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Inhibition of β-adrenergic receptor kinase prevents rapid homologous desensitization of β_2-adrenergic receptors

(A431 cells/permeabilization/receptor phosphorylation/heparin)

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ABSTRACT **Homologous (agonist-specific) desensitization of β-adrenergic receptors (βARs) is accompanied by and appears to require phosphorylation of the receptors. We have recently described a novel protein kinase, βAR kinase, which phosphorylates βARs *in vitro* in an agonist-dependent manner. This kinase is inhibited by two classes of compounds, polyanions and synthetic peptides derived from the β_2-adrenergic receptor (β_2AR). In this report we describe the effects of these inhibitors on the process of homologous desensitization induced by the β-adrenergic agonist isoproterenol. Permeabilization of human epidermoid carcinoma A431 cells with digitonin was used to permit access of the charged inhibitors to the cytosol; this procedure did not interfere with the pattern of isoproterenol-induced homologous desensitization of β_2AR-stimulated adenylyl cyclase. Inhibitors of βAR kinase markedly inhibited homologous desensitization of β_2ARs in the permeabilized cells. Inhibition of desensitization by heparin, the most potent of the polyanion inhibitors of βAR kinase, occurred over the same concentration range (5–50 nM) as inhibition of purified βAR kinase assessed in a reconstituted system. Inhibition of desensitization by heparin was accompanied by a marked reduction of receptor phosphorylation in the permeabilized cells. Whereas inhibitors of βAR kinase inhibited homologous desensitization, inhibitors of protein kinase C and of cyclic-nucleotide-dependent protein kinases were ineffective. These data establish that phosphorylation of βARs by βAR kinase is an essential step in homologous desensitization of the receptors. They further suggest a potential therapeutic value of inhibitors of βAR kinase in inhibiting agonist-induced desensitization.**

Occupancy of a wide variety of hormone and neurotransmitter receptors by agonists often leads to a loss of receptor responsiveness to subsequent stimulation by agonist. This phenomenon is generally termed homologous desensitization. This is in contrast to the heterologous form of desensitization, which is defined as a loss of receptor responsiveness caused by agonist occupancy of other receptors. Homologous desensitization has been most thoroughly studied for the β-adrenergic receptor (βAR)–adenylyl cyclase system (1). Homologous desensitization of βARs is accompanied by receptor phosphorylation (2, 3). We have described a cAMP-independent kinase, termed βAR kinase, that specifically phosphorylates the agonist-occupied forms of the β_2-adrenergic receptor (β_2AR) and α_2-adrenergic receptor (4, 5) as well as light-activated rhodopsin (6). Phosphorylation of the β_2AR by βAR kinase might trigger the process of functional uncoupling from the stimulatory guanine nucleotide binding protein, G_s (7).

Although several findings suggest an important role of βAR kinase in homologous desensitization, direct evidence for this hypothesis has been lacking. We have described two classes of inhibitors of this enzyme: polyanions such as heparin (8) and several synthetic peptides derived from proposed intracellular regions of the β_2AR (J.L.B., C. Staniszewski, J. Onorato, M.J.L., H. G. Dohlman, M.G.C., and R.J.L., unpublished data). Such inhibitors should allow an analysis of the contribution of βAR kinase to homologous desensitization. However, since all of these inhibitors are charged and therefore are presumably unable to cross the plasma membrane, techniques are required to allow the inhibitors to gain access to the cytosol while simultaneously preserving the phenomenology of homologous desensitization. In this study we report that permeabilized human epidermoid carcinoma A431 cells show an unaltered pattern of homologous desensitization of the β_2AR and that this desensitization is prevented by inhibitors of βAR kinase.

