British Journal of Pharmacology (2001) 132, 1477–1484

www.nature.com/bjp

Agonist trafficking of $G_{i/o}$-mediated α_{2A}-adrenoceptor responses in HEL 92.1.7 cells

*,[1]Jyrki P. Kukkonen, [2]Christian C. Jansson & [1]Karl E.O. Åkerman

[1]Department of Physiology, Division of Cell Physiology, Uppsala University, BMC, P.O. Box 572, S-75123 Uppsala, Sweden and [2]Åland Central Hospital, Laboratory, FIN-22100 Mariehamn, Finland

1 The ability of 19 agonists to elevate Ca^{2+} and inhibit forskolin-induced cyclic AMP elevation through α_{2A}-adrenoceptors in HEL 92.1.7 cells was investigated. Ligands of catecholamine-like- (five), imidazoline- (nine) and non-catecholamine-non-imidazoline-type (five) were included.

2 The relative maximum responses were similar in both assays. Five ligands were full or nearly full agonists, six produced 20–70% of the response to a full agonist and the remaining eight gave lower responses (<20%) so that their potencies were difficult to evaluate.

3 Marked differences in the potencies of the agonists with respect to the two measured responses were seen. The catecholamines were several times less potent in decreasing cyclic AMP than in increasing Ca^{2+}, whereas the other, both imidazoline and ox-/thiazoloazepine ligands, were several times more potent with respect to the former than the latter response. For instance, UK14,304 was more potent than adrenaline with respect to the cyclic AMP response but less potent than adrenaline with respect to the Ca^{2+} response.

4 All the responses were sensitive to pertussis toxin-pretreatment. Also the possible role of PLA_2, β-adrenoceptors or ligand transport or metabolism as a source of error could be excluded. The results suggest that the active receptor states produced by catecholamines and the other agonists are markedly different and therefore have different abilities to activate different signalling pathways.
British Journal of Pharmacology (2001) **132,** 1477–1484

Keywords: α_2-adrenoceptors; Ca^{2+}; cyclic AMP; HEL 92.1.7 cells; agonist trafficking; G protein; $G_{i/o}$; fura-2

Abbreviations: B-HT 920, 2-amino-6-allyl-5,6,7,8-tetrahydro-4H-thiazolo-(5,4-d)-azepine; B-HT 933, 2-amino-6-ethyl-4,5,7,8-tetrahydro-6H-oxazolo-[5,4-d]-azepine; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $\Delta[Ca^{2+}]_i$, change in intracellular Ca^{2+} concentration; clonidine, 2-(2,6-dichloroaniline)-2-imidazoline; *p*-I-clonidine, 2-([2,6-dichloro-4-iodophenyl]imino)imidazoline; desipramine, 10,11-dihydro-N-methyl-5H-dibenz(b,f)azepine-5-propanamine; detomidine, 4(5)-(2,3-dimethylbenzyl)imidazole; FCS, foetal calf serum; guanabenz, 1-(2,6-dichlorobenzylideneamino)guanidine; guanfacine, N-(aminoiminomethyl)-2,6-dichlorobenzenacetamide; (−)-isoproterenol, (−)-1-(3′,4′-dihydroxyphenyl)-2-isopropylaminoethanol; D-medetomidine, (+)-(S)-4-(1-[2,3-dimethylphenyl]ethyl)-1H-imidazole; moxonidine, 4-chloro-N-(4,5-dihydro-1H-imidazol-2-yl)-6-methoxy-2-methyl-5-pyrimidinamine; naphazoline, 4,5-dihydro-2-(1-naphthalenylmethyl)-1H-imidazole; nialamide, N-isonicotinoyl-N′-(*β*-[N-benzylcarboxamido]ethyl)hydrazine; oxymetazoline, (3-[(4,5-dihydro-1H-imidazol-2-yl-)methyl]-6-[1,1-dimethylethyl]-2,4-dimethylphenol ; (S)-(−)-propranolol, (S)-1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol; quinacrine, 6-chloro-9-([4-diethylamino]-1-methylbutyl)amino-2-methoxy-acridine; RX821002, 2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline; TBM, TES buffered medium; TES, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino) ethane sulphonic acid; tizanidine, 5-chloro-4-(2-imidazolin-2-yl-amino)-2,1,3-benzothiadiazole; UK14,304, 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine; xylazine, N-(2,6-dimethylphenyl)-5,6-dihydro-4H-1,3-thiazin-2-amine

