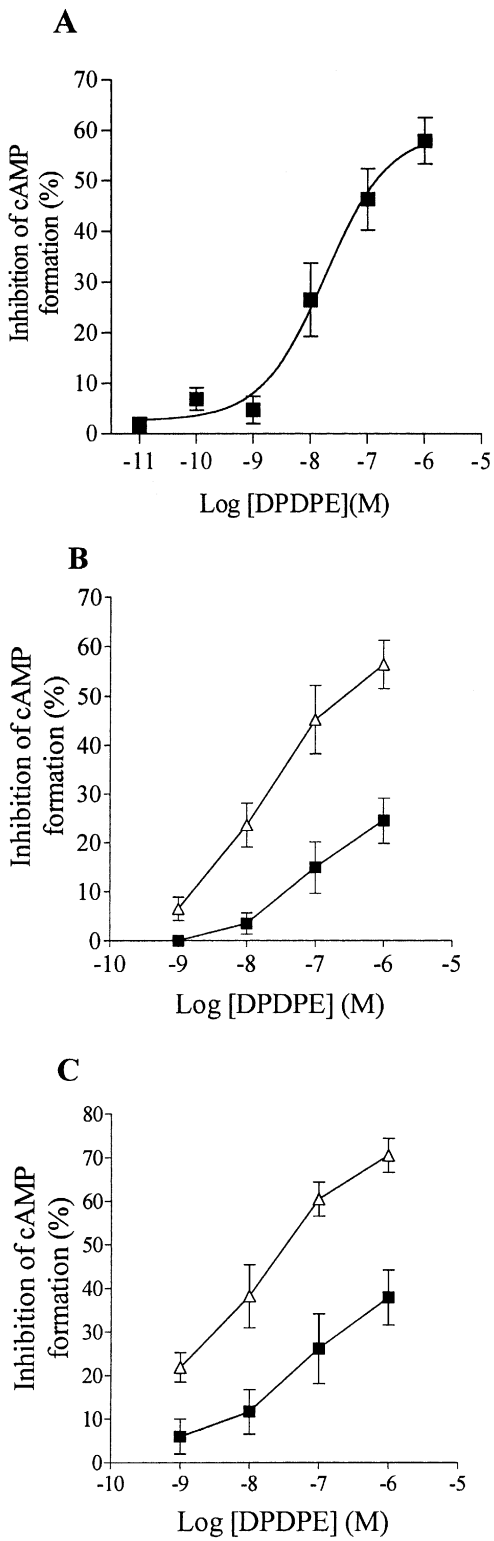

Fig. 1. DPDPE-mediated inhibition of cAMP accumulation, and desensitization of DPDPE-mediated inhibition of cAMP accumulation in NG108-15 cells. (A) Concentration-dependent inhibition of 300 nM iloprost-stimulated cAMP accumulation by DPDPE in wild-type NG108-15 cells. The EC_{50} for DPDPE-mediated inhibition of iloprost-stimulated cAMP formation from the fitted curve was 18 nM, and maximum inhibition was 60%. (B) Concentration-dependent inhibition of 300 nM iloprost-stimulated cAMP accumulation in cells pretreated with vehicle (△) or 100 nM DPDPE (■) for 5 min. Iloprost-stimulated cAMP accumulations (i.e. with basal accumulation subtracted) were 161 ± 49 and 152 ± 55 pmol cAMP mg^{-1} protein in control and 5 min DPDPE-pretreated cells, respectively. (C) Concentration-dependent inhibition of 300 nM iloprost-stimulated cAMP accumulation in cells pretreated with vehicle (△) or 100 nM DPDPE (■) for 15 min. Iloprost-stimulated cAMP accumulations (i.e. with basal accumulation subtracted) were 235 ± 27 and 264 ± 23 pmol cAMP mg^{-1} protein in control and 15 min DPDPE-pretreated cells, respectively. DPDPE-induced inhibition of iloprost-stimulated cAMP accumulation was calculated as described in Section 2. Values are means ± S.E.M., $n = 4$. The DPDPE-mediated inhibition of cAMP accumulation was significantly less in cells pretreated with DPDPE ($P < 0.05$; two-way analysis of variance).

