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Functional assessment of recombinant human α_2-adrenoceptor subtypes with Cytosensor microphysiometry

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Abstract

We applied the Cytosensor Microphysiometry system to study the three human α_2-adrenoceptor subtypes, α_{2A}, α_{2B} and α_{2C}, expressed in Chinese hamster ovary (CHO) cells, and assessed its potential in the quantitative monitoring of agonist activity. The natural full agonist, (−)-noradrenaline, was used to define agonist efficacy. The imidazole derivative dexmedetomidine was a potent full agonist of all three receptor subtypes. The imidazolines clonidine and UK 14,304 (5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine) appeared to be partial agonists at α_{2B}-adrenoceptors ($E_{max} \approx 60\%$ of (−)-noradrenaline) but full agonists at α_{2A}- and α_{2C}-adrenoceptors. The responses mediated by all three α_2-adrenoceptor subtypes were partly inhibited by the sodium–hydrogen (Na^+/H^+) exchange inhibitor, MIA (5-(*N*-methyl-*N*-isobutyl)-amiloride). The agonist responses were totally abolished by pretreatment with pertussis toxin in cells with α_{2A}- and α_{2C}-adrenoceptors, and partly abolished in cells with α_{2B}-adrenoceptors. The residual signal in α_{2B}-cells was sensitive to the intracellular Ca^{2+} chelator, BAPTA (1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid acetoxymethyl ester). Cholera toxin (which acts on G_s-proteins) had no effect on the agonist responses. The results suggest that the extracellular acidification responses mediated by all three human α_2-adrenoceptor subtypes are dependent on Na^+/H^+ exchange and $G_{i/o}$ pathways, and that α_{2B}-adrenoceptors are capable of coupling to another, $G_{i/o}$-independent and Ca^{2+}-dependent signaling pathway.

Keywords: α_2-Adrenoceptor; Functional activity; Microphysiometry; CHO (Chinese hamster ovary) cell; G-protein; Signal transduction

1. Introduction

The α_2-adrenoceptors (α_{2A}, α_{2B} and α_{2C}) are members of a large family of cell membrane receptors which mediate their signals through heterotrimeric guanine nucleotide binding regulatory proteins (G-proteins). The α_2-adrenoceptors have been demonstrated to be capable of mediating signals to several different cellular effectors, including adenylyl cyclases, ion channels (K^+ and Ca^{2+}) and phospholipases (A_2, C and D) (Limbird, 1988; Cotecchia et al., 1990; Duzic and Lanier, 1992; MacNulty et al., 1992). In most cases, α_2-adrenergic regulation of these pathways is sensitive to pertussis toxin, suggesting that GTP-binding proteins in the G_i/G_o subfamily mediate these effects (Kurose et al., 1991; Limbird et al., 1995). An exception to this pertussis toxin-sensitivity is activation of adenylyl cyclase, which appears to be mediated by G_s (Pohjanoksa et al., 1997). On the other hand, pertussis toxin-sensitive G-proteins, $G_{i\alpha 2}$ and $G_{i\alpha 3}$, have been shown to mediate 5-HT_{1A}-receptor activation of Na^+/H^+ exchange in transfected Chinese hamster ovary (CHO) cells (Garnovskaya et al., 1997). Also other forms of heterotrimeric GTP-binding proteins, $G_{\alpha q}$, $G_{\alpha 12}$ and $G_{\alpha 13}$, have been shown to activate Na^+/H^+ exchange in mammalian cells through protein kinase C-dependent and -independent signaling pathways (Dhanasekaran et al., 1994; Lin et al., 1996; Orlowski and Grinstein, 1997). The coupling of α_2-adrenoceptor subtypes to regulation of Na^+/H^+ exchange has not been investigated in a standardized test model such as transfected CHO cells.

