Activation and Deactivation Kinetics of $\alpha_{2A}$- and $\alpha_{2C}$-Adrenergic Receptor-activated G Protein-activated Inwardly Rectifying K$^+$ Channel Currents*

Although G protein-coupled receptor-mediated signaling is one of the best studied biological events, little is known about the kinetics of these processes in intact cells. Experiments with neurons from $\alpha_{2A}$-adrenergic receptor knockout mice suggested that the $\alpha_{2A}$-receptor subtype inhibits neurotransmitter release with higher speed and at higher action potential frequencies than the $\alpha_{2C}$-adrenergic receptor. Here we investigated whether these functional differences between presynaptic $\alpha_{2A}$-adrenergic receptor subtypes are the result of distinct signal transduction kinetics of these two receptors and their coupling to G proteins. $\alpha_{2A}$- and $\alpha_{2C}$-receptors were stably expressed in HEK293 cells at moderate (~2 pmol/mg) or high (17–24 pmol/mg) levels. Activation of G protein-activated inwardly rectifying K$^+$ (GIRK) channels was similar in extent and kinetics for $\alpha_{2A}$- and $\alpha_{2C}$-receptors at both expression levels. However, the two receptors differed significantly in their deactivation kinetics after removal of the agonist norepinephrine. $\alpha_{2C}$-Receptor-activated GIRK currents returned much more slowly to base line than did $\alpha_{2A}$-stimulated currents. This observation correlated with a higher affinity of norepinephrine at the murine $\alpha_{2C}$-receptor than at the $\alpha_{2A}$-receptor subtype and may explain why $\alpha_{2C}$-adrenergic receptor subtypes are especially suited to control sympathetic neurotransmission at low action potential frequencies in contrast to the $\alpha_{2A}$-receptor subtype.

G protein-coupled receptors (GPCRs) transfer a large diversity of extracellular signals into the cell interior, including light, neurotransmitters, and hormones. Although GPCRs represent some of the best studied signaling molecules, relatively little information exists about the kinetics of signal transduction by these receptors (except for rhodopsin) in intact cells. However, more detailed knowledge about the kinetic properties of GPCR signal transduction would be of particular interest to investigators, whether these functional differences between presynaptic $\alpha_{2A}$- and $\alpha_{2C}$-receptors (1). In mouse atria, the $\alpha_{2A}$-subtype inhibited norepinephrine release at high stimulation frequencies whereas the $\alpha_{2C}$-receptor operated at lower levels of sympathetic nerve activity. Moreover, inhibition of norepinephrine release mediated by the $\alpha_{2A}$-subtype occurred much faster than inhibition by the $\alpha_{2C}$-receptor. These findings indicate that two presynaptic receptors in the inhibitory feedback loop of transmitter release may differentially regulate synaptic transmission. Several explanations may account for these functional differences. $\alpha_{2}$-Adrenergic receptor subtypes have been shown to differ in their signal transduction, agonist-dependent internalization and receptor trafficking, subcellular localization, and binding to intracellular scaffolding proteins (2–11).

$\alpha_{2}$-Adrenergic receptors are essential regulators of the sympathetic and central nervous system (12–15). Three subtypes of $\alpha_{2}$-adrenergic receptors have been identified in different species (12). Recently, transgenic mouse models lacking individual $\alpha_{2}$-receptor subtypes have been generated to define the physiological significance and therapeutic potential of these receptor subtypes (for reviews, see Refs. 16–18). $\alpha_{2}$-Receptors are essential constituents of a negative feedback loop regulating presynaptic neurotransmitter release (15). Experiments with gene-targeted mice lacking individual $\alpha_{2}$-adrenergic receptors have demonstrated that two $\alpha_{2}$-receptor subtypes, $\alpha_{2A}$ and $\alpha_{2C}$, serve as presynaptic regulators in the sympathetic nervous system and in the central nervous system (1, 19). Mice lacking both of these receptors had enhanced circulating norepinephrine levels and developed cardiac hypertrophy and failure (1).