Introduction

G protein-coupled receptors are able to couple to a multitude of signal transduction pathways. Some diversity arises from the specific coupling of certain G proteins to certain responses, e.g. $G_{\alpha q}$ to stimulation of phospholipase Cβ, $G_{\alpha s}$ to stimulation of adenylyl cyclase and $G_{\alpha i}$ to inhibition of adenylyl cyclase. Additional diversity is dependent on the coupling of the same G protein to several responses either through the same subunit (e.g. coupling of $G_{\beta\gamma}$ to stimulation of phospholipase Cβ and inhibition of Ca^{2+} channels) or through the different subunits released in equal amounts by the G protein activation (e.g. coupling of $G_{\alpha i}$ to inhibition of adenylyl cyclase and $G_{\beta\gamma}$ to stimulation of phospholipase Cβ). The prevailing division of G protein-coupled receptors to G_q-, G_s- and $G_{i/o}$-coupled receptors is based on the signalling through G protein α-subunits, though great differences in the ability of different G protein-coupled receptors to couple to different subfamilies of G proteins have become apparent (reviewed in Gudermann *et al*., 1996): for instance, human thyrotropin receptor interacts with at least 10 different G proteins from all four subfamilies (G_s, G_i, G_q, G_{12}) (Laugwitz *et al*., 1996) whereas for most other receptors the spectrum is much more limited. There are several subtypes of G proteins in all the subfamilies, e.g. in the most diverse $G_{i/o}$-subfamily nine α-subunits, six of which

*Author for correspondence at: Department of Physiology, Division of Cell Physiology, Uppsala University, BMC, P.O. Box 572, S-75123 Uppsala, Sweden; E-mail: jkukkone@fysiologi.uu.se

are more or less ubiquitous and three tissue specific, have been cloned. Additional functional diversity is created by the coupling of one G_{α}-subunit to different $G_{\beta\gamma}$ subunits. The ability of the receptors to activate different members of this G protein subfamily has recently received increasing attention. Direct and indirect evidence indicates that $\alpha_{2A/D}$-adrenoceptors can couple to at least G_{i2}, G_{i3} and G_{o1} (Simonds *et al*., 1989; Gerhardt & Neubig, 1991; McClue *et al*., 1992; Remaury *et al*., 1993; Yang & Lanier, 1999) whereas α_{2C}-adrenoceptors may couple to G_{i1}, G_{o1} or G_{o2} (Duzic *et al*., 1992). When reconstituted in phospholipid vesicles together with G proteins, both α_{2A} and α_{2C} activated members of the $G_{i/o}$ subfamily with the efficacy order of $G_{i3} > G_{i1} \geqslant G_{i2} > G_{o1}$ (Kurose *et al*., 1991).

It has previously been observed that different agonists can couple to separate signalling pathways with different efficacy *via* the same receptors (reviewed in Kenakin, 1995). Such behaviour can often be explained by differences in stoichiometries at the receptor-G protein or G protein-effector level. In such cases the order of efficacy for the different ligands should be the same irrespective of the response measured, and only dictated by differences in the respective ligands' intrinsic efficacy (Kenakin, 1995). However, it has sometimes been observed that the order of efficacy between ligands is reversed for different responses (Boddeke, 1991; Krumins & Barber, 1997; Berg *et al*., 1998). This cannot be explained by the reasoning above. On the contrary, it has been taken as an indication that different agonists can either induce (induction theory) or stabilize (selection theory) different active receptor conformations (Kenakin, 1995; Leff *et al*., 1997). If these enriched different active conformations will preferentially activate specific G proteins, a distinct activation of different signalling pathways by different agonists will be observed. This phenomenon has been termed 'agonist trafficking' (of receptor signals) (Kenakin, 1995).

The natural ligands for α_2-adrenoceptors are the catecholamines noradrenaline and adrenaline. They usually behave as full agonists, but their potencies (EC_{50}) are often lower than the potencies of some common synthetic imidazolines, like UK14,304 (5-bromo-N-[4,5-dihydro-1H-imidazol-2-yl]-6-quinoxalinamine), D-medetomidine ([+]-[S]-4-[1-(2,3-dimethylphenyl)ethyl]-1H-imidazole HCl) and clonidine (2-[2,6-dichloroaniline]-2-imidazoline HCl). This has been seen with respect to the most often measured response, inhibition of stimulated cyclic AMP production (Jones *et al*., 1987; Voigt *et al*., 1991; Jansson *et al*., 1994; Pohjanoksa *et al*., 1997), but also with respect to GTPγS binding as well as cyclic AMP and Ca^{2+} elevation (Jansson *et al*., 1995; Holmberg *et al*., 1998; Jasper *et al*., 1998; Kukkonen *et al*., 1998). The reason for this may simply be the higher binding affinity of the imidazolines (Jansson *et al*., 1994; 1995; Pohjanoksa *et al*., 1997; Jasper *et al*., 1998). The other groups of synthetic ligands include dichlorophenylguanidines guanabenz (1-[2,6-dichlorobenzylideneamino]guanidine) and guanfacine (N-[aminoiminomethyl]-2,6-dichlorobenzenacetamide), ox-/thiazoloazepines B-HT 920 (2-amino-6-allyl-5,6,7,8-tetrahydro-4H-thiazolo-[5,4-d]-azepine di-HCl) and B-HT 933 (2-amino-6-ethyl-4,5,7,8-tetrahydro-6H-oxazolo-[5,4-d]-azepine di-HCl) and the thiazine xylazine (N-[2,6-dimethylphenyl]-5,6-dihydro-4H-1,3-thiazin-2-amine HCl), which all usually behave as partial agonists, though the dichlorophenylguanidines can be very potent (Jasper *et al*., 1998).