Continuous monitoring of extracellular pH with the Cytosensor Microphysiometry system allows the detection and quantitation of functional responses from living cells (McConnell et al., 1992). The microphysiometry assay continuously monitors the extracellular pH surrounding cells in culture, and reports receptor activation by measuring increases in extracellular acidification rate, occurring

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in response to agonist stimulation. Several receptor types have been shown to produce responses upon agonist activation allowing quantitative pharmacological analysis, including the use of modulators and antagonists (Chio et al., 1993; Castro et al., 1996; Brown et al., 1997; Garnovskaya et al., 1997; Smith et al., 1998; Wood et al., 1999).

We have now performed the first application of this method to study the three human α_2-adrenoceptor subtypes α_{2A}, α_{2B} and α_{2C}, heterologously expressed in stably transfected CHO cell lines. The natural full agonist, (−)-noradrenaline, was used to determine maximal responsivity. The experiments were performed using four different agonists, (−)-noradrenaline, clonidine, dexmedetomidine and 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine (UK 14,304). We were interested in identifying the precise signal transduction pathways utilized by α_2-adrenoceptor subtypes in recombinant CHO cells. Experiments involving pharmacological perturbations of the signal transduction pathways of CHO cells were therefore performed. Our results support the usefulness of this new assay for agonist characterization and for studies on coupling and signaling mechanisms of α_2-adrenoceptors.

2. Materials and methods

2.1. Materials

[^{3}H]RX821002 (2-(2-methoxy-1,4-benzodioxan-2-yl)-2-imidazoline) was from Amersham (Buckinghamshire, UK; specific activity 52 Ci/mmol). BAPTA (1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid acetoxymethyl ester), MIA (5-(*N*-methyl-*N*-isobutyl)-amiloride) and UK 14,304 were from Research Biochemicals (Natick, MA, USA). Dexmedetomidine was a gift from Orion-Farmos (Turku, Finland). The following compounds were obtained from Sigma (St. Louis, MO, USA): (−)-noradrenaline (bitartrate salt), clonidine, Geneticin® (G418), pertussis toxin and cholera toxin. Cell culture reagents were supplied by Gibco (Gaithersburg, MD, USA). Other chemicals were of analytical or reagent grade, and were purchased from commercial suppliers.

2.2. Transfection and cell culture

Adherent CHO cells (American Type Culture Collection, Rockville, MD, USA) were cultured in αMEM (α-minimum essential medium) supplemented with 2 mM glutamine, 26 mM $NaHCO_3$, 5% heat-inactivated fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. The cells were grown in 5% CO_2 at 37°C. Cells were harvested into chilled phosphate-buffered saline, pelleted and suspended in αMEM for Cytosensor Microphysiometry. The pMAMneo-based (Clontech Laboratories, Palo Alto, CA, USA) expression constructs were transfected into CHO cells with the Lipofectin® reagent kit (Gibco, Paisley, UK). For each transfection 3 μg plasmid DNA was used per 5×10^4 cells. Neomycin (G418) resistant clones (750 μg/ml) were selected and examined for their ability to bind the α_2-adrenoceptor antagonist [^{3}H]RX821002. The transfected cells chosen for further experiments were subsequently maintained in 250 μg/ml G418 (Pohjanoksa et al., 1997).

2.3. Radioligand binding

The receptor densities (B_{max}) and the corresponding radioligand K_d values for the cell clones were determined in saturation binding experiments using [^{3}H]RX821002 as radioligand (Halme et al., 1995). No specific binding of [^{3}H]RX821002 was observed in non-transfected CHO cells. Two expression levels of each receptor, approximately 0.3–0.7 pmol/mg total cellular protein (''low'') and 1.3–3.0 pmol/mg (''high''), were chosen for functional studies. They were: B_{max} 1.3 ± 0.2 pmol/mg, K_d 0.89 ± 0.27 nM (high) and B_{max} 0.3 ± 0.02 pmol/mg, K_d 0.48 ± 0.08 nM (low) for α_{2A}-adrenoceptors, B_{max} 2.6 ± 0.5 pmol/mg, K_d 4.37 ± 0.55 nM (high) and B_{max} 0.54 ± 0.18 pmol/mg, K_d 2.20 ± 0.08 nM (low) for α_{2B}-adrenoceptors and B_{max} 3.0 ± 0.4 pmol/mg, K_d 1.28 ± 0.12 nM (high) and B_{max} 0.65 ± 0.12 pmol/mg, K_d 0.98 ± 0.13 nM (low) for α_{2C}-adrenoceptors (Pohjanoksa et al., 1997; Peltonen et al., 1998).