We therefore sought a simple experimental model to investigate potential differences in the speed of $\alpha_{2A}$- and $\alpha_{2C}$-receptor-mediated signaling and the role of receptor levels for signaling kinetics. We have determined the properties of these receptor subtypes to inhibit N-type Ca$^{2+}$ channels or activate G protein-activated inwardly rectifying K$^+$ (GIRK) channels in a heterologous expression system in human embryonic kidney (HEK293) cells. We found that, at similar levels of expression, $\alpha_{2A}$- and $\alpha_{2C}$-receptors did not differ in their activation kinetics, but the deactivation after receptor stimulation by norepinephrine was significantly slower for $\alpha_{2C}$- than for $\alpha_{2A}$-receptors. This finding is consistent with a higher affinity of norepinephrine for $\alpha_{2C}$- than for $\alpha_{2A}$-receptors. Different deactivation kinetics may account for part of the functional differences between presynaptic $\alpha_{2}$-adrenergic receptor subtypes to enhance the power of synaptic regulation and plasticity.

**EXPERIMENTAL PROCEDURES**

$\alpha_{2}$-Receptor-expressing Cell Lines and Transfection Procedure—HEK293 cells were stably transfected with the murine $\alpha_{2A}$- and $\alpha_{2C}$-Received for publication, September 7, 2001
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adrenergic receptors using a pcDNA3 vector (2). After selection with 200 μg/ml G418 (Life Technologies, Inc.), stable cell clones were screened by radioligand binding and homogeneity of receptor expression was controlled by immunofluorescence (2). α2A-AR or α2C-AR-expressing cell lines were transiently transfected with a bicistronic expression vector encoding for GIRK1 and GIRK4 (kindly provided by Dr. L.Y. Jan, Howard Hughes Medical Institute, University of California, San Francisco, CA) as well as with pH3 plasmids encoding the CD8 receptors (0.15 μg/5-cm dish; kindly provided by Dr. G Yellen, Harvard University, Cambridge, MA) using the Effectene transfection kit (Qiagen, Hilden, Germany). For GIRK deactivation studies, either rat RGS4 (in pcDNA3.1) or empty pcDNA3 vector (0.5 μg/5-cm dish) was added to the transfection reaction. 18–24 h after transfection, cells were replated onto poly-L-lysine-coated cell culture dishes and were used for experiments 40–50 h after transfection. Successfully transfected cells were detected by binding Dynabeads coated with anti-CD8 antibodies (Dynal ASA, Oslo, Norway) to the cells (20). For measurement of N-type Ca2+ channels, G1A1 cells (Ref. 21; cells provided by Dr. R.J. Miller with permission from SIBIA Neuroscience, La Jolla, CA) were transiently transfected with pcDNA3-α2A or pcDNA3-α2C (1 μg/5-cm dish) and pH3-CD8 (0.15 μg/5-cm dish) using Effectene.

**Immunofluorescence Staining—**Immunofluorescence detection of α2C-adrenergic receptor was performed as described previously (2) using α2A- and α2C-adrenergic receptor subtype-specific antibodies (2, 22) and secondary anti-mouse Alexa (Molecular Probes, Leiden, Netherlands) and anti-mouse Cy2 antibodies (Dianova, Hamburg, Germany).

**Radioligand Binding—**Expression levels of α2-adrenergic receptors were determined after lysis of cells with hypotonic buffer (5 mM Tris, 2 mM EDTA, pH 7.4) as described (23). [3H]RX21002 (Amersham Pharmacia Biotech, Freiburg, Germany) was used as radioligand, and non-specific binding was determined by addition of 1 μM atipamezole (Orion Corp., Turku, Finland). For competition assays, 4 nM [3H]RX21002 was used with equal amounts of α2A- and α2C-receptors (150 fmol) (24).

**Electrophysiology—**Inward currents through GIRK channels were measured by whole cell patch recording similar to that described previously (25). Briefly, whole cell membrane currents were measured at −90 mV using K+-based and agonist-induced currents were initiated by fast superfusion of the cells using a solenoid valve operated superfusion system (26). Solution exchange occurred within less than 150 ms. Voltage ramps (from 120 mV to +60 mV in 500 ms, every 10 s) were used to determine current-voltage relationships. Barium currents through N-type Ca2+ channels were measured as described (27). Depolarization-evoked Ba2+ currents were measured every 10 s using 25-ms test pulses of +10 mV (holding potential was −90 mV). All measurements were performed at room temperature.

