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Comparative rates of desensitization of β-adrenergic receptors by the β-adrenergic receptor kinase and the cyclic AMP-dependent protein kinase

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ABSTRACT **Three separate processes may contribute to rapid β-adrenergic receptor desensitization: functional uncoupling from the stimulatory guanine nucleotide-binding protein G_s, mediated by phosphorylation of the receptors by two distinct kinases, the specific β-adrenergic receptor kinase (βARK) and the cyclic AMP-dependent protein kinase A (PKA), as well as a spatial uncoupling via sequestration of the receptors away from the cell surface. To evaluate the relative importance and potential role of the various processes in different physiological situations, a kinetic analysis of these three mechanisms was performed in permeabilized A431 epidermoid carcinoma cells. To allow a separate analysis of each mechanism, inhibitors of the various desensitization mechanisms were used: heparin to inhibit βARK, the PKA inhibitor peptide PKI to inhibit PKA, and concanavalin A treatment to prevent sequestration. Isoproterenol-induced phosphorylation of β_2 receptors in these cells by βARK occurred with a $t_{1/2}$ of <20 sec, whereas phosphorylation by PKA had a $t_{1/2}$ of about 2 min. Similarly, βARK-mediated desensitization of the receptors proceeded with a $t_{1/2}$ of <15 sec, and PKA-mediated desensitization with a $t_{1/2}$ of about 3.5 min. Maximal desensitization mediated by the two kinases corresponded to a reduction of the signal-transduction capacity of the receptor/adenylyl cyclase system by about 60% in the case of βARK and by about 40% in the case of PKA. Receptor sequestration was much slower ($t_{1/2}$ of about 10 min) and involved no more than 30% of the cell surface receptors. It is concluded that βARK-mediated phosphorylation is the most rapid and quantitatively most important factor contributing to the rapid desensitization. This rapidity of the βARK-mediated mechanism makes it particularly well suited to regulate β-adrenergic receptor function in rapidly changing environments such as the synaptic cleft.**

Desensitization is a cellular process in which a response to a continuous or repeated stimulus attenuates with time. The β-adrenergic receptor/adenylyl cyclase system is one of the most widely studied models of desensitization phenomena (1–3). Several biochemical mechanisms have been shown to contribute to desensitization of β-adrenergic receptors. These include (*i*) a rapid functional uncoupling of the receptors from the signal-transducing protein, the stimulatory guanine nucleotide-binding protein G_s; (*ii*) rapid sequestration of the receptors away from the cell surface into an as yet ill-defined membrane compartment where they are physically separated from G_s; and (*iii*) a slow reduction of the total cellular receptor complement, a process that is called down-regulation. The rapid functional uncoupling of receptors and G_s can be triggered by the phosphorylation of the receptors either by protein kinase A (PKA) or by the β-adrenergic receptor kinase (βARK), a specific cytosolic kinase that phosphorylates only the active, agonist-occupied form of several G-protein-coupled receptors (2, 4). Receptor phosphorylation by βARK promotes the binding of another protein, called β-arrestin, and this interaction appears to result in uncoupling of receptors and G_s (5). No such cofactor has been identified for PKA-mediated desensitization of β-adrenergic receptors.

Recently, a dissection of the three rapid desensitization mechanisms—uncoupling by PKA, uncoupling by βARK, and sequestration—has been undertaken by two different approaches. One approach relies on the expression of mutated β_2 receptors where the presumed sites of phosphorylation by either PKA or βARK were disrupted (6). The other technique uses selective inhibitors of each of the three processes (7, 8). Both approaches have led to similar conclusions regarding the extent of desensitization by either PKA or βARK: the two kinases can mediate about the same maximal extent of desensitization. When the desensitization is measured as the loss of signal-transduction capacity of the receptor system (9), the action of each of the two kinases individually can result in about 50% desensitization. However, PKA-mediated desensitization occurs at about 100-fold lower agonist concentrations than βARK-mediated desensitization. Similar conclusions regarding the high agonist sensitivity of PKA-mediated desensitization have been drawn from studies using S49 mouse lymphoma cells defective in PKA (10).

