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Differential Control of Adrenal and Sympathetic Catecholamine Release by α_2-Adrenoceptor Subtypes

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In the adrenergic system, release of the neurotransmitter norepinephrine from sympathetic nerves is regulated by presynaptic inhibitory α_2-adrenoceptors, but it is unknown whether release of epinephrine from the adrenal gland is controlled by a similar short feedback loop. Using gene-targeted mice we demonstrate that two distinct subtypes of α_2-adrenoceptors control release of catecholamines from sympathetic nerves (α_{2A}) and from the adrenal medulla (α_{2C}). In isolated mouse chromaffin cells, α_2-receptor activation inhibited the electrically stimulated increase in cell capacitance (a correlate of exocytosis), voltage-activated Ca^{2+} current, as well as secretion of epinephrine and norepinephrine. The inhibitory effects of α_2-agonists on cell capacitance, voltage-activated Ca^{2+} currents, and on catecholamine secretion were completely abolished in chromaffin cells isolated from α_{2C}-receptor-deficient mice. *In vivo*, deletion of sympathetic or adrenal feedback control led to increased plasma and urine norepinephrine (α_{2A}-knockout) and epinephrine levels (α_{2C}-knockout), respectively. Loss of feedback inhibition was compensated by increased tyrosine hydroxylase activity, as detected by elevated tissue dihydroxyphenylalanine levels. Thus, receptor subtype diversity in the adrenergic system has emerged to selectively control sympathetic and adrenal catecholamine secretion via distinct α_2-adrenoceptor subtypes. Short-loop feedback inhibition of epinephrine release from the adrenal gland may represent a novel therapeutic target for diseases that arise from enhanced adrenergic stimulation. (*Molecular Endocrinology* 17: 1640–1646, 2003)

ADRENOCEPTORS FORM THE interface between the endogenous catecholamines, epinephrine and norepinephrine, and a wide array of target cells in the body to mediate the biological effects of the sympathetic nervous system. To date, nine different adrenoceptor subtypes have been identified (three α_1, three α_2, three β receptors) (1). Transgenic mouse models have recently become very valuable in the identification of the physiological significance of individual adrenergic receptor subtypes *in vivo* (2–7).

The adrenergic system can be divided into three parts: adrenergic neurons in the central nervous system, sympathetic neurons that innervate many organs and tissues, and the adrenal medulla. Norepinephrine is the principal neurotransmitter of sympathetic neurons, whereas epinephrine, and to a smaller degree also its precursor norepinephrine, is secreted from the chromaffin cells of the adrenal medulla into the circulation (8). Thus, the actions of norepinephrine are mostly restricted to the sites of release from sympathetic nerves, whereas epinephrine acts as a hormone to activate cardiovascular and metabolic responses via the blood stream (9). As adrenal chromaffin cells and sympathetic neurons are both derived from a common neural crest precursor (10), it may be hypothesized that both cell types share similar patterns of regulation, including feedback control of neurotransmitter release.

Norepinephrine released from sympathetic nerves not only activates adrenoceptors on effector cells, but also stimulates presynaptic adrenoceptors to inhibit further transmitter secretion (11, 12). α_{2A}- and α_{2C}-adrenergic receptors were previously identified as presynaptic inhibitory receptors that control the release of norepinephrine from peripheral sympathetic nerves and central catecholaminergic neurons *in vitro* (13–15). Furthermore, the deletion of the α_{2A}- and α_{2C}-adrenergic receptor subtypes caused increased susceptibility to development of heart failure after chronic pressure overload *in vivo* (16). In contrast, it is unknown at present whether secretion of catecholamines from the adrenal medulla is under similar feedback control. To assess the existence and physiological relevance of feedback regulation in the two limbs of the sympathetic system, we used mice lacking individual α_2-adrenoceptor subtypes (14, 17). Here we demonstrate for the first time that two distinct α_2-receptor subtypes control sympathetic nerve (α_{2A}) and adrenal (α_{2C}) catecholamine release and that autocrine feedback reg-

Abbreviations: $[Ca^{2+}]_i$, Intracellular calcium concentration; L-dopa, 3-(3,4-dihydroxyphenyl)-L-alanine; α_2-KO, α_2-receptor knockout; WT, wild-type.

