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α_{2B}-Adrenoceptors couple to Ca^{2+} increase in both endogenous and recombinant expression systems

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Abstract

The ability of cloned human α_{2B}-adrenoceptors heterologously expressed in Sf9 cells and endogenous α_{2B}-adrenoceptors in NG 108-15 neuroblastoma × glioma cells to couple to increase of intracellular Ca^{2+} was studied. Ca^{2+} increases in NG 108-15 cells were detectable but slight, whereas those in α_{2B}-adrenoceptor-expressing Sf9 cells were greater. In the latter, the maximum Ca^{2+} increase correlated positively, and the EC_{50}-value of noradrenaline negatively, with the receptor expression density. The order of potency of the agonists was D-medetomidine ([D]-4-[5]-[1-(2,3-dimethylphenyl)ethyl]-1*H*-imidazole) > noradrenaline ≈ clonidine > oxymetazoline, with clonidine and UK14,304 (5-bromo-*N*-[4,5-dihydro-1*H*-imidazole-2-yl]-6-quinoxalinamine) being weak partial agonists. In Sf9 cells Ca^{2+} increases consisted of concomitant mobilization from an intracellular store and influx of extracellular Ca^{2+}. In these cells α_{2B}-adrenoceptor stimulation also increased the inositol 1,4,5-trisphosphate mass. We conclude that α_{2B}-adrenoceptors can couple to intracellular Ca^{2+} increases which may involve prior activation of phospholipase C. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: α_{2B}-Adrenoceptor; Ca^{2+} signaling; Fura-2; Sf9; Baculovirus expression system; NG 108-15

1. Introduction

Traditionally, α_1-adrenoceptors were thought to couple to inositol phospholipid hydrolysis and Ca^{2+} mobilization and α_2-adrenoceptors to couple to inhibition of adenylyl cyclase (Limbird, 1988). More recently it has become clear that α_2-adrenoceptors can also couple to other responses such as increase of cAMP and cytosolic Ca^{2+}. In some cases the Ca^{2+} increase has been due to activation of cation channels (Aburto et al., 1993; Lepretre and Mironneau, 1994; Musgrave and Seifert, 1995) but also Ca^{2+} mobilization from the intracellular stores has been shown in HEL human erythroleukemia cells (Michel et al., 1989a; Dorn et al., 1997), human platelets (Kagaya et al., 1992), rat cerebral astrocytes (Salm and McCarthy, 1990; Enkvist et al., 1996), and smooth muscle cells (Aburto et al., 1993; Erdbrugger et al., 1993). Since most of the studies have not characterized the α_2-adrenoceptor subtype involved, little information is available about a possible subtype-selective coupling of α_2-adrenoceptors to Ca^{2+} increases. However, the Ca^{2+} increases in HEL cells and astrocytes have been shown to occur via α_{2A}-adrenoceptors (Michel et al., 1989a; Enkvist et al., 1996), whereas in porcine aortic smooth muscle cells two distinct subtypes may be involved, which can be differentiated based on their prazosin sensitivity (Erdbrugger et al., 1993). Although IP_3 (inositol 1,4,5-trisphosphate) increases in response to α_2-adrenoceptor stimulation were not detected in the initial studies in HEL cells (Michel et al., 1989a), they were later indicated by the sensitivity of the Ca^{2+} elevations to phospholipase C inhibitors and by direct IP_3 measurements both in these cells (Åkerman et al., 1996; Dorn et al.,

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1997) and in rat cerebral astrocytes (Enkvist et al., 1996). Unlike the IP_3 and Ca^{2+} increases mediated by α_1-adrenoceptors, the IP_3 and Ca^{2+} increase mediated by α_2-adrenoceptors has been shown to be sensitive to pertussis toxin pretreatment, suggesting involvement of $G_{i/o}$ proteins.

The cloning of all three pharmacologically characterized α_2-adrenoceptor subtypes (α_{2A}, α_{2B}, α_{2C}) has allowed their heterologous expression in a variety of cells, including Chinese hamster lung fibroblasts (Cotecchia et al., 1990), COS-7 (from CV-1 Origin, SV40) monkey kidney fibroblasts (Cotecchia et al., 1990), Chinese hamster ovary cells (Eason et al., 1992), S115 mouse mammary tumor cells (Jansson et al., 1994) and Sf9 insect ovary cells (Oker-Blom et al., 1993; Jansson et al., 1995). Heterologously expressed α_{2A}-adrenoceptors have been shown to couple to Ca^{2+} increases in Chinese hamster ovary and COS-7 cells (Dorn et al., 1997). α_{2C}-adrenoceptors also can weakly stimulate inositol phospholipid hydrolysis in Chinese hamster lung fibroblasts and COS-7 cells (Cotecchia et al., 1990) although no Ca^{2+} elevations have been measured. Indirect evidence of α_{2B}-adrenoceptor-mediated Ca^{2+} increases has been obtained in PC-12 pheochromocytoma cells transfected with α_{2B}-adrenoceptors (Duzic and Lanier, 1992).

Therefore the aim of the present study was to systematically investigate the ability of cloned α_2-adrenoceptor subtypes to promote Ca^{2+} increases upon recombinant expression. For this purpose we have chosen the transient expression in Sf9 cells as a high efficiency expression system which enables studies at different expression levels (Kukkonen et al., 1996). These cells express G_i-, G_s- and G_q-like G proteins as determined using antibodies directed against rat G proteins (Butkerait et al., 1995). As essentially only α_{2B}-adrenoceptors couple to Ca^{2+} elevation in this expression system, their pharmacology was characterized. Further measurements were made in a cell line which endogenously expresses α_{2B}-adrenoceptors, NG 108-15 neuroblastoma × glioma cells (Bylund et al., 1988; Bylund and Ray Prenger, 1989; McClue and Milligan, 1990; Wilson et al., 1991).

2. Materials and methods

Experiments on Sf9 cells were performed at the Åbo Akademi University and the experiments on NG 108-15 cells at the Universitätsklinikum Essen.

