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The effect of inhibitors of receptor internalization on the desensitization and resensitization of three G_s-coupled receptor responses

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1 Many G protein-coupled receptors (GPCRs) are known to internalize following agonist exposure, however the relative importance of this mechanism for the desensitization and resensitization of different GPCRs is unclear. In the present study, we have pretreated NG108-15 cells with hypertonic sucrose or concanavalin A (con A), to investigate the effects of these inhibitors of internalization on the agonist-induced desensitization and subsequent resensitization of three G_s-coupled receptor responses.

2 Incubation of cells with sucrose or con A did not affect subsequent acute agonist stimulation of the A_{2A} adenosine receptor or the agonist-induced desensitization of this receptor response. However, the resensitization of the A_{2A} adenosine receptor response following agonist removal was abolished in the presence of sucrose or con A.

3 Sucrose or con A treatment affected neither the desensitization nor resensitization of IP-prostanoid receptor responsiveness. On the other hand con A but not sucrose reduced the agonist-induced desensitization of secretin receptor responsiveness. However, secretin receptor responsiveness did not resensitize within the time period studied whether or not inhibitors of internalization were present.

4 These results indicate that receptor internalization appears to subserve different functions for different GPCRs.

Keywords: G protein-coupled receptor; internalization; desensitization; resensitization; sucrose; concanavalin A; NG108-15 cells

Introduction

There is now compelling evidence to indicate that many G protein-coupled receptors (GPCRs) rapidly internalize following agonist addition (reviewed in Koenig & Edwardson, 1997). In a number of cases the agonist-activated receptors cluster in clathrin-coated pits before being internalized to early endosomes (von Zastrow & Kobilka, 1992; Goodman *et al*., 1996), although there is evidence that some GPCRs may internalize *via* non-clathrin pathways (Zhang *et al*., 1996). Early studies on the β_2-adrenoceptor suggested that agonist-induced internalization may contribute to desensitization (Cheung *et al*., 1989), but it is now clear that in most cases internalization is not involved in rapid desensitization of this receptor. Rather, experiments using inhibitors of internalization indicate that resensitization of β_2-adrenoceptor responsiveness does not occur without internalization (Yu *et al*., 1993; Pippig *et al*., 1995; Zhang *et al*., 1997).

The agonist-induced desensitization of a number of other GPCRs also seems to occur independently of internalization (Ng *et al*., 1995; Garland *et al*., 1996; Szekeres *et al*., 1998). However, for some GPCRs, the relationship between desensitization, internalization and resensitization may not be so straightforward. For example, although inhibition of internalization does not affect desensitization of the m4 muscarinic receptor, it actually speeds up resensitization (Bogatkewitsch *et al*., 1996). Furthermore, rapid receptor internalization may be partly responsible for the agonist-induced desensitization of secretin (Holtmann *et al*., 1996) and somatostatin receptor responses (Beaumont *et al*., 1997). It is therefore questionable that the phenomenon of internalization subserves the same function with all GPCRs. In the present study we have investigated this further by determining the effects of two inhibitors of internalization, hypertonic sucrose and concanavalin A (con A), on the agonist-induced desensitization and subsequent resensitization of A_{2A} adenosine, IP-prostanoid and secretin receptor responses. These three GPCRs are natively expressed in NG108-15 cells and couple *via* G_s to adenylyl cyclase activation (Williams & Kelly, 1994; Mundell *et al*., 1997; Mundell & Kelly, 1998). The results indicate that inhibitors of internalization have dramatically different effects on the desensitization and resensitization of these three G_s-coupled receptors.

Methods

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, 100 units ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin, and supplemented with 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. For culture of NG108-15 cells stably transfected with pMEP4 vector or pMEP4 containing the coding sequence of bovine GRK2 (Benovic *et al*., 1986), the above medium was supplemented with 100 $\mu g\ ml^{-1}$ hygromycin (Mundell *et al*., 1998). For culture of NG108-15 cells stably transfected with pCMVneo vector or pCMVneo containing the dominant negative mutant form of GRK2 (Kong *et al*., 1994), the above medium was supplemented with 200 $\mu g\ ml^{-1}$ geneticin (Mundell *et al*., 1997).

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Cell treatments

Cells were grown as monolayers in 25 cm^2 culture flasks, with sucrose, con A and receptor agonists being added directly to the culture medium. For each experiment, a number of flasks were used so that cells could be harvested before desensitization, following the desensitization period, and during the resensitization period. Following the desensitization period, medium was removed and the cell monolayer given 3×2 ml rapid washes with warm culture medium $\pm$ sucrose or con A. For resensitization, 2 ml of fresh medium $\pm$ sucrose or con A was added to the cells.