MATERIALS AND METHODS

[α-^{32}P]ATP, [γ-^{32}P]ATP, [^{3}H]cAMP, ^{125}I-labeled cyanopindolol (^{125}I-cyanopindolol), and ^{125}I-cyanopindolol diazirine were obtained from New England Nuclear; heparin (H-3125 from porcine mucosa), from Sigma. 1-5(isoquinoline sulfonyl)-2-methylpiperazine (designated H-7), from Calbiochem; and digitonin, from Gallard Schlessinger. The peptide corresponding to residues 1–24 of the heat-stable inhibitor of cAMP-dependent protein kinase [PKI-(1–24) tetracosapeptide; ref. 9] and the β_2AR-(57–71) pentadecapeptide (Ala-Ile-Ala-Lys-Phe-Glu-Arg-Leu-Gln-Thr-Val-Thr-Asn-Tyr-Phe; ref. 10) and β_2AR-(59–69) undecapeptide (Ala-Lys-Phe-Glu-Arg-Leu-Gln-Thr-Val-Thr-Asn) were chemically synthesized.

Phosphorylation of the Purified β_2AR by Purified βAR Kinase. β_2ARs from hamster lung were purified by affinity chromatography and HPLC to >95% homogeneity (11). βAR kinase was purified from bovine cerebral cortex by precipitation with ammonium sulfate, followed by chromatography on Ultrogel AcA34, DEAE-Sephacel, and CM-Fractogel to >75% purity as described (8, 12). Purified β_2ARs were inserted into phosphatidylcholine vesicles by chromatography on Extracti-gel, followed by polyethylene glycol treatment and centrifugation at 280,000 × *g* for 90 min (13). Phosphorylation of the reconstitu ted receptors by purified βAR kinase was done in the presence of 50 μM (−)-isoproterenol as described (12). Subsequently, the samples were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis on 10% gels (14). The phosphorylated β_2ARs were visualized by autoradiography, and the corresponding bands were cut out and their content of ^{32}P quantified.

Permeabilization of A431 Cells. Human epidermoid carcinoma A431 cells were grown to about 95% confluency in

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Abbreviations: βAR and β_2AR, β- and β_2-adrenergic receptor; PKI-(1–24), peptide corresponding to amino acids 1–24 of the heat-stable inhibitor of cAMP-dependent protein kinase; β_2AR-(57–71) and β_2AR-(59–69), peptides corresponding to amino acids 57–71 and 59–69, respectively, of human β_2AR.

Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were harvested with collagenase, washed three times with calcium-free phosphate-buffered saline (PBS), then washed twice in 150 mM potassium glutamate/10 mM Hepes/5 mM EGTA/7 mM $MgCl_2$, pH 7.1 (KG buffer), and finally resuspended in KG buffer supplemented with 5 mM glucose and 2 mM ATP (KG-A buffer) at a density of 4×10^7 cells per ml.

The concentration of digitonin required to permeabilize the cells was found to vary considerably with cell density. At 4×10^7 cells per ml, about 0.015% digitonin was necessary to achieve permeabilization of >95% cells as assessed by staining with trypan blue. In all experiments, digitonin was added stepwise until >95% of the cells were trypan blue positive.

Desensitization of β_2ARs in A431 Cells. Permeabilized cells in KG-A buffer (or, for controls, intact cells in PBS) were incubated with or without 1 μM (−)-isoproterenol for 10 min or the indicated times (4×10^7 cells per ml). The incubation was terminated by addition of 10 vol of ice-cold KG buffer (or PBS, respectively), followed by centrifugation at $1000 \times g$ for 5 min. After three identical washes, cells were disrupted with a Polytron homogenizer in 5 mM Tris·HCl, pH 7.4/2 mM EDTA. Crude membranes were prepared by spinning the supernatant after low-speed centrifugation ($1000 \times g$ for 5 min) at $40,000 \times g$ for 20 min.

Adenylyl cyclase activity in the membranes was determined as described by Salomon *et al.* (15). The free Mg^{2+} concentration in the assay was 4 mM. The incubation lasted for 20 min at 37°C; accumulation of [^{32}P]cAMP was linear over this time period.