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ulation of epinephrine secretion in the adrenal gland is necessary to control circulating epinephrine levels.

RESULTS AND DISCUSSION

Regulation of Plasma and Urine Catecholamine Levels by α_2-Adrenoceptors

Norepinephrine and epinephrine levels were determined in the plasma of anesthetized wild-type (WT) and α_2-receptor knockout (α_2-KO) mice. Surprisingly, plasma catecholamines were differentially affected by deletion of the different α_2-receptor subtypes (Fig. 1). Deletion of the α_{2A}-receptor led to increased plasma levels of norepinephrine whereas epinephrine concentrations were selectively elevated in α_{2C}-receptor-deficient mice (Fig. 1B). These findings were confirmed in urine samples collected over a period of 24 h in awake, freely moving animals (Fig. 1, C and D). Excretion (24 h) of norepinephrine was increased by 155% in α_{2A}-KO mice and epinephrine excretion was enhanced by 95% in α_{2C}-KO animals. Urine volume did not differ between WT and α_{2B}- or α_{2C}-KO mice, but was significantly elevated in α_{2A}-KO mice ($+95 \pm 15\%$, α_{2A}-KO *vs.* WT; $P < 0.05$).

These observations suggest that secretion of catecholamines from peripheral sympathetic nerves and from the adrenal medulla is differentially regulated by α_{2A}- and α_{2C}-adrenoceptors (Fig. 1). In the peripheral nervous system, epinephrine is exclusively synthesized by adrenal chromaffin cells, whereas sympathetic nerves are the primary source of circulating norepinephrine (9). Whereas α_2-mediated feedback inhibition of sympathetic norepinephrine release has been well documented (for recent reviews, see Refs. 2, 18, and 19), it is unclear whether epinephrine secretion from adrenal chromaffin cells is subject to similar feedback regulation. Early reports suggested that high concentrations of circulating epinephrine can inhibit the secretion of catecholamines from perfused dog adrenals (20, 21), but studies on isolated adrenal chromaffin or pheochromocytoma cells have yielded conflicting results (22–25). Under certain experimental conditions, the nonselective α-receptor antagonist, phenoxybenzamine, increased release of catecholamines from rat adrenal gland *in vivo* (26). However, it was suggested that this effect was due to activation of inhibitory α-adrenoceptors on cholinergic nerve endings rather than by direct effects on chromaffin cells (12).

Expression of α_{2C}-Adrenoceptors in Adrenal Chromaffin Cells

Before investigating the function of isolated mouse chromaffin cells, we analyzed adrenal histology and expression patterns of α_2-adrenoceptor subtypes. Adrenal glands from male α_{2C}-receptor-deficient mice were significantly larger than organs from WT or α_{2A}- or α_{2B}-KO mice [α_{2C}-KO adrenal weight: $+31 \pm 8\%$ *vs.* WT; $P < 0.05$; α_{2C}-KO, n = 14; WT, n = 12]. Cross-sections of adrenal glands did not reveal any structural defects in specimens from α_2-receptor-deficient mice (Fig. 2), except for mild hypertrophy of chromaffin cells from α_{2C}-KO mice (Fig. 2C; cross-sectional area $+28 \pm 5\%$, α_{2C}-KO *vs.* WT). In isolated chromaffin cells from WT mice, only α_{2C}-receptor mRNA expression could be readily detected (Fig. 2D). α_{2A}- or α_{2B}-receptor mRNA could not be amplified reproducibly from chromaffin cells that were either isolated from WT mice or from α_{2C}-receptor-deficient animals (Fig. 2D). Thus, genetic deletion of the α_{2C}-