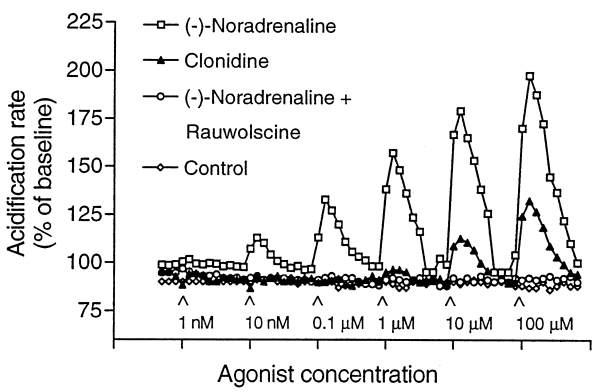

Fig. 1. Acidification rate data taken from Cytosoft, the Cytosensor software. CHO cells expressing the α_{2B}-adrenoceptor subtype (2.6 pmol/mg protein) were exposed to increasing concentrations of (−)-noradrenaline, clonidine and (−)-noradrenaline with 100 μM rauwolscine. Non-transfected CHO cells were similarly exposed to increasing concentrations of (−)-noradrenaline (control). Cells were exposed to successive 50 s applications of agonist, at the concentrations indicated, and a 30 min wash was employed between successive agonist exposures. Increases in acidification rate due to agonist exposure are expressed as % increase over normalized baseline. Data are representative of three to six separate experiments. Each division of the abscissa represents 50 s agonist application and 30 min wash.

2.4. Measurement of extracellular acidification rate

Extracellular acidification rates were measured using a four-channel Cytosensor Microphysiometry instrument (Molecular Devices., Menlo Park, CA, USA). Cultured CHO cells were seeded into 12-mm capsule cups at 3×10^5 cells/cup and incubated in 5% CO_2 at 37°C for 20 h. The capsule cups were loaded into the sensor chambers of the instrument and the chambers were perfused with running medium (bicarbonate-free αMEM supplemented with 2 mM glutamine, 26 mM NaCl, 50 U/ml penicillin and 50 μg/ml streptomycin), at a flow rate of 100 μl/min. Agonists were diluted into running medium and perfused through a second fluid path. Valves directed the flow from either fluid path to the sensor chamber. During each 2 min pump cycle, the pump was on for 1 min 20 s and was then switched off for the remaining 40 s. The pH of the running medium was recorded from 1:25 to 1:55 min. The pump was started at 2 min to start the next cycle. Cells were exposed to agonists for 50 s. A 15 to 30 min wash period was employed between successive agonist exposures. This stimulation protocol was validated in preliminary experiments. The rate of acidification of the chamber was calculated by the Cytosoft program (Molecular Devices). All four chambers of the instrument had identical values for the response in mV s^{-1}/change in pH units, i.e., 61 mV s^{-1}/pH unit. Changes in the rate of acidification were calculated as the difference between the maximum effect after agonist addition and the average of three measurements taken immediately before agonist addition.

In some experiments, confluent cells were preincubated with pertussis toxin (200 ng/ml) (Garnovskaya et al.,