Patch pipettes were fabricated from borosilicate glass capillaries (GF-150-10, Harvard Apparatus, Edenbridge, United Kingdom) using a horizontal puller (P-95, Sutter Instruments, Novato, CA). The DC resistance of the filled pipettes ranged from 2 to 5 megohms. Membrane currents were recorded using either an EPC 9 patch clamp amplifier (GF-150-10, Harvard Apparatus, Edenbridge, United Kingdom) using a whole cell patch clamp technique. The time course of the activation of GIRK currents (representative current trace) by the α2-adrenergic phenylephrine were determined. Ba2+ currents through N-type Ca2+ channels were elicited every 10 s by a depolarizing voltage pulse from −90 mV to +10 mV for 25 ms. Application of the agonist 1, 2, 3, 4, 5, and 10 s prior to a voltage pulse allowed us to follow the time course of the current inhibition. For comparison both α2C-receptor-mediated responses were normalized to the maximal response.

**RESULTS**

**Ca2+ Channel Inhibition and GIRK Activation by α2-Adrenergic Receptors—**The ability of α2A-adrenergic receptor subtypes α2A and α2C to activate GIRK currents or to inhibit N-type Ca2+ currents was initially tested in HEK293 cells transiently expressing either one of the receptor subtypes and GIRK channels or in HEK cells stably expressing N-type Ca2+ channels composed of α1D/β2/αδ and α1B/δ-channel subunits (G1A1 cells; Ref. 21). Activation of both adrenergic receptors, α2A and α2C, led to inhibition of N-type Ca2+ currents (Fig. 1) and to activation of GIRK channels (Fig. 1). The time course of the phenylephrine-induced inhibition of Ba2+ currents through N-type Ca2+ channels was similar for the two α2-receptor subtypes (data not shown). In addition, the time course of the activation of GIRK currents was indistinguishable from the time course of the phenylephrine-induced inhibition of N-type Ca2+ channels (Fig. 1). However, independent of the effector system, the time courses of α2-adrenergic responses were quite variable in this transient expression system, possibly because of cell to cell variability of the expression levels of the receptors. Therefore, we chose to investigate the signaling of both receptor subtypes in stable HEK293 cell lines expressing defined amounts of receptor. From a total of 48 cell lines with −0.1–50 pmol of receptor/mg of membrane protein, four cell lines were chosen for further experiments (Fig. 2). An intermediate level (2 pmol of receptor/mg of membrane protein) and a high level (17–24 pmol of receptor/mg of membrane protein) of receptor expression were used for all experiments. Lower expression levels of α2-receptors resulted in less reproducible Ca2+ channel inhibition or GIRK channel activation, respectively (data not shown). As previous studies had indicated that α2A- and α2C-receptors may differ in their plasma membrane targeting (2, 11), all cell clones used for this study were screened by immunofluorescence staining and only clones with plasma membrane localization of α2-receptors were included for further study (Fig. 2, c and d). As the GIRK channel activation kinetics by α2-receptors had been found to be similar to the kinetics of α2-receptor-mediated inhibition of N-type Ca2+ channels, and as measurement of GIRK current activation allowed for a higher temporal resolution of the α2-response, we...
focused on these channels for detailed kinetic analysis of α2-receptor signaling.