Phosphorylation of β_2ARs in Permeabilized A431 Cells. Permeabilized cells (4×10^8) in 10 ml of KG-A buffer containing 1 mCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP were incubated with or without 1 μM (−)-isoproterenol (and other compounds as indicated) for 10 min at 37°C. All buffers used in these experiments contained 5 μg of soybean trypsin inhibitor, 5 μg of leupeptin, and 10 μg of benzamidine per ml and 0.1 mM phenylmethylsulfonyl fluoride. The reaction was terminated by addition of 30 ml of 150 mM potassium glutamate/5 mM EDTA/10 mM sodium phosphate, pH 7.1, at 0°C and centrifugation at $1000 \times g$ for 5 min. After three identical washing steps, crude membranes were prepared as above. The membranes were solubilized with 2% digitonin, and β_2ARs were purified by affinity chromatography with alprenolol-Sepharose (11). Equal amounts of β_2ARs from each sample (≈0.5 pmol, determined by radioligand binding using ^{125}I-cyanopindolol) were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis as described above. Gels were fixed in 40% (vol/vol) methanol and 15% (vol/vol) acetic acid and dried prior to autoradiography.

In addition, purified β_2ARs in these experiments were identified by photoaffinity labeling with ^{125}I-cyanopindolol diazirine. Aliquots of the samples (≈60 fmol of β_2ARs) were desalted over Sephadex G-50 and incubated for 2 hr at 20°C with 200 pM of the ligand with or without 10 μM alprenolol. Free ligand was removed by desalting as above, and the samples were irradiated with UV light for 3 min. The samples were loaded on the same gel as the samples described above.

Data Analysis. Concentration–response curves were analyzed by nonlinear curve-fitting to the Hill equation as described (16). Adenylyl cyclase activity was expressed as the percentage of the activity in the presence of 10 mM NaF. Since NaF stimulates adenylyl cyclase via the stimulatory guanine nucleotide binding protein, G_s, this normalizes for effects that occur at the level of G_s or the cyclase (i.e., effects that represent heterologous desensitization). Desensitization was assessed by measuring the loss of maximal stimulation by isoproterenol [determined with 10 μM (−)-isoproterenol] and was calculated as [1 − stimulation (desensitized)/stimulation (control)] × 100. For example, a decrease from 60% to 40% of NaF-stimulated activity corresponds to a desensitization of [1 − (40/60)] × 100 = 33%.

RESULTS

A variety of permeabilization techniques were tested for their ability to provide constant and reproducible access to the