2.1. Plasmid constructs, viruses and cells

The recombinant baculovirus containing the genes encoding α_{2A}- and α_{2B}-adrenoceptors, respectively, were produced by transfection of the constructs pLucGRBac1-α_{2A} and pLucGRBac1-α_{2B} with wild-type *Ac*NPV DNA into Sf9 cells as described in detail previously (Karp et al., 1992; Jansson et al., 1995). The recombinant baculovirus containing the α_{2C}-adrenoceptor-coding gene was produced as described by Oker-Blom et al. (1993).

Spodoptera frugiperda (Sf9) insect cells were grown in TNMFH medium (Grace's medium supplemented with lactalbumin hydrolyzate and yeastolate; pH 6.3, 9 mM $CaCl_2$) supplemented with 100 U/ml penicillin (Nordvacc Media, Sweden), 80 U/ml streptomycin (Nordvacc Media), 2.5 μg/ml fungizone (Gibco, UK) and 10% (v/v) heat inactivated fetal calf serum (Gibco) essentially as described by Summers and Smith (1987). Cultures were maintained in suspension at 22°C using glass spinner bottles (Bellco, USA). For the infections Sf9 cells were plated out on plastic culture dishes (∅96 mm; Greiner, Germany). After adhesion (15 min) the cell monolayer was infected with virus in TNMFH medium (pH 6.3). The multiplicity of infection was at least 5 (5 virus particles per cell). Eight hours post infection the medium was replaced with TNMFH medium (pH 7.4, 1.5 mM $CaCl_2$ (Kukkonen et al., 1996)). The change in pH is likely to facilitate acquisition of the physiological ligand binding properties by the receptors (Birdsall et al., 1989). Some batches of cells were pretreated with pertussis toxin after the infection to assess a possible involvement of pertussis toxin-sensitive G proteins. Since high pertussis toxin concentrations seem to be needed to obtain significant ADP-ribosylation of insect G proteins (Butkerait et al., 1995; see also Jansson et al., 1995) and the applicable preincubation time was limited to a maximum of 24 h, a concentration of 500 ng/ml was used.

NG 108-15 cells were kindly provided by Drs. Ian Musgrave and Günther Schulz from the Department of Pharmacology at the Freie Universität Berlin (Berlin, Germany). The cells were cultured in DMEM medium (Dulbecco's modified Eagle medium; Gibco) supplemented with 100 μM hypoxanthine, 1 μM aminopterin, 20 μM thymidine, 2 mM glutamine, 100 U/ml penicillin, 80 U/ml streptomycin and 10% (v/v) fetal calf serum in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Some batches of cells were pretreated with pertussis toxin after the infection to assess a possible involvement of pertussis toxin-sensitive G proteins. Since longer incubations than with Sf9 cells were possible a concentration of 100 ng/ml was used for 72 h.

2.2. Reagents

Br-cAMP (8-Bromoadenosine 3′:5′-cyclic monophosphate), clonidine, EGTA, forskolin, (−)-noradrenaline, oxymetazoline, pertussis toxin, phentolamine, prostaglandin E_2, rauwolscine and theophylline were purchased from Sigma (St. Louis, MO, USA). Digitonin was from Merck (Darmstadt, Germany), fura-2 acetoxymethyl ester and BAPTA (1,2-bis[*o*-aminophenoxy]ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) acetoxymethyl ester from Molecular Probes (Eugene, OR, USA) and UK 14,304 (5-bromo-*N*-[4,5-dihydro-1*H*-imidazol-2-yl]-6-quinoxalinamine) from

RBI (Natick, MA, USA). L- and D-medetomidine ([L/D]-4-[5]-[1-(2,3-dimethylphenyl)ethyl]-1H-imidazole) were generously provided by Orion-Corporation Orion-Pharma (Turku, Finland). [^{3}H]rauwolscine (82.3 Ci/mmol) was purchased from DuPont NEN (Boston, MA, USA).

2.3. Ca^{2+}-measurements with Sf9 insect cells

The fluorescent Ca^{2+}-indicator fura-2 was used to monitor changes in intracellular Ca^{2+} (Grynkiewicz et al., 1985) essentially as described in (Kukkonen et al., 1996). Briefly, the cells were gently detached by suspension in Ca^{2+}-free HEPES buffered medium (HBM), spun down and resuspended in HBM (129.7 mM NaCl, 5.44 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 7.3 mM NaH_2PO_4, 63 mM sucrose and 20 mM HEPES; pH adjusted to 7.4 with NaOH (Kukkonen et al., 1996)). The cells were loaded with 4 μg/ml fura-2 acetoxymethyl ester in HBM at 25°C for 20 min, washed once with Ca^{2+}- free HBM and stored on ice as pellets. Intracellular free calcium was measured as follows: One pellet (about 5×10^5 cells) was resuspended in 350 μl HBM at 25°C and the cell suspension was placed in a stirred quartz microcuvette in a thermostated cell-holder in a fluorescence spectrophotometer. Fluorescence was monitored either with a Hitachi F-4000 or a Hitachi F-2000 fluorescence spectrophotometer at wavelengths 340 nm (excitation) and 505 nm (emission). The single wavelength method was preferred to the double wavelength method because the pronounced leak of fura-2 out of the Sf9 cells during the experiments, and the subsequent increase in extracellular fura-2 fluorescence, made the calculation of the ratio uncertain. The single wavelength method was applicable here since (i) all the experiments could be calibrated using 60 μg/ml digitonin, which gives the maximum value for fluorescence (F_{max}) and 10 mM EGTA, which gives the minimum value for fluorescence (F_{min}), (ii) no absorbance/fluorescence artifacts were observed with any of the compounds used and (iii) all the α_2 agonist signals could be inhibited using α_2 antagonists. The free Ca^{2+}-concentration was calculated from the fluorescence (F) using the equation

$$[Ca^{2+}]_i = (F - F_{min})/(F_{max} - F) \times 224 \text{ nM}$$

in which the extracellular fura-2 fluorescence is subtracted from the F values.