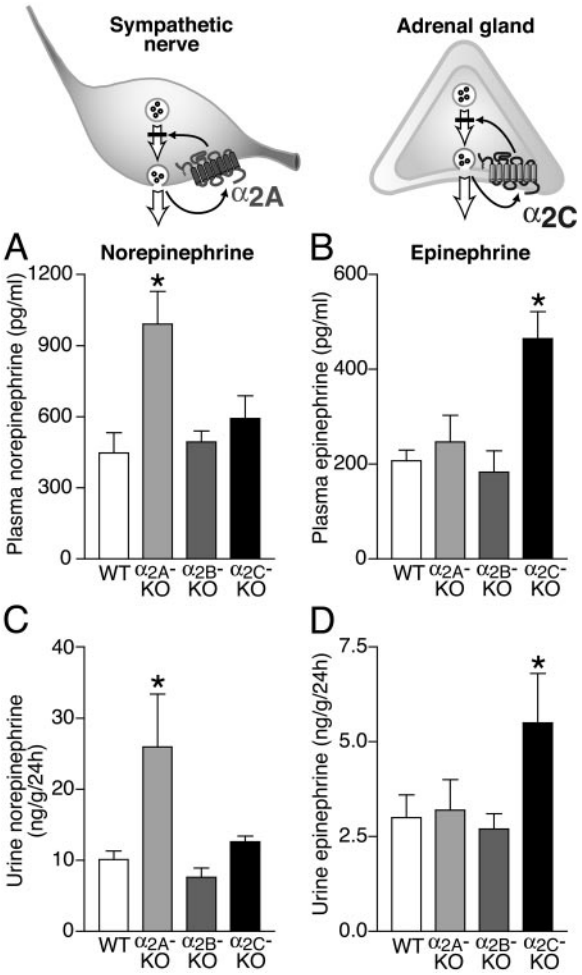

Fig. 1. Plasma and Urine Catecholamine Levels in α_2-Adrenoceptor-Deficient Mice

Norepinephrine (A and C) was found to be elevated in blood plasma (obtained under anesthesia) and in urine (collected in metabolic cages over 24 h) from mice lacking α_{2A}-adrenoceptors as compared with WT mice. In contrast, epinephrine levels were significantly higher in α_{2C}-adrenoceptor-deficient mice when compared with the other genotypes (B and D). These results suggest that α_{2A}- and α_{2C}-receptors differentially control catecholamine release from sympathetic nerves and from adrenal chromaffin cells, respectively (*schematic drawing*). *, $P < 0.05$, KO *vs.* WT, means ± SEM, n = 13–25.

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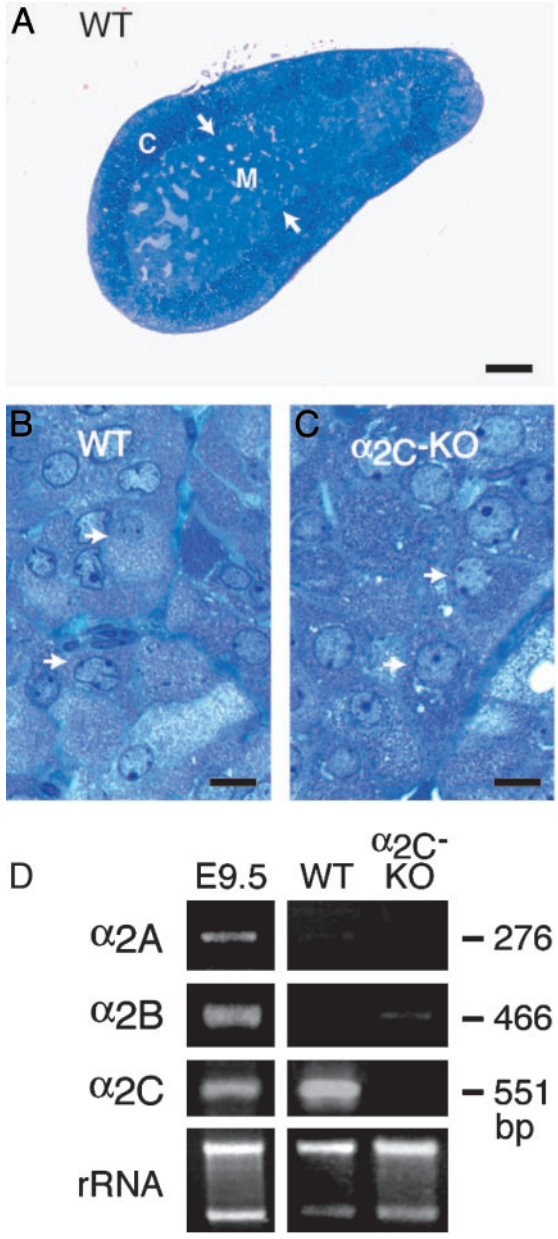