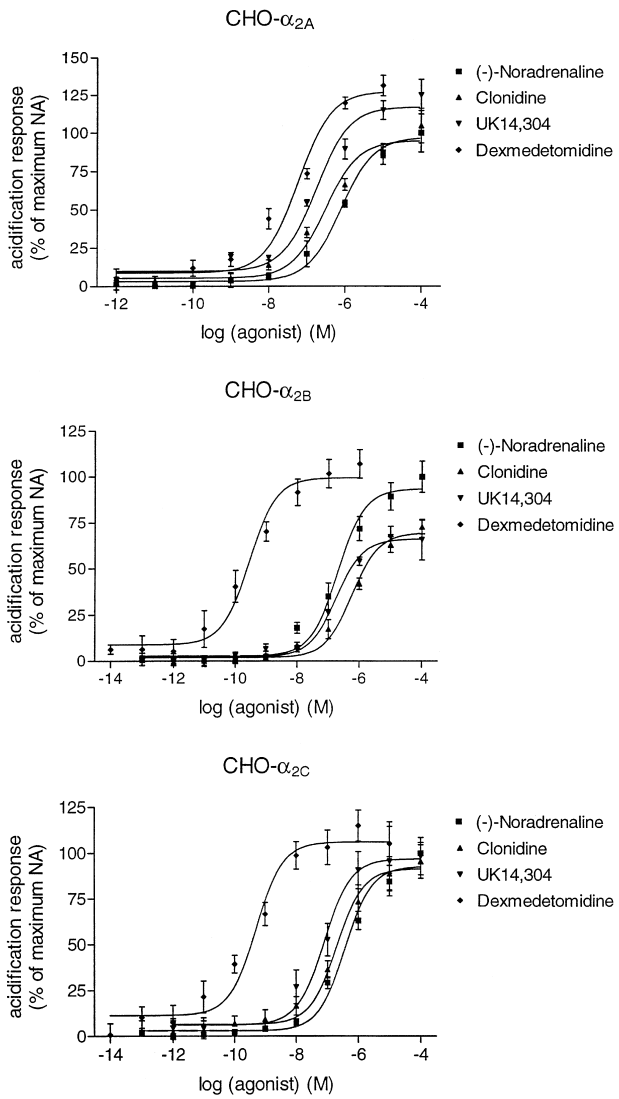

Fig. 2. Concentration–response curves generated from the extracellular acidification rate data. The responses relative to 100 μM (−)-noradrenaline (NA) were calculated for α_{2A}, α_{2B} and α_{2C} adrenoceptor subtypes with ''high'' (1.3–3.0 pmol/mg protein) receptor densities. Values are means ± S.E. from three to six separate experiments.

1997; Peltonen et al., 1998) or cholera toxin (1 μg/ml) (Shimegi et al., 1994) for 18–20 h before the capsule cups were loaded into the sensor chambers. Other test substances, such as the Na^+/H^+exchange inhibitor, MIA and the intracellular Ca^{2+}chelator, BAPTA, were added 15 min (MIA, 10 μM) (Garnovskaya et al., 1997) or 30 min (BAPTA, 50 μM) (Jansson et al., 1991) before agonists. Baseline acidification rates were normalised to 100% and changes due to agonist exposure were calculated as percent increases over normalized baseline. Control responses were recorded in the presence of a maximally effective concentration of (−)-noradrenaline (100 μM) at the start and end of each agonist concentration–effect experiment. No desensitization was observed.

2.5. Calculations

EC_{50} and maximal response (E_{max}) values were calculated using the curve-fitting programme GraphPad PRISM (GraphPad Software, San Diego, CA, USA). The results are expressed as means ± S.E. of three to six separate experiments. Student's *t*-test with two-tailed probabilities, or one-way analysis of variance (ANOVA) together with the Student–Newman–Keuls-test were used to test the statistical significance of the observed differences between group means.