In some experiments single cells were investigated using image analysis. For these experiments the cells were grown on circular glass coverslips (Ø22 mm) and loaded with 5 μg/ml fura-2 acetoxymethyl ester at 37°C in HBM for 20 min. The experiments were performed and the data were analyzed using an Intracellular Imaging InCyt2™ fluorescence imaging system. The cells were excited by alternating wavelengths of 340 and 380 nm using narrow-band excitation filters and fluorescence was measured through a 430 nm dichroic mirror and a 510-nm barrier filter with a Cohu CCD camera. Two ratioed images were acquired per second.

2.4. Inositol 1,4,5-trisphosphate (IP_3) measurements with Sf9 cells

Sf9 cells were infected as for Ca^{2+} measurement. The cells were gently scraped off 24 h post infection and washed twice with Ca^{2+}-free HBM. The cells were suspended in HBM and the cell number was adjusted to 10^7 cells/ml by counting in a Bürker chamber. IP_3 was extracted with perchloric acid essentially as described by Palmer et al. (1986). The basal IP_3 content was measured as such or after preincubation with 100 μM forskolin (1 min), 2 mM 8-Br-cAMP (2 min), 20 nM ionomycin (1 min) or 30 μM BAPTA acetoxymethyl ester (20 min). In all the cases above the noradrenaline-stimulated (100 μM) IP_3 increase also was measured after 1-min incubation (to match the time course of the Ca^{2+} increase). The reactions were terminated by mixing 100 μl of cell suspension with 25 μl ice-cold 20% (v/v) perchloric acid. The samples were incubated on ice for 20 min, after which the protein was sedimented by centrifugation at $2000 \times g$ for 15 min at 4°C. The supernatants were neutralized with 1.5 M KOH containing 60 mM HEPES and 0.5 M Tris–HCl (pH 9) was added to a final concentration of 0.1 M (final pH ≈ 9). The resulting $KClO_4$ sediment was removed by centrifugation at $2000 \times g$ for 15 min at 4°C and the supernatants were collected and stored frozen. The IP_3 concentrations of the samples were determined using the 'D-*myo*-Inositol 1,4,5-triphosphate (IP_3) [^{3}H]assay system' (Amersham, Buckinghamshire, UK) as recommended by the manufacturer.

2.5. Ca^{2+} measurements with NG 108-15 cells

The intracellular Ca^{2+} concentrations were determined as previously described for HEL cells using fura-2 (Michel et al., 1989a; Feth et al., 1992). Briefly, NG 108-15 cells were resuspended at 10^6 cells/ml in buffer containing 120 mM NaCl, 5 mM KH_2PO_4, 5 mM magnesium acetate, 1 mM $CaCl_2$, 1 mg/ml glucose and 20 mM HEPES, pH 7.4. Excitation of the cell suspension was performed in a Hitachi F-2000 fluorescence spectrophotometer in an alternating manner at 340 and 380 nm with emission being measured at 510 nm. Raw data were converted into intracellular Ca^{2+} concentration, using the double wavelength ratio method (Grynkiewicz et al., 1985) with software supplied by the fluorescence spectrophotometer manufacturer.

2.6. Radioligand binding

The Sf9 cells were infected and preadapted to pH 7.4 in the same way as the cells used in the Ca^{2+} measurements.

The cells were gently scraped off 16–26 h post-infection and washed once with Ca^{2+}-free HBM. The cells were suspended in the required volume of the HBM and the reactions were started by adding 250 μl of this suspension to 250 μl of the reaction mixture containing HBM + 30 nM [^{3}H]rauwolscine. This concentration was enough to produce 80–90% saturation of the binding sites based on the binding affinities of 3–8 nM determined for different subtypes of α_2-adrenoceptors. Phentolamine (10 μM) was used to determine non-specific binding. An aliquot of cell suspension was used for protein assay (Bradford, 1976) with bovine serum albumin as standard. Samples were continuously agitated during the incubation. The reactions were allowed to proceed for 30 min at 22°C, a time which was sufficient to reach maximum binding. The reactions were terminated by rapid filtration through printed filtermat B filters (Wallac Oy, Turku, Finland) with four subsequent washes with ice-cold HBM using a Tomtec Harvester 96 (Tomtec, Orange, CO). After the filter had been dried in an oven, a MeltiLex B/HS melt-on scintillator sheet (Wallac, Turku, Finland) was melted on it. The radioactivity was determined by counting with a Wallac Microbeta scintillation counter.

NG 108-15 cells were homogenized in 20 mM ice-cold $NaHCO_3$ solution (10^8 cells/4 ml) with an Ultra-Turrax for 10 s at full speed and thereafter 2 × 20 s at 2/3 speed. The homogenates were filtered through four layers of medical gauze and centrifuged at 1000 × *g* for 20 min at 4°C. The supernatants were collected and pelleted at 50,000 × *g* for 20 min at 4°C. α_2-adrenoceptors were identified with [^{3}H]rauwolscine as the radioligand in saturation and competition binding assays as described previously (Michel et al., 1989b). Briefly, NG 108-15 cell membranes (60–100 μg protein/tube) were incubated with [^{3}H]rauwolscine ± prazosin, oxymetazoline or noradrenaline in 50 mM Tris/0.5 mM EDTA (pH 7.4) for 60 min at 25°C. Non-specific binding was determined with 10 μM phentolamine. The protein concentration was determined as above.

2.7. Data analysis

The data are shown as means ± S.E.M. of *n* number of determinations. Statistical significance was calculated with Student's two-tailed *t*-test. Non-linear curve-fitting was performed with SigmaPlot 4.1 (Jandel Scientific, Corto Madera, CA, USA; Sf9 cells) or InPlot (GraphPad Software, San Diego, CA, USA; NG 108-15 cells).