Adenylyl cyclase assay

Cells were harvested in 10 ml of ice-cold PBS and pelleted by centrifugation at $200 \times g$ for 1 min. The resulting pellets were washed twice in 10 ml of ice-cold PBS and frozen at $-70°C$ until required. Adenylyl cyclase activity was measured by a protein binding assay (Brown *et al*., 1971). Cell pellets were thawed and homogenised in a glass dounce homogeniser containing ice-cold homogenization buffer (0.3 M sucrose, 25 mM Tris, pH 7.4). A 40 μl sample of homogenate was then added to 30 μl premix buffer (final assay concentration 50 mM Tris, pH 7.5, 5 mM Mg^{2+}, 1 mM ATP, 1 μM GTP, 250 μM 4-(3-Butoxy-4-methoxybenzyl) imidazolidin-2-one (Ro201724) as phosphodiesterase inhibitor, 20 mM creatine phosphate and 130 u ml^{-1} creatine phosphokinase) and 30 μl of drug at the relevant concentration. The tubes were incubated at 37°C for 10 min and the reaction terminated by the addition of 20 μl of 100% trichloroacetic acid and the tubes placed on ice for 10 min. Precipitated protein was pelleted by centrifugation at $2{,}900 \times g$ for 20 min at 4°C and 50 μl of the resulting supernatant added to 50 μl of 1 M NaOH and 200 μl of 50 mM Tris, pH 7.4, 4 mM EDTA (TE buffer); 50 μl of this solution was then added to fresh tubes containing 100 μl TE buffer, 100 μl [^{3}H]-cyclic AMP in TE buffer (about 20,000 c.p.m.) and 100 μl of binding protein in TE buffer (to give final concentration of $\sim$750 μg protein ml^{-1}; prepared from bovine adrenal cortex). Tubes containing 100 μl of standard concentrations of cyclic AMP (0.125–20 pmol) were used to construct a standard curve. After 2 h incubation at 4°C, 200 μl of TE buffer containing charcoal (Norit GSX; 50 mg ml^{-1} final concentration) and bovine serum albumin (2 mg ml^{-1} final concentration) were added and 15 min later the tubes were centrifuged at $2{,}900 \times g$ for 20 min at 4°C. The resulting supernatant was transferred into vials for liquid scintillation counting. Standard curve data were fitted to a logistic expression (GraphPad Prism; GraphPad Software) and the unknowns read off. Protein content of homogenates was determined (Bradford, 1976) and adenylyl cyclase activity expressed as pmol cyclic AMP min^{-1} mg^{-1} protein.