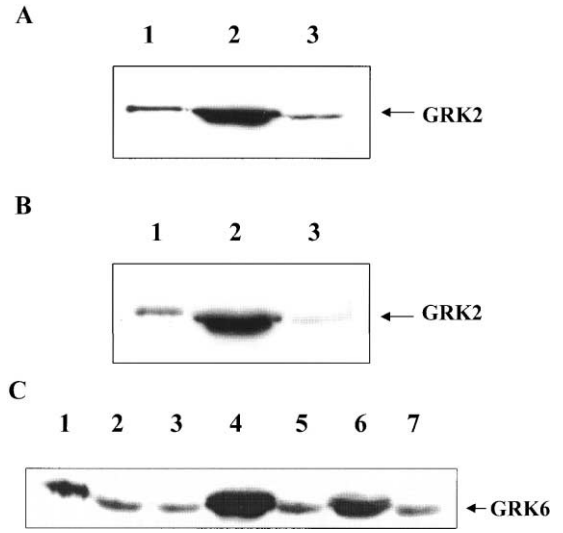

Fig. 2. Western blot analysis of GRKs in transformed NG108-15 cells. (A) Cells overexpressing wild-type GRK2. Whole cell lysates were subjected to SDS-PAGE followed by Western transfer and immunoblotting with a monoclonal antibody that recognises GRK2. Lane 1, 20 ng of purified GRK2; lane 2, 40 μg B7 lysate; lane 3, 40 μg P1 plasmid control lysate. (B) Cells expressing DNM GRK2. Whole cell lysates were subjected to SDS-PAGE followed by Western transfer and immunoblotting with a monoclonal antibody that recognises GRK2. Lane 1, 20 ng of purified GRK2; lane 2, 40 μg D19 lysate; lane 3, 40 μg P10 plasmid control lysate. (C) Cells overexpressing wild-type GRK6. Whole cell lysates were subjected to SDS-PAGE followed by Western transfer and immunoblotting with a polyclonal antibody that recognises GRK6. Lane 1, 20 ng of purified GRK6; lanes 2 and 3, 40 μg lysates from transformed cell lines that showed little or no GRK6 overexpression (not used further in this study); lane 4, 40 μg GRK6/7 lysate; lane 5, 40 μg P3 plasmid control lysate; lane 6, 40 μg GRK6/14 lysate; lane 7, 40 μg P2 plasmid control lysate.

function by nonlinear regression using Graphpad Prism, to allow determination of K_d and B_{max} values.

2.6. Data analysis

Data were analysed by two-way analysis of variance or Student's *t*-test, using the Graphpad Prism statistics programme. Results were considered significant if $P < 0.05$.