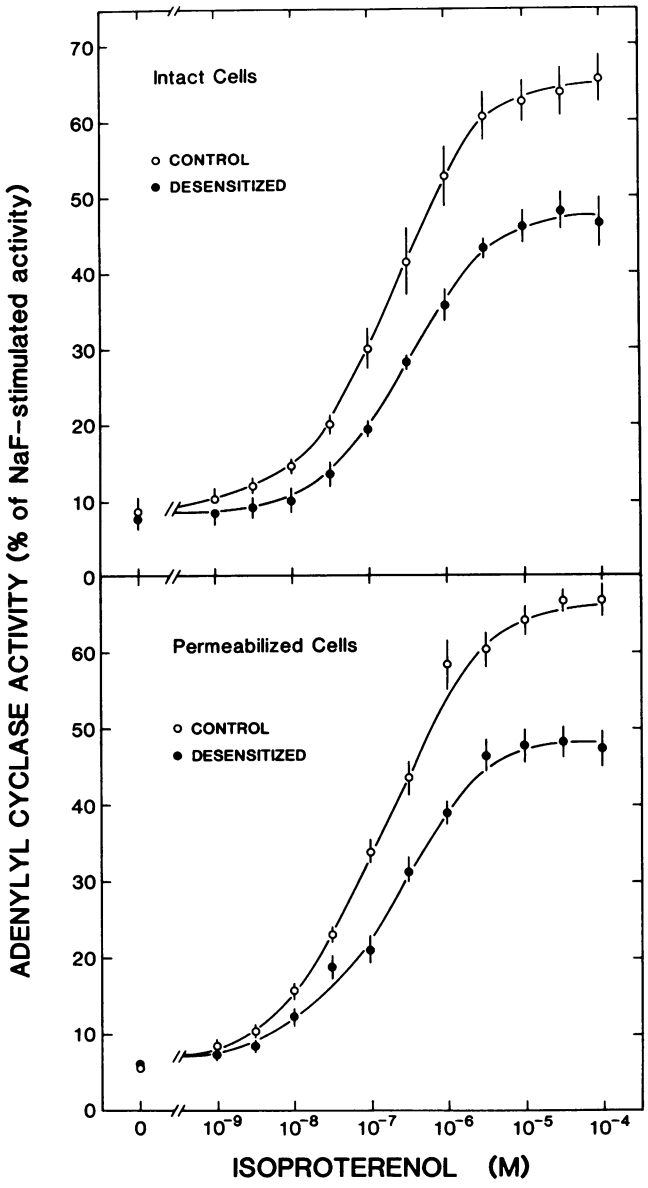

FIG. 1. Desensitization of β_2ARs in intact (*Upper*) and permeabilized (*Lower*) A431 cells. Cells were incubated for 10 min with (desensitized) (•) or without (control) (○) 1 μM (−)-isoproterenol as detailed. Adenylyl cyclase activities of membranes prepared from the cells were measured in the presence of various concentrations of (−)-isoproterenol and were expressed as percent of the activity in the presence of 10 mM NaF. Values for EC_{50}, maximal stimulation over basal (E_{max}, in percent of activity with 10 mM NaF), and activity in the presence of 10 mM NaF (=100%, in pmol of cAMP per mg of protein per min) were as follows: for control intact cells they were 200 nM (EC_{50}), 56% (E_{max}), and 84 ± 2 pmol/mg/min (100%); for desensitized intact cells they were 300 nM (EC_{50}), 40% (E_{max}), and 70 ± 7 pmol/mg/min (100%); for control permeabilized cells they were 130 nM (EC_{50}), 62% (E_{max}), and 86 ± 2 pmol/mg/min (100%); for desensitized permeabilized cells they were 190 nM (EC_{50}), 43% (E_{max}), and 69 ± 3 pmol/mg/min (100%). Data are means ± SEM of three independent experiments.

cytosol while leaving homologous desensitization unaltered. These included scrape-loading (17), electropermeabilization (18), and permeabilization with staphylococcal α toxin (19) and different detergents. Permeabilization with digitonin gave the most consistent results and was used for all future experiments. Fig. 1 shows that permeabilization of A431 cells with digitonin did not change the pattern of homologous desensitization to isoproterenol (1 μM). Isoproterenol-induced stimulation of adenylyl cyclase in membranes from cells incubated with 1 μM isoproterenol for 10 min at 37°C was reduced in both potency and maximal effect as compared with membranes of control cells. In both intact and permeabilized cells, the extent of maximal stimulation over basal activity was reduced by the pretreatment from ≈60% to ≈40% of NaF-stimulated activity, corresponding to a desensitization of ≈30%.

Fig. 2 shows the time course of desensitization in permeabilized cells in the absence or presence of two inhibitors of βAR kinase, heparin (100 nM) and a peptide corresponding to the first intracellular loop of the human β_2AR [β_2AR-(57–71), 100 μM]. In the absence of inhibitors, desensitization occurred with a half-time of <5 min. β_2AR-(57–71) markedly slowed the rate of desensitization, and desensitization was almost completely abolished by 100 nM heparin. Neither compound affected desensitization in intact cells (data not shown).

It has been shown that heparin and its analogues may alter stimulation of adenylyl cyclase at micromolar concentrations (20). To ascertain that the inhibition of desensitization by heparin was not due to a reduced formation of cAMP, we determined accumulation of [^{32}P]cAMP from [α-^{32}P]ATP added to permeabilized cells. The presence of up to 1 μM heparin did not reduce [^{32}P]cAMP formation either in the presence or the absence of isoproterenol. Thus, our data suggest that compounds that inhibit βAR kinase might slow down or suppress homologous desensitization.