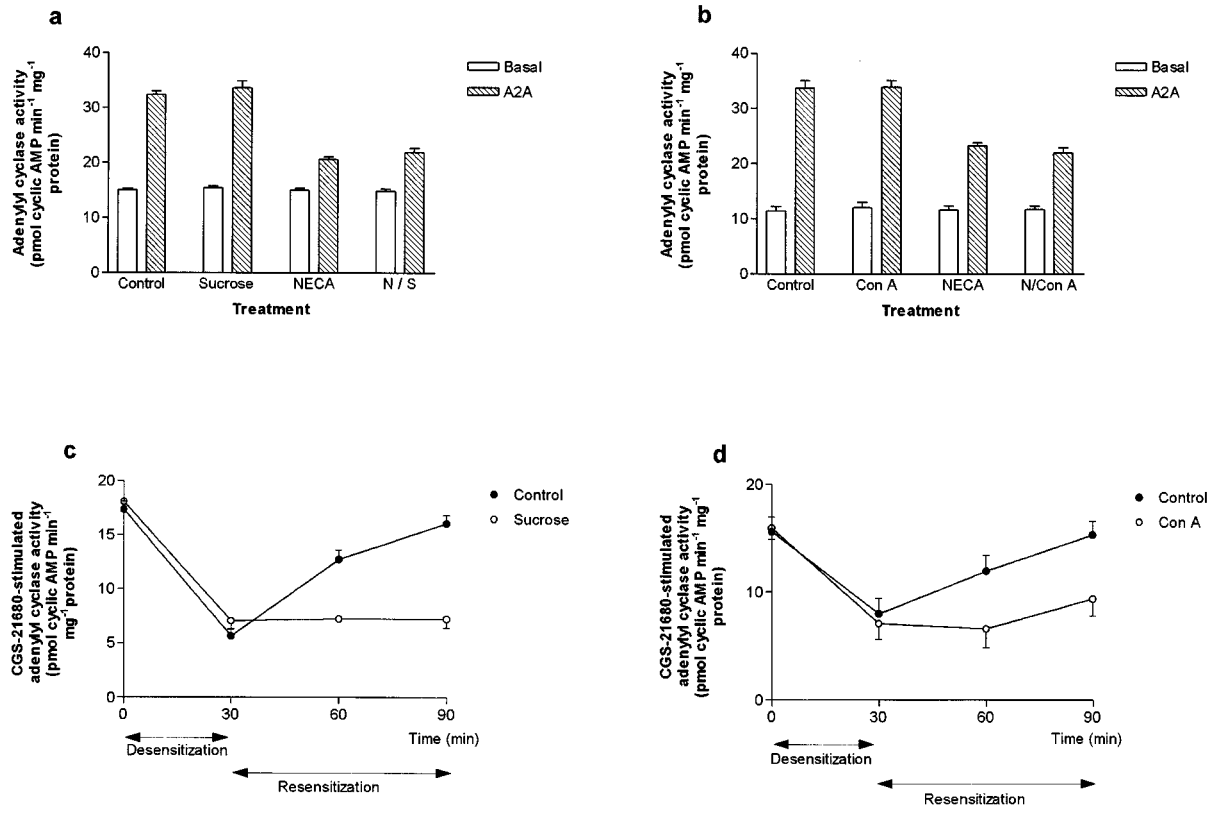

Figure 1 Effect of sucrose (a and c) or con A (b and d) on the agonist-induced desensitization and resensitization of A_{2A} adenosine receptor responsiveness. a and b: Cells were pretreated with or without sucrose (0.4 M; a) or con A (0.25 mg ml^{-1}; b) for 15 min. At this time point some cells were harvested (control and sucrose in a and control and con A in b) whilst the remaining cells were incubated for a further 30 min in the presence of NECA (10 μM) and in the continued presence (N/S) or absence (NECA) of sucrose or in the continued presence (N/con A) or absence (NECA) of con A before being harvested. Results show either basal adenylyl cyclase activity or that in the presence of 3 μM CGS-21680. Values are means $\pm$ s.e.mean for four separate experiments. c and d: Cells were pretreated with or without sucrose or con A for 15 min. At this time point (0), NECA (10 μM) was added for 30 min in the continued presence or absence of sucrose or con A. Following this time, cells were washed and reincubated with fresh medium for a further 60 min, again in the continued presence or absence of sucrose or con A. Cells were harvested at time points 0, 30, 60 and 90 min. Results in c and d show 3 μM CGS-21680-stimulated adenylyl cyclase activity (basal activity subtracted), and are values $\pm$ s.e.mean from four separate experiments. During the resensitization period, A_{2A} adenosine receptor-stimulated adenylyl cyclase activity in the presence of sucrose or con A was significantly less than in the absence of these agents ($P < 0.05$, ANOVA).

Statistics

Where appropriate, statistical significance of different values was assessed by Student's *t*-test or by 2-way ANOVA using the GraphPad InStat or Prism programmes.

Materials

[8-^{3}H]-cyclic AMP (925 GBq mmol^{-1}) was obtained from Amersham International plc. Cell growth medium and geneticin were from GIBCO BRL, hygromycin from Boehringer Mannheim Ltd., and 2-(*p*-carboxyethyl)phenylamino-5′-N-carboxamidoadenosine (CGS-21680) from Research Biochemicals International. Iloprost was a generous gift from Schering AG (Berlin) and the protein assay reagent was from Pierce and Warriner. All other reagents and drugs were obtained from the Sigma Chemical Co.

Results

Inhibitors of internalization and A_{2A} adenosine receptor function

Pretreatment of NG108-15 cell monolayers with hypertonic sucrose (0.4 M; 15 min) or con A (0.25 mg ml^{-1}; 15 min) did not affect basal adenylyl cyclase activity or the ability of the A_{2A} adenosine receptor agonist CGS-21680 (3 μM) to activate adenylyl cyclase in cell homogenates (Figure 1a,b). Although NG108-15 cells express both A_{2A} and A_{2B} adenosine receptors, this concentration of agonist selectively activates A_{2A} adenosine receptors (Mundell & Kelly, 1998). Furthermore, inclusion of sucrose or con A in the culture medium before and during agonist pretreatment (NECA; 10 μM for 30 min), did not affect the degree of A_{2A} adenosine receptor desensitization achieved (around 60%; Figure 1). Following removal of agonist, A_{2A} adenosine receptor responsiveness resensitized to control levels within an hour. However, inclusion of either sucrose or con A during the resensitization period completely blocked the recovery of A_{2A} adenosine receptor responsiveness (Figure 1c,d).