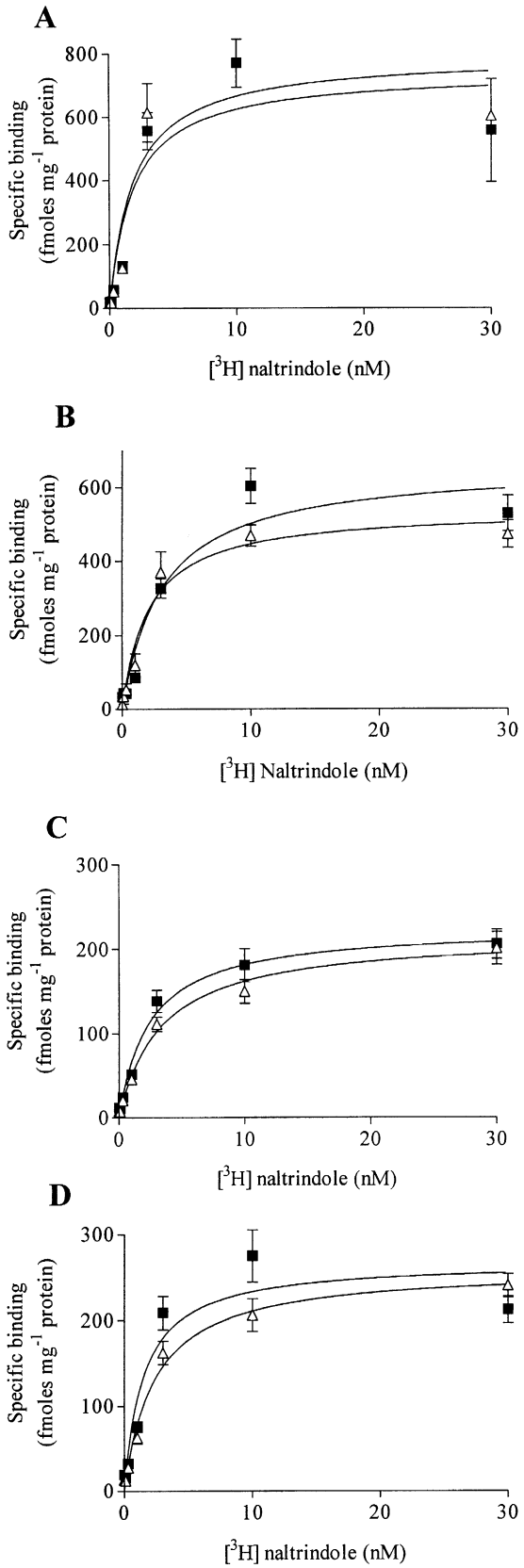

Fig. 3. Specific binding of [^{3}H]naltrindole to δ-opioid receptors in membranes prepared from transformed NG108-15 cells. (A) Plasmid control (P10, △) or DNM GRK2 cells (D19, ■); (B) plasmid control (P1, △) or wild-type GRK2-overexpressing cells (B7, ■); (C) plasmid control (P3, △) or GRK6-overexpressing cells (GRK6/7, ■); (D) plasmid control (P2, △) or GRK6-overexpressing cells (GRK6/14, ■). Nonspecific binding was determined in the presence of 10 μM naloxone. Values are means ± S.E.M., $n = 3$–4. B_{max} (fmol mg^{-1} protein) from fitted curves were: P10, 794 ± 63; D19, 754 ± 153; P1, 544 ± 37; B7, 659 ± 56; P3, 223 ± 25; GRK6/7, 230 ± 22; P2, 261 ± 14; GRK6/14, 269 ± 22. Although there were no significant differences in B_{max} values between experimental pairs, the B_{max} values for P10, D19, P1 and B7 were higher than those for P3, GRK6/7, P2 and GRK6/14 ($P < 0.05$). K_d values (nM) from fitted curves were: P10, 2.0 ± 0.2; D19, 1.9 ± 0.4; P1, 2.3 ± 0.6; B7, 3.0 ± 0.2; P3, 3.7 ± 0.9; GRK6/7, 2.7 ± 0.7; P2, 2.4 ± 0.1; GRK6/14, 1.9 ± 0.2.

3. Results

3.1. δ-Opioid receptor desensitization in WT NG108-15 cells

The δ-opioid receptor agonist DPDPE induced a concentration-dependent inhibition of iloprost (300 nM)-stimulated cAMP production in wild-type NG108-15 cells, with an IC_{50} of 18 nM (Fig. 1A). Maximal inhibition of approximately 60% of the iloprost (300 nM) response was achieved at around 1 μM DPDPE. Pretreatment of wild-type cells with 100 nM DPDPE for either 5 or 15 min produced a marked reduction in the subsequent ability of DPDPE to inhibit cAMP formation when compared to nonpretreated cells (Fig. 1B and C). For example, after 5 min pretreatment with 100 nM DPDPE, the subsequent ability of 1 μM DPDPE to inhibit cAMP accumulation was reduced from 40–50% to 10–20%. Furthermore, DPDPE pretreatment did not affect subsequent iloprost-stimulated cAMP accumulation; iloprost-stimulated cAMP accumulations were 235 ± 27 and 264 ± 23 pmol cAMP mg^{-1} protein in control and 15 min DPDPE-pretreated cells, respectively. Since DPDPE-induced desensitization appeared to be maximum after 5 min of pretreatment, all subsequent desensitization experiments were undertaken with a 5-min DPDPE pretreatment protocol.

3.2. Western blotting of NG108-15 cell lysates for GRKs, and analysis of [^{3}H]naltrindole binding to stably transfected cells

Previous Western blot analysis indicated that wild-type NG108-15 cells express GRK2, GRK3 and GRK6 (Mundell et al., 1997). In the present experiments, NG108-15 cells were employed which stably overexpress wild-type GRK2 (Mundell et al., 1998), DNM GRK2 (Mundell et al., 1997) or wild-type GRK6 (Fig. 2A–C). Compared to respective plasmid-transfected controls, B7 cells overexpress wild-type GRK2 by at least 20-fold (Fig. 2A), D19 cells express DNM GRK2 by at least 20-fold over endogenous wild-type GRK2 (Fig. 2B), and GRK6/7 and GRK6/14 overexpress GRK6 by at least 30- and 20-fold over endogenous wild-type GRK6, respectively (Fig. 2C).

