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Protean Agonism at α_{2A}-Adrenoceptors

CHRISTIAN C. JANSSON, JYRKI P. KUKKONEN, JOHNNY NÄSMAN, GE HUIFANG, SIEGFRIED WURSTER, RAIMO VIRTANEN, JUHA-MATTI SAVOLA, VIC COCKCROFT, and KARL E. O. ÅKERMAN

Department of Biochemistry and Pharmacy (C.C.J., J.P.K., G.H.), Turku Centre for Biotechnology (J.P.K.), Åbo Akademi University, Turku, Finland, Department of Pharmacology and Clinical Pharmacology, University of Turku, Turku, Finland (C.C.J., G.H., S.W.), Department of Physiology and Medical Biophysics, Uppsala University, Uppsala, Sweden (J.P.K., K.E.O.Å, J.N.), and Orion-Corporation, Orion-Pharma, Turku, Finland (S.W., R.V., J.-M.S., V.C.)

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ABSTRACT

The coupling of the endogenously expressed α_{2A}-adrenoceptors in human erythroleukemia cells (HEL 92.1.7) to Ca^{2+} mobilization and inhibition of forskolin-stimulated cAMP production was investigated. The two enantiomers of medetomidine [(±)-[4-(1-[2,3-dimethylphenyl]ethyl)-1*H*-imidazole]HCl] produced opposite responses. Dexmedetomidine behaved as an agonist in both assays (i.e., it caused Ca^{2+} mobilization and depressed forskolin-stimulated cAMP production). Levomedetomidine, which is a weak agonist in some test systems, reduced intracellular Ca^{2+} levels and further increased forskolin-stimulated cAMP production and therefore can be classified as an inverse agonist. A neutral ligand, MPV-2088, antagonized responses to both ligands. Several other, chemically diverse α_2-adrenergic ligands also were tested. Ligands that could promote increases in Ca^{2+} levels and inhibition of cAMP production could be classified as full or partial agonists. Their effects could be blocked by the α_2-adrenoceptor antagonist rauwolscine and by pertussis toxin treatment. Some typical antagonists such as rauwolscine, idazoxan, and atipamezole had inverse agonist activity like levomedetomidine. The results suggest that the α_{2A}-adrenoceptors in HEL 92.1.7 cells exist in a precoupled state with pertussis toxin-sensitive G proteins, resulting in a constitutive mobilization of intracellular Ca^{2+} and inhibition of cAMP production in the absence of agonist. This constitutive activity can be antagonized by inverse agonists such as levomedetomidine and rauwolscine. Levomedetomidine can be termed a "protean agonist" because it is capable of activating uncoupled α_2-adrenoceptors in other systems and inhibiting the constitutive activity of precoupled α_2-adrenoceptors in HEL 92.1.7 cells. With this class of compounds, the inherent receptor "tone" could be adjusted, which should provide a new therapeutic principle in receptor dysfunction.

Many G protein-coupled receptors undergo spontaneous activation in the absence of agonist (Allen *et al.*, 1991; Lefkowitz *et al.*, 1993; Barker *et al.*, 1994; Samama *et al.*, 1994; Tian *et al.*, 1994; Tiberi and Caron, 1994; Milligan *et al.*, 1995). Basal activity has previously been detected in cells and in membrane preparations from cells endogenously expressing opioid receptors (Costa and Hertz, 1989). In experimental systems, spontaneous activity can be achieved through receptor mutation, overexpression, or G protein overexpression (Lefkowitz *et al.*, 1993; Milligan *et al.*, 1995) as has been shown for adrenergic, dopaminergic, and 5-hydroxytryptamine receptors (Allen *et al.*, 1991; Ren *et al.*, 1993; Barker *et al.*, 1994; Samama *et al.*, 1994; Tiberi and Caron, 1994; Chidiac *et al.*, 1996). The spontaneous receptor activity can be demonstrated using antagonists that produce an inverse response. Two types of antagonists are found, neutral antagonists and negative antagonists (Costa and Hertz, 1989). Antagonists with negative activity have also been termed inverse agonists. A new class of G protein-coupled receptor ligands called "protean agonists" has been proposed on theoretical grounds. Depending on the basal