3. Results

3.1. Studies on Sf9 cells

Receptor expression level and IP_3 formation were measured simultaneously in Sf9 cells expressing α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors (Fig. 1). Significant IP_3 increases in response to noradrenaline stimulation were measured in the α_{2B}-adrenoceptor-expressing cells after all the infection times (24, 36 and 48 h post infection). The α_{2A}-adrenoceptors also increased IP_3 at the highest expression level (48 h post-infection). No IP_3 increases were detected with α_{2C}-adrenoceptors, but their expression level was also significantly lower than the expression level of the other two subtypes.

Similar results were observed for Ca^{2+} measurements (data not shown) although long infection times could not be used, as this resulted in a high basal Ca^{2+} and a large variability in signals probably due to general adverse effects of the infection. This has previously been shown for muscarinic receptor-expressing Sf9 cells (Kukkonen et al., 1996). Therefore, Ca^{2+} measurements were performed at 24 h post-infection. At this time, the receptor densities were 0.3 ± 0.03, 2.3 ± 0.01 and 0.3 ± 0.07 pmol/mg protein (as determined from three to four batches of cells) in α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor-expressing cells, respectively. In non-infected and wild-type virus-infected cells, the specific [^{3}H]rauwolscine binding amounted to approximately 0.05 ± 0.005 and 0.1 ± 0.02 pmol/mg protein (as

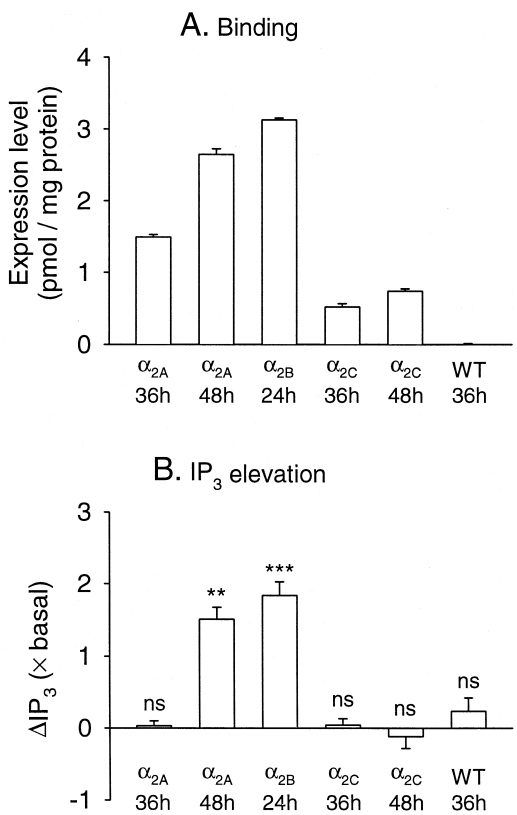

Fig. 1. Receptor expression levels (A) and IP_3 responses to 100 μM noradrenaline (B) in Sf9 cells expressing different α_2-adrenoceptor constructs after different infection times. WT stands for wild-type virus-infected cells. ΔIP_3 (× basal) = ($IP_{3/elevated}$-$IP_{3/basal}$)/$IP_{3/basal}$. Results are from one batch of cells done in quadruplicate; similar results were obtained for other batches also with respect to Ca^{2+} increase (data not shown).

determined from three batches of cells), respectively. The binding to α_{2A}- and α_{2C}-adrenoceptors with short infection times thus barely exceeded the binding to the wild-type virus-infected cells. The basal $[Ca^{2+}]_i$ was similar (115 ± 32 nM; $n = 33$) in cells expressing α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors (24 h post-infection), as well as in wild-type virus-infected (24 h post-infection) and in non-infected cells. The agonists, noradrenaline, D-medetomidine, clonidine and oxymetazoline (100 μM each), did not significantly alter $[Ca^{2+}]_i$ in non-infected or wild-type virus-infected Sf9 cells. As shown in Fig. 2A the addition of noradrenaline to cells infected with recombinant α_{2B}-adrenoceptor-expressing virus caused a long-lasting increase in fura-2 fluorescence, indicating a sustained rise in $[Ca^{2+}]_i$. Only very small changes in fura-2 fluorescence were observed in the cells infected with α_{2A}- or α_{2C}-adrenoceptor-containing constructs (data not shown). All further experiments were thus performed with α_{2B}-expressing Sf9 cells at 24 h post-infection. A transient Ca^{2+} response to noradrenaline was seen (approximately 50% of the signal in the presence of external Ca^{2+}) after chelation of external Ca^{2+} by addition of 10 mM EGTA (Fig. 2B), indicating that a large part of the signal consisted of Ca^{2+} mobilization from the intracellular stores, in agreement with the IP_3 increase detected. IP_3 increases in α_{2B}-expressing cells were thus investigated in more detail.

As phosphoinositolphospholipid-specific phospholipase C is a Ca^{2+}-dependent enzyme, the effect of clamping of $[Ca^{2+}]_i$ was tested. This was performed to investigate whether the IP_3 rise would be secondary to Ca^{2+} increase. A 20-min preincubation with 30 μM of BAPTA acetoxymethyl ester which, in hydrolyzed form, chelates intracellular Ca^{2+} thus preventing any rise in $[Ca^{2+}]_i$, abolished the noradrenaline-induced increase in IP_3 mass (Fig. 3G,H). The Ca^{2+} ionophore ionomycin (20 nM) caused a Ca^{2+} increase similar to that with 100 μM noradrenaline (data not shown) but did not affect IP_3 formation; however, the IP_3 response to noradrenaline added 1 min later was significantly enhanced (Fig. 3I,J). On the contrary, the elevation of intracellular cAMP with 100 μM of the adenylyl cyclase activator forskolin (1-min preincubation) or directly with 2 mM of the cell-permeable cAMP analogue 8-Br-cAMP (2-min preincubation) affected neither the basal intracellular IP_3 level nor the noradrenaline-induced elevation (Fig. 3C,D and E,F, respectively).