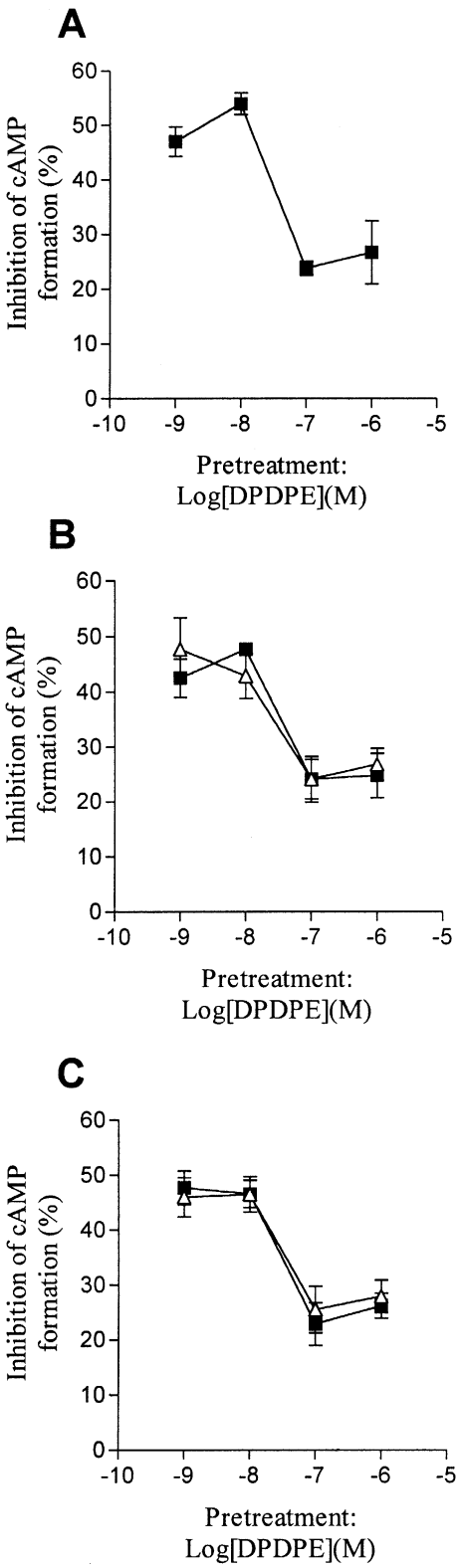

Fig. 4. Lack of effect of overexpression of wild-type GRK2 or DNM GRK2 on δ-opioid receptor desensitization. (A) Wild-type (nontransfected) NG108-15 cells were pretreated with increasing concentrations of DPDPE (10^{-9}–10^{-6} M). After 5 min, cells were washed and rechallenged with DPDPE (100 nM) in the presence of iloprost (300 nM) for 10 min. (B) Plasmid control (P1, △) and wild-type GRK2-overexpressing cells (B7, ■) were pretreated, washed and challenged as described in A. Iloprost-stimulated cAMP accumulations (i.e. with basal accumulation subtracted) were 271 ± 25 and 201 ± 9 pmol cAMP mg^{-1} protein in nonpretreated P1 and B7 cells, respectively. (C) Plasmid control (P10, △) and DNM GRK2 (D19, ■)-overexpressing cells were pretreated, washed and challenged exactly as described in A. Iloprost-stimulated cAMP accumulations (i.e. with basal accumulation subtracted) were 190 ± 25 and 167 ± 31 pmol cAMP mg^{-1} protein in nonpretreated P10 and D19 cells, respectively. DPDPE-induced inhibition of iloprost-stimulated cAMP accumulation was calculated as described in Section 2. Values are means ± S.E.M., $n = 4$. The DPDPE pretreatment-induced loss of subsequent DPDPE responsiveness was the same in B7 and D19 cells as compared to respective plasmid controls.

Saturation binding experiments with the δ-opioid receptor antagonist ligand [^{3}H]naltrindole were then undertaken to determine whether GRK manipulation alters δ-opioid receptor binding in NG108-15 cells. The specific binding of [^{3}H]naltrindole to δ-opioid receptors in membranes prepared from all the transformed NG108-15 cell lines used was of high affinity and saturable (Fig. 3A–D). As compared to respective plasmid-transfected controls, overexpression of wild-type GRK2, DNM GRK2 or wild-type GRK6 in NG108-15 cells did not alter the affinity or extent of [^{3}H]naltrindole binding to cell membranes. However, [^{3}H]naltrindole binding to membranes from GRK6-overexpressing cells and respective plasmid controls was less than that of membranes from P1, B7, P10 and D19 cells.