GRK2 manipulation and resensitization of A_{2A} adenosine receptor responsiveness

Resensitization of A_{2A} adenosine receptor responsiveness was also investigated in NG108-15 cells overexpressing wild type GRK2 (Mundell *et al*., 1998) or a dominant negative mutant form of GRK2 (Mundell *et al*., 1997). The former (B7 cells) express wild type GRK2 at levels 20–30 fold over non-transfected or plasmid-transfected control cells (P1), whereas in the latter cells (D28 cells), A_{2A} adenosine receptor desensitization is inhibited by overexpression of mutant GRK2. In both B7 and P1 cells, NECA pretreatment (10 μM; 30 min) produced marked desensitization of subsequent 3 μM CGS-21680-stimulated adenylyl cyclase activity, the degree of desensitization being slightly greater in B7 than P1 cells (Figure 2a). However, the rate of resensitization of A_{2A} adenosine receptor responsiveness was the same in B7 and P1 cells. Furthermore, and similar to non-transfected NG108-15 cells, hypertonic sucrose completely blocked the resensitization of A_{2A} adenosine receptor responsiveness in B7 cells (compare Figure 1c with Figure 2b). Finally, resensitization was examined in cells overexpressing dominant negative mutant GRK2 (D28) and plasmid-transfected controls (P7). It is difficult to obtain the same level of desensitization in the two clones, since A_{2A} adenosine receptor desensitization is reduced in D28 cells. In an attempt to circumvent this, D28 and P7 cells were pretreated with a high agonist concentration for a prolonged period (100 μM NECA for 1 h). Even under

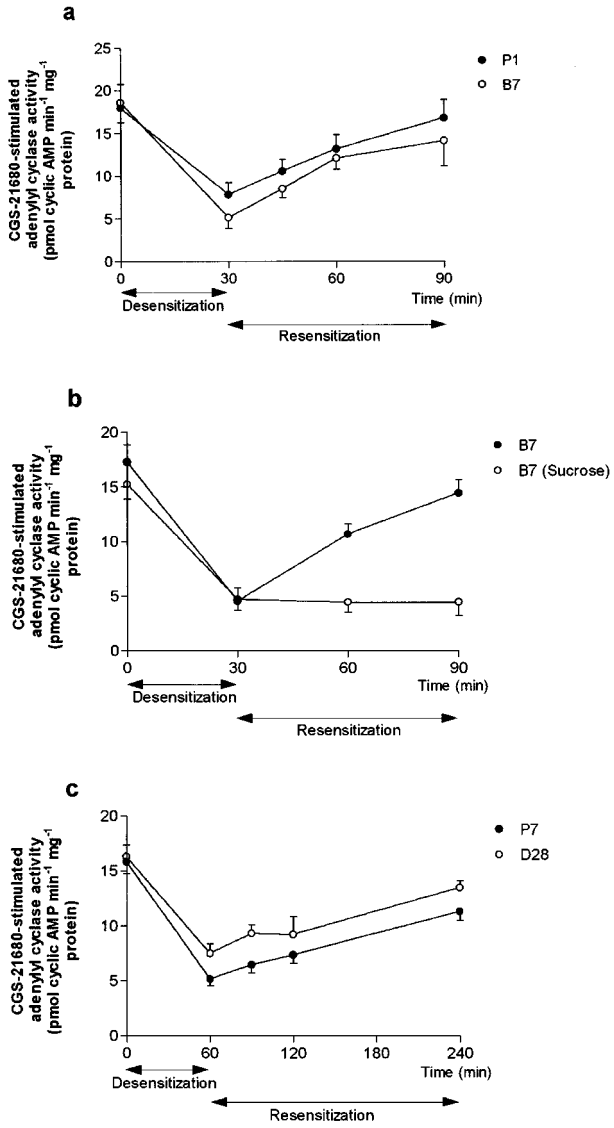

Figure 2 Desensitization and resensitization of A_{2A} adenosine receptor responsiveness in NG108-15 cells overexpressing wild type GRK2 or dominant negative mutant GRK2. (a) GRK2 overexpressing (B7) or plasmid-transfected cells (P1) were pretreated with NECA (10 μM) for 30 min. Following this time, cells were washed and reincubated with fresh medium for a further 60 min. Cells were harvested at time points, 0, 30, 45, 60 and 90 min. Shown is 3 μM CGS-21680-stimulated adenylyl cyclase activity (basal activity subtracted), and values are means±s.e.mean from four separate experiments. (b) GRK2 overexpressing (B7) cells were pretreated with or without sucrose for 15 min. At this time point (0), NECA (10 μM) was added for 30 min in the continued presence or absence of sucrose. Following this time, cells were washed and reincubated with fresh medium for a further 60 min, again in the continued presence or absence of sucrose. Cells were harvested at time points 0, 30, 60 and 90 min. Shown is 3 μM CGS-21680-stimulated adenylyl cyclase activity (basal activity subtracted), and values are means±s.e.mean from four separate experiments. (c) Dominant negative mutant GRK2 overexpressing (D28) or plasmid-transfected cells (P7) were pretreated with NECA (100 μM) for 60 min. Following this time, cells were washed and reincubated with fresh medium for a further 180 min. Cells were harvested at time points 0, 60, 90, 120 and 240 min. Shown is 3 μM CGS-21680-stimulated adenylyl cyclase activity (basal activity subtracted), and values are means±s.e.mean from four separate experiments.

these conditions, desensitization is somewhat less in D28 cells (Figure 2c). However, P7 and D28 cells still appear to resensitize at similar rates, although somewhat slower than in other experiments, probably due to the longer period of desensitization.