The other agonists tested also increased $[Ca^{2+}]_i$. Concentration-response curves were made for noradrenaline, clonidine, D-medetomidine, L-medetomidine, and oxymetazoline (Fig. 4, Table 1). While D-medetomidine and oxymetazoline had an activity similar to that of noradrenaline, clonidine and UK 14,304 were partial agonists and

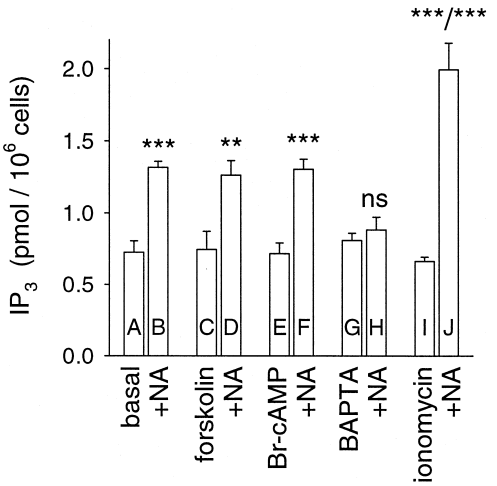

Fig. 3. IP_3 content of α_{2B}-expressing Sf9 cells. IP_3 content was determined under basal conditions and after addition of 100 μM noradrenaline (NA), both in control cells and in the cells pretreated with 100 μM forskolin (1 min), 2 mM 8-Br-cAMP (2 min), 20 nM ionomycin (1 min) or 30 μM BAPTA acetoxymethyl ester (20 min). The results are from one experiment performed in triplicate. The experiment was repeated three times with qualitatively similar results. The significance for the noradrenaline-stimulated level are indicated with respect to the corresponding basal level (i.e., for B against A, D against C, F against E, H against G), except for noradrenaline in the presence of ionomycin (J), for which the significance is indicated with respect to the basal ionomycin (I)/ noradrenaline control (B). ns: non-significant ($P > 0.05$); ∗∗: $P < 0.01$; ∗∗∗: $P < 0.001$.

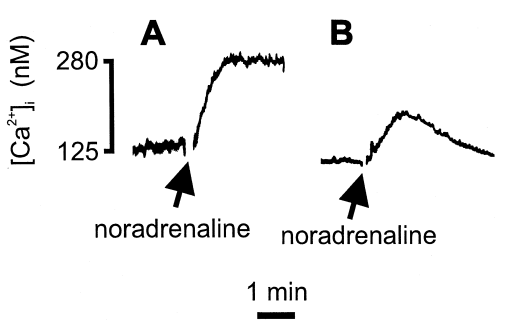

Fig. 2. Noradrenaline-induced $[Ca^{2+}]_i$ increases in α_{2B}-expressing Sf9 cells. 100 μM noradrenaline (NA) was added as indicated by arrows in the presence of extracellular Ca^{2+} (A) and following its chelation by addition of 10 mM EGTA (B). Results are from a representative experiment.

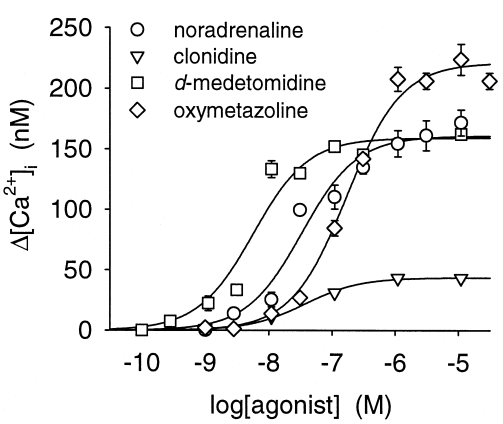

Fig. 4. Representative concentration–response curves for different agonists in Sf9 cells expressing α_{2B}-adrenoceptors. The experiment was performed with one batch of cells in triplicate.

Table 1
The changes in $[Ca^{2+}]_i$ on agonist stimulation of Sf9 cells expressing the α_{2B}-subtype as measured with fura-2

Agonist	$\Delta[Ca^{2+}]_i$ (nM)	EC_{50} (nM)
Noradrenaline	132.1 ± 47.7	35.6 ± 16.9
Clonidine	44.1 ± 12.6	46.0 ± 27.7
D-Medetomidine	160.3 ± 44.0	3.71 ± 2.54
L-Medetomidine	0	–
Oxymetazoline	171.7 ± 60.7	177 ± 26
UK 14,304	52.8 ± 21.1	n.d.

$\Delta[Ca^{2+}]_i$ is the maximum change in $[Ca^{2+}]_i$ and EC_{50} the concentration of agonist producing half-maximal stimulation.
The results are from several independent batches of cells (at least three parallel recordings per batch), with the exception of the EC_{50} value for clonidine (from one batch), and are shown as means ± S.E.M.
n.d. = not determined.

L-medetomidine was without any effect. The order of potency of the agonists was D-medetomidine > noradrenaline ≈ clonidine > oxymetazoline: Correct assessment of the EC_{50} value for clonidine and UK 14,304 was, however, difficult because of the low maximum response. The α_2-selective antagonist rauwolscine had a competitive inhibitory constant (K_i) of 5.2 ± 2.1 nM ($n = 14$; calculated from the noradrenaline concentration–response relations in the presence of different concentrations of rauwolscine). The Ca^{2+} response to noradrenaline was insensitive to pretreatment with pertussis toxin (500 ng/ml, 24 h; data not shown). There was also no effect of cholera toxin pretreatment (20 μg/ml, 16 h) on the noradrenaline-induced Ca^{2+} increase (data not shown) although the basal cAMP level was elevated several-fold (see also Jansson et al., 1995).