3.3. Effect of GRK2 or DNM GRK2 overexpression on δ-opioid receptor desensitization

In wild-type NG108-15 cells, pretreatment with DPDPE for 5 min concentration dependently induced desensitization to subsequent challenge with the δ-opioid agonist, the EC_{50} for desensitization lying between 10 and 100 nM DPDPE (Fig. 4A). Desensitization of the DPDPE-mediated inhibition of cAMP formation reached approximately 50% after pretreatment with 0.1 and 1.0 μM DPDPE. To determine whether GRK2 plays a role in δ-opioid receptor desensitization, we examined the effects of overexpression of wild-type GRK2 or DNM GRK2 on DPDPE-induced desensitization. However, neither an increase nor a de-

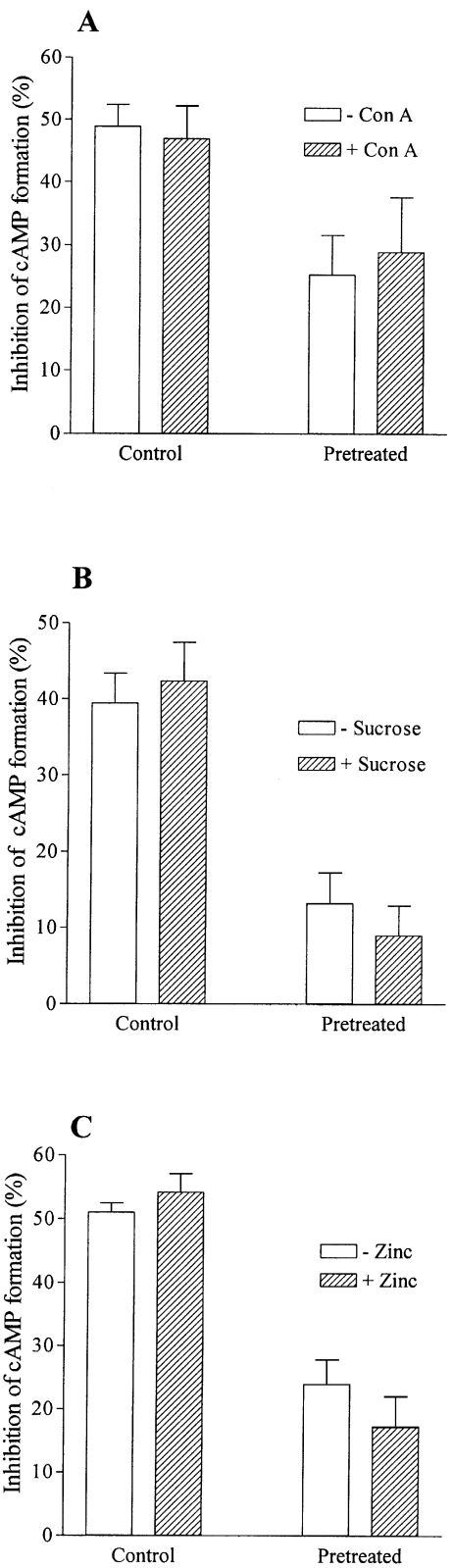

Fig. 5. Lack of effect of inhibitors of receptor internalization (A,B), and zinc (C), on δ-opioid receptor desensitization in wild-type NG108-15 cells. (A) Cells were preincubated with either vehicle or concanavalin A (0.25 mg ml^{-1}) for 15 min. After this time, cells were exposed to either vehicle (control) or DPDPE (100 nM, pretreated) for a further 5 min in the continued presence or absence of concanavalin A. Cells were then washed and rechallenged with DPDPE (100 nM) in the presence of iloprost (300 nM) for 10 min. Iloprost-stimulated cAMP accumulations (i.e. with basal accumulation subtracted) were 144 ± 33 and 138 ± 24 pmol cAMP mg^{-1} protein in cells not pretreated or pretreated with concanavalin A, respectively. (B) Cells were preincubated with either vehicle or sucrose (0.4 M) for 15 min. After this time, cells were exposed to either vehicle (control) or DPDPE (100 nM, pretreated) for a further 5 min in the continued presence or absence of sucrose. Cells were then washed and rechallenged with DPDPE (100 nM) in the presence of iloprost (300 nM) for 10 min. Iloprost-stimulated cAMP accumulations (i.e. with basal accumulation subtracted) were 160 ± 16 and 87 ± 33 pmol cAMP mg^{-1} protein in cells not pretreated or pretreated with sucrose, respectively. (C) Cells were preincubated with either vehicle or zinc (250 μM) for 15 min. After this time, cells were exposed to either vehicle (control) or DPDPE (100 nM, pretreated) for a further 5 min in the continued presence or absence of zinc. Cells were then washed and rechallenged with DPDPE (100 nM) in the presence of iloprost (300 nM) for 10 min. Iloprost-stimulated cAMP accumulations (i.e. with basal accumulation subtracted) were 275 ± 53 and 313 ± 57 pmol cAMP mg^{-1} protein in cells not pretreated or pretreated with zinc, respectively. DPDPE-induced inhibition of iloprost-stimulated cAMP accumulation was calculated as described in Section 2. Values are means ± S.E.M, $n = 3$–5. The DPDPE pretreatment-mediated reduction in inhibition of cAMP formation was not different for concanavalin A-, sucrose-, or zinc-treated cells as compared to respective controls.