3. Results

3.1. Agonist activities

Functional activation of α_2-adrenoceptors in intact CHO cells was assessed with Cytosensor Microphysiometry using four different agonists, (−)-noradrenaline, clonidine, dexmedetomidine and UK 14,304. The natural full agonist, (−)-noradrenaline (100 μM), was used as a reference compound to determine maximal responsivity. The response to (−)-noradrenaline was blocked by the α_2-adrenoceptor antagonist, rauwolscine (100 μM), and was absent in non-transfected CHO cells (Fig. 1). The imidazole derivative dexmedetomidine was a potent full agonist of all three receptor subtypes; its efficacy actually exceeded that of (−)-noradrenaline in some cases ($P < 0.001$ for α_{2A}), as shown in Fig. 2 and Table 1. The imidazolines clonidine and UK 14,304 appeared to be partial agonists at α_{2B}-adrenoceptors ($E_{max} \approx 60\%$ of (−)-noradrenaline; $P < 0.005$) (Fig. 2.) but full agonists at α_{2A}- and α_{2C}-adrenoceptors. Also the efficacy of UK 14,304 exceeded that of (−)-noradrenaline at α_{2A}-adrenoceptors ($P < 0.01$). The rank order of agonist potency was dexmedetomidine > UK 14,304 > clonidine > (−)-noradrenaline for α_{2A}- and α_{2C}-adrenoceptor subtypes, and dexmedetomidine > UK 14,304 ≥ (−)-noradrenaline > clonidine for the α_{2B}-adrenoceptor subtype.

Table 1
Comparison of half-maximal effective concentrations (EC_{50}) and maximal effects (E_{max}) of agonists at ''high'' (1.3–3.0 pmol/mg) expression levels of each receptor subtype (α_{2A}, α_{2B} and α_{2C}), in transfected CHO cell lines in the Cytosensor Microphysiometry assay. Values are means ± S.E. from three to six separate experiments
The EC_{50} values were estimated from log concentration–response curves by non-linear regression analysis, and E_{max} values are relative to a maximally effective concentration of (−)-noradrenaline (NA, 100 μM).

Agonist	K_i (nM)[a]	EC_{50} (nM)	E_{max} (% of NA)
CHO-α_{2A}			
(−)-Noradrenaline	277 ± 145	756 ± 107	100 ± 2
Clonidine	17.2 ± 1.54	283 ± 142	105 ± 11
Dexmedetomidine	2.20 ± 0.25	67.1 ± 5	132 ± 7[b]
UK 14,304	12.9 ± 1.77	159 ± 49	125 ± 10[c]
CHO-α_{2B}			
(−)-Noradrenaline	500 ± 206	200 ± 57	100 ± 9
Clonidine	56.0 ± 12.6	578 ± 190	73 ± 8[d]
Dexmedetomidine	3.33 ± 1.41	0.29 ± 0.01	107 ± 8
UK 14,304	525 ± 38	178 ± 11	66 ± 11[d]
CHO-α_{2C}			
(−)-Noradrenaline	256 ± 102	369 ± 101	100 ± 6
Clonidine	96.9 ± 8.69	199 ± 154	95 ± 9
Dexmedetomidine	2.97 ± 1.78	0.50 ± 0.17	115 ± 9
UK 14,304	361 ± 101	78.2 ± 39	98 ± 10

[a] K_i-values published by Pohjanoksa et al. (1997), except for UK 14,304, which was determined in the current study.
[b] $P < 0.001$ compared to (−)-noradrenaline.
[c] $P < 0.01$ compared to (−)-noradrenaline.
[d] $P < 0.005$ compared to (−)-noradrenaline.

The relative maximal effect (E_{max}) of these compounds (compared to (−)-noradrenaline) and their potency (EC_{50}) values were similar in CHO cells with ''low'' and ''high'' (0.3–0.7 or 1.3–3.0 pmol/mg total cellular protein) expression levels of α_2-adrenoceptor subtypes (data not shown). In absolute terms, the increases in extracellular acidification rates (μV s^{-1}) induced by (−)-noradrenaline were different for these two receptor densities, ''low'' and ''high''. Thus, in α_{2A} maximal extracellular acidification rate increases were 30–40 μV s^{-1} (''low'') and 70–90 μV s^{-1} (''high''), in α_{2E} 40–50 μV s^{-1} (''low'') and 80–100 μV s^{-1} (''high''), and in α_{2C} 40–50 μV s^{-1} (''low'') and 90–110 μV s^{-1} (''high'').

3.2. Signal transduction pathways

Intact cells were pretreated with the inhibitors, MIA, pertussis toxin and cholera toxin, and with the intracellular Ca^{2+}chelator, BAPTA, and with their different combinations, to assess the roles of some possible signal transduction pathways in the receptor responses in the Cytosensor Microphysiometry system. Changes in acidification rates were induced with 1 μM dexmedetomidine in pretreated and control cell chambers.