Fig. 2. Adrenal Gland Morphology and α_2-Adrenoceptor Expression in WT and α_{2C}-Adrenoceptor-Deficient Mice

Cross-section through the adrenal gland of a male WT mouse (A) and high-magnifications of adrenal medulla chromaffin cells of WT (B) or α_{2C}-KO (C) mice. Chromaffin cells in the adrenal cortex of α_{2C}-KO mice showed normal morphology but appeared enlarged by 28% in their cross-sectional area as compared with WT cells (B *vs.* C). C, Adrenal cortex; M, adrenal medulla; *arrow*, border between adrenal cortex and medulla. *Bars,* 300 μm (A); 5 μm (B and C). D, Isolated chromaffin cells from WT mice expressed α_{2C}-adrenoceptors as detected by RT-PCR but no α_{2A}- or α_{2B}-adrenoceptors. In α_{2C}-deficient chromaffin cells, no compensatory expression of α_{2A}- or α_{2B}-receptor was detectable. As a possible control, RT-PCR signals for α_{2A}-, α_{2B}-, and α_{2C}-receptors from WT mice at embryonic day 9.5 (E9.5) are shown (42). No signals were observed in the absence of reverse transcriptase (not shown). *Lower panel*, Ethidium bromide-stained gel of 18S and 28S RNA after extraction from cells.

receptor gene was not accompanied by a compensatory up-regulation of α_{2A}- or α_{2B}-receptor expression. In the human adrenal gland, α_{2C}-adrenoceptors are also the predominating α_2-receptor subtype (27, 28).

Tissue epinephrine and norepinephrine levels did not differ in adrenal glands from WT and α_2-adrenoceptor-deficient mice (data not shown). However, 3-(3,4-dihydroxypheny)-L-alanine (L-dopa) content was significantly elevated in α_{2C}-KO adrenal glands as compared with WT adrenals (Fig. 3A), reflecting an increased activity of tyrosine hydroxylase which catalyzes the initial rate-limiting step of catecholamine biosynthesis (9). Similarly, L-dopa levels were elevated in cardiac tissue from mice lacking the α_{2A}-receptor subtype (Fig. 3B), confirming that increased catecholamine release from adrenal chromaffin cells or sympathetic nerves causes a compensatory increase in catecholamine biosynthesis.