In situations where high agonist concentrations are present, such as within the synaptic cleft, the sensitivity of the desensitization response to low agonist concentrations would seem to be of little importance. Instead, the speed at which desensitization is achieved would appear to be more critical. Thus, the most rapid mechanism will be the most important in these systems. The present study describes a kinetic analysis of the three rapid mechanisms leading to β-receptor desensitization. Since thus far the critical amino acids of the receptor required for sequestration have not been identified and since, therefore, receptor sequestration cannot be abolished by the mutagenesis approach, we used the technique of inhibition of the three mechanisms by inhibitors (8). The inhibitors used were heparin for βARK (8, 11), the PKA inhibitor peptide (PKI) of the rabbit muscle protein inhibitor for PKA (12), and concanavalin A (Con A) pretreatment of cells for sequestration (13). Although these inhibitors are clearly not entirely specific, we have shown previously (8) that they are specific in the context of β-receptor desensitization—i.e., all of them affect only one of the three rapid desensitization mechanisms.

MATERIALS AND METHODS

Permeabilization of A431 Cells. Human epidermoid carcinoma A431 cells were grown to ≈95% confluency in Dulbecco's modified Eagle's medium supplemented with 10%

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Abbreviations: βARK, β-adrenergic receptor kinase; PKA, protein kinase A; PKI, PKA inhibitor peptide.

fetal bovine serum. Cells were harvested with collagenase and washed twice with 10 ml of calcium-free phosphate-buffered saline (PBS) with intervening centrifugations at 300 × *g* for 5 min. If cells were to be pretreated with Con A, they were incubated with Con A (0.25 mg/ml) in PBS at 37°C for 20 min and then again washed with PBS. The cells were then washed twice with 150 mM potassium glutamate/10 mM Hepes/5 mM EGTA/7 mM $MgCl_2$, pH 7.1 (KG buffer). They were finally resuspended in KG buffer with 5 mM glucose and 2 mM ATP (KG-A buffer) at a density of 4×10^7 cells per ml. Permeabilization was achieved by stepwise addition of 1% digitonin until permeabilization of >95% of the cells was achieved as assessed by staining with trypan blue (see ref. 7 for details).

Phosphorylation of β_2-Adrenergic Receptors in Permeabilized A431 Cells. The phosphorylation of β_2 receptors in permeabilized A431 cells was measured essentially as described (7, 8). In brief, Con A-pretreated cells were permeabilized in KG-A buffer, and 1 μM PKI or 1 μM heparin was added to inhibit PKA or βARK, respectively. The cells were incubated at 37°C, and [γ-^{32}P]ATP [1 mCi (37 MBq) per tube] was added 1 min before the addition of 10 μM (−)-isoproterenol. After various incubation times the reactions were stopped by the addition of ice-cold KG buffer containing 0.15 μM okadaic acid (14) to inhibit phosphatase activity during the receptor isolation. The receptors were then purified by affinity chromatography, electrophoresed in an SDS/12% polyacrylamide gel, and visualized by autoradiography.

Desensitization of A431 Cells. The permeabilized cells suspended in KG-A buffer were incubated with or without 1 μM PKI or 1 μM heparin to inhibit PKA or βARK, respectively, before (−)-isoproterenol was added to each tube. The cells were incubated with 10 μM (−)-isoproterenol for various times. The reaction was terminated by addition of 20 ml of ice-cold KG buffer, and the cells were washed three times in the same buffer. Cells were lysed in 5 mM Tris·HCl, pH 7.4/2 mM EDTA and the pellet was resuspended in 75 mM Tris·HCl, pH 7.4/5 mM $MgCl_2$/2 mM EDTA. Adenylyl cyclase activity in these crude membranes was determined as described by Salomon *et al.* (15); incubation was at 37°C for 20 min.

Determination of Receptor Sequestration. Agonist-induced sequestration was measured in nonpermeabilized cells, since earlier studies showed no effect of either permeabilization or the inhibition of receptor phosphorylation on receptor sequestration, and since its determination in nonpermeabilized cells is more accurate (8). A431 cells were exposed to 10 μM (−)-isoproterenol in PBS at 37°C for various times, and then placed on ice and washed three times with ice-cold PBS. The percentage of sequestered receptors was then determined as described (6). In brief, the receptor concentration was measured in the cells by binding with [^{125}I]iodopindolol, using 1 μM (−)-propranolol to define the total number of receptors and 0.3 μM CGP 12177 to define cell surface receptors.