The HEL 92.1.7 human erythroleukaemia cell line expresses α_{2A}-adrenoceptors which elevate intracellular free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) (Michel *et al*., 1989; Musgrave & Seifert, 1995; Kukkonen *et al*., 1997; Jansson *et al*., 1998) and inhibit stimulated adenylyl cyclase activity (McKernan *et al*., 1987; Jansson *et al*., 1998) through $G_{i/o}$-type G proteins. In this study we address the issue of possible differential signalling through different active receptor states produced by different agonists. This is performed by measurement of Ca^{2+} and cyclic AMP responses to 17 different α_2-adrenoceptor agonists belonging to five different chemical classes.

Methods

Cell culture

HEL 92.1.7 cells, obtained from the ATCC (Rockville, MD, U.S.A), were grown in suspension culture in RPMI-1640 medium supplemented with 7.5% heat-inactivated foetal calf serum (FCS; Gibco, Paisley, U.K.), 100 u ml^{-1} penicillin (Sigma, St Louis, MO, U.S.A.) and 80 u ml^{-1} streptomycin (Sigma) as described previously (Kukkonen *et al*., 1997). Cells were harvested by centrifugation for 5 min at $250 \times g$. When the effect of pertussis toxin-pretreatment was investigated, the cells were treated with 100 ng ml^{-1} pertussis toxin for 24 h. As a control for cell viability, the lack of the inhibitory effect of pertussis toxin-pretreatment on the Ca^{2+} elevation induced by P2Y-purinoceptor stimulation with 10 μM ATP was used.

Drugs

[^{3}H]-Adenine and [^{14}C]-cyclic AMP were from Amersham (Buckinghamshire, U.K.). (−)-Adrenaline, clonidine, desipramine (10,11-dihydro-N-methyl-5H-dibenz[b,f]azepine-5-propanamine), guanabenz, guanfacine, 3-isobutyl-1-methylxanthine, (−)-isoproterenol ([−]-1-[3′,4′-dihydroxyphenyl]-2-isopropylaminoethanol), naphazoline (4,5-dihydro-2-[1-naphthalenylmethyl]-1H-imidazole), nialamide (N-isonicotinoyl-N′-[β-(N-benzylcarboxamido)ethyl]hydrazine), (−)-noradrenaline, (±)-*p*-octopamine, oxymetazoline (3-[(4,5-dihydro-1H-imidazol-2-yl-)-methyl]-6-[1,1-dimethylethyl]-2,4-dimethylphenol HCl), pertussis toxin, phentolamine, (S)-(−)-propranolol ([S]-1-[isopropylamino]-3-[1-naphthyloxy]-2-propanol HCl), quinacrine (6-chloro-9-[(4-diethylamino)-1-methylbutyl]amino-2-methoxy-acridine), tizanidine (5-chloro-4-[2-imidazolin-2-yl-amino]-2,1,3-benzothiadiazole) and xylazine were from Sigma. α-Methyl-noradrenaline, B-HT 920, B-HT 933, dopamine, *p*-I-clonidine (2-[(2,6-dichloro-4-iodophenyl)imino]imidazoline HCl), rauwolscine and UK14,304 were from RBI (Natick, MA, U.S.A.) and digitonin from Merck AG (Darmstadt, Germany). Fura-2 acetoxymethylester was from Molecular Probes (Eugene, OR, U.S.A.). D-Medetomidine and detomidine (4[5]-[2,3-dimethylbenzyl]imidazole HCl) were from Orion-Corporation Orion-Pharma (Turku, Finland). Moxonidine (4-chloro-N-[4,5-dihydro-1H-imidazol-2-yl]-6-methoxy-2-methyl-5-pyrimidinamine) and RX821002 (2-[2-methoxy-1,4-benzodioxan-2-yl]-2-imidazoline [methoxy idazoxan]) were kind gifts from Dr Birgit Brueggemann (Beiersdorf-Lily GmbH, Hamburg, Germany) and Dr Corinne Gelhay (Pierre Fabre, Castres, France), respectively.

Media

The TES buffered medium (TBM) consisted of (mM): NaCl 137, KCl 5, $CaCl_2$ 1, glucose 10, $MgCl_2$ 1.2, KH_2PO_4 0.44, $NaHCO_3$ 4.2 and 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino) ethane sulfonic acid (TES) 20 adjusted to pH 7.4 with NaOH.

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

The fluorescent Ca^{2+}-indicator fura-2 was used to monitor changes in $[Ca^{2+}]_i$ as described previously for HEL 92.1.7 cells (Kukkonen *et al*., 1997; Jansson *et al*., 1998) and for CHO cells (Kukkonen *et al*., 1998). Fluorescence measurements were performed with a Hitachi F-4000 fluorescence spectrophotometer at the wavelengths 340 nm (excitation) and 505 nm (emission), with a Hitachi F-2000 fluorescence spectrophotometer at the wavelengths 340/380 nm (excitation) and 505 nm (emission) or with a PTI QuantaMaster QM1 fluorescence spectrophotometer at the wavelengths 340/360/380 nm (excitation) and 505 nm (emission). The dye responses were calibrated by sequential addition of digitonin (60 μg ml^{-1}) and EGTA (10 mM) at the end of the experiment to obtain the maximum (F_{max}) and minimum (F_{min}) fluorescence values, respectively. The extracellular fura-2 concentration was measured by first adding EGTA and then digitonin. The intracellular free Ca^{2+} concentration was calculated from the fluorescence values (F) obtained at 340 nm as in Kukkonen *et al*. (1997) or from the 340/360 nm data.