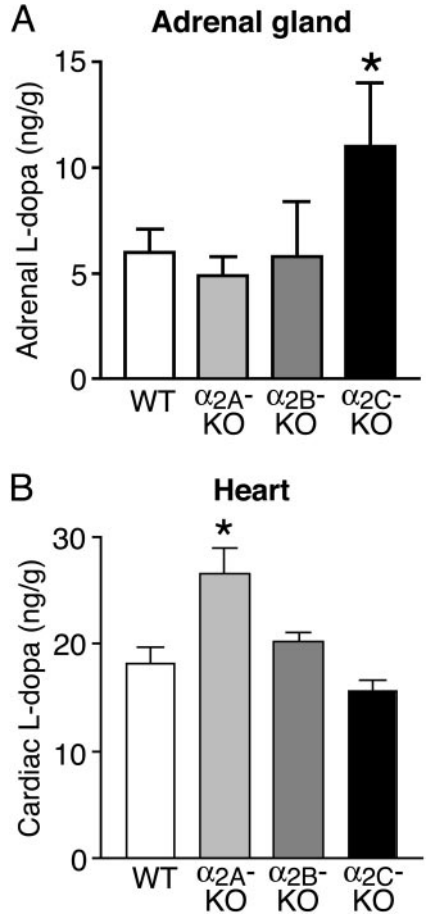

Fig. 3. Elevated Tissue L-dopa Levels in α_2-Adrenoceptor-Deficient Mice

Adrenal gland (A) and cardiac (B) concentrations of L-dopa were determined as an indicator of tyrosine hydroxylase activity. Adrenal L-dopa levels were selectively elevated in mice lacking α_{2C}-adrenoceptors, whereas L-dopa in the heart was significantly increased only in α_{2A}-KO mice. *, $P < 0.05$ KO *vs.* WT; n = 6–8 animals per genotype; means ± SEM.

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In Vitro Regulation of Exocytosis and Ca^{2+} Currents in Isolated Chromaffin Cells

To test whether in the mouse, adrenal α_2-adrenoceptors can, in fact, inhibit catecholamine secretion, effects of α_2-adrenergic receptor stimulation by norepinephrine and the non-subtype-selective α_2-receptor agonist UK14,304 on isolated mouse chromaffin cells were investigated (Fig. 4). Using whole-cell capacitance measurements, we found that depolarization led to an increase in cell capacitance due to fusion of release-competent secretory vesicles with the plasma membrane in WT chromaffin cells (Fig. 4A). This capacitance increase could be significantly attenuated by the α_2-receptor agonist UK14,304 (Fig. 4, B and F) or by norepinephrine (data not shown). Furthermore, UK14,304 decreased peak Ca^{2+} currents in WT chromaffin cells (Fig. 4, D and G). Without α_2-receptor stimulation, depolarization-induced increases in cell capacitance and voltage-activated calcium currents did not differ between cells isolated from WT or α_{2C}-deficient mice (Fig. 4, F and G). However, the inhibitory effects of the α_2-agonist UK14,304 on both parameters were completely abolished in cells from α_{2C}-deficient mice. Thus, α_2-adrenergic receptor mediated inhibition of catecholamine secretion from chromaffin