The Ca^{2+} response to noradrenaline was directly dependent on the expression level: when the receptor density, $\Delta[Ca^{2+}]_{max}$ (the maximum Ca^{2+} increase), and EC_{50} were measured within the time frame 16–24 h post-infection (when the increase in receptor density is most pronounced (data not shown) and the adverse effects of infection have not yet reduced Ca^{2+} elevation, Kukkonen et al., 1996), $\Delta[Ca^{2+}]_{max}$ increased while the EC_{50} value decreased with increasing receptor number (Fig. 5). To investigate the relative receptor expression levels in individual cells, the Ca^{2+} responses were measured using an image analysis system. The proportion of responding cells was usually more than 80% on each coverslip. This is illustrated in Fig. 6 where 16 of the 17 cells reacted to 100 μM noradrenaline with an increase in $[Ca^{2+}]_i$. The responses were very similar in the different cells.

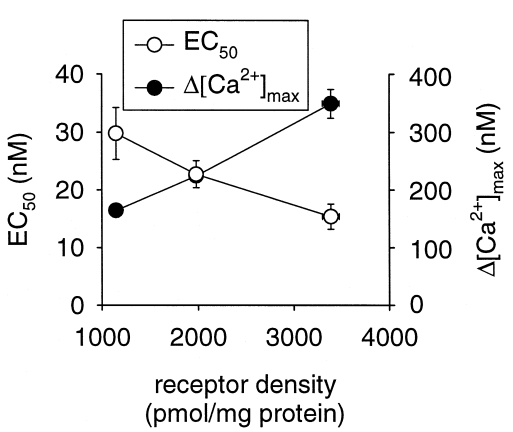

Fig. 5. The effect of the receptor expression level on noradrenaline-induced (100 μM) $[Ca^{2+}]_i$ increases in α_{2B}-expressing Sf9 cells. The receptor expression level, EC_{50} and maximal change in $[Ca^{2+}]_i$ were measured 16, 20 and 24 h post-infection. The data points are from a single experiment performed in quadruplicate. The experiment was repeated twice with similar results.

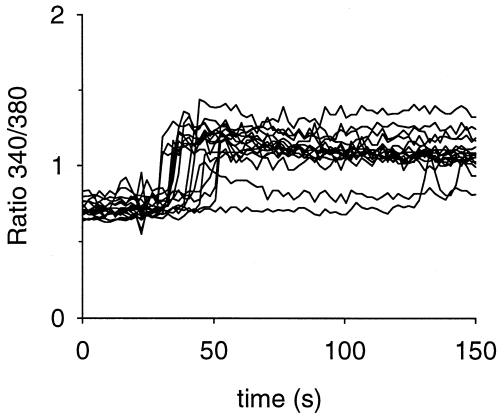

Fig. 6. Increase in Ca^{2+} in response to noradrenaline (100 μM) in individual Sf9 cells infected with baculovirus containing α_{2B}-adrenoceptor gene 24 h post-infection. The time course of the reactivity in 17 cells on one representative coverslip is shown.

3.2. Studies on NG 108-15 cells

In NG 108-15 cells [^{3}H]rauwolscine detected a single class of high affinity binding sites ($K_d = 2.3 \pm 0.6$ nM, $B_{max} = 0.243 \pm 0.035$ pmol/mg protein, $n = 8$). In competition binding studies, oxymetazoline had an about thirty times lower affinity (776 ± 89 nM) than prazosin (25 ± 1 nM). The prazosin/oxymetazoline ratio of 0.03 confirms the presence of a homogeneous population of α_{2B}-subtype of α_2-adrenoceptors (Bylund et al., 1988).

The basal $[Ca^{2+}]_i$ in NG 108-15 cells was 83 ± 2 nM ($n = 350$). Noradrenaline increased intracellular Ca^{2+} with a time course similar to that seen in α_{2B}-adrenoceptor-expressing Sf9 cells (Fig. 7). The maximal Ca^{2+} increase in response to noradrenaline was small (25.6 ± 1.3 nM, $n = 18$) and its EC_{50}-value was 109 ± 34 nM ($n_H = 0.52 \pm 0.08$; determined from nine batches of cell; Fig. 7).

To ensure that the responses observed were not caused by activation of α_1- or β-adrenoceptors, the effect of different adrenoceptor antagonists on the Ca^{2+} increases was tested. The noradrenaline-stimulated (100 μM) Ca^{2+} increases in NG 108-15 cells were not affected by 10 μM

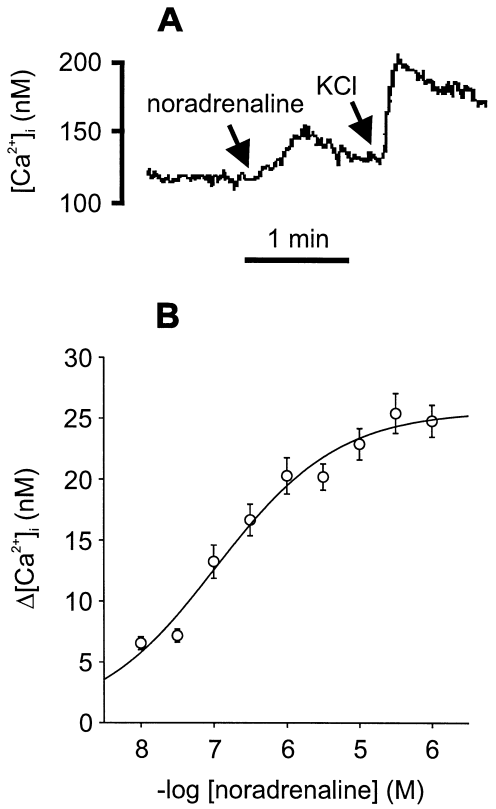

Fig. 7. Elevation of intracellular Ca^{2+} in NG 108-15 cells in response to noradrenaline. The upper panel shows a representative experiment, using 100 μM noradrenaline and 30 mM KCl as indicated. The lower panel shows the concentration-response curve for noradrenaline as determined from nine batches of cells. The calculated EC_{50} value is 109 nM.

propranolol and were antagonized about equally effectively by yohimbine and prazosin, suggesting the presence of α_{2B}-adrenoceptors ($n = 6–9$). Pertussis toxin treatment (100 ng/ml, 3 days) reduced the Ca^{2+} response by 65 ± 5% ($P < 0.01$).