Data Analysis. Data were analyzed by nonlinear curve fitting (16). Kinetic data were fitted to monoexponential equations, and concentration–response curves to the Hill equation. Desensitization experiments were analyzed essentially as described (6, 8). In brief, the adenylyl cyclase activity in each experiment was normalized to the activity in the presence of 1 mM forskolin. The maximum activity elicited by (−)-isoproterenol in nondesensitized cells was then set to 100%, and all the other values were expressed relative to this activity. Desensitization was quantitated as the loss of signal-transduction capacity, τ, as detailed earlier (9). All curves of one set of desensitization experiments were fitted simultaneously to the equation (17)

$$E = E_m \frac{\tau[A]}{(K_A + [A]) + \tau[A]},$$

with E denoting the effect, E_m the maximum possible effect, [A] the agonist concentration, and K_A the dissociation constant of the agonist–receptor complex. τ is a parameter describing the signal-transduction capacity of the system and is estimated individually for each curve, whereas all the other parameters are shared. τ_0 denotes the τ value under control conditions; $[1 - (\tau/\tau_0)] \times 100$ is then taken as a measure of desensitization (in percent). Intuitively, this parameter can best be described as the percentage of receptors that need to be destroyed to give an equivalent loss of receptor-stimulated adenylyl cyclase activity.

RESULTS

PKA and βARK are thought to trigger agonist-induced desensitization of β-adrenergic receptors by phosphorylation of the receptors. To measure the kinetics of each of the two reactions, we measured β_2-receptor phosphorylation in permeabilized A431 cells under conditions where neither receptor sequestration nor phosphorylation of the receptors by the other kinase can occur. To do this, sequestration was prevented by pretreating the cells with Con A; we have shown

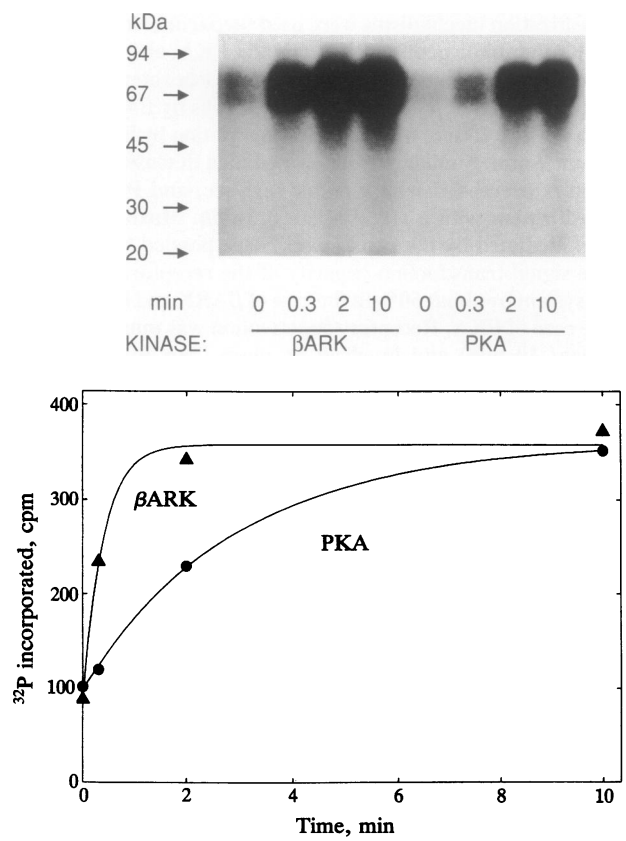

FIG. 1. Agonist-induced phosphorylation of β_2 receptors in permeabilized A431 cells by βARK and PKA. Con A-pretreated, digitonin-permeabilized cells were incubated with [γ-^{32}P]ATP, 10 μM (−)-isoproterenol, and either 1 μM PKI (to measure βARK-mediated phosphorylation) or 1 μM heparin (to measure PKA-mediated phosphorylation). Reactions were terminated after the indicated times, and the receptors were isolated by affinity chromatography and electrophoresed in an SDS/12% polyacrylamide gel. (*Upper*) Autoradiogram of a gel. (*Lower*) Radioactivity incorporated into the β_2 receptors, in cpm per band. $t_{1/2}$ values were obtained by curve fitting to a monoexponential equation: βARK (▲), 16 sec; PKA (●), 2.1 min. Data are means of two separate experiments.