Inhibitors of internalization and IP-prostanoid receptor function

Pretreatment of NG108-15 cells with hypertonic sucrose (0.4 M; 15 min) or con A (0.25 mg ml^{-1}; 15 min) did not affect the ability of the IP-prostanoid receptor agonist iloprost (1 μM) to activate adenylyl cyclase in cell homogenates (Figure 3a,b). Furthermore, inclusion of sucrose or con A in the culture medium before and during agonist pretreatment (iloprost; 1 μM for 30 min), did not affect the degree of IP-prostanoid receptor desensitization achieved (around 75%; Figure 3). Following removal of agonist, IP-prostanoid receptor responsiveness resensitized to near control levels within 2 h; inclusion of either sucrose or con A during the resensitization period had no effect on the recovery of IP-prostanoid receptor responsiveness (Figure 3c, d).

Inhibitors of internalization and secretin receptor function

Pretreatment of NG108-15 cells with hypertonic sucrose (0.4 M; 15 min) or con A (0.25 mg ml^{-1}; 15 min) did not affect the ability of secretin (0.1 μM) to acutely activate adenylyl cyclase in cell homogenates (Figure 4a,b). Inclusion of sucrose in the incubation medium before and during agonist pretreatment (secretin; 0.1 μM for 5 min) did not significantly affect the degree of secretin receptor desensitization achieved (around 80%; Figure 4a), although a small reversal was evident. However, inclusion of con A in the incubation medium before and during secretin pretreatment markedly reversed secretin receptor desensitization (Figure 4b). Following removal of agonist, and unlike the other two receptor responses studied, secretin receptor responsiveness failed to resensitize during the incubation period (1 h). Furthermore, inclusion of neither sucrose nor con A during the resensitization period affected the continuing desensitization of secretin receptor responsiveness (Figure 4c,d).

Discussion

The β_2-adrenoceptor has been widely employed as a prototype receptor to investigate GPCR function (Hausdorff *et al*., 1990). This receptor undergoes rapid agonist-induced desensitization due to phosphorylation by protein kinase A and GRKs (reviewed in Ferguson *et al*., 1996). Phosphorylation by GRKs causes arrestins to bind to the phosphorylated receptor, thus uncoupling receptor and G-protein (Lohse *et al*., 1990). Agonist-induced internalization of the β_2-adrenoceptor follows phosphorylation, and is due in part to the interaction between