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ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; IBMX, 3-isobuthyl-1-methyl-xanthine; TES, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid; TBM, TES-buffered medium; clonidine, 2-(2,6-dichloroaniline)-2-imidazoline HCl; guanabenz, 1-(2,6-dichlorobenzylideneamino)guanidine); guanfacine, *N*-(aminoiminomethyl)-2,6-dichlorobenzenacetamide; idazoxan, (±)-2-(1,4-benzodioxan-2-yl)-2-imidazoline HCl; oxymetazoline, (3-[4,5-dihydro-1*H*-imidazol-2-yl]-methyl)-6-(1,1-dimethylethyl)-2,4-dimethylphenol HCl; propranolol, (±)-1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol HCl; naphazoline, 4,5-dihydro-2-(1-naphthalenylmethyl)-1*H*-imidazole; xylazine, *N*-(2,6-dimethylphenyl)-5,6-dihydro-4*H*-1,3-thiazin-2-amine HCl; rauwolscine, 17α-hydroxy-20α-yohimban-16β-carboxylic acid methyl ester HCl; tizanidine, 5-chloro-4-(2-imidazolin-2-yl-amino)-2,1,3-benzothiadiazole; atipamezole, 4(5)-(2-ethyl-indan-2-yl)imidazole HCl; detomidine, 4(5)-(2,3-dimethylbenzyl)imidazole]HCl; medetomidine, (±)-[4-(1-[2,3-dimethylphenyl]ethyl)-1*H*-imidazole]HCl; dexmedetomidine, (+)-(*S*)-4-(1-[2,3-dimethylphenyl]ethyl)-1*H*-imidazole]HCl; levomedetomidine (−)-(*R*)-4-(1-[2,3-dimethylphenyl]ethyl)-1*H*-imidazole HCl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

activity of the receptor, these should either act as agonists or inverse agonists (Kenakin, 1995). The rationale put forward by Kenakin (1995) has been that a protean agonist is a weak agonist (i.e., having a low efficacy). The basal receptor activity produces an active receptor state (R*) that competes with the ligand-induced active state (AR*) for the G protein. As AR* has a lower affinity for the G protein than R* a factual decrease (i.e., inverse agonism by the ligand) will be observed when there is a substantial basal formation of the R*. In the absence of a marked spontaneous production of R*, a protean agonist will act like a normal agonist. The results of Chidiac *et al*. (1996) have recently shown the existence of protean agonists in practice.

The HEL 92.1.7 cell line shows robust increases in $[Ca^{2+}]_i$ (Michel *et al*., 1989; Musgrave and Seifert, 1995), as well as inhibition of adenylyl cyclase in response to agonist stimulation of the endogenous α_{2A}-adrenoceptor (McKernan *et al*., 1987). Both responses are sensitive to pertussis toxin and hence mediated by $G_{i/o}$-type G protein. In this study, precoupling of the receptors was probed by measurement of changes in $[Ca^{2+}]_i$ and cAMP levels in response to a variety of α_2-adrenoceptor ligands.

Materials and Methods

Drugs

[^{3}H]Adenine and [^{14}C]cAMP were from Amersham (Buckinghamshire, UK). (−)-Epinephrine, clonidine, desipramine (10,11-dihydro-*N*-methyl-5*H*-dibenz[*b*,*f*]azepine-5-propanamide), guanabenz, guanfacine, idazoxan, IBMX, naphazoline, (−)-norepinephrine, prazosin, oxymetazoline, pertussis toxin, propranolol, quinacrine (6-chloro-9-[(4-diethylamino)-1-methylbutyl]amino-2-methoxy-acridine), tizanidine, and xylazine were from Sigma Chemical (St. Louis, MO). α-Methyl-noradrenaline, *p*-iodo-clonidine (2-[(2,6-dichloro-4-iodophenyl)imino]imidazoline HCl), rauwolscine, and UK14,304 (5-bromo-6-[2-imidazoline-2-ylamino]quinoxaline) were from RBI (Natick, MA). Fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR). Atipamezole, detomidine, MPV-2088 ([(−)-4-(5-fluoro-2,3-dihydro-1*H*-inden-2-yl)-1*H*-imidazole]HCl), medetomidine, and its two isomers dexmedetomidine and levomedetomidine were from Orion-Corporation, Orion-Pharma (Turku, Finland).

