Cell-type Specific Targeting of the α2C-Adrenoreceptor

EVIDENCE FOR THE ORGANIZATION OF RECEPTOR MICRODOMAINS DURING NEURONAL DIFFERENTIATION OF PC12 CELLS

We have previously shown differences in the intracellular targeting of α2A (α2A) and α2C (α2C)-adrenoreceptors expressed in the same cell line (von Zastrow, M., Link, R., Daunt, D., Barsh, G., and Kobilka, B. (1993) J. Biol. Chem. 268, 763-766; Daunt, D. A., Hurt, C., Hein, L., Kallio, J., Feng, F., and Kobilka, B. K. (1997) Mol. Pharmacol. 51, 711-720). α2A-Adrenoreceptors reside primarily in the plasma membrane in HEK 293 cells, while co-expressed α2C-adrenoreceptors are found mainly in an intracellular compartment. Since α2C-adrenoreceptors are expressed primarily in the brain, we compared the intracellular targeting of α2C-adrenoreceptors in two neuroendocrine cell lines with the targeting in three epithelial cell lines and one fibroblast cell line. In transiently transfected COS7 cells, and in stably transfected normal rat kidney cells, Madin-Darby canine kidney cells, and Rat1 fibroblasts, a significant proportion of α2A-adrenoreceptor detected by immunocytochemistry co-localized with markers for both the endoplasmic reticulum and the cis/trans-Golgi compartments. In contrast, both PC12 cells and AtT20 cells efficiently targeted α2C-adrenoreceptors to the plasma membrane. Ligand binding and Western blot analyses indicate that intracellular receptor in normal rat kidney cells is functional and undergoes normal post-translational processing. In PC12 cells the expressed α2C-adrenoreceptors become concentrated in neurite outgrowths in discrete regions of the plasma membrane having a high density of F-actin following treatment with nerve growth factor. These findings provide evidence for cell-type specific factors that facilitate the targeting of the G protein-coupled receptors to the plasma membrane.

G protein-coupled receptors mediate transmission of information across the plasma membrane by activation of membrane-associated G-proteins that couple to intracellular effector systems. Although most G protein-coupled receptor ligands are confined to the extracellular space, for some G protein-coupled receptors, such as the thrombin receptor, thyrotropin-releasing hormone receptor, and the α2C-adrenoreceptor, a significant proportion of the receptor population is found in intracellular compartments. In the case of thrombin receptors, evidence suggests that the intracellular pool of receptors may serve as a reservoir, capable of restoring functional, non-cleaved thrombin receptors to the plasma membrane after the cell has been exposed to thrombin (3). A minor fraction of thyrotropin-releasing hormone receptors expressed in HEK cells are targeted to the plasma membrane while a larger fraction is found in an intracellular compartment and are non-functional (4). However, when the thyrotropin releasing hormone receptors were expressed in two pituitary cell lines (GH3 and GHY cells) the receptors were found predominantly in the plasma membrane and to be functional by binding assays.

For the α2C-adrenoreceptor, the functional status and role of an intracellular pool is less clear. This is particularly interesting in light of α2C-adrenoreceptor overall structural and functional similarity to the other α2-adrenoreceptor subtypes, α2A- and α2B-adrenoreceptors (for reviews, see Ref. 5). Several reports have documented the presence of a large intracellular distribution of α2C-adrenoreceptors when either transiently or stably transfected into a variety of cell lines (1, 2, 6). Furthermore, the levels of expression achieved for the α2C-adrenoreceptors in cells have been comparable to the expression level obtained for the other two α2-adrenoreceptor subtypes in these same cell lines (7, 8). This would indicate that the functional expression of the three α2-adrenoreceptor subtypes occurs at similar efficiencies. In addition, the three α2-adrenoreceptors have been reported to have comparable signaling characteristics in that they all couple to Gi/α proteins, inhibit adenyl cyclase (7, 8), and their stimulation can lead to activation of a mitogen-activated protein kinase pathway (9, 10).

A recent report described the expression of α2C-adrenoreceptors in Madin-Darby canine kidney cells and found the receptors to be in the plasma membrane and in two intracellular compartments, endoplasmic reticulum, and trans-Golgi network (6). We have studied the trafficking of α2C-adrenoreceptor in Rat1 fibroblasts and determined that the intracellular pool of receptors was localized in the endoplasmic reticulum and in the cis/trans-Golgi compartments. Unlike the thrombin receptor, there is no cycling of the α2C-adrenoreceptor between this intracellular pool and the plasma membrane in these cells (2). These observations taken together suggest that the α2C-adrenoreceptor may be improperly folded or poorly processed and thereby retained in the ER.

1 The abbreviations used are: ER, endoplasmic reticulum; NRK, normal rat kidney; NGF, nerve growth factor; PBS, phosphate-buffered saline; HA, hemagglutinin; BiP, immunoglobulin heavy chain-binding protein.

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the α₂C-adrenoreceptor subtype and may play some functional role in the cell, or may be due to the lack of expression of specific processing or transport factors (e.g. chaperones) in the cell lines examined to date.