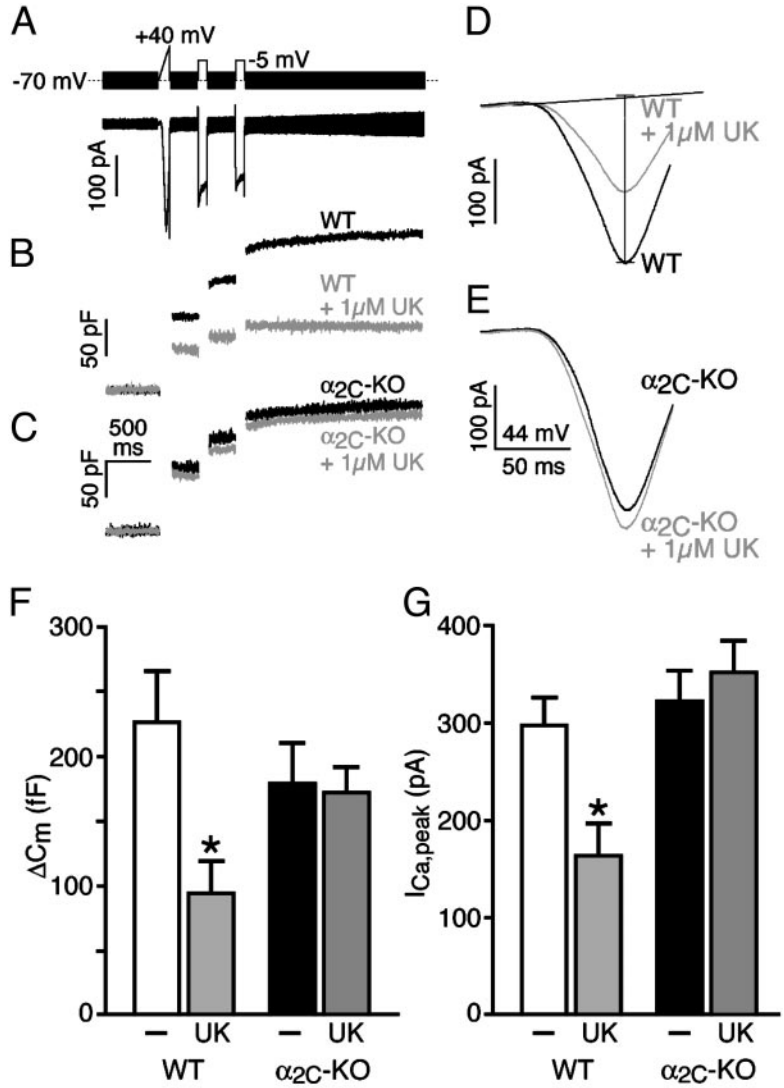

Fig. 4. Inhibition of Exocytosis and Voltage-Activated Calcium Currents by α_{2C}-Adrenoceptors in Mouse Adrenal Chromaffin Cells

Voltage protocol applied to stimulate secretion (A, *upper panel*) and a typical voltage clamp recording (A, *lower panel*). Average capacitance traces (B and C) and average Ca^{2+} currents evoked by ramp depolarization (D and E). UK, α_2-Agonist UK14,304. In isolated WT mouse chromaffin cells, the α_2-agonist UK14,304 inhibited the depolarization-evoked capacitance increase (B and F) as well as the peak Ca^{2+} current (D and G). The inhibitory effect of the α_2-agonist on cell capacitance and calcium currents was completely abolished in chromaffin cells from α_{2C}-receptor-deficient mice (C, E, F, and G). *, $P < 0.01$, means ± SEM (WT, n = 20; WT + UK, n = 19; KO, n = 22; KO + UK, n = 22).

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cells likely proceeds by a Ca^{2+}-dependent process that is similar to the feedback inhibition observed in adrenergic nerve terminals.

The basal (prestimulus) intracellular calcium concentration $[Ca^{2+}]_i$ regulates the number of fusion-competent vesicles (29, 30) and may also modify the size of calcium currents due to the resulting differences in driving force for calcium influx. To rule out the unlikely possibility that the differences seen between different genotypes in chromaffin cells were due to differences in basal $[Ca^{2+}]_i$ we monitored $[Ca^{2+}]_i$ during the experiments described above by introducing the calcium-sensitive dye fura-2 into the cells through the patch pipette. Basal cytosolic calcium concentrations did not differ between genotypes and were not affected by α_2-receptor stimulation ($[Ca^{2+}]_i$ WT: 145 ± 26 nM; WT + UK14,304: 173 ± 20 nM; α_{2C}-KO: 147 ± 23 nM; α_{2C}-KO + UK14,304: 135 ± 22 nM).

α_{2C}-Adrenergic Receptors Inhibit Adrenal Secretion of Epinephrine and Norepinephrine