crease in GRK2 activity affected DPDPE-mediated desensitization of δ-opioid inhibition of cAMP formation (Fig. 4B and C).

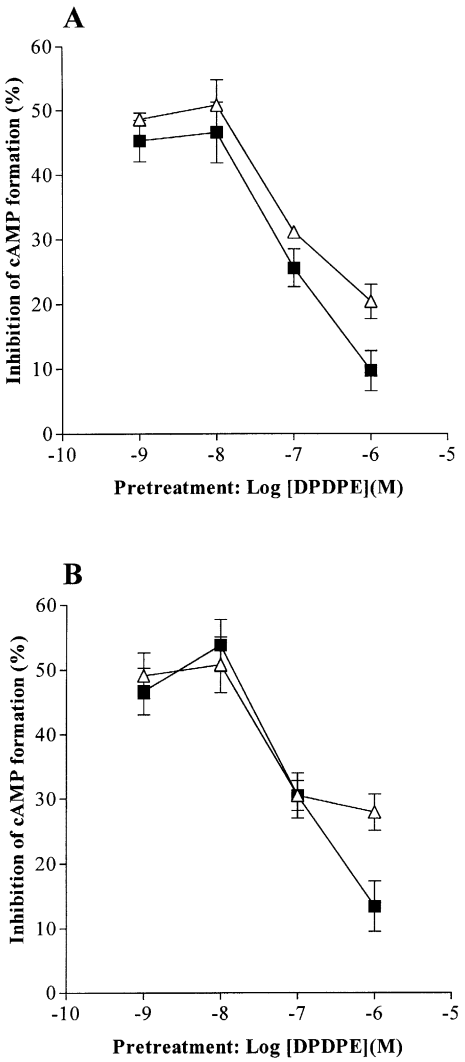

Fig. 6. GRK6 overexpression increases δ-opioid receptor desensitization in NG108-15 cells. (A) Plasmid control (P3, △) or GRK6-overexpressing cells (GRK6/7, ■) were pretreated with increasing concentrations of DPDPE (10^{-9}–10^{-6} M). After 5 min, cells were washed and rechallenged with DPDPE (100 nM) in the presence of iloprost (300 nM) for 10 min. Iloprost-stimulated cAMP accumulations (i.e. with basal accumulation subtracted) were 220 ± 19 and 199 ± 28 pmol cAMP mg^{-1} protein in nonpretreated P3 and GRK6/7 cells, respectively. (B) Plasmid control (P2, △) and GRK6 (GRK6/14, ■)-overexpressing cells were pretreated, washed and rechallenged exactly as described in part A. Iloprost-stimulated cAMP accumulations (i.e. with basal accumulation subtracted) were 172 ± 25 and 213 ± 27 pmol cAMP mg^{-1} protein in nonpretreated P2 and GRK6/14 cells, respectively. DPDPE-induced inhibition of iloprost-stimulated cAMP accumulation was calculated as described in Section 2. Values are means ± S.E.M., $n = 4$–5. The maximal desensitization induced by DPDPE pretreatment was greater in GRK6-overexpressing cells as compared to plasmid-transfected controls. From individual fitted curves for each experiment, maximal inhibition of cAMP formation in P3 cells was 20.4 ± 2.7% and in GRK6/7 cells 7.4 ± 2.9% ($P < 0.05$). Similarly, maximal inhibition of cAMP formation in P2 cells was 23.9 ± 2.6% and in GRK6/14 cells 12.0 ± 2.2% ($P < 0.05$).