Measurement of intracellular cyclic AMP

The growth medium of confluent cultures was replaced with fresh medium supplemented with 2.5 μCi ml^{-1} of [^{3}H]-adenine. After incubation for 2 h the cells were collected, spun down and resuspended in TBM supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) and incubated for 10 min at 37°C. When the effect of α_2- or β-adrenoceptor antagonists, the phospholipase A_2 inhibitor quinacrine, the monoamine oxidase inhibitor nialamide and catecholamine reuptake inhibitor desipramine were tested, they were preincubated together with 3-isobutyl-1-methylxanthine. The reactions were started by pipetting the cell suspension ($\approx 8 \times 10^6$ cells ml^{-1}) in 96-well plates (Nunc 269620, Nunc A/S, Roskilde, Denmark). The compounds to be investigated (α_2-adrenoceptor agonists and forskolin) had previously been diluted in TBM and dispensed in a volume of 100 μl; this, together with 50 μl of cell suspension per well, gave a total reaction volume of 150 μl. The reactions were allowed to proceed for 10 min at 37°C, after which they were terminated by centrifugation at $1100 \times g$ for 1 min, rapid decanting of the supernatants and addition of 200 μl ice-cold 0.33 M perchloric acid per well. The plates were frozen down to −20°C, thawed, and the cell debris were spun down ($1100 \times g$, 15 min). The extent of conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP was determined by sequential Dowex/alumina chromatography of the supernatants. [^{14}C]-cyclic AMP tracer in 0.75 ml 0.33 M perchloric acid (about 1000 c.p.m.) was added into each Dowex column together with the samples. Radioactivity was determined by liquid scintillation counting (Wallac 1410, Wallac, Turku, Finland) in Optiphase HiSafe 3. Conversion to [^{3}H]-cyclic AMP was calculated as a percentage of total eluted [^{3}H]-ATP and was normalized to the recovery of [^{14}C]-cyclic AMP tracer (generally 70%).

Data analysis

Values are given as mean ± s.e.mean; n refers to the number of batches of cells on which the measurements were performed. Non-linear curve-fitting was performed using SigmaPlot for Windows 4.00 (Jandel Scientific, Corte Madera, CA, U.S.A.). The difference in the ability of each agonist to activate Ca^{2+} and cyclic AMP response was evaluated by calculating the group averages of $EC_{50\text{-}Ca^{2+}}$/$EC_{50\text{-cyclic AMP}}$ and performing between-group comparisons (catecholamines versus imidazolines and ox-/thiazoloazepines) using the Student's two-tailed non-paired t-test.

Results

Ca^{2+}

The basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was 97 ± 7 nM (n = 25). As also reported before, α_2-adrenoceptor activation resulted in Ca^{2+} elevations that consisted of a transient release and a sustained influx (data not shown; see also Michel *et al*., 1989; Jansson *et al*., 1998). Nineteen agonists were tested for their ability to induce Ca^{2+} elevation, and for 14 of them, a good estimate of the EC_{50} for the maximum 'spike' response was obtained (Table 1; Figure 1). Adrenaline was clearly the most efficacious of the agonists with an approximately 4.6 fold elevation of basal $[Ca^{2+}]_i$. HEL 92.1.7 cells have previously been reported to express β-adrenoceptors with high affinity for the β-antagonist propranolol (Michel *et al*., 1989). These receptors did not seem to interfere with the α_{2A}-mediated Ca^{2+} elevation since all the agonist-mediated Ca^{2+} elevations were abolished with 10 μM RX821002, 10 μM rauwolscine or by pertussis toxin-pretreatment. Propranolol (10 or 100 μM) caused a slight right shift in the concentration-response curves of the ligands, probably due to its affinity for α_{2A}-adrenoceptors (Gerhardt *et al*., 1990; see also below). We also tested the effect of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine in the Ca^{2+} assay: 150 μM 3-isobutyl-1-methylxanthine had no effect on the EC_{50} value or the maximum response.