To show that these effects are indeed due to inhibition of βAR kinase, we compared the concentration dependence for inhibition of βAR kinase activity in a reconstituted system with that for inhibition of β_2AR desensitization by heparin (Fig. 3). Heparin inhibited phosphorylation of pure reconstituted β_2ARs by βAR kinase with an IC_{50} value of 6 nM and caused almost complete inhibition at 30–100 nM (Fig. 3 *Upper*). Desensitization of β_2ARs in permeabilized A431 cells was inhibited by heparin with an IC_{50} value of 20 nM, and inhibition was virtually complete at concentrations above 100 nM. Thus, the concentration–response curves for the two effects are similar.

If the inhibition of desensitization by heparin is in fact caused by inhibition of βAR kinase, then it would be expected that heparin would reduce the isoproterenol-induced phosphorylation of β_2ARs in permeabilized cells. Therefore, we incubated permeabilized A431 cells with [γ-^{32}P]ATP with or without 1 μM isoproterenol in the absence or presence of 1 μM heparin. Incubation time (10 min) and conditions were the same as in the desensitization experiments reported in Fig. 3. In the absence of heparin, isoproterenol caused a 5-fold increase in the phosphorylation of the β_2ARs (Fig. 4*a*). Heparin (1 μM) markedly reduced this increase while not affecting basal phosphorylation. Quantitation by scanning of the autoradiograms showed that heparin reduced the isopro-

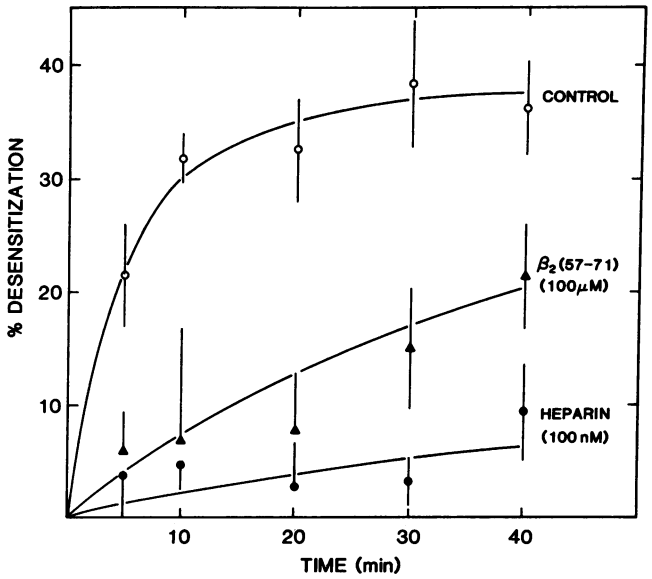

FIG. 2. Time course of β_2AR desensitization in permeabilized A431 cells. Permeabilized cells were incubated for the indicated periods of time with 1 μM (−)-isoproterenol alone (control) or in the presence of 100 μM of the peptide β_2AR-(57–71) [β_2(57–71)] or 100 nM heparin. Desensitization was measured as the percent loss of stimulation by 10 μM (−)-isoproterenol of adenylyl cyclase activity in membranes. Data are means ± SEM of six (control) or three independent experiments.