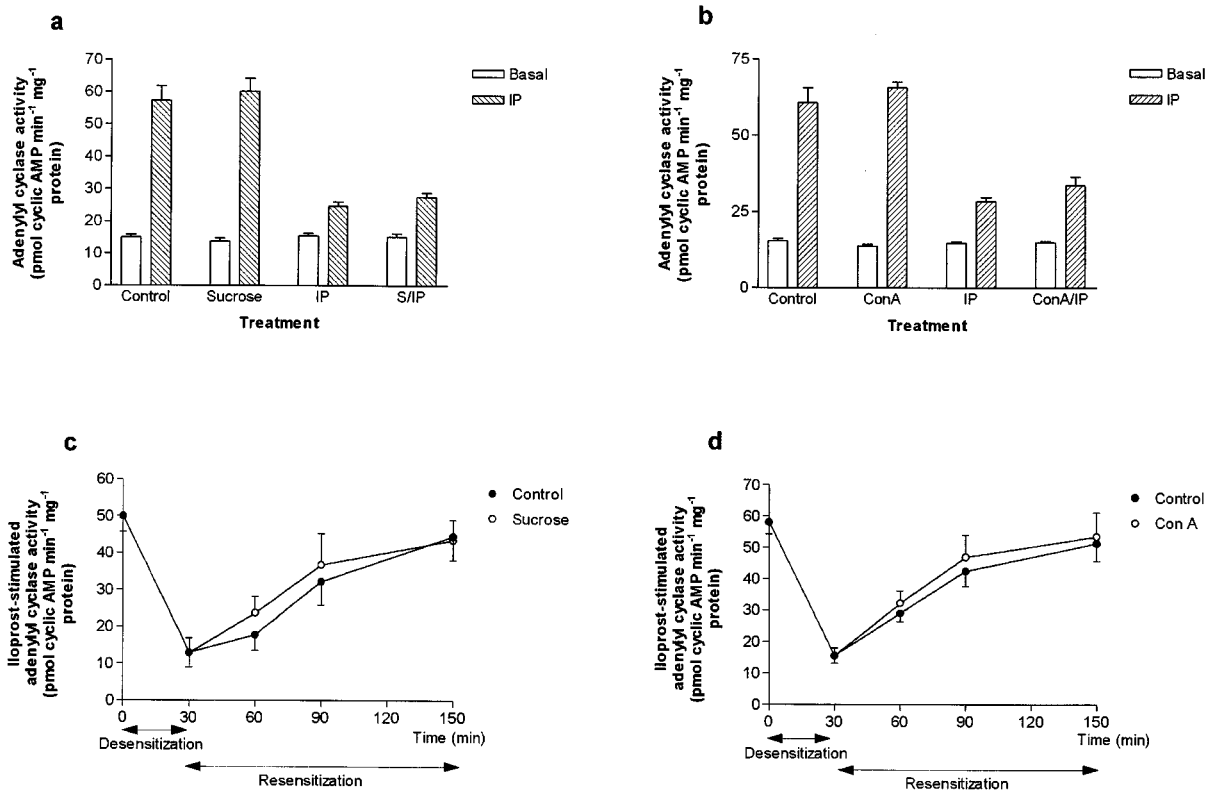

Figure 3 Effect of sucrose (a and c) or con A (b and d) on the agonist-induced desensitization and resensitization of IP-prostanoid receptor responsiveness. a and b: Cells were pretreated with or without sucrose (0.4 M; a) or con A (0.25 mg ml^{-1}; b) for 15 min. At this point some cells were harvested (control and sucrose in a and control and con A in b) whilst others were incubated for a further 30 min in the presence or absence of iloprost (1 μM) and in the continued presence (S/IP) or absence (IP) of sucrose or in the continued presence (con A/IP) or absence (IP) of con A before being harvested. Results show either basal adenylyl cyclase activity or that in the presence of 1 μM iloprost. Values are means $\pm$ s.e.mean from four separate experiments. c and d: Cells were pretreated with or without sucrose or con A for 15 min. At this time point (0), iloprost (1 μM) was added for 30 min in the continued presence or absence of sucrose or con A. Following this time, cells were washed and reincubated with fresh medium for a further 120 min, again in the continued presence or absence of sucrose or con A. Cells were harvested at time points 0, 30, 60, 90 and 150 min. Results in c and d show 1 μM iloprost-stimulated adenylyl cyclase activity (basal activity subtracted), and are values $\pm$ s.e.mean for four separate experiments.