3.4. Effect of inhibitors of receptor internalization, and zinc, on δ-opioid receptor desensitization

To determine a possible role for receptor internalization in δ-opioid receptor desensitization, NG108-15 cells were pretreated with either concanavalin A (0.25 mg ml^{-1}) or hypertonic sucrose (0.4 M). However, these pretreatments did not reverse DPDPE-mediated desensitization (Fig. 5A and B). Zinc has been reported to inhibit GRK2 activity (Benovic et al., 1987), and so in the present study, we employed it to further investigate a possible role for GRK2 in δ-opioid receptor desensitization. However, zinc (250 μM) also did not reverse DPDPE-induced desensitization (Fig. 5C).

3.5. Effects of GRK6 overexpression on δ-opioid receptor desensitization

Finally, to examine the potential role of GRK6 in δ-opioid receptor desensitization in NG108-15 cells, plasmid (P3 and P2) or GRK6-overexpressing cells (GRK6/7 and GRK6/14; see Fig. 2C) were pretreated for 5 min with DPDPE (1 nM–1 μM). When subsequently rechallenged with 100 nM DPDPE, the maximal desensitization of the DPDPE response was greater in GRK6-overexpressing cells as compared to plasmid-transfected controls (Fig. 6).

4. Discussion

Previous studies have implicated a role for phosphorylation in the agonist-induced desensitization of δ-opioid receptors (reviewed in Beaumont and Henderson, 1999; Law et al., 2000b). In NG108-15 cells also, phosphorylation appears to underlie rapid desensitization of the δ-opioid receptor. For example, the desensitization of δ-opioid receptor-mediated Ca^{2+} channel inhibition in NG108-15 cells is reversed by coapplication of the GRK blocker heparin, but not by manipulation of protein kinase A and protein kinase C activity (Morikawa et al., 1998). In addition, the desensitization of δ-opioid receptor-mediated inhibition of cAMP formation in NG108-15 cells is reversed by the nonselective protein kinase inhibitor staurosporine (Cai et al., 1996). Furthermore, the phosphorylation and desensitization of δ-opioid receptors expressed in human embryonic kidney 293 (HEK293) cells is enhanced or inhibited by coexpression of GRK2 or DNM GRK2, respectively (Pei et al., 1995). Together these findings suggest that GRK2 mediates the desensitization of endogenous δ-opioid receptors, particularly since NG108-15 cells express substantial quantities of this kinase (Mundell et al., 1997). In this study therefore, we employed a molecular biological approach to determine whether or not GRK2 is responsible for mediating the rapid desensitization of endogenous δ-opioid receptors in NG108-15 cells.

We have previously shown that the desensitization of adenosine A_{2A} and A_{2B} receptor responsiveness in NG108-15 cells is inhibited or enhanced by overexpression of DNM GRK2 or wild-type GRK2, respectively (Mundell et al., 1997, 1998). However, using the same stably transformed cell lines, we were unable to detect any effect of GRK2 manipulation on δ-opioid receptor-mediated desensitization. Whereas we previously found that GRK2 overexpression greatly enhanced the sensitivity of the adenosine A_{2A} receptor response to agonist-induced desensitization, in the present study, there was no change in the agonist concentration dependency of δ-opioid receptor-mediated desensitization, even though Western blotting confirmed that the cells markedly overexpress GRK2. Furthermore, we found that zinc, an inhibitor of GRK2 activity (Benovic et al., 1987) that inhibits adenosine A_2 receptor desensitization in NG108-15 cells (Mundell and Kelly, 1998), also did not affect δ-opioid receptor-mediated desensitization. This suggests that GRK2 is not critical for rapid agonist-induced δ-opioid receptor desensitization in NG108-15 cells. Indeed, the δ-opioid receptor can undergo significant desensitization in the absence of detectable agonist-induced receptor phosphorylation (Zhao et al., 1997; Wang et al., 1998; Law et al., 2000a; Maestri-El Kouhen et al., 2000), indicating that multiple mechanisms may contribute to δ-opioid receptor desensitization. Interestingly, whereas we reported that adenosine A_2 receptors in NG108-15 cells are regulated by GRK2, we have found the agonist-induced desensitization of other endogenous GPCRs in this cell line, such as the somatostatin (Beaumont et al., 1998), secretin and prostanoid IP receptor (Mundell et al., 1997, 1998), and now the δ-opioid receptor, remains unaltered with changes in GRK2 activity. Thus, there appears to be substantial selectivity of GRK2 for endogenously expressed GPCR substrates, the molecular basis of which is at present unknown.