Cell Culture

HEL 92.1.7 cells obtained from the American Culture Collection (Rockville, MD) were grown in suspension culture in RPMI-1640 medium supplemented with 7.5% heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 units/ml penicillin (Sigma), and 50 μg/ml streptomycin (Sigma) in 5% CO_2 at 37°. Cells were harvested by centrifugation for 5 min at 250 × *g*. In some experiments, cells were pretreated by adding 500 ng/ml pertussis toxin to the culture medium 24 hr before the assay.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ in cells was determined using Fura-2 (Grynkiewicz *et al*., 1985). The cells were loaded with Fura-2 acetoxymethyl ester (4 μg/ml) in TBM (137 mM NaCl, 5 mM KCl, 10 mM glucose, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 20 mM TES adjusted to pH 7.4), supplemented with 1 mM $CaCl_2$, for 20 min at 37°. Thereafter, the cells were washed once, centrifuged, and resuspended in TBM supplemented with 100 μM $CaCl_2$ and kept at room temperature until measurement of $[Ca^{2+}]_i$. About 10^6 cells were pelleted, and fluorescence measurements were initiated by resuspending the cells in 350 μl of TBM, supplemented with 1 mM $CaCl_2$, at 37°. The cell suspension was placed in a quartz microcuvette in a thermostated cell holder. The cells were continuously stirred during the experiments with a magnetic stirrer. Fluorescence measurements were performed with a Hitachi F-4000 fluorescence spectrophotometer at the wavelengths 340 nm (excitation) and 505 nm (emission) or with a Hitachi F-2000 fluorescence spectrophotometer with the dual-wavelength optional function at the wavelengths 340/380 nm (excitation) and 505 nm (emission). The dye responses were calibrated by sequential addition of digitonin (60 μg/ml) and EGTA (10 mM) at the end of the experiment to obtain maximal (F_{max}) and minimal (F_{min}) fluorescence values, respectively. The extracellular Fura-2 concentration was measured by first adding EGTA and then digitonin. The $[Ca^{2+}]_i$ was calculated from the fluorescence values (F) obtained at 340 nm using the equation $[Ca^{2+}]_i = (F - F_{min})/(F_{max} - F) \times 224$ nM, in which the extracellular Fura-2 fluorescence is subtracted from the F values.

Measurement of Intracellular cAMP

The growth medium of confluent cultures was replaced with serum-free medium supplemented with 5 μCi/ml [^{3}H]adenine. After incubation for 2 hr, the cells were collected, pelleted, and washed once with TBM, supplemented with 1 mM $CaCl_2$. Thereafter, the cells were resuspended and divided into aliquots of ~10^6 cells in 0.8 ml of the same medium. The cells were preincubated with 0.5 mM IBMX (a phosphodiesterase inhibitor), 100 μM propranolol (a β-adrenoceptor antagonist), and 150 μM quinacrine (a phospholipase A_2 inhibitor) for 10 min at 37°. Forskolin (10 μM) and agonists in different concentrations (1 nM to 100 μM) were added. After 10 min, the cells were centrifuged for 1 min at 10,000 × *g*, the medium was immediately removed, and the reaction was terminated by resuspension in 1 ml of 0.33 M perchloric acid, containing about 1600 cpm [^{14}C]cAMP. The extent of conversion of [^{3}H]ATP to [^{3}H]cAMP was determined by sequential Dowex/alumina ion exchange to isolate cAMP (Salomon *et al*., 1974). Conversion to [^{3}H]cAMP was expressed as a percentage of total eluted tritium and was normalized to the recovery of [^{14}C]cAMP tracer (generally 70%). Radioactivity was determined by liquid scintillation counting (Wallac 1410; Wallac Oy, Turku, Finland) in Optiphase HiSafe 3.

Radioligand Binding

Preparation of the homogenates. Recombinant Shionogi S115 mouse mammary tumor cell lines expressing the human α_{2A} (α_2-C10), α_{2B} (α_2-C2), or α_{2C}-adrenoceptor (α_2-C4) subtypes were cultured in a hollow fiber bioreactor, harvested, and washed by centrifugation as described by Ala-Uotila *et al*. (1994). The cell pellet was suspended in 50 mM Tris/5 mM EDTA (pH 7.5 at 4°) and homogenized in Teflon-glass homogenizer (10 strokes with 1000 rpm, Potter S). Cell homogenate was then centrifuged twice at 47,800 × *g* for 30 min at 4° with resuspension by two strokes in Tris/EDTA buffer. The final pellet was resuspended in incubation buffer (50 mM KH_2PO_4 buffer, pH 7.5 at 25°) and distributed in aliquots to be stored at −80° for later use.

Preparation of the rat and rabbit liver followed what has been described previously (Garcia-Sainz *et al*., 1992). After removal, tissue was cleaned from the connective tissue and washed in buffer of 0.25 M sucrose with 5 mM HEPES (pH 7.4 at 4°), minced with scissors and frozen in liquid nitrogen to be stored at −80°. Frozen liver pieces were thawed and homogenized (Potter S, 500 rpm, eight strokes with Teflon pestle) in 0.25 M sucrose supplemented with 5 mM HEPES and 10 mM EDTA (pH 7.4 at 4°) as well as with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml bacitracin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, and 2 μg/ml soybean trypsin inhibitor; Sigma). Stock solution of phenylmethylsulfonyl fluoride was made in isopropanol freshly before use. Liver suspension was centrifuged for 5 min at 600 × *g* at 4°, supernatant was poured through a double cheesecloth, and the pellet was homogenized, centrifuged, and filtered as above. The supernatants were combined and centrifuged for 15 min at 47,800 × *g* at 4°. After resuspension in ice-cold 50 mM Tris buffer with 10 mM EDTA (pH 7.4 at 30°), the homogenate was

incubated for 30 min at 30° to remove endogenous catecholamines and then centrifuged as above.