Acetylcholine-stimulated secretion of epinephrine could be inhibited by the α_2-agonist UK14,304 in chromaffin cells from WT, α_{2A}-KO, and α_{2B}-KO mice but not in cells from α_{2C}-deficient mice (Fig. 5). Similarly, the secretion of norepinephrine from isolated chromaffin cells was inhibited by an α_2-agonist in WT cells but not in α_{2C}-receptor-deficient cells (not shown). In WT chromaffin cells, epinephrine secretion induced by 80 mM K^+-mediated depolarization was inhibited by the α_2-agonist UK14,304 by 72 ± 9%. Basal catecholamine release and the degree of acetylcholine-induced stimulation of release did not differ between WT and α_2-KO chromaffin cells (not shown). These findings demonstrate that in isolated chromaffin cells as in the adrenal, the α_{2C}-adrenergic receptor subtype is the dominant regulator of catecholamine secretion. Due to their slower deactivation kinetics and higher affinity for catecholamines (31), α_{2C}-receptors may be ideal receptors to sense and control the hormone epinephrine, whereas low-affinity α_{2A}-receptors act as fast inhibitory regulators at the sympathetic nerve terminals (14, 32). Taken together, these results not only confirm that the α_{2A}-subtype is the predominant receptor involved in the presynaptic regulation of norepinephrine from sympathetic nerves, but also definitely establish that the α_{2C}-adrenergic receptor is an essential feedback regulator of epinephrine release from the adrenal gland.

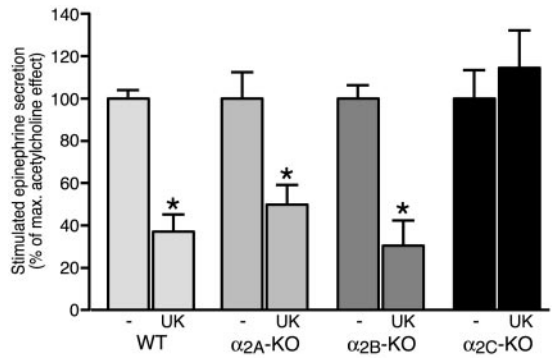

Fig. 5. Inhibition of Epinephrine Secretion by α_{2C}-Adrenoceptors in Mouse Adrenal Chromaffin Cells

Acetylcholine-induced epinephrine secretion from WT or α_{2A}-KO and α_{2B}-KO chromaffin cells was inhibited by the α_2-agonist UK14,304. In chromaffin cells from α_{2C}-KO mice, no effect of the α_2-receptor agonist was observed (n = 4–6). *, $P < 0.05$, agonist *vs.* control; means ± SEM.

The regulatory mechanism by which α_{2C}-receptors control epinephrine secretion from chromaffin cells includes, at least, inhibition of calcium channels. The pathway is therefore most likely similar to the feedback mechanism that operates in adrenergic neurons: α_{2C}-adrenoceptors that are localized in the plasma membrane activate inhibitory G proteins ($G_{i/o}$). $\beta\gamma$-Subunits released from G proteins may directly inhibit voltage-gated calcium channels (41). However, further studies are required to test whether additional intracellular pathways contribute to α_{2C}-receptor-mediated feedback regulation of epinephrine secretion. For example, in the present study we have not addressed the question of whether vesicle maturation (*e.g*. the number of fusion-competent vesicles) may also be regulated by α_{2C}-receptor-mediated feedback.

Conclusions

This is, to our knowledge, the first report demonstrating that α_{2C}-adrenoceptors control secretion of the stress hormone epinephrine via an autocrine feedback loop in adrenal chromaffin cells and that genetic disruption of this feedback mechanism leads to enhanced blood levels of epinephrine.

The paradigm for endocrine feedback control is the hypothalamic-pituitary system, which releases tropic hormones to control peripheral endocrine glands, including the gonads, thyroid gland, and adrenal cortex. The conventional view is that hormones produced in the peripheral organs (*e.g.* glucocorticoids, thyroid hormone) feed back on the release of the hypothalamic-pituitary tropic hormones (*e.g.* CRH, TRH) via a long feedback circuit. However, recent experimental evidence suggests that, even in these systems, ultrashort feedback loops operate to control the tropic hormones, *e.g*. TRH inhibits its own synthesis (33). Ultrashort feedback control of GnRH may actually be required for pulsatile secretion of GnRH release from hypothalamic neurons (34). These examples illustrate that short and long feedback circuits operate together in the regulation of endocrine secretion. In this regulatory network, short feedback loops may have greater physiological and pathophysiological relevance than previously appreciated. Thus, α_{2C}-adrenoceptors may represent novel therapeutic targets to attenuate or prevent the development of diseases that arise from overactivity of the adrenergic system.