earlier that this prevents the sequestration process also in permeabilized cells (8). We have also shown earlier that in the presence of both PKI and heparin there is almost no agonist-induced phosphorylation of β_2 receptors in this model, suggesting that the agonist-induced phosphorylation of β_2 receptors in this system is almost completely due to the action of these two kinases (8). Thus, βARK-mediated phosphorylation can be measured in the presence of PKI, so that PKA is inactive, and PKA-mediated phosphorylation can be measured in the presence of heparin, so that βARK is inactive. Under these conditions, isoproterenol induces phosphorylation of the β_2 receptors in the permeabilized A431 cells with two very different kinetics (Fig. 1). In the presence of PKI, a rapid pattern of phosphorylation is observed that must be attributed to βARK: half-maximal phosphorylation is observed after <20 sec, and almost no further increase in receptor phosphorylation is seen after 2 min. PKA-mediated receptor phosphorylation, which is measured in the presence of heparin, is much slower: half-maximal phosphorylation is achieved after about 2 min. In spite of these kinetic differences between βARK- and PKA-mediated phosphorylation, however, the maximal extent of phosphorylation is similar, resulting in a 3- to 4-fold increase of ^{32}P incorporation into the receptors.

Using the same conditions we then measured agonist-induced desensitization mediated by PKA or βARK. Fig. 2 shows the pattern of desensitization caused by βARK (i.e., when the desensitization was done in the presence of PKI), and Fig. 3 the same for PKA-mediated desensitization (i.e., with desensitization in the presence of heparin). Again, cells had been pretreated with Con A, so that there should be no contribution of receptor sequestration. βARK-mediated desensitization occurs rapidly; there is a significant rightward shift and a drop of the maximum of the concentration–response curve even after 15 sec, and most of the desensitization process occurs within the first 2 min. In contrast, PKA-mediated desensitization seems to be a more gradual process that only begins to become apparent after 30 sec and is still continuing at 10 min.

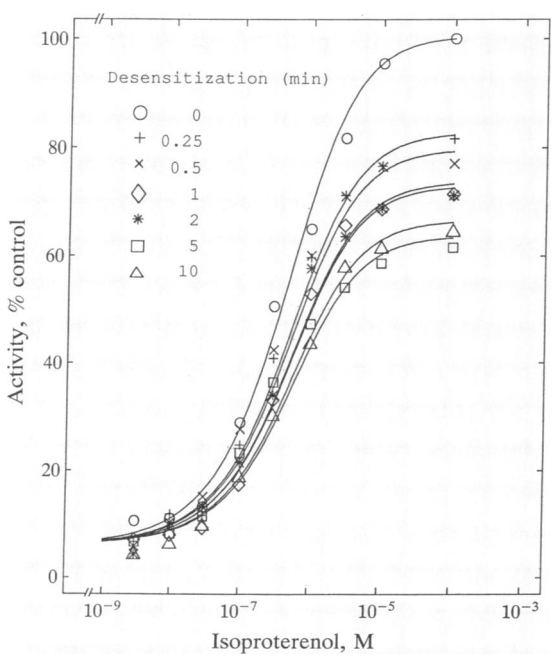

FIG. 2. βARK-mediated desensitization of β_2 receptors in permeabilized A431 cells. Con A-pretreated permeabilized cells were desensitized in the presence of 1 μM PKI with 10 μM (−)-isoproterenol for the indicated times. (−)-Isoproterenol-stimulated adenylyl cyclase activity was determined in the membranes. Curves were generated by simultaneous curve fitting. Data are means of three independent experiments.