Differentiation of NG 108-15 cells by 4-day treatment with a combination of 10 μM prostaglandin E_1 and 1 mM of the phosphodiesterase inhibitor theophylline (Campbell et al., 1990; Wilson et al., 1991), or by culturing in medium containing only 1% serum (Wilson et al., 1991) did not result in greater noradrenaline-stimulated Ca^{2+} increases (data not shown).

4. Discussion

Depending on the tissue where they are expressed, α_2-adrenoceptors may induce either inhibitory or stimulatory responses (reviewed in Ruffolo et al., 1993; Hieble et al., 1995). Historically, adrenoceptors have been subdivided into α_1-, α_2- and β-adrenoceptors based not only on use of selective drugs but also on preferred coupling mechanisms. In this scheme, signal transduction by α_2-adrenoceptors was believed to occur via inhibition of adenylyl cyclase (reviewed in Limbird, 1988; Regan and Cotecchia, 1992). In addition, inhibition of Ca^{2+} currents may explain many of the inhibitory responses in neurons (Holz et al., 1986; Bley and Tsien, 1990). Inhibitory mechanisms are, however, unlikely to account for many of the primarily stimulatory physiological effects, such as smooth muscle contraction, platelet aggregation and secretion, found to be mediated by these receptors. Consequently, α_2-adrenoceptors have been found to couple to increases in cAMP (Fraser et al., 1989; Eason et al., 1992; Pepperl and Regan, 1993; Jansson et al., 1994, 1995) and Ca^{2+} (Michel et al., 1989a; Salm and McCarthy, 1990; Kagaya et al., 1992; Aburto et al., 1993; Lepretre and Mironneau, 1994). Whether Ca^{2+} increase is a generalized mechanisms for signaling through α_2-adrenoceptors or whether the response is specific for certain subtypes of α_2-adrenoceptors has not been studied systematically.

The α_{2B}-adrenoceptor has been shown to be different from the other two α_2-adrenoceptors in that it couples more strongly to a pertussis toxin-insensitive stimulation of cAMP production (Duzic and Lanier, 1992: PC-12; Pepperl and Regan, 1993: JEG-3; Jansson et al., 1994: S115; Jansson et al., 1995: Sf9). It is thus of interest that the α_{2B}-adrenoceptor in particular seems to be able to couple to Ca^{2+} increase. This may, however, be due to the higher expression level of the α_{2B}-adrenoceptor than of the other two subtypes, although it should be noted that the response is still seen at lower α_{2B}-adrenoceptor expression levels. It is unfortunate that the measurements of Ca^{2+} increases could not be performed after long infection times (48 h, 72 h) in α_{2A}- and α_{2C}-expressing cells because of the loss of the viability of the cells. Ca^{2+} increases could also be demonstrated with endogenous α_{2B}-adrenoceptors in NG 108-15 neuroblastoma × glioma cells with a lower receptor density as compared to the Sf9 cells, although the Ca^{2+} increases in NG 108-15 cells were much smaller than those in α_{2B}-adrenoceptor-expressing Sf9 cells. Nevertheless, we have now shown for the first time coupling of α_{2B}-adrenoceptors to Ca^{2+} increase.

The relationship between the receptor expression level and the response in Sf9 cells was also examined in this study. The IP_3 and binding values for α_{2A}-expressing cells 48 h post-infection and for α_{2B}-expressing cells 24 h post-infection seem to correlate well whereas, somewhat surprisingly, no IP_3 increase was seen in α_{2A}-expressing cells 36 h post-infection. Similarly, the relationship between receptor number and maximum Ca^{2+} increase or the EC_{50} for noradrenaline in α_{2B}-expressing cells was not linear. The response is often a product of binding and several amplification steps, the latter of which are usually added together to be described with one hyperbolic equation. The simplifications most often used for the amplification are linear and hyperbolic amplification. The latter gives results which are very similar to those we now saw (Fig. 5) and that are generally seen in physiological systems (e.g., Whaley et al., 1994). In addition, further non-

linearity may be caused by cell type-dependent limitations in the accessibility of some of the components involved in the signal transduction cascade (Stickle and Barber, 1989). Therefore, the non-linearity between receptor number and maximum Ca^{2+} increase or EC_{50} value is by no means unexpected. In the case of IP_3 measurements, however, it is more likely that the apparent non-linearity of α_{2A} expression and response is a result of the lower detection limit of the IP_3 assay than of the Ca^{2+} assay. For this reason no attempt was made to measure IP_3 elevations in NG 108-15 cells.