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MATERIALS AND METHODS

α_2-Adrenoceptor-Deficient Mice

The generation of the mouse lines lacking α_2-adrenoceptor subtypes has been described previously (13, 17, 35). All animal procedures were approved by the responsible university and government authorities (Protocol No. 621-2531.01-28/01).

Catecholamine Determination

Catecholamines were measured in plasma obtained from tribromoethanol-anesthetized mice, from urine samples that were collected over a 24-h period in metabolic cages, or from the supernatant of chromaffin cells cultured *in vitro*. Adrenal and cardiac catecholamine levels were determined after extraction as described previously (13). Epinephrine, norepinephrine, and L-dopa were quantified by HPLC combined with electrochemical detection (14).

Chromaffin Cells

To obtain chromaffin cells, adrenal glands from 8- to 12-wk-old mice were incubated in Locke's solution containing 1 mg/ml collagenase (>200 U/ml; Biochrom KG, Berlin, Germany) (36). Cells were resuspended in HEPES-buffered Tyrode's solution (for determination of catecholamine release) or in enriched DMEM and plated on glass coverslips (for electrophysiological experiments). Enriched DMEM (Linaris, Wertheim, Germany) contained: 2.2 g/liter $NaHCO_3$, 4.5 g/liter D-glucose, 1.028 g/liter L-glutamine, 10 ml/liter insulin-transferrin-selenium-X (Life Technologies, Inc., Gaithersburg, MD), penicillin and streptomycin Life Technologies, Inc.). For detection of α_2-adrenoceptor expression, RNA was isolated from 100–150 chromaffin cells using the RNAqueous 4PCR kit (Ambion, Austin, TX). RT-PCR was performed with 0.2 μg of RNA as described previously (35, 37, 38).

Electrophysiological Measurements and Determination of $[Ca^{2+}]_I$

The bath solution contained (in mM): 145 NaCl, 2.8 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 mg/ml D-glucose (pH 7.2) (osmolarity, 310 mosmol/liter). After the whole-cell patch was established, the cell was perfused locally with a solution containing: 25 tetraethylammonium chloride, 48 Na-gluconate, 60 Na-acetate, 2.8 K-gluconate, 10 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1 mg/ml D-glucose, 600 nM tetrodotoxin (pH 7.2) (osmolarity was adjusted to 310 mosmol/liter). The pipette solution contained: 70 Cs-glutamate, 30 CsCl, 8 NaCl, 0.125 $CaCl_2$, 32 HEPES, 2 MgATP, 0.3 GTP, 0.5 fura-2 (Molecular Probes, Eugene, OR) (pH 7.2) (osmolarity was adjusted to 300 mosmol/liter). The calculated free $[Ca^{2+}]_i$ was approximately 80 nM.

Conventional whole-cell recordings were performed at 30 C with sylgard-coated 3–5 MΩ pipettes (Kimax-51; Kimble Products, Vineland, NY) 1–2 d after cell preparation. Access resistance ranged from 5–20 MΩ. An EPC-9 patch clamp amplifier was used together with the Pulse software package (HEKA Electronics, Lambrecht, Germany). Capacitance measurements were performed using the Lindau-Neher technique as described previously (39). A 1000-Hz, 70-mV peak-to-peak sinusoid voltage stimulus was superimposed onto a direct current holding potential of minus 70 mV. Currents were filtered at 3 kHz and sampled at 12 kHz. A triple depolarization voltage protocol was applied 2 min after the whole-cell configuration was established and after 2-min local perfusion. Peak current was estimated from the first (ramp) depolarization after leak substraction as indicated in Fig. 2A, *right panel*. Cell size, leak current, and access resistance were not different between the groups (data not shown). Fluorescence measurements were conducted as described previously (40).

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M.B. and G.N. have contributed equally to this manuscript.

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