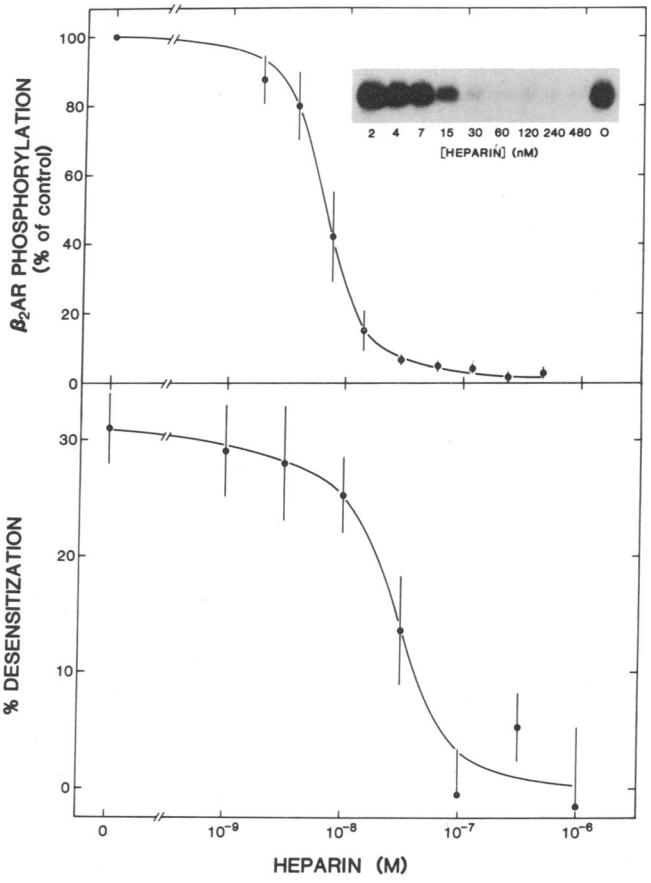

FIG. 3. Inhibition by heparin of β_2AR phosphorylation by βAR kinase in a reconstituted system (*Upper*) and of β_2AR desensitization in permeabilized A431 cells (*Lower*). (*Upper*) Purified reconstituted β_2ARs were phosphorylated by purified βAR kinase with [γ-^{32}P]ATP in the presence of various concentrations of heparin. Curve-fitting gave an IC_{50} value of 6.1 ± 2.0 nM. (*Inset*) Relevant section of a representative autoradiogram. Data are means ± SEM of three independent experiments. (*Lower*) Permeabilized A431 cells were desensitized by incubation with 1 μM (−)-isoproterenol for 10 min at 37°C in the presence of various concentrations of heparin. Desensitization was measured as the percent loss of stimulation by 10 μM (−)-isoproterenol of adenylyl cyclase activity in membranes. Curve-fitting gave an IC_{50} value of 21 ± 4 nM. Data are means ± SEM of four independent experiments.

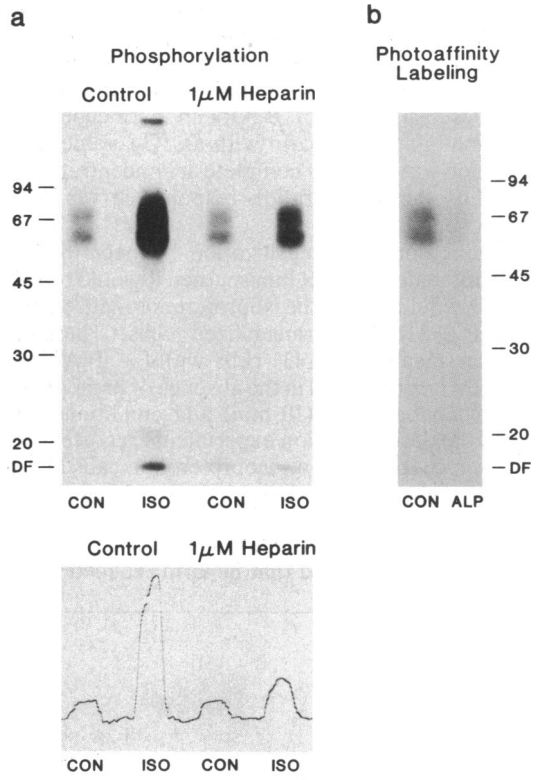

FIG. 4. Phosphorylation of β_2ARs in permeabilized A431 cells. Permeabilized cells were incubated with [γ-^{32}P]ATP without (CON) or with (ISO) 1 μM (−)-isoproterenol in the absence or presence of 1 μM heparin. β_2ARs were solubilized, purified by affinity chromatography, and electrophoresed on a 10% sodium dodecyl sulfate/polyacrylamide gel, each lane containing 0.5 pmol of receptor. (*Upper Left*) Autoradiogram obtained after a 10-day exposure at −70°C. (*Lower Left*) Transverse densitometric scan of the autoradiogram. (*Right*) An aliquot of purified receptors from cells incubated without (−)-isoproterenol or heparin was photoaffinity-labeled with ^{125}I-cyanopindolol diazirine in the absence (CON) or presence (ALP) of 10 μM alprenolol, followed by electrophoresis on the same gel (60 fmol per lane) and autoradiography. Similar results were obtained in two other experiments.