α2-Receptor-mediated Activation of GIRK Currents—Activation of α2-receptors by norepinephrine led to a concentration-dependent increase in steady-state GIRK currents (Fig. 3). In cells expressing similar levels of receptors, the concentration-response curves were indistinguishable for the α2A- and the α2C-receptor. For both receptor subtypes, the potency for norepinephrine was closely correlated with the density of receptor expression. A 10-fold increase in receptor expression led to a similar increase in the potency of norepinephrine to activate steady-state GIRK currents. However, both parameters were not only dependent on the agonist concentration, but also correlated with the receptor expression level independent of the receptor subtype, e.g. the half-time for GIRK current activation by 10 nM norepinephrine was determined (Fig. 4). With increasing concentrations of norepinephrine (10 nM to 10 µM), the time for half-maximal GIRK activation decreased progressively. At 2 pmol/mg of α2-receptor expression, the half-times for GIRK activation were similar for α2A- and α2C-receptors at all three norepinephrine concentrations tested. Only at high receptor levels and at 10 µM norepinephrine, concentrations 104 times the EC50, the activation of GIRK currents was significantly faster when activated by α2A-receptors (218 ± 13 ms) as compared with α2C-receptors (503 ± 29 ms, p < 0.05) (Fig. 4).

The activation kinetics of norepinephrine-induced GIRK currents were not only dependent on the agonist concentration, but also correlated with the receptor expression level independent of the receptor subtype, e.g. the half-time for GIRK current activation by 10 nM norepinephrine decreased from 5.3 ± 1.1 s to 2.1 ± 0.3 s when α2A-receptor expression was increased from 2 pmol/mg to 17 pmol/mg (similar results were obtained for the α2C-receptor, Fig. 4). Thus, with the possible exception of maximal stimulation of high levels of α2-receptors, no subtype-specific differences could be identified for activation kinetics or steady-state GIRK currents. However, both parameters were greatly dependent on the level of receptor expression for the two α2-receptor subtypes.

Deactivation Kinetics of GIRK Currents—To search for subtype-specific differences in off-rates of α2-receptor-activated GIRK transients, the time course of deactivation of GIRK currents was monitored after rapid removal of norepinephrine (Fig. 5). For both α2-receptors, GIRK currents completely returned to base-line values within 60 s. However, a significant difference remained between the deactivation time courses of the two receptor subtypes; α2A-stimulated GIRK currents de-
activated 2–3.5 times faster than α2C-stimulated GIRK currents (Fig. 5, b and c). This difference was the same at high receptor levels (Fig. 5, a and b) and at moderate levels (Fig. 5c). For both receptor subtypes, the half-time for deactivation was similar at moderate and at high levels of receptor expression.

To test whether different receptor affinities for norepinephrine are responsible for the observed deactivation kinetics, competition binding experiments were performed with HEK cells expressing α2A- or α2C-receptors. The IC50 values for norepinephrine were higher at the α2A-receptor (5.03 μM) than at the α2C-subtype (0.77 μM), indicating that, indeed, norepinephrine has a higher affinity for the α2C-receptor than for the α2A-receptor (Fig. 5d).

The higher affinity of norepinephrine for the α2C-receptor compared with the α2A-receptor suggested a slower time constant for the unbinding of the agonist from the α2C-receptor. We hypothesized this phenomenon to be responsible for the slower deactivation kinetics of the α2C-receptor-mediated responses. To rule out that the slower deactivation of the α2C-adrenergic receptor was caused by a post-receptor mechanism, the regulator of G protein-signaling protein, RGS4, was transiently overexpressed in these cells (Fig. 6). RGS4 has been shown to accelerate deactivation of G proteins 100–1000-fold (28) and to substantially accelerate GIRK current deactivation after agonist removal (29, 30). If indeed the slower deactivation of α2C-receptors were the result of decreased G protein inactivation, RGS4 overexpression should lead to enhanced turn-off rates of the G protein. However, addition of RGS4 did not affect the deactivation of α2-receptor-activated GIRK currents (Fig. 6).

Interestingly, phenylephrine, an α-adrenergic receptor agonist with very low affinity, showed significantly faster deactivation of GIRK currents than norepinephrine, when used at equally potent concentrations (Fig. 6). These findings indicate that the agonist affinity rather than intrinsic receptor-G protein interaction determined the turning off rates of the α2-receptor evoked responses.

**DISCUSSION**

Our study represents the first in which activation and deactivation of G protein signaling in intact cells were determined in dependence of defined amounts of receptor expression. No subtype-dependent differences were found in steady-state GIRK currents and their activation kinetics between α2A- and α2C-receptors expressed at moderate or at high levels in HEK293 cells. However, for both receptor subtypes, a 10-fold increase in receptor expression was correlated with a 2.5–3-fold acceleration of the GIRK current activation for both receptor subtypes.