Binding assays. In saturation binding experiments with S115 cells, 10 concentrations of ^{3}H-rauwolscine (77.9 or 80.5 Ci/mmol; New England Nuclear Research Products, Boston, MA) were incubated with the S115 cell homogenate (20–50 μg of total protein per tube) in a total volume of 0.25 ml in triplicate with or without presence of 100 μM oxymetazoline (to indicate nonspecific binding of the radioligands) in 50 mM KH_2PO_4 (pH 7.5 at 25°). In competition binding experiments, the radioligands were incubated at concentrations close to their K_D value with the cell and tissue suspension (protein concentration as above) with or without presence of a test compound in a total volume of 0.25 ml. Nonspecific binding was determined using 100 μM oxymetazoline as described above. After a 30-min incubation at 25°, incubation was terminated by rapid filtration (TomTec 96 harvester) through prewet GF/B glass-fiber filter mats (Wallac Oy) and three washes with ice-cold 10 mM Tris (pH 7.7 at 4°). After drying, a solid scintillate (Meltilex; Wallac) was melted on filter mats, and the radioactivity was measured (BetaPlate; Wallac Oy) with 35% efficiency for tritium.

In a saturation binding experiment with rat and rabbit liver, 10 concentrations of [^{3}H]prazosin (74.4 Ci/mmol; New England Nuclear) were incubated with the liver homogenate (100–200 μg of total protein per tube) in a total volume of 0.25 ml in triplicate with or without presence of 10 μM phentolamine (Sigma; to indicate nonspecific binding of the radioligand) in 50 mM Tris (pH 7.7 at 25°). In competition binding experiments, the radioligand was incubated at concentrations close to its K_D value with the tissue suspension (protein concentration as above) with or without presence of a test compound in a total volume of 0.25 ml. Nonspecific binding was determined using 10 μM phentolamine as described above. After a 30-min incubation at 25°, incubation was terminated by rapid filtration, the filters were washed and dried, and the radioactivity was measured as above. For all the binding experiments, protein determinations were done using a colorimetric assay (Bradford, 1976) with bovine serum albumin as standard.

Twitch Responses in Vas Deferens

All animal experimentation was approved by the local laboratory animal care committee (approval no. 132). Rats of Sprague-Dawley strain were from B&K (Stockholm, Sweden). The rats were housed in groups of four or five in the same cage, under standard conditions (20–22°, light/dark cycle with lights on between 6 a.m. and 6 p.m.) with free access to water and food. Action of levomedetomidine on peripheral presynaptic α_2-adrenoceptors *in vitro* was investigated in the prostatic portion of the rat vas deferens as described previously (Virtanen *et al.*, 1988). In brief, the prostatic portions of the vas deferens of male rats (200–300 g) were suspended under a resting tension of 0.5 *g* weight in an organ bath containing Kreb's solution supplemented with 1 μM (±)-propranolol and 20 nM desipramine. Twitch responses were induced by stimulation of intramural nerves (0.2 Hz, 2 msec, supramaximal voltage) and recorded as above.

Calculations

All results were analyzed using Prism (GraphPAD Software, San Diego, CA). Statistical analysis was carried out by one-way analysis of variance followed by Dunnett's test. $p < 0.05$ was considered to be statistically significant. The results are expressed as mean ± standard error.

Results

Effects on $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ in resting HEL 92.1.7 cells was 97 ± 7 nM (13 batches of cells). The two enantiomers of medetomidine had opposite effects on $[Ca^{2+}]_i$; dexmedetomidine caused a transient increase in $[Ca^{2+}]_i$ and levomedetomidine caused a transient reduction (Fig. 1). The dose-response relation of these two opposite effects is shown in Fig. 2. In both cases, an apparent saturation of the response was seen with calculated EC_{50} values of 18 ± 6 and 761 ± 502 nM for dexmedetomidine and levomedetomidine, respectively. The effect of levomedetomidine was preserved after several washes of the cells (data not shown), indicating that the basal receptor activity was not caused by the presence of an endogenous α_2 agonist.