Cyclic AMP

The basal conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP was 0.17 ± 0.04% (n = 3). Forskolin (10 μM) increased the basal cyclic AMP production to 5.16 ± 0.31% (n = 3). The same 19 α_2-adrenoceptor agonists were tested for the inhibition of forskolin-induced cyclic AMP production. Ten of them gave responses that were large enough to allow determination of the EC_{50} (Table 1; Figure 1). The most efficacious agonist was once again adrenaline, although this response was only slightly higher than the response to the other strong agonists, noradrenaline, α-methyl-noradrenaline, moxonidine and UK14,304. There was a good correlation with the maximum responses measured in the Ca^{2+} assay (Table 1; Figure 2A). On the contrary, the EC_{50} values were clearly different. When

Table 1 The maximum responses (E_{max}) and EC_{50} values for Ca^{2+} elevation and cyclic AMP decrease elicited by different α_2-adrenoceptor ligands

	Ca^{2+} elevation		*Cyclic AMP decrease*	
Drug	*E_{max}* (nM $\Delta[Ca^{2+}]$)	*EC_{50}* (nM)	*E_{max}* (% inhibition)	*EC_{50}* (nM)
Adrenaline	360±22	15±9	76±2	2600±1200
Moxonidine	273±14	1200±200	73±1	240±16
UK14,304	261±37	39±10	73±2	15±1
α-Methyl-noradrenaline	258±12	84±42	71±2	2200±900
Noradrenaline	242±13	56±14	72±2	2600±1000
D-Medetomidine	158±33	18±6	49±1	3.3±0.1
Dopamine	>100	>10,000	35±4	>5000
B-HT 920	85±11	1300±800	44±1	190±55
B-HT 933	72±6	12,000±5000	46±2	7100±1100
p-I-clonidine	71±12	28±7	27±3	7.9±3.8
Clonidine	66±7	130±2	22±1	40±15
Xylazine	39±34	2900±500	0±11	–
Detomidine	28±24	19±23	3±11	–
Guanfacine	27±11	330±30	8±8	–
Tizanidine	24±7	510±420	1±2	–
Guanabenz	23±9	270±310	−6±12	–
Oxymetazoline	21±36	–	13±8	–
Naphazoline	18±12	140±90	−4±13	–
Octopamine	0	–	0	–

The values, mean±s.e.mean, are from triplicate determinations ($n=3-6$).

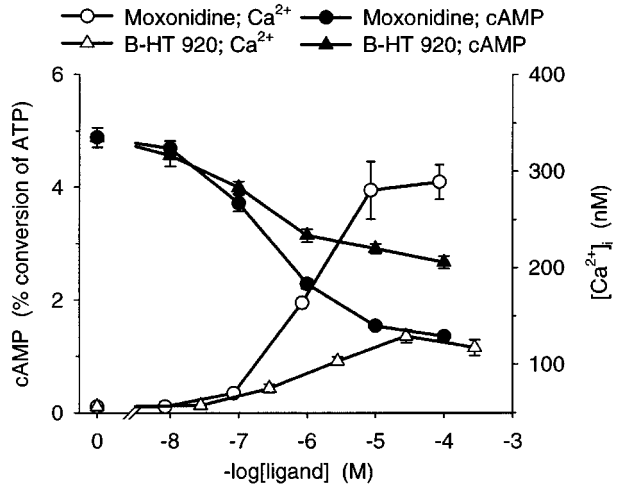

Figure 1 Ca^{2+} and cyclic AMP responses to two ligands, moxonidine (an imidazoline) and B-HT 920 (an oxazoloazepine), as mean±s.e.mean of three determinations in triplicate.

EC_{50} values for the Ca^{2+} and cyclic AMP responses were compared graphically (Figure 2B) the agonists were clearly divided into two groups. Linear regression of the data from catecholamine agonists, which are consistently more potent for inducing Ca^{2+} elevation compared to cyclic AMP inhibition, yields a potency ratio (Ca^{2+} versus cyclic AMP) of 59, whereas the corresponding ratio for imidazoline and ox-/thiazoloazepine agonists, which are more potent against the cyclic AMP response, is 0.27. The difference in $EC_{50\text{-Calcium}}/EC_{50\text{-cyclic AMP}}$ between the groups is highly significant ($P<0.01$). Thus, low concentrations of catecholamines couple exclusively to Ca^{2+} elevation whereas low concentrations of imidazolines and ox-/thiazolazepines preferentially couple to inhibition of cyclic AMP production.

All the responses were inhibited with pertussis toxin-treatment, as they also were inhibited by 10 μM RX 821002. β-adrenoceptors could once again be excluded: 10 μM propranolol, which should have a prominent effect on the previously reported high affinity β-adrenoceptors in HEL cells (Michel *et al*., 1989), caused a similar dextral shift in the concentration-response curves (average of 10 agonists 1.6±0.6 times) to that in the Ca^{2+} measurements. The K_i calculated from this, 16 μM, is in the agreement with the K_i of 23±7 μM for propranolol reported for human α_{2A}-adrenoceptors (Gerhardt *et al*., 1990). Thus no β-adrenoceptors interfered with the α_{2A}-adrenoceptor-mediated responses in the HEL cells used in this study. No response was obtained to isoproterenol (β-adrenoceptor agonist), suggesting that functional β-adrenoceptors are scarce in the HEL cells used in this study. Catecholamine breakdown (monoamine oxidase) and uptake inhibitors nialamide and desipramine (10 μM each), respectively, did not have any effect on the concentration-response curves. To remove possible phospholipase A_2-dependent generation of other messengers we included 150 μM of the cell permeable phospholipase A_2 inhibitor quinacrine (see, for example, Fraser *et al*., 1989) in the assay. This treatment did not change the conclusion of agonist trafficking of α_2-adrenoceptor responses.