terenol-induced phosphorylation by 50–80% in three experiments (Fig. 4*a Lower*). Although some receptor degradation appeared to occur (as evidenced by a band of lower molecular weight), photoaffinity labeling with ^{125}I-cyanopindolol diazirine confirmed that the purified phosphorylated bands represent β_2ARs (Fig. 4*b*). Thus, inhibition of β_2AR desensitization by heparin is paralleled by an inhibition of isoproterenol-induced receptor phosphorylation.

Finally, we compared the effects of other kinase inhibitors and of analogues of the β_2AR peptide both on phosphorylation of reconstituted β_2ARs by βAR kinase and on desensitization in permeabilized A431 cells (Table 1). The peptide PKI-(1–24), which inhibits cAMP-dependent protein kinase with a K_i value of 5×10^{-9} M (9), had no effect at concentrations up to 1 μM on either βAR kinase activity or desensitization. H7, an inhibitor of protein kinase C and cyclic nucleotide-dependent protein kinases with K_i values of about 5 μM (21), also did not affect either βAR kinase activity or desensitization at 1 μM and 10 μM. At 100 μM it caused ≈20% inhibition of both processes.

Whereas the 15-amino-acid peptide representing the first intracellular loop of the human β_2AR at a concentration of 100 μM caused significant inhibition of βAR kinase activity, the central 11-amino-acid segment was virtually inactive in this respect. In parallel, the 15-amino-acid peptide markedly inhibited desensitization (see also Fig. 2), whereas the shorter peptide did not affect it. These data confirm a correlation between inhibition of βAR kinase and inhibition of homologous desensitization.

Table 1. Inhibition of βAR kinase and of desensitization by kinase inhibitors and peptides of the β_2AR

Compound	Conc., μM	Inhibition of βAR kinase, %	Inhibition of desensitization, %
Kinase inhibitors			
Heparin	0.1	96 ± 1	102 ± 16
PKI-(1–24)	1	0 ± 9	0 ± 12
H7	1	3 ± 4	0 ± 4
	10	2 ± 7	7 ± 5
	100	19 ± 6	22 ± 14
β_2AR peptides			
β_2AR-(57–71)	100	80 ± 13	52 ± 12
β_2AR-(59–69)	100	0 ± 6	3 ± 12

Inhibition of βAR kinase and desensitization were measured as shown in Fig. 3. βAR kinase activity under control conditions corresponds to 6.5 ± 1.6 pmol of phosphate incorporated during a 30-min incubation. Desensitization under control conditions was 29 ± 4% as defined in *Materials and Methods*. Data are means ± SEM of at least three experiments. Conc., concentration.

DISCUSSION

Homologous or agonist-specific desensitization of β_2ARs is associated with receptor phosphorylation (1). Several protein kinases have been shown to be capable of phosphorylating purified β_2ARs *in vitro*, including protein kinase C (22), cAMP-dependent protein kinase (23), and βAR kinase (4). We have previously suggested that βAR kinase is the kinase responsible for homologous desensitization of β_2ARs. This hypothesis was based on three observations. First, homologous desensitization occurs in variants of S49 lymphoma cells, which are deficient in either the stimulatory guanine nucleotide binding protein G_s (cyc$^-$) or the cAMP-dependent protein kinase (kin$^-$), so that it must be independent of stimulation of adenylyl cyclase or activation of cAMP-dependent protein kinase (3). Second, phosphorylation of reconstituted purified β_2ARs by βAR kinase results in impaired interaction with G_s. This process may require in addition binding of an analogue of arrestin, a protein that binds to and thereby inactivates phosphorylated rhodopsin (7, 24). Third, removal of potential phosphorylation sites for βAR kinase in the carboxyl terminus of the human β_2AR by mutagenesis markedly delays the onset of homologous desensitization (25). However, all of these lines of evidence are indirect and do not prove either that βAR kinase-mediated phosphorylation of β_2ARs occurs *in vivo* or that it is of functional relevance. In contrast, inhibition of βAR kinase enzymatic activity and examination of its consequences in intact cells is a more direct approach to documenting the role of this enzyme. The identification of inhibitors of βAR kinase (8) has provided the tools needed for such an investigation. The use of permeabilized cells, which retain their pattern of homologous desensitization to isoproterenol, has allowed us to assess the effects of βAR kinase inhibition on βAR desensitization.