Twenty different α_2-adrenoceptor ligands were tested for effects on $[Ca^{2+}]_i$. Most of the agonists tested gave significant transient increases in $[Ca^{2+}]_i$ (Fig. 3). To investigate whether the rise in $[Ca^{2+}]_i$ is a result of influx from the external medium or mobilization from intracellular stores, some of the experiments were performed in Ca^{2+}-free medium (nominally Ca^{2+}-free TBM plus 100 μM EGTA). The increases induced by epinephrine, norepinephrine, dexmedetomidine, and clonidine were reduced by 30–40% by Ca^{2+} removal (data not shown). For all ligands tested, the changes in $[Ca^{2+}]_i$ were dose dependent (data not shown). Epinephrine was most active although it was closely followed by norepinephrine and UK14,304 (Fig. 3). The increases in $[Ca^{2+}]_i$ by 10 μM concentration of the different agonists could be blocked by the α_2-adrenoceptor antagonist rauwolscine (1 μM) and by pertussis toxin pretreatment (500 ng/ml, 24 hr) (data not shown), whereas essentially no effect was seen with the α_1-antagonist prazosin; its affinity was calculated to be >2 μM with respect to noradrenaline-induced Ca^{2+} elevation. Two of the tested antagonists, rauwolscine and idazoxan, caused a reduction in $[Ca^{2+}]_i$ in a similar manner as levomedetomidine and were thus classified as inverse agonists (Fig. 3). One α_2-adrenoceptor ligand, MPV-2088, had no effect on $[Ca^{2+}]_i$.

Oxymetazoline, an α_{2A}-selective ligand with a weak agonistic effect, caused a small Ca^{2+} elevation. Prior (5 min in advance) application of oxymetazoline inhibited both the Ca^{2+}-decreasing effect of levomedetomidine and the Ca^{2+}-elevating effect of dexmedetomidine. The calculated affinities for the inhibition of the responses were not significantly different (10 ± 1 and 50 ± 25 nM, respectively; three determinations).

Effects on cAMP. In nonstimulated HEL 92.1.7 cells, the conversion of [^{3}H]ATP to [^{3}H]cAMP was 0.21 ± 0.06% (16 batches of cells), and this conversion was stimulated 7–10-fold by forskolin. The same ligands that were used in the Ca^{2+} experiments were tested for their ability to modify forskolin-stimulated cAMP production. In the same way as with the Ca^{2+} measurements, the ligands could be classified as agonists, neutral ligands, and inverse agonists. The rela-

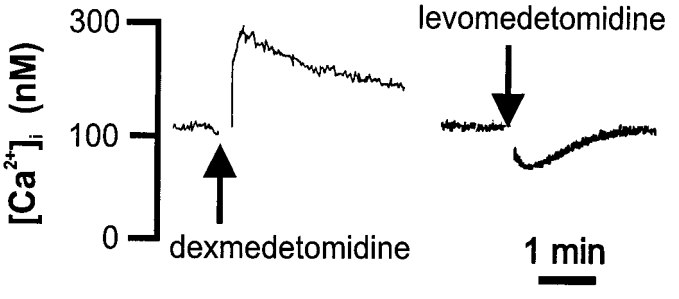

Fig. 1. The effects of the two isomers of medetomidine, dexmedetomidine and levomedetomidine, on the $[Ca^{2+}]_i$. A tracing of raw fluorescence after the addition of 10 μM dexmedetomidine or 10 μM levomedetomidine, typical of experiments from which $[Ca^{2+}]_i$ was calculated, is shown.

tive maximum effects obtained in the cAMP assay closely followed those obtained in Ca^{2+} measurements, with epinephrine, norepinephrine, and UK14,304 being full agonists and the typical antagonists rauwolscine, atipamezole, and idazoxan being inverse antagonists (Fig. 3). For the agonists detomidine, tizanidine, guanabenz, guanfacine, xylazine, oxymetazoline, and naphazoline, no significant effects on cAMP production could be seen. The inhibitory effects by 10 μM of the agonists could be blocked by rauwolscine (10 μM), MPV-2088 (10 μM), and pertussis toxin pretreatment (500 ng/ml, 24 hr).

Again, the two enantiomers of medetomidine had opposite effects regarding intracellular cAMP level. Dexmedetomidine inhibited forskolin-stimulated cAMP production, but with levomedetomidine, a potentiation of cAMP production was seen (Fig. 4). The EC_{50} values for dexmedetomidine and levomedetomidine were 17 ± 0.9 and 390 ± 200 nM respectively (six batches of cells). MPV-2088 had no effect on the forskolin-stimulated cAMP production, but it blocked the effects of both levomedetomidine and dexmedetomidine (Figs. 3 and 5). Pertussis toxin pretreatment abolished the stimulation of cAMP production by levomedetomidine, as well as the inhibitory effect by dexmedetomidine, suggesting that both effects are transduced through $G_{i/o}$-type G proteins (Fig. 5).

Agonist activity of levomedetomidine in vas deferens. Twitch responses of the prostatic portion of the rat vas deferens were inhibited by cumulative concentrations (at 2-min interval) of levomedetomidine with a pD_2 value of 7.76 ± 0.19 (five batches of cells). The maximum response to levomedetomidine was 30% of the response to dexmedetomidine.