Discussion

The results of this study show a difference in the ability of chemically different α_2-adrenoceptor agonists to activate two signalling pathways. The catecholamine ligands are more potent in the Ca^{2+} assay than in the cyclic AMP assay, whereas the imidazoline and ox-/thiazoloazepine ligands are more potent in the cyclic AMP than in the Ca^{2+} assay. Unfortunately, the catecholamine-like ligands possessing a lower number of hydroxyl groups (dopamine and octopa-

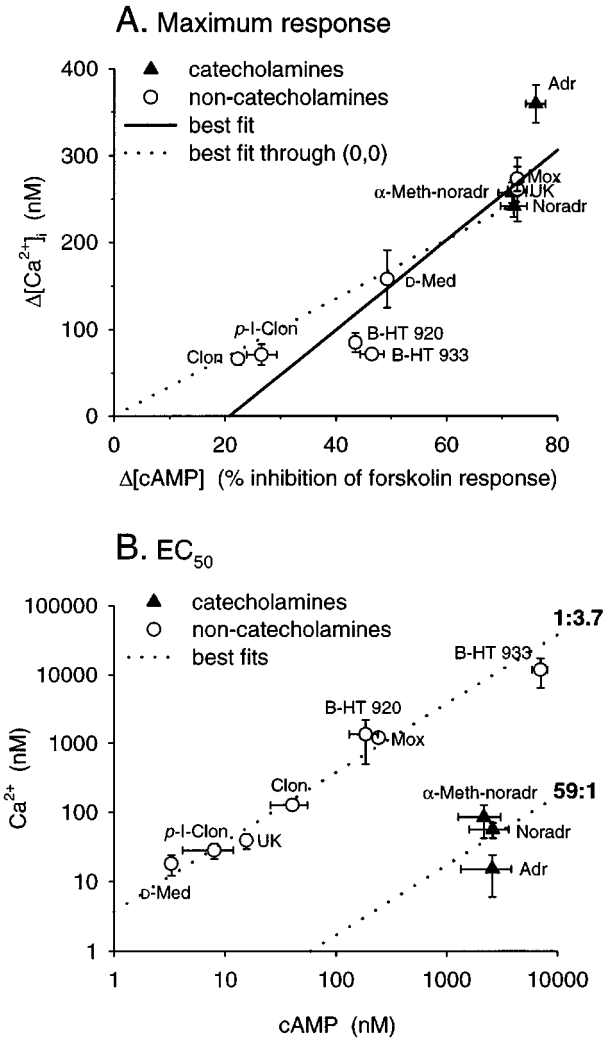

Figure 2 Correlation of the abilities of α_2-adrenoceptor agonists to induce cyclic AMP decrease and Ca^{2+} elevation. (A) The maximum responses; (B) the EC_{50} values. Also drawn are the best linear fit (solid line) and the best linear fit forced to origo (dotted line) in (A) and the relationships 1 : 3.7 and 59 : 1 in (B). The drawn relationships of indicated value are based on the average of logarithms of $EC_{50\text{-}Ca^{2+}}/EC_{50\text{-cyclic AMP}}$ for the corresponding ligand groups. The overall r (calculated for the logarithmic data) for (A) is 0.903 and for (B) 0.437. The overall $EC_{50\text{-}Ca^{2+}}/EC_{50\text{-cyclic AMP}}$ in (B) is 2.84 ± 0.79. Catecholamines alone have $EC_{50\text{-}Ca^{2+}}/EC_{50\text{-cyclic AMP}}$ of 0.0219 ± 0.0095 and the imidazolines and ox-/thiazoloazepines 4.05 ± 0.72 (significance for difference between groups: $P<0.01$).