The responses mediated by α_{2A}- and α_{2C}-adrenoceptors were totally abolished by pretreatment of intact CHO cells

with pertussis toxin ($P < 0.001$), and were partly abolished also in cells with α_{2B}-adrenoceptors (72%) ($P < 0.001$). This indicated that the observed acidification rate increases were either entirely or partly (α_{2B}) due to the coupling of α_2-adrenoceptors to pertussis toxin-sensitive G-proteins (G_i/G_o). The residual signal in α_{2B}-cells was sensitive to the intracellular Ca^{2+} chelator, BAPTA (50 μM). BAPTA alone inhibited α_2-adrenoceptor-mediated acidification responses to 1 μM dexmedetomidine by 65% in α_{2A}- ($P < 0.005$), by 71% in α_{2B}- ($P < 0.001$) and by 70% in α_{2C}-expressing cells ($P < 0.005$). The responses to 1 μM dexmedetomidine mediated by all three α_2-adrenoceptor subtypes were partly inhibited by the Na^+/H^+exchange inhibitor, MIA (10 μM). The maximal inhibitory effect with 10 μM MIA was 71% for α_{2A} ($P < 0.005$), 62% for α_{2B} ($P < 0.005$) and 51% for α_{2C} ($P < 0.005$) (Fig. 3). In addition, the inhibitory effects of BAPTA and MIA added simultaneously were 63%–77% for all three subtypes.

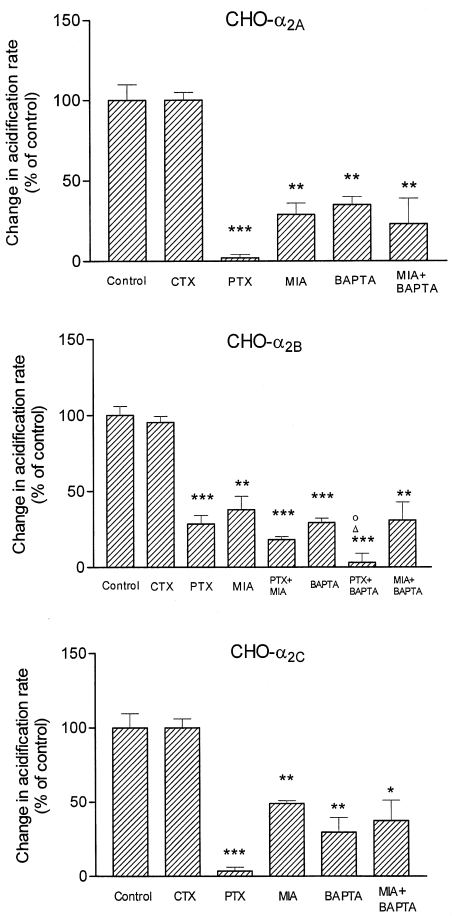

Fig. 3. The functional responses mediated by all three α_2-adrenoceptor subtypes (1.3–3.0 pmol/mg protein) were fully, partly or not at all inhibited by pertussis toxin (200 ng/ml), cholera toxin (1 μg/ml), MIA (10 μM) or BAPTA (50 μM). Changes in acidification rates were induced with 1 μM dexmedetomidine in pretreated and control cell chambers. Values are means ± S.E. from three to five separate experiments. $^{***}P < 0.001$; $^{**}P < 0.005$; $^{*}P < 0.01$ compared to control; $^{o}P < 0.005$ vs. BAPTA and $^{\Delta}P < 0.005$ vs pertussis toxin.

Cholera toxin (which acts on G_s-proteins) had no statistically significant effect on extracellular acidification responses to 1 μM dexmedetomidine, indicating no mediation of the effect through G_s-proteins.

4. Discussion

This study demonstrated that transfected human α_2-adrenoceptor subtypes are coupled to regulation of proton extrusion rate (extracellular acidification) in intact CHO cells. Microphysiometry (McConnell et al., 1992) proved to be useful in the analysis of functional coupling of agonist stimulation at the three α_2-adrenoceptor subtypes.