MPV-2088. Because MPV-2088 was the only tested α_2 antagonist that was neutral, its effect on the levomedetomidine-induced elevation and dexmedetomidine-induced decrease of cAMP level was determined. As expected, it blocked the increase in cAMP production by levomedetomidine as well as the reduction in cAMP production by dexmedetomidine (Fig. 5). In addition, MPV-2088 showed high selectivity for α_2-adrenoceptors in binding experiments: in recombinant S115 cells, its binding affinity (K_D) was 0.30 ± 0.07 nM for α_{2A} (six determinations in triplicate), 1.7 ± 0.14 nM for α_{2B} (five determinations in triplicate), and 0.95 ± 0.03 nM for α_{2C} (three determinations in triplicate). The binding affinity for MPV-2088 for the α_1-adrenoceptor was 169 ± 21 nM as determined in rat liver (four determinations in triplicate) and

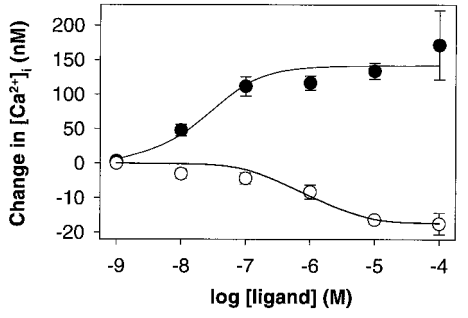

Fig. 2. The dose-response curves for dexmedetomidine (●) and levomedetomidine (○) with respect to the modulation of the $[Ca^{2+}]_i$. The basal level of $[Ca^{2+}]_i$ is subtracted from each point. The values are mean ± standard error from three independent experiments. Observe the nonlinear ordinate.

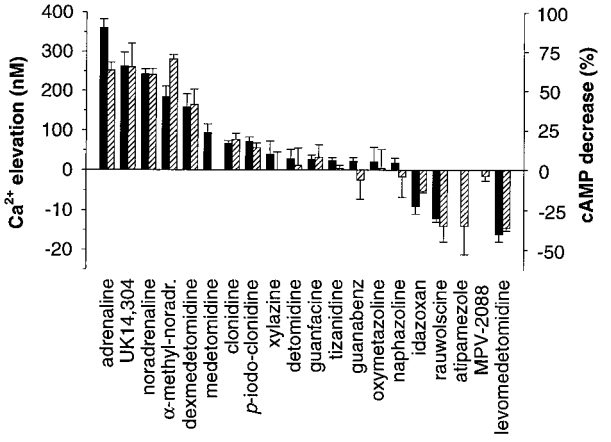

Fig. 3. Effects of 10 μM of different α_2-adrenoceptor ligands on the intracellular level of Ca^{2+} (*left ordinate*, ■) and on the forskolin-stimulated cAMP production (*right ordinate*, ▨). The basal $[Ca^{2+}]_i$ and the forskolin-stimulated increase in cAMP production are taken as 0; negative Ca^{2+} elevation and negative cAMP decrease therefore mean Ca^{2+} decrease and cAMP elevation, respectively. The values are mean ± standard error from three independent experiments performed in triplicate. Ca^{2+} responses to medetomidine and atipamezole were not tested. Observe the nonlinear left ordinate.

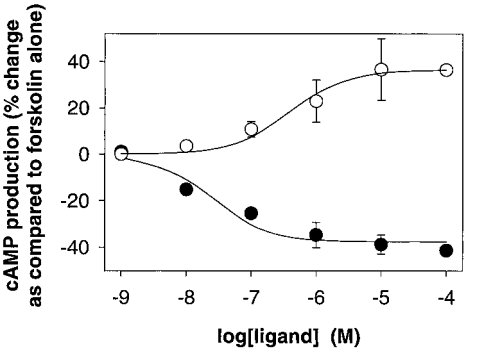

Fig. 4. The effects of dexmedetomidine (●) and levomedetomidine (○) on the cAMP production in the presence of 10 μM forskolin. The forskolin-stimulated increase in cAMP production is taken as 0. Negative values indicate inhibition and positive values further stimulation of forskolin-stimulated cAMP production. The values are mean ± standard error from three independent experiments performed in triplicate.