For some GPCRs, agonist-induced internalization appears to represent a mechanism of desensitization (Lohse et al., 1990). Indeed, we have recently shown that the desensitization of endogenous somatostatin and muscarinic M_4 acetylcholine receptor responses in NG108-15 cells is blocked by hypertonic sucrose, which inhibits clathrin-mediated internalization (Beaumont et al., 1998; Holroyd et al., 1999). To test whether a similar mechanism contributes to δ-opioid receptor desensitization, wild-type NG108-15 cells were pretreated with either the plant lectin concanavalin A, or hypertonic sucrose, both treatments being known to block receptor internalization (Lohse et al., 1990; Holroyd et al., 1999). However, these treatments did not reverse δ-opioid receptor desensitization, suggesting that internalization is not involved in rapid desensitization of this receptor response. Recent studies have shown that internalization of heterologously expressed δ-opioid receptors can contribute to agonist-induced desensitization, but our results indicate this is not the case for endogenously expressed δ-opioid receptors, at least in NG108-15 cells. Indeed, in human SK-N-BE neuroblastoma cells, treatment with concanavalin A or hypertonic sucrose blocked the resensitization of endogenous δ-opioid receptor responses in this cell type (Hasbi et al., 2000), indicating the likelihood that, as with many GPCRs, internalization represents mainly a mechanism whereby δ-opioid receptors become dephosphorylated and resensitized before recycling to the plasma membrane in a resensitized state (Krueger et al., 1997).

The results of the present study leave open the role of phosphorylation in the agonist-induced desensitization of δ-opioid receptor responsiveness in NG108-15 cells. However, DNM GRK2 has been shown to reduce the agonist-induced phosphorylation and desensitization of δ-opioid receptors expressed in HEK293 cells (Pei et al., 1995). Furthermore, in a recent study, mutation of S^{363} in the C-terminus of the δ-opioid receptor markedly reduced agonist-induced phosphorylation and desensitization of the receptor (Law et al., 2000a; Maestri-El Kouhen et al., 2000). Apart from GRK2, NG108-15 cells express other GRKs such as GRK3 and GRK6 (Mundell et al., 1997; Willets et al., 1999). We therefore attempted to overexpress these two kinases in NG108-15 cells to ascertain their potential role in δ-opioid receptor desensitization. For reasons that are at present unclear, we were unable to obtain stably transfected NG108-15 cell clones that significantly overexpressed GRK3. Instead therefore, we investigated δ-opioid receptor desensitization in NG108-15 cells that stably overexpressed GRK6. In these cells, there was an increase in maximal δ-opioid receptor desensitization as compared to plasmid-transfected controls, implicating GRK6 in this process. Indeed it is possible that endogenous GRK6 mediates the δ-opioid receptor desensitization observed in nontransfected NG108-15 cells, although further studies with DNM GRK6 or GRK6 antisense constructs would be needed to substantiate this. Interestingly, a very recent study (Aiyar et al., 2000) reports that GRK6 antisense expression inhibits the agonist-induced desensitization of CGRP receptor responsiveness, whereas GRK2 antisense expression does not. These results lend further support to the idea that there is marked selectivity of GRKs for different GPCRs. For example in our own studies in NG108-15 cells, GRK6 appears to regulate secretin but not adenosine A_2 receptor responsiveness in these cells (Willets et al., 2000), and the present results also implicate GRK6 in δ-opioid receptor desensitization. Finally, it is possible that the increased δ-opioid receptor desensitization could be due to a GRK6-induced alteration in δ-opioid receptor number. However, [^{3}H]naltrindole binding to NG108-15 cell membranes indicated no difference in δ-opioid receptor expression between matched clonal pairs. On the other hand, some of the clones examined had significantly higher B_{max} values than others. The reasons for this are at present unclear, but may relate to the fact that the P10, D19, P1 and B7 clones used in this study had undergone many more passages than P3, GRK6/7, P2

and GRK6/14 clones. Thus, changes in δ-opioid receptor expression due to passage number could have occurred. Nonetheless, this does not detract from the conclusion that GRK2 or GRK6 manipulation does not alter δ-opioid receptor expression when compared to matched plasmid controls.

In conclusion, we find no evidence to implicate GRK2 or receptor internalization in the agonist-induced desensitization of endogenous δ-opioid receptor responsiveness in NG108-15 cells. Instead, another receptor kinase such as GRK6 may be involved, and warrants further investigation.

Acknowledgements

This work was supported by a project grant from the Medical Research Council.

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