Guanine nucleotide effects on agonist binding are a characteristic of G-protein-linked α_2-adrenoceptors (Jansson et al., 1994; Halme et al., 1995). At the second messenger level, adenylyl cyclase inhibition has been classically described as a response to activation of α_2-adrenoceptor subtypes (Pohjanoksa et al., 1997). Since neither binding assays nor adenylyl cyclase responses alone give sufficient information about agonist–receptor interactions, the specific functional characteristics of each α_2-adrenoceptor subtype have been further elucidated in different assays monitoring G-protein activation (Virolainen et al., 1997; Peltonen et al., 1998) and intracellular Ca^{2+} responses (Kukkonen et al., 1997; Soini et al., 1998), and now with the Cytosensor Microphysiometry assay system.

In the current study, clear differences in efficacy at the α_{2B}-adrenoceptor subtype could be observed between the full agonists (−)-noradrenaline and dexmedetomidine and the partial agonist clonidine. Also UK 14,304 appeared to be a partial agonist at α_{2B}-adrenoceptors. The result for UK 14,304 at α_{2B}-adrenoceptors was unexpected, because it has been a full agonist in adenylyl cyclase assays (Jansson et al., 1994; Pohjanoksa et al., 1997). However, in line with the present results, it was recently shown with a [^{35}S]GTPγS binding assay and CHO cell membranes that UK 14,304 acts as full agonist at the α_{2A}-adrenoceptor subtype but as a partial agonist at α_{2B}- and α_{2C}-adrenoceptors (Peltonen et al., 1998). The rank orders of potency determined for the tested α_2-adrenoceptor agonists in stimulating increases in extracellular acidification rate in CHO cells were in general agreement with those determined in studies on the inhibition of forskolin-stimulated adenylyl cyclase activity (Pohjanoksa et al., 1997), and GTPγS-binding assays (Jasper et al., 1998; Peltonen et al., 1998). GTPγS-binding assays give direct information on receptor-mediated G-protein activation, in contrast to the currently employed Microphysiometry technique, which measures the production of acidic metabolites by complex living cell systems.

The potency and relative efficacy (compared to (−)-noradrenaline) of agonists were not dependent on receptor density in Cytosensor Microphysiometry assays. A high

receptor density (1.3–3.0 pmol/mg) did not interfere with discrimination between full and partial agonists compared to cells with a lower receptor density (0.3–0.7 pmol/mg), as has previously been the case in adenylyl cyclase assays (Pohjanoksa et al., 1997). At α_{2A}-adrenoceptors, dexmedetomidine and UK 14,304 were capable of inducing greater maximal responses than the endogenous ligand (−)-noradrenaline. One possible explanation for this is that the intensity of the response may be related to the relative affinities of the agonist for the various active receptor conformations. Alternatively, the different responses may be related to the kinetics of the drug–receptor interactions.

Our results indicate that the α_{2B}-adrenoceptor subtype clearly differs from the other two α_2-adrenoceptor subtypes in its coupling to second messenger systems. We conclude that all three α_2-adrenoceptor subtypes caused receptor-mediated, concentration-dependent increases in extracellular acidification rate, which were dependent on pertussis toxin-sensitive G-proteins (G_i), availability of intracellular Ca^{2+} and a Na^+/H^+ exchange mechanism, but did not involve the action of G_s-proteins. In addition, no marked differences in the proportional involvement of these signaling pathways could be seen between the receptor subtypes, with the exception of the α_{2B}-adrenoceptor subtype, which was markedly pertussis toxin-insensitive compared to the other two subtypes.

In contrast to assays measuring changes in second messenger concentrations, the current Microphysiometry assay gives direct information on receptor-mediated functional activation in complex living cell systems. Because the system permits rapid bioassays from living, intact cells, it appears to offer significant advantages for possible applications in basic research and in biotechnology (McConnell et al., 1992). Our results support the usefulness of this new assay for agonist characterization and for screening of new α_2-adrenoceptor ligands.

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