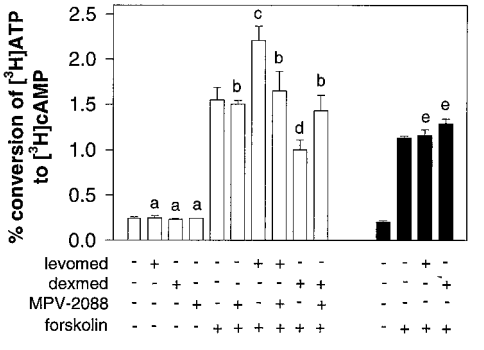

Fig. 5. Effect of 10 μM levomedetomidine (*levomed*), 10 μM dexmedetomidine (*dexmed*), and 10 μM MPV-2088 on cAMP production in the absence and presence of 10 μM forskolin in the control cells (□) and in the cell pretreated with pertussis toxin (500 ng/ml, 24 hr; ■). The values are mean ± standard error from three independent experiments performed in triplicate. Statistical significance was assessed against the basal level in control cells (*a*, $p > 0.05$, not significant), to the forskolin-stimulated level in control cells (*b*, $p > 0.05$, not significant; *c*, $p < 0.05$; *d*, $p < 0.01$), and to the forskolin-stimulated level in pertussis toxin-pretreated cells (*e*, $p > 0.05$, not significant).

645 ± 229 nM in rabbit liver (four determinations in triplicate).

Discussion

The results of the current study show that the two isomers of medetomidine, dexmedetomidine and levomedetomidine, have opposite effects on signal transduction through the endogenous α_2-adrenoceptors in HEL 92.1.7 cells. Dexmedetomidine acted as an agonist (by elevating $[Ca^{2+}]_i$ and inhibiting cAMP production) as has been shown in many other systems (Savola and Virtanen, 1991; Jansson *et al.*, 1994a, 1994b, 1995; Pohjanoksa *et al.*, 1997). On the contrary, levomedetomidine acted as an inverse agonist by reducing $[Ca^{2+}]_i$ and enhancing cAMP production. In many other systems, levomedetomidine has been a weak partial α_2-adrenoceptors agonist on the level of adenylyl cyclase (α_A, α_B, α_C in S115 cells: Jansson *et al.*, 1994a; α_{2A}, α_{2C} in PS12 cells: Jansson *et al.*, 1994b; α_{2B} in Sf9 cells: Jansson *et al.*, 1995; α_{2C} in CHO cells: Pohjanoksa *et al.*, 1997). *In vivo* levomedetomidine has shown agonistic effect by increasing sleeping time (Savola and Virtanen, 1991). The agonistic character of levomedetomidine was also seen in the inhibition of the contraction of rat vas deferens (effect on $\alpha_{2A/D}$-adrenoceptors; Smith and Docherty, 1992; Smith *et al.*, 1992) shown in the current study. On the contrary, in HEL 92.1.7 cells levomedetomidine was an inverse agonist. The inverse agonistic effect seen in here was sensitive to rauwolscine and MPV-2088 and to pertussis toxin. This together with the previously reported high selectivity of levomedetomidine for α_2 receptors (Virtanen *et al.*, 1988) suggests that the inverse effect reported in the current study is indeed transduced by α_2-adrenoceptors.

The antagonists rauwolscine, atipamezole, and idazoxan induced similar responses in the $[Ca^{2+}]_i$ and/or cAMP accumulation assays as levomedetomidine. Because these antagonists showed negative activity, they can be classified as inverse agonists. The antagonist MPV-2088 showed no activity, although it has nanomolar or even subnanomolar affinity for α_2-adrenoceptors. Both the agonistic and inversely agonistic effects were blocked by MPV-2088 and by pertussis toxin treatment, indicating that these signals are transmitted through α_2-adrenoceptors and $G_{i/o}$-type G proteins. The effects of the inverse agonists on cAMP production could only be seen after forskolin treatment. This is probably due to the low basal adenylyl cyclase activity and correspondingly small effect of the active $G_{i/o}$-type G proteins in the absence of forskolin. After activation of adenylyl cyclase with forskolin, the constitutive activity of the $G_{i/o}$-type G proteins and the subsequent effect of inverse agonists on it are revealed.

The reason for the transient nature of the inverse Ca^{2+} signal to inverse α_2 agonists is unknown. We, however, assume this to be a result of a feedback regulatory mechanism activated to protect the cells from the possibly noxious $[Ca^{2+}]_i$ caused by the constitutive receptor activity. The Ca^{2+} levels would remain elevated due to the capacitative Ca^{2+} entry (Putney and Bird, 1993) but would be limited by the activation of the plasma membrane Ca^{2+}-ATPase by Ca^{2+}-calmodulin (Scharff, 1981). If this was the case with HEL 92.1.7 cells, the cells would not have to have a notably elevated $[Ca^{2+}]_i$ despite the constitutive receptor activity. Binding of inverse agonist would thus lead to a decrease in spontaneous receptor activity, resulting in an undershoot of $[Ca^{2+}]_i$ due to the activated Ca^{2+}-ATPase. The slow return to the basal level would be due to the dissociation of the calmodulin from the Ca^{2+}-ATPase, which has a half-time of minutes (Scharff and Foder, 1982; Åkerman *et al.*, 1985). Whether this is the actual mechanism can only be speculated. However, it is clear that the inverse Ca^{2+} signal is caused by α_2-receptor inhibition as (1) it is observed with both rauwolscine and levomedetomidine and (2) the signal to levomedetomidine is inhibited both by rauwolscine and MPV-2088.