The source of the α_{2B}-adrenoceptor-mediated Ca^{2+} increase in Sf9 cells was investigated using the Ca^{2+} chelator EGTA. The noradrenaline-stimulated Ca^{2+} elevation occurred partly independently of extracellular Ca^{2+}, indicating mobilization from intracellular sources. This pattern of a small and transient release and a subsequent large and sustained influx seems to be typical of Sf9 cells as it has been observed earlier for heterologously expressed muscarinic receptors (Kukkonen et al., 1996). An intracellular Ca^{2+} source is also used on α_2-adrenoceptor stimulation in HEL cells (Michel et al., 1989a), astrocytes (Enkvist et al., 1996) and smooth muscle cells (Erdbrugger et al., 1993), although in these mammalian cells release seems to play a greater part in the total Ca^{2+} signal, resulting in a clearly biphasic Ca^{2+} signal with a large, fast and transient release followed by a smaller and more sustained influx. This is probably seen in NG 108-15 cells also, although the source of the Ca^{2+} increase has not been investigated. Intracellular Ca^{2+} mobilization is usually a result of the activation of phosphoinositolphospholipid-specific phospholipase Cβ, leading to production of IP_3 which stimulates the release of Ca^{2+} from intracellular stores (Berridge, 1987). Our data also show that α_{2B}-adrenoceptor stimulation can induce IP_3 formation as previously shown for recombinant α_{2A}- and α_{2C}-adrenoceptors in Chinese hamster lung fibroblasts and COS-7 cells (Cotecchia et al., 1990) and for endogenous α_{2A}-adrenoceptors in HEL cells (Åkerman et al., 1996; Dorn et al., 1997) and in rat cerebral astrocytes (Enkvist et al., 1996). This seems to be mediated by a Ca^{2+}-dependent phospholipase C since the intracellular Ca^{2+} chelating agent, BAPTA, blocked the noradrenaline-induced IP_3 increase and the Ca^{2+} ionophore, ionomycin, potentiated it. On the other hand, ionomycin-induced Ca^{2+} increase did not affect IP_3 formation per se. This is similar to the situation in reconstituted systems, where Ca^{2+} is essentially needed for the stimulation of phospholipase Cβ with G protein, but is itself a weak stimulant (Waldo et al., 1991). It thus seems unlikely that the α_2-adrenoceptor-induced IP_3 formation in Sf9 cells would be secondary to Ca^{2+} increase; it is, however, impossible to judge from these data whether Ca^{2+} increase is secondary to IP_3 increase—as is thought to happen in most cases—or whether these two second-messenger responses are independent of each other. If the α_{2B}-adrenoceptor-induced IP_3 formation does not occur secondary to Ca^{2+} elevation it could still be secondary to changes in intracellular cAMP (Volpe and Alderson-Lang, 1990). However, our data argue against this possibility since neither the adenylyl cyclase activator, forskolin, nor the cell-permeable cAMP analogue, 8-Br-cAMP, affected the noradrenaline-induced IP_3 formation.

It was of interest to compare the agonist profiles for noradrenaline, D-medetomidine and clonidine obtained with respect to cAMP elevation by Jansson et al. (1995) and with respect to Ca^{2+} increase in the present study. Although the EC_{50} values obtained in the Ca^{2+} assay were 10- to 100-fold lower, there is a direct correlation between both the maximum responses and EC_{50}-values. Unexpectedly, UK 14,304 which functions in most systems as a full agonist induced a very small Ca^{2+} increase. Eason et al. (1994) have demonstrated that UK 14,304 functions as a full agonist for α_{2B}-adrenoceptor-mediated inhibition of adenylate cyclase but is a very weak inducer of the coupling of the same receptor to the stimulation of cAMP production. The previously reported coupling of α_{2B}-adrenoceptors to increase instead of reduction of the cAMP level (Jansson et al., 1995) suggests coupling of these receptors to G_s rather than G_i in Sf9 cells. Some normally G_s coupling receptors, like the luteinizing hormone receptor, adenosine A_{2B} receptor and *Drosophila* dopamine receptor (DopR99B) are able to increase $[Ca^{2+}]_i$ in *Xenopus* oocytes (Gudermann et al., 1992; Yakel et al., 1993; Feng et al., 1996). Whether this Ca^{2+} increase occurs through G_s, or for example G_q, is not known. However, as cholera-toxin pretreatment did not have any effect on the basal or noradrenaline-stimulated Ca^{2+} levels, at least G_s is not likely to be involved in the Ca^{2+} increase in the α_{2B}-expressing Sf9 cells.

The mechanism underlying α_{2B}-adrenoceptor-mediated Ca^{2+} increases is thus not completely clear. In the case of the receptors coupling to pertussis toxin-sensitive G proteins this is thought to be due to phospholipase C activation by βγ subunits from $G_{i/o}$ (Lee et al., 1993; Smrcka and Sternweis, 1993; Wu et al., 1993). This has recently been suggested to be the mechanism for the α_{2A}-mediated Ca^{2+} elevation in COS-7 cells (Dorn et al., 1997). Similarly, mixed α_2-adrenoceptor subtype-mediated Ca^{2+} elevations in porcine aortic smooth muscle cells (Erdbrugger et al., 1993) and α_{2A}-adrenoceptor-mediated Ca^{2+} increase in HEL cells (Michel et al., 1989a), human platelets (Kagaya et al., 1992) and astrocytes (Enkvist et al., 1996) involve a pertussis toxin-sensitive G protein. Our experiments with NG 108-15 cells demonstrate that the Ca^{2+} increase involves a pertussis toxin-sensitive G protein. We have not been able to obtain consistent inhibition of noradrenaline-induced Ca^{2+} increases by pertussis toxin in the Sf9 cell line. Whether a pertussis toxin-sensitive G protein exists in Sf9 cells is debatable, as no (Quehenberger et al., 1992), or only partial, effects on signal transduction have been observed with this toxin (Ng et al., 1993; Jansson et al., 1995). The presence of α-subunits of

G_i-proteins as well as of other G proteins has been demonstrated in these cells, using immunoblotting with antibodies against rat G proteins (Butkerait et al., 1995). Therefore it is evident that there are G proteins very similar to their mammalian counterparts in Sf9. The resistance to pertussis may thus be due to other factors, such as lack of cell surface receptors for the toxin or low susceptibility of the insect G proteins to ADP-ribosylation by pertussis toxin. The latter possibility is supported by the high concentrations of pertussis toxin needed to ADP-ribosylate Sf9 G proteins even in the membrane preparation (Butkerait et al., 1995). It is thus still conceivable that this response is mediated by $G_{i/o}$. Together, our data demonstrate that the α_{2B}-adrenoceptors—when heterologously expressed in Sf9 cells—can couple to the phosphoinositol-second messenger system leading to mobilization of intracellular Ca^{2+}. The agonist pharmacology with respect to Ca^{2+} elevation is similar to that of the previously described cAMP increase by these receptors. Also the endogenous α_{2B}-adrenoceptors in NG 108-15 cells couple to increase of intracellular Ca^{2+}.

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