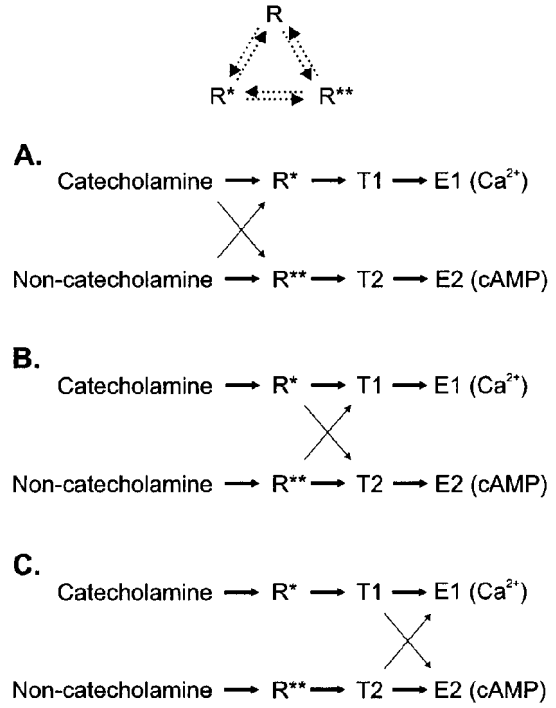

Figure 3 A schematic representation of the hypothesis with three somewhat different models, which are all possible based on the results. R is the inactive and R* and R** the two active receptor conformations, T1 and T2 are two signal transducers (e.g. G proteins) and E1 and E2 are the separate signal cascades which finally lead to a generation of a second messenger response (Ca^{2+} and cyclic AMP, respectively). The solid arrows indicate the direction of the response (the thicker the arrow, the stronger the coupling) and the dotted arrows in the top scheme the interconvertibility of the receptor conformations. In (A) a single receptor conformation only couples to a single transducer, which then couples to a single second messenger cascade. Catecholamines bind more strongly to R* whereas the other ligands bind more strongly to R** leading to a stronger activation of E1 (Ca^{2+}) or E2 (cyclic AMP) response, respectively. In (B) the agonist trafficking of the receptor signals is accomplished at the level of receptor-transducer-coupling: the catecholamine-activated receptor (R*) is more effective in coupling to T1 (and thus to E1) whereas the non-catecholamine-activated receptor (R**) is more effective in its coupling to T2 (and thus to E2). In the third scheme, (C), a single receptor conformation only couples to a single transducer (as in A), which then couples to both second messenger cascades. The agonist trafficking of the receptor signals is brought about by the different ability of transducers to activate different cascades: the catecholamine-activated transducer T1 is more effective in coupling to E1 whereas the non-catecholamine-activated transducer T2 is more effective in its coupling to E2. The schemes (B) and (C) are essentially similar to the scheme presented in Berg *et al.* (1998), though somewhat more complex.

mine) did not give any useful information due to low potency or lack of efficacy, respectively. Altogether, there is a 180 fold difference between the groups in the relative abilities to cause these responses. The greater ability of non-catecholamine ligands to reduce cyclic AMP is also seen in the markedly higher efficacy of the weak partial ox-/thiazoloazepine ligands B-HT 920 and B-HT 933 with respect to the cyclic AMP response, though the same is not seen with imidazolines (clonidine, *p*-I-clonidine, D-medetomidine, UK14,304, moxonidine) which follow rather well the 1 : 1 relationship. This suggests that there might be differences even between imidazolines and ox-/thiazoloazepines.

To ensure that the results obtained corresponded to a true agonist trafficking of receptor responses, we performed a series of control experiments. In these experiments we could show that neither the previously reported β-adrenoceptors (Michel *et al.*, 1989) nor any putative catecholamine uptake or breakdown mechanisms affected the responses measured. We also excluded the possibility that the activation of an intracellular system, phospholipase A_2, could be causing the observed effect by using a phospholipase A_2 inhibitor, quinacrine. Nor was any agonist concentration-curve biphasic or shallow, which would have been indicative of differential coupling to several pathways.

The difference in the efficiency of different agonists towards Ca^{2+} and cyclic AMP responses shows that different agonists

can preferentially activate particular signalling pathways. The difference in the chemical structure of the agonists suggests that this is caused by a stabilization/induction of partially different receptor conformations.

Altogether, the results of the present study show that chemically different ligands can activate different signalling pathways with different efficacy. Our present results, together with our previous results as well as results from many other groups with HEL 92.1.7 cells, indicate that all the responses are mediated (i) by α_2- and not by other adrenoceptors or any other receptors and (ii) by only the α_{2A}-subtype (McKernan *et al*., 1987; Michel *et al*., 1989; Musgrave & Seifert, 1995; Jansson *et al*., 1998). Therefore, our results demonstrate that ligand-specific trafficking of signals is seen with this receptor. The situation is most obvious with ligands like adrenaline and clonidine, the former of which is 8.7 times more potent than the latter with respect to the Ca^{2+} elevation, whereas clonidine decreases cyclic AMP 65 times more potently than adrenaline. The obvious hypothesis is that the diverging signalling for the chemically different ligands is caused by enrichment of different active receptor states, which have different ability to activate different G proteins. The possibilities for divergence are schematically illustrated in Figure 3. The receptor (R) exists in two interconvertible active conformations (R* and R**), which can be differently activated by chemically different ligands. These active receptor conformations activate signal transducers (T1 and T2; e.g. G proteins), the activation of which finally leads to activation of separate second messenger-generating pathways (E1 and E2). The agonist trafficking of the receptor signals is accomplished at the different levels of signal transduction cascade: agonist–receptor-state-coupling (A), receptor-state–transducer-coupling (B) or transducer–second-messenger-cascade-coupling (C). All of these schemes are possible in the light of the results. The nature of the signal transducers cannot be directly resolved. However, a major part could be played by the G proteins. The previously mentioned results with the partial agonists D-medetomidine and B-HT 920 suggest that not only are catecholamines and synthetic ligands trafficking the signals in a different way, but that there may also be differences in the trafficking properties between the chemically different synthetic ligands. This is also indicated in previous studies. Two imidazoline ligands, UK14,304 and *p*-I-clonidine, may activate $G_{\alpha i2}$ and $G_{\alpha i3}$ to a different extent *via* $\alpha_{2A/D}$ (Gerhardt & Neubig, 1991); *p*-I-clonidine is also usually found to be a partial agonist whereas UK 14,304 is a full agonist for $\alpha_{2A/D}$ (Gerhardt *et al*., 1990; Jansson *et al*., 1998). Noradrenaline, UK14,304, oxymetazoline and clonidine are full agonists with respect to the $G_{\alpha o1}$ but only the two first are full agonists with respect to the $G_{\alpha i1}$ activation (Yang & Lanier, 1999). Thus the puzzling results in the present study, where responses putatively mediated by a single G protein subfamily ($G_{i/o}$) show ligand-dependent trafficking, may be explained by differential activation of different members of this family, playing different roles in intracellular signal transduction. Previous results have shown that α_{2A}-adrenoceptors can couple to two different G protein families, namely $G_{i/o}$ and G_s (Eason *et al*., 1992; Pepperl & Regan, 1993). Even agonist trafficking has been shown, although no general conclusions of the activation profile of the ligands of different chemical classes on these responses can be drawn (Eason *et al*., 1994; Airriess *et al*., 1997; Brink *et al*., 2000). This shows that α_{2A}-adrenoceptors are able to adopt different conformations to activate different G proteins; in some cases distinct intracellular domains of the receptor have been shown to be responsible for activation of different G proteins (Eason & Liggett, 1996).