Prototypes of the two classes of inhibitors, heparin for the polyanions, and β_2AR-(57–71) for the receptor peptides, both caused a concentration-dependent inhibition of homologous desensitization. Under assay conditions where primarily homologous desensitization is measured, heparin was able to completely inhibit desensitization to isoproterenol. Similar concentrations of heparin were required to inhibit βAR kinase and desensitization, and inhibition of desensitization was accompanied by a marked reduction of β_2AR phospho-

rylation in the permeabilized cells. Of several kinase inhibitors and receptor peptides tested, only those that inhibited βAR kinase also inhibited desensitization. Taken together, these data strongly suggest that inhibition of βAR kinase leads to a loss of homologous desensitization and, hence, that phosphorylation of β_2ARs by βAR kinase causes this form of desensitization.

Heparin did not affect basal βAR phosphorylation in permeabilized cells (see Fig. 4). In addition, the modest level of heterologous desensitization observed in this study, as evidenced by a loss of NaF-stimulated adenylyl cyclase activity (see the legend to Fig. 1), was also not affected by heparin (data not shown). These observations suggest that neither basal βAR phosphorylation nor heterologous desensitization are mediated by βAR kinase. In addition, our data support previous findings that protein kinase C and cAMP-dependent protein kinase do not play a major role in homologous desensitization, since inhibitors of these kinases did not affect this process. However, both kinases might cause basal βAR phosphorylation, and it appears plausible that cAMP-dependent protein kinase is responsible for the residual isoproterenol-induced phosphorylation seen in permeabilized cells in the presence of heparin (Fig. 4). This residual phosphorylation did not promote homologous desensitization as assessed by a decrease in the maximal stimulation by isoproterenol. However, it may be associated with a shift of the EC_{50} value of isoproterenol without markedly altering the maximal effect (W. P. Hausdorff, M. Bouvier, B. F. O'Dowd, G. P. Irons, M.G.C., and R.J.L., unpublished data; see also Fig. 1). Such cAMP-dependent protein kinase-mediated effects on desensitization have been reported to be more apparent under different assay conditions (26).

The lack of effect of H7 on isoproterenol-induced homologous desensitization led Toews *et al.* (27) to speculate that phosphorylation of βARs is not required for desensitization. However, our data show that even high concentrations of H7 (100 μM) inhibit βAR kinase only slightly. Two other kinases have been shown to be inhibited by heparin, casein kinase II (28), and low density lipoprotein receptor kinase (29). Neither kinase is likely to be involved in βAR desensitization because casein kinase II does not phosphorylate βARs (23) and casein kinase II and low density lipoprotein receptor kinase have similar substrate specificity.

The effects of inhibition of βAR kinase on homologous desensitization are similar to those of removal of potential phosphorylation sites for βAR kinase from the carboxyl terminus of the β_2AR by mutagenesis (25). In all cases, homologous desensitization at short time periods (≤15 min) is virtually abolished. This confirms the notion that the serine- and threonine-rich carboxyl terminus contains most or all of the phosphorylation sites for βAR kinase.

In summary, our data strongly support the hypothesis that phosphorylation of β_2ARs by βAR kinase is indeed responsible for homologous desensitization. The observation that this process can be inhibited by inhibitors of βAR kinase offers new perspectives for the analysis and pharmacological alteration of desensitization. It should be expected that membrane-permeable inhibitors of βAR kinase will block homologous desensitization in intact cells or tissues. Such agents might be of value in clinical situations where desensitization to β-adrenergic agonists is a therapeutic problem.

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