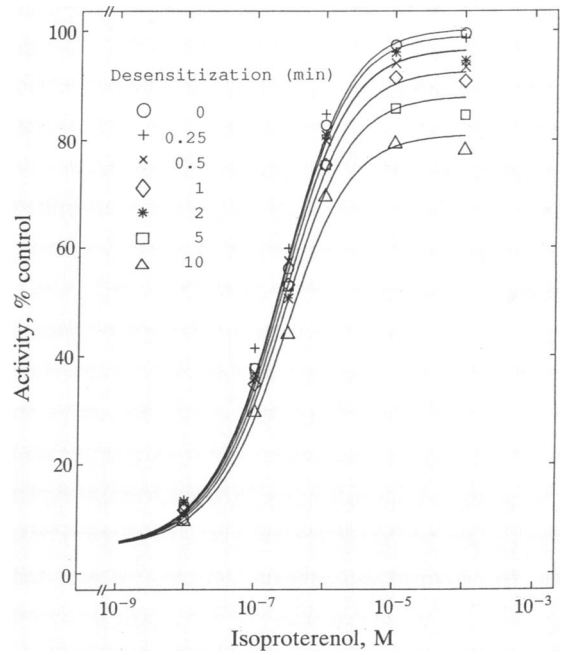

FIG. 3. PKA-mediated desensitization of β_2 receptors in permeabilized A431 cells. Con A-pretreated permeabilized cells were desensitized in the presence of 1 μM heparin with 10 μM (−)-isoproterenol for the indicated times. (−)-Isoproterenol-stimulated adenylyl cyclase activity was determined in the membranes. Curves were generated by simultaneous curve fitting. Data are means of four independent experiments.

A quantitative analysis of these experiments was done by calculating desensitization as the loss of signal-transduction capacity, τ. This was done by simultaneous curve fitting of all the data of each set of experiments and calculation of a desensitization value as described in *Materials and Methods*. Fig. 4 gives the time course of this parameter of desensitization for the βARK- and the PKA-mediated processes. A $t_{1/2}$ of <15 sec is calculated for the βARK-mediated desensitization, together with a maximal extent of desensitization of >60%. For PKA-mediated desensitization a $t_{1/2}$ of about 3.5 min and a maximal effect of about 40% are estimated. These

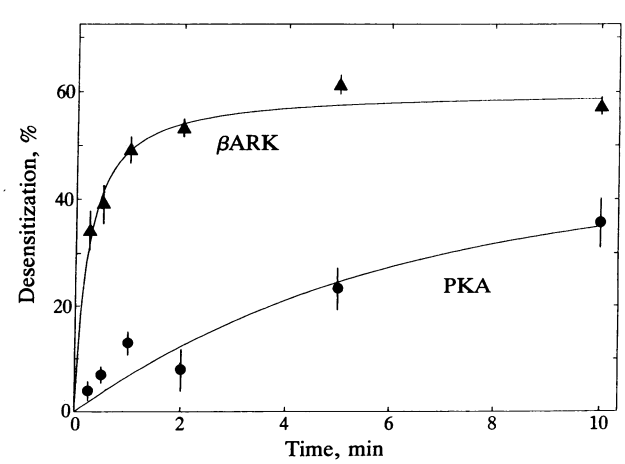

FIG. 4. Time course of βARK- and PKA-mediated desensitization in permeabilized A431 cells. The desensitization parameters, $[1 - (\tau/\tau_0)] \times 100$, were obtained from curve fitting to the data shown in Figs. 2 and 3. Maximal desensitization and $t_{1/2}$ were obtained from monoexponential curve fitting: βARK (▲), 61% and 13 sec; PKA (●), 42% and 3.5 min.