Levomedetomidine differs markedly from rauwolscine and idazoxan in that the latter two compounds, tested in many different systems, always act as antagonists. The inverse response to a ligand that in some systems acts as a weak agonist on the same subtype is possible to explain by using the model of Kenakin (1995). A protean agonist will normally produce an activated receptor state that has, however, lower affinity for the G protein than spontaneously produced active receptor state. In systems in which there is little spontaneous formation of the activated receptor state, the receptor-activating property of the ligand will therefore be seen and positive agonism will be observed. On the other hand, when there is a considerable spontaneous formation of the active receptor state, the lower efficacy of the ligand will result in inverse agonism (Kenakin, 1995). Altogether, the results with levomedetomidine indicate that this compound is a "protean" ligand, having the ability to act as an agonist (a weak such) in systems with little or no constitutive receptor activity and as an inverse agonist in systems with receptors expressing high constitutive activity. The possibility that a coupling to different G proteins in different cells would be causing the normal versus inverse agonism observed cannot be excluded. This, however, might also be the case in a system where both agonistic and inversely agonistic responses are seen in the same cells (e.g., at different receptor expression levels). Both the agonistic (S115 cells, Jansson *et al.*, 1994a; Sf9 cells, Jansson *et al.*, 1994b) and inverse agonistic effects (the current study) are seen as an effect on the adenylyl cyclase function, suggesting that levomedetomidine can transduce both agonistic and antagonistic signals through α_{2A} receptors and α_i G proteins, although the subtypes of α_i are unknown. Also, the high affinity of oxymetazoline for inhibition of levomedetomidine- and dexmedetomidine-induced signals and the low affinity of prazosin suggest the involvement of α_{2A} receptors both in the Ca^{2+} decrease and increase in HEL 92.1.7 cells.

The structural basis for the opposite actions of levomedetomidine and dexmedetomidine should be considered. In the case of the rigid agonist UK14,304 (a full α_{2A}-adrenoceptor agonist) and other conformationally restricted α_2-adrenoceptor agonists, a left-handed kink with the chiral center between the imidazoline ring and the quinoxaline nucleus is seen (Hancock *et al.*, 1988; Munck *et al.*, 1994). This defines that the activated receptor must accommodate for a pronounced left-handed kink of the rigid ligand. Because the two enantiomers, which are mirror images, produce opposite effects, the conformation of the binding cavity should change drastically on transition from the inactivated to the activated state. This model is in agreement with recent studies on the β_2-adrenoreceptors using fluorescence tagging in which a range of ligands produced spectral shifts that correlated with

their functional activity (Gether *et al.*, 1995). Compounds usually considered to be antagonists behaved as inverse agonists and showed opposite fluorescence changes compared with agonists. This suggests that native receptors can access a neutral conformation when no ligand is bound. These conformational changes in the binding cavity may well be related to the previously reported conformational changes in rhodopsin involving helix number III (Alkorta and Du, 1994; Farahbakhsh *et al.*, 1995) and to the *cis/trans*-isomerization of retinal in rhodopsin where the kinked *cis* conformation is associated with the active state of the G protein and the linear *trans* conformation is silent (Arnis and Hofmann, 1995).

The protean agonistic properties of levomedetomidine [i.e., weak agonism in some systems (Jansson *et al.*, 1994a, 1994b, 1995) and inverse agonism in others] can be explained by the inherent tonus of the receptor-signal transduction pathway (Kenakin, 1995). With the recent realization that receptors can have spontaneous activity both in normal and in pathological situations (Milano *et al.*, 1994), protean agonists represent a new and promising class of targeted drug therapy. This applies to cases in which a constitutively active mutated receptor or possible overactivity of G protein-modulatory proteins (Sato *et al.*, 1996; Dohlman and Thorner, 1997) can cause pathophysiological elements of disease. It will be beneficial to be able to restore the tonus of receptor activation without interfering significantly with the normal activation of the receptor. There is a basic difference in the action of the antagonist-type inverse agonists and protean agonists: the former inhibit the activation of the silent, "normal," receptors, whereas the latter will activate silent receptors. Both types of inverse agonism may be beneficial in different pathological situations.

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Send reprint requests to: Karl E. O. Åkerman, Uppsala University, Department of Physiology and Medical Biophysics, BMC, P.O. Box 572, S-75123 Uppsala, Sweden. E-mail: karl.akerman@fysiologi.uu.se