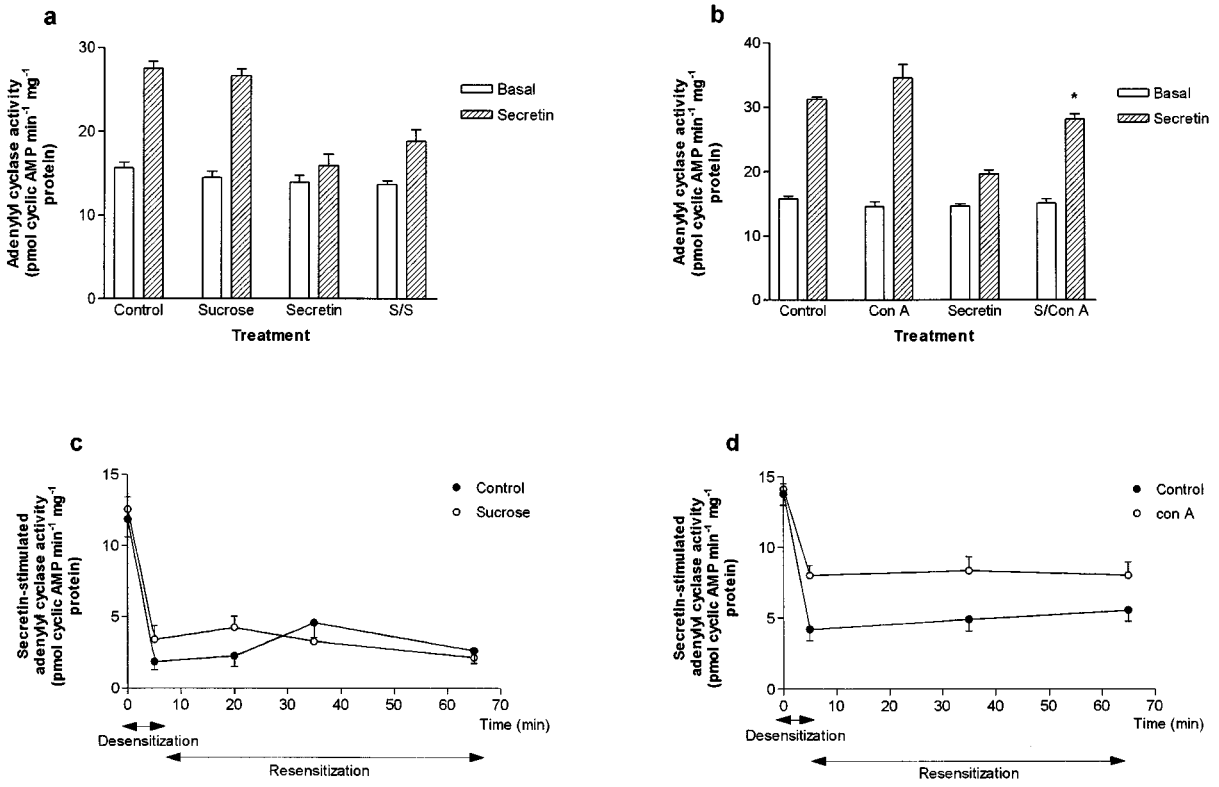

Figure 4 Effect of sucrose (a and c) or con A (b and d) on the agonist-induced desensitization and resensitization of secretin receptor responsiveness. a and b: Cells were pretreated with or without sucrose (0.4 M; a) or con A (0.25 mg ml^{-1}; c) for 15 min. At this time point some cells were harvested (control and sucrose in a and control and con A in b) whilst others were incubated for a further 5 min in the presence or absence of secretin (0.1 μM) and in the continued presence (S/S) or absence (Secretin) of sucrose or in the continued presence (S/con A) or absence (Secretin) of con A before being harvested. Results show either basal adenylyl cyclase activity or that in the presence of 0.1 μM secretin. Values are means $\pm$ s.e.mean from four separate experiments. *Desensitization for secretin in presence of con A significantly less than secretin alone, $P<0.05$, Student's t-test. c and d: Cells were pretreated with or without sucrose or con A for 15 min. At this time point (0), secretin (0.1 μM) was added for 5 min in the continued presence or absence of sucrose or con A. Following this time, cells were washed and reincubated with fresh medium for a further 60 min, again in the continued presence or absence of sucrose or con A. Cells were harvested at time points 0, 5, 20, 35 and 65 min. Results in c and d show 0.1 μM secretin-stimulated adenylyl cyclase activity (basal activity subtracted), and are values $\pm$ s.e.mean from four separate experiments. Throughout the desensitization and resensitization period, secretin-stimulated adenylyl cyclase activity in the presence of con A was significantly higher than in cells incubated without con A ($P<0.05$, ANOVA).

arrestins and clathrin, thus targeting the phosphorylated receptor to clathrin-coated pits and eventually vesicles (Goodman *et al.*, 1996). Upon internalization, vesicles termed endosomes (von Zastrow & Kobilka, 1992) are thought to be the site of receptor dephosphorylation. A reduction in pH of the endosome stimulates phosphatase activity (Krueger *et al.*, 1997) and hence the dephosphorylated receptor is recycled back to the plasma membrane for resensitization of response. It is likely that with prolonged periods of agonist stimulation, internalized receptors become down regulated rather than being recycled back to the plasma membrane (Ferguson *et al.*, 1996), and a recent study indicates that the initial pathway for this process involves, in part, clathrin-coated pits (Gagnon *et al.*, 1998).

A key question is whether other GPCRs follow the same pattern of regulation, i.e. phosphorylation, desensitization, internalization, dephosphorylation and resensitization. It is clear that some GPCRs appear to do so (Ishii *et al.*, 1998), but there is evidence that muscarinic m4, secretin and somatostatin receptors do not follow this general pattern (Bogatkewitsch *et al.*, 1996; Holtmann *et al.*, 1996; Beaumont *et al.*, 1998). In the present study, we investigated the effects of two inhibitors of internalization, hypertonic sucrose and con A, on the desensitization and resensitization of three GPCRs expressed in the NG108-15 cell line. An advantage of this approach is that all three receptors are endogenously expressed in the same cell line and thus the results are directly comparable.

Hypertonic sucrose inhibits receptor internalization by reversibly blocking the formation of clathrin coated pits (Heuser *et al.*, 1989). Con A is a plant lectin which binds to and cross-links glycosylated proteins such as GPCRs (Lis & Sharon, 1986), and can inhibit the agonist-induced internalization of β_2-adrenoceptors (Pippig *et al.*, 1995) and m3 muscarinic receptors (Szekeres *et al.*, 1998). At concentrations employed in these previous studies, the two agents did not affect A_{2A} adenosine receptor desensitization, but abolished the resensitization of A_{2A} adenosine receptor responsiveness. Although we have not directly measured the internalization of A_{2A} adenosine receptors in the present study, the most likely explanation is that these agents block receptor internalization, thus preventing the dephosphorylation of receptors and resensitization of the response. Agonist-induced phosphorylation and internalization of the canine A_{2A} adenosine receptor stably expressed in Chinese Hamster Ovary cells has been demonstrated (Palmer *et al.*, 1994). Although we have no direct evidence that the A_{2A} adenosine receptor in NG108-15 cells is phosphorylated, overexpression of dominant negative mutant GRK2 does inhibit desensitization of this receptor response (Mundell *et al.*, 1997). Thus the A_{2A} adenosine receptor appears to behave similarly to the β_2-adrenoceptor in