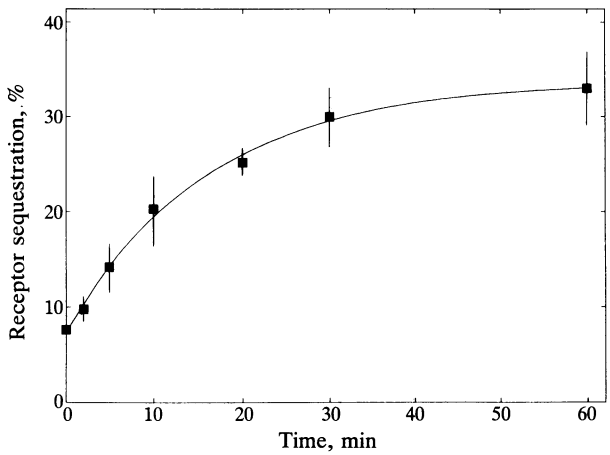

FIG. 5. Agonist-induced sequestration of β_2 receptors in A431 cells. Cells were incubated for the indicated times with 10 μM (−)-isoproterenol. Sequestered and total cellular receptors were determined. Curve fitting gave the following values: basal sequestration, 7%; maximal sequestration, 34%; $t_{1/2}$, 11 min. Data are means of four independent experiments.

kinetic values are in good agreement with those found for the phosphorylation reactions catalyzed by the two kinases (Fig. 1).

To compare these kinetics with those of receptor sequestration, we also measured the latter process in the same cells (Fig. 5). It is readily apparent that this process is much slower, with a $t_{1/2}$ of >10 min. In agreement with earlier data (8), the amount of receptor sequestration is relatively limited. Even after 60 min, only about 30% of the cell surface receptors are sequestered.

DISCUSSION

Agonist-induced desensitization of β-adrenergic receptors occurs via a complex set of mechanisms. Three separate rapid mechanisms have been delineated in earlier studies, and their quantitative contribution to the overall loss of signal-transduction capacity has been analyzed. These studies have shown that PKA-mediated desensitization occurs in response to remarkably low agonist concentrations (6, 8, 10), whereas βARK-mediated desensitization and sequestration exactly parallel the receptor occupancy curve; i.e., they are half-maximal when 50% of the receptors are agonist-occupied (8).

Apart from the agonist concentration dependence of these mechanisms, their relative rate is a second important parameter. In fact, in the presence of high agonist concentrations, such as those present in the synaptic cleft, the kinetics of these processes are far more critical than fractional receptor occupancy to determination of their relative roles. So far, very little is known about the kinetics of these three processes. Early data by Waldo *et al.* (13) indicated that functional uncoupling of receptors preceded sequestration. No differentiation between PKA- and βARK-mediated uncoupling has been reported.

The present study provides a kinetic analysis of these mechanisms. The most striking finding is the rapidity of the βARK-mediated receptor phosphorylation process, which occurs with a $t_{1/2}$ of about 15 sec. Within the experimental errors, βARK-mediated desensitization has the same time course. This suggests that the steps leading to receptor phosphorylation are the rate-limiting steps, whereas the subsequent steps, which include binding of β-arrestin, must be very rapid. PKA-mediated phosphorylation and desensitization are about 10-fold slower than the βARK-mediated processes. Again, PKA-mediated phosphorylation and desensitization have a similar time course.

In the present study, the extent of desensitization caused by βARK was somewhat higher than that caused by PKA, whereas in an earlier study using the same model, about the same maximal effect was observed (8). In a study using the approach of eliminating the respective phosphorylation sites (6), a slightly more pronounced desensitization was seen when the βARK sites were intact as compared with the PKA sites, a result that is more in line with the present findings.

In contrast to the phosphorylation reactions, receptor sequestration is a relatively slow process. In earlier experiments we have shown that the quantitative contribution of sequestration to desensitization is a minor one (8). The slow kinetics of this process cast further doubt on the hypothesis that the main role of receptor sequestration is to desensitize receptors.

Since βARK-mediated phosphorylation and desensitization require relatively high agonist concentrations, we have suggested (8) that these processes might be of particular importance for synaptic receptors, which are exposed to high concentrations of catecholamines. The high speed of these processes found in the present study is in good agreement with this hypothesis. Since catecholamine concentrations in the synaptic cleft change much more rapidly than the low, hormonal concentrations acting on nonsynaptic receptors, a rapid regulatory mechanism is required. Correspondingly, the slower PKA-mediated processes seem better suited to regulate responsiveness at nonsynaptic receptors. At synaptic receptors, PKA-mediated desensitization might provide a less volatile mechanism regulating receptor function over more extended periods of time. These hypotheses provide an emerging picture of the distinct biochemical as well as physiological roles of the different mechanisms regulating β-adrenergic receptor responsiveness.

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