Most interestingly, α2A- and α2C-adrenergic receptors differed significantly in their deactivation kinetics. This finding may explain part of the functional differences between the two α2-receptor subtypes, which have been identified in mouse sympathetic neurons in vitro (1). At high stimulation frequencies of isolated mouse atria, norepinephrine release was primarily controlled by the α2A-receptor subtype, whereas at lower frequencies the α2C-receptor was the principle regulator of transmitter release (1).

Deactivation of norepinephrine-activated GIRK currents was significantly faster for the α2A- than for the α2C-receptor. This finding is consistent with the higher affinity (and thus slower dissociation kinetics) of norepinephrine for the α2C-subtype than for the α2A-receptor subtype (31). Deactivation of the α2C-receptor-induced GIRK currents occurred with faster kinetics, when the low affinity agonist phenylephrine was used instead of the endogenous agonist, norepinephrine. In addition, enhancing the rate of G protein inactivation by overexpressing RGS4 in these cells did not alter GIRK deactivation. Taken together, these results indicate that agonist dissociation rather than receptor-G protein interaction or a post-receptor effect was responsible for the slower turn-off rate of the norepinephrine-activated α2C-receptor.

The finding of similar activation kinetics is in contrast to in vitro observations in intact sympathetic neurons where endogenous α2A-receptors can inhibit presynaptic transmitter release significantly faster than α2C-receptors (1). Several possibilities remain to explain this discrepancy between the HEK293 cell data of this study and the situation in intact neurons. Expression levels may differ between α2-receptor subtypes. Indeed, in the central nervous system, 80% of α2-adrenergic receptors belong to the α2C-subtype, whereas only 10% are α2A-receptors (32). The α2C-receptor is restricted to very few brain nuclei and thus does not contribute significantly to total brain α2-receptor binding. However, these data are based on radioligand binding experiments in membrane homo-

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**Fig. 5.** Deactivation kinetics of α2-adrenergic receptor-activated GIRK currents after removal of norepinephrine. GIRK currents were first activated via α2A- or α2C-receptors by superfusing the cells with norepinephrine until reaching a steady state, and the time course of GIRK current deactivation was then measured in response to rapid washout of norepinephrine (a, representative recordings). The times until reaching half-maximal deactivation were summarized (b) and were expressed as means ± S.E. (n = 5–8). c, norepinephrine displaced the α2-receptor antagonist, [3H]RX821002, at lower concentrations from α2C- than from α2A-receptors (Kd for α2A, 5.0 μM; for α2C, 0.8 μM).
Selective targeting of α2C-receptor subtypes to specific membrane domains has been shown in a number of different cell types (2, 9, 11). Whereas α2A-receptors were always targeted to the plasma membrane, α2C-receptors were found in addition in an intracellular membrane compartment (22). Specific targeting of α2A* and α2C-receptors to distinct plasma membrane domains in intact neurons may account for differences in signaling kinetics because of their distance from the neurotransmitter release site or because of specific interaction with distinct intracellular messenger systems. Different modulatory pathways have been implicated in presynaptic inhibition of neurotransmission by G protein-coupled receptors (33), which also differ in their signaling kinetics. In sympathetic neurons norepinephrine, via Go, slowed the kinetics of Ca2+ current activation and a second, sustained reduction in Ca2+ current, further studies are required to test whether indeed α2A* and α2C-receptors in intact neurons in vivo differ in their subcellular localization and/or intracellular signaling pathways. These studies demonstrate that subtype-specific differences in receptor signaling kinetics may be important avenues for future study to further understand the functional significance of GPCR subtype diversity.

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FIG. 6. Receptor rather than G protein deactivation limits deactivation kinetics of α2C-adrenergic receptor-activated GIRK currents after removal of norepinephrine. GIRK currents in cells expressing α2C-receptors were determined (see representative current recording in a) and summarized data shown in b; n = 7–8). In addition, the effect of coexpression of RGS4 on the deactivation kinetics of currents activated by 10 nM norepinephrine was analyzed (n = 4).
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