terms of complete dependance on internalization for resensitization to occur (Yu *et al*., 1993; Pippig *et al*., 1995). We also investigated the effects of altered levels of GRK2 activity on resensitization of A_{2A} adenosine receptor responsiveness. However, neither overexpression of wild type GRK2 nor inhibition of GRK2 activity by overexpression of dominant negative mutant GRK2, affected the rate of A_{2A} adenosine receptor resensitization. We have previously shown that these manipulations increase (Mundell *et al*., 1998) or decrease (Mundell *et al*., 1997), respectively, the rate of agonist-induced desensitization of A_{2A} adenosine receptor responsiveness in these cells, but it appears that the rate of resensitization is unaffected, presumably reflecting the fact that phosphorylated A_{2A} adenosine receptors internalize and resensitize normally irrespective of levels of GRK2 activity.

In contrast to the A_{2A} adenosine receptor response, resensitization of IP-prostanoid receptor responsiveness was unaffected by hypertonic sucrose or con A. This indicates either that desensitization and resensitization of this response does not require receptor internalization, or that the internalization occurs by a mechanism refractory to the two inhibitors employed. We (Williams & Kelly, 1994) and others (Adie *et al*., 1992) have previously demonstrated that in NG108-15 cells, desensitization of IP-prostanoid receptor responsiveness involves concurrent down regulation of receptor and $G_{s\alpha}$ from the cell membrane, but the present results suggest that this process may not be inhibited by sucrose or con A. The somewhat longer recovery period for IP-prostanoid receptor responsiveness as compared to that for the A_{2A} adenosine receptor, may reflect the time needed for recruitment of newly synthesized IP-prostanoid receptors and $G_{s\alpha}$ to the cell membrane.

Desensitization of secretin receptor responsiveness appears to involve rapid agonist-induced receptor internalization (Holtmann *et al*., 1996). Although the GRK-mediated phosphorylation and desensitization of secretin receptors expressed at high levels in HEK 293 cells has been recently demonstrated (Shetzline *et al*., 1998), our own studies on natively expressed receptors in NG108-15 cells indicate that secretin receptor desensitization is not modified by manipulation of GRK2 levels or activity (Mundell *et al*., 1997; Mundell *et al*., 1998). Since we found con A to block desensitization of secretin receptor responsiveness then it is possible that rapid internalization actually mediates the desensitization of this receptor response. Direct interference with the binding of secretin to its receptor seems unlikely since con A pretreatment did not affect acute activation of adenylyl cyclase by secretin. Unlike con A, hypertonic sucrose did not clearly reduce desensitization of secretin receptor responsiveness. This suggests that if the secretin response desensitizes *via* receptor internalization, then clathrin coated pits are not involved since hypertonic sucrose has been clearly shown to block this mechanism in different cell types (Daukas & Zigmond, 1985; Heuser *et al*., 1989; Slowiejko *et al*., 1996). Indeed, GPCRs can internalize *via* pathways independent of clathrin/dynamin (Zhang *et al*., 1996; Vogler *et al*., 1998; Lee *et al*., 1998) including cavaeolae (Feron *et al*., 1997; de Weerd & Leeb-Lundberg, 1997), which are not blocked by hypertonic sucrose (Roettger *et al*., 1995). The lack of any secretin receptor resensitization in the time scale examined was an unexpected and novel finding of this study. The reasons for this are unclear, but possibilities include that secretin remains bound to the receptor, or that rapid down regulation of the receptor protein occurs. Further studies will be required to resolve this.

Other factors need to be considered when comparing the effects of inhibitors of internalization on the desensitization and resensitization of A_{2A} adenosine, IP-prostanoid and secretin receptors. For example, the relative levels of expression of these three G_s-coupled receptors in this cell line is unknown, and consequently it is possible that differences in receptor expression and receptor reserve influence the effects of hypertonic sucrose and con A on loss and recovery of these responses. Furthermore, it is conceivable that hypertonic sucrose and con A prevent reassociation of some receptors and G-proteins in the plasma membrane, rather than, or as well as, receptor internalization. Finally, the glycosylation state of the receptors may affect the ability of con A to interact with each; in this respect it is interesting that whereas the secretin receptor has five putative sites for N-glycosylation (Ishihara *et al*., 1991), the A_{2A} adenosine receptor and IP-prostanoid receptor each have two (Fink *et al*., 1992; Namba *et al*., 1994).

In conclusion we have demonstrated that the desensitization and resensitization of three GPCRs endogenously expressed in the same cell type and coupled to the same G-protein can be differentially modified by inhibitors of receptor internalization. It will be interesting in future studies to determine the molecular mechanisms underlying these distinct effects.

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