The significance of expression of several members of the $G_{i/o}$ subfamily is rather unclear. In particular, there has been much speculation on the role of G_o. $G_{\alpha o}$ does not inhibit adenylyl cyclase (except for type 1), whereas $G_{\alpha i1}$, $G_{\alpha i2}$ and $G_{\alpha i3}$ do (Taussig *et al*., 1994). Some effects on ion channels seem to rely on G_o (Diverse-Pierluissi *et al*., 1995; Valenzuela *et al*., 1997) and novel forms of regulation of intracellular signalling have been suggested (Hajdu-Cronin *et al*., 1999; Jordan *et al*., 1999; Posner *et al*., 1999). Also, different $G_{\alpha i}$ subtypes may differ in signalling. In Rat1 cells the α_{2A}-mediated cyclic AMP decrease is completely dependent on $G_{\alpha i2}$ although both $G_{\alpha i2}$ and $G_{\alpha i3}$ are activated by the receptor (McClue *et al*., 1992). Furthermore, the regulation of different members of the $G_{i/o}$ family by RGS proteins may be different (Diverse-Pierluissi *et al*., 1999; Posner *et al*., 1999; Cavalli *et al*., 2000). Differential $G_{\beta\gamma}$ coupling of these two subtypes has been suggested by, for example, Diverse-Pierluissi *et al*. (1995).

The role of Ca^{2+} as an intracellular mediator regulated by α_2-adrenoceptors is at the moment unclear. It contradicts the traditional role of α_2-adrenoceptors as inhibitory receptors. Under normal circumstances, α_2-adrenoceptor mediated Ca^{2+} elevations have been measured in smooth muscle preparations (Aburto *et al*., 1993; Lepretre & Mironneau, 1994). Otherwise, α_2-receptor signalling may be directed towards Ca^{2+} elevation under special conditions. For instance, we have shown that treatment of rat cerebral astrocytes with cyclic AMP, which mimics the 'reactive astrocyte'-phenotype, increases the α_{2A}-adrenoceptor expression and α_2-mediated Ca^{2+} elevations (Enkvist *et al*., 1996). More recently, our results have suggested that the α_2-adrenoceptor response can be redirected from the inhibitory, cyclic AMP-decreasing response to a stimulatory response, Ca^{2+} elevation, by the G_q-coupled P2Y-purinoceptors (Åkerman *et al*., 1998). α_2-adrenoceptors may thus be switched to stimulatory responses as part of normal cellular signalling but putatively also under pathological conditions. In this context the observed difference in the ligands' ability to activate the inhibitory (cyclic AMP decrease) and stimulatory responses (Ca^{2+} increase) may also be interesting from the therapeutic point of view.

The pharmacotherapeutic aim of the receptor research is usually to obtain a ligand with a good receptor subtype selectivity. This is evidently not always enough: for instance, in the case of α_2-adrenoceptors, the α_{2A} subtype is responsible for most of the α_2-receptors' very diverse biological functions. In this study we are suggesting that additional selectivity for certain responses can be obtained provided (i) that they are mediated by distinct pathways (i.e. G proteins) and (ii) that these distinct pathways can be activated by pathway-selective ligands. Selective activation of signalling cascades is a new therapeutic principle in line with the other novel ideas of pharmacological intervention of the intracellular signalling pathways.

This study was funded by The Lars Hierta Foundation, The Åke Wiberg Foundation, The Magnus Ehrnrooth Foundation, Oy Veikkaus Ab, The Medical Research Council of Sweden and The Cancer Research Fund of Sweden. We acknowledge Drs Johnny Näsman and Tomas Holmqvist for critical comments during the manuscript preparation, and Drs Birgit Brueggemann (Beiersdorf-Lily GmbH, Hamburg, Germany) and Corinne Gelhay (Pierre Fabre, Castres, France) for the supply of moxonidine and RX821002, respectively.

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(Received November 27, 2000
Revised January 12, 2001
Accepted January 19, 2001)