To explore the later possibility, we have investigated the targeting of α₂C-adrenoreceptors in several cell lines including two of neuronal lineage. The α₂C-adrenoreceptors are primarily expressed in the central nervous system and recent studies of α₂C adrenoreceptor knockout mice demonstrate this receptor subtype plays a role in modulating several aspects of behavior (5, 14, 15) and in regulating catecholamine release from sympathetic neurons in the heart (16, 17). Our results provide evidence for cell-type specific targeting of G protein-coupled receptors and for the development of receptor microdomains during neuronal differentiation of PC12 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Normal rat kidney (NRK) cells, Rat1 fibroblast cells, and COS7 cells were cultured at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Inc., Calabasa, CA) and gentamicin (25 μg/ml; Roche Molecular Biochemicals, Indianapolis, IN). PC12 cells were cultured at 37°C with 10% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% equine donor serum and 5% bovine calf serum (Hyclone). Murine α₂C-adrenoreceptor was subcloned into the pCDNA3 expression vector. A 12CA5 epitope was added to the end of the amino terminus by using an oligonucleotide linker-adapter into the NcoI site of the 5' coding sequence of the receptor. Cells were transfected with 12CA5 epitope tagged α₂C-adrenoreceptor by either calcium phosphate precipitation or electroporation. Stably transfected cells were obtained by growing cells in the presence of 0.25 to 0.8 mg/ml G418 (Life Technologies, Inc., Grand Island, NY). The neuronal induction of PC12 cells was accomplished by the addition of 50 ng/ml nerve growth factor (NGF) to the media.

Relative α₂C-adrenoreceptor expression level for each of the stably transfected cell lines was determined by saturation binding with the α₂C-adrenoreceptor antagonist, [³H]RX521002. Briefly, cell membrane preparations were made by Polytron homogenization in a hypotonic lysis buffer (10 mM Tris and 1 mM EDTA, pH 7.4). The cell homogenate was centrifuged at 1,000 x g for 5 min to remove nuclear debris and intact cells. The recovered supernatant was centrifuged at 10,000 x g for 90 min to obtain cell membranes. The membrane pellet was then suspended in binding buffer (75 mM Tris, 12.5 mM MgCl₂, and 10,000× differential labeling of the surface membrane preparations were made by Polytron homogenization in a hypotonic lysis buffer (10 mM Tris and 1 mM EDTA, pH 7.4). The cell homogenate was centrifuged at 1,000 x g for 5 min to remove nuclear debris and intact cells. The recovered supernatant was centrifuged at 10,000 x g for 90 min to obtain cell membranes. The membrane pellet was then suspended in binding buffer (75 mM Tris, 12.5 mM MgCl₂, and 10,000× 10 mM EDTA, pH 7.4) and stored at

**Immunocytochemistry**—Cells were seeded and grown on sterile glass coverslips coated with poly-lysine 2 days before studying. After various treatments, cell preparations were fixed for 5 min with either 4% paraformaldehyde at room temperature or cold methanol at 20°C. Following fixation cells were rinsed three times with phosphate-buffered saline (PBS) supplemented with calcium (Ca²⁺) and magnesium (Mg²⁺). A blocking agent composed of 5% dry milk, 50 mM HEPES, pH 7.4, in PBS was used to reduce nonspecific antibody activity. The nonionic detergent, Nonidet P-40 (Sigma), was added to a final concentration of 0.2% in the blocking agent to permeabilize cells fixed with paraformaldehyde. All antibody applications of fixed specimens were done in the presence of blocking agent for 1 h at room temperature. For differential labeling of the surface versus the combined intracellular and surface α₂C-adrenoreceptors, cells were labeled with the monoclonal antibody, 12CA5, against the amino terminus HA epitope (BalCo Berkeley Antibody Co., Richmond, CA) at 1:500 dilution for 1 h at room temperature in Dulbecco's modified Eagle's medium with 40 mM HEPES, pH 7.4, and 0.4% bovine serum albumin. After surface labeling, cells were washed three times with ice-cold PBS and fixed with 4% paraformaldehyde for 5 min at room temperature. After fixation, the cells were washed three times with PBS to remove unbound antibody and then treated with a blocking agent with 0.2% Nonidet P-40 for 30 min. The cell preparation was incubated with the affinity purified C4 rabbit polyclonal antibody. The endoplasmic reticulum of the cells was labeled with either BIP, a luminal protein, or calnexin (Stress-Gen, Victoria, B.C., Canada), an integral membrane protein. The cis/medial Golgi compartment was labeled with a mouse monoclonal antibody recognizing the resident protein, mannosidase II (BabCo Berkeley Antibody Co.). A rabbit polyclonal antibody (William Brown, Cornell Uni-

**Western Blotting**—Immunoblot analysis of α₂C-adrenoreceptor processing was done using either whole cell lysate or membrane preparation immunoprecipitates. Cells as described above were lysed in hypotonic lysis buffer (10 mM Tris and 1 mM EDTA, pH 7.4). The collected cell lysate was prepared using PBSTDS buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in PBS, pH 7.4). Cells were grown on a 10-cm dish until 70–80% confluent and were solubilized with 1 ml of PBSTDS buffer at 4°C for 30 min. Following solubilization, the nuclear fraction of the lysate was removed by high speed centrifugation at 14,000 rpm for 15 min at 4°C. Whole cell lysate from each of the cell lines was stored at −70°C. Membrane preparations made for ligand binding experiments were solubilized with 1% SDS buffer. Protein concentration for each sample was determined using Bio-Rad protein assay.

For examination of post-translational processing of each cell type, 80–100 µg of membrane protein was solubilized in 1% SDS. The endoglycosidase H reaction buffer was made to have a final concentration of 0.5% Nonidet P-40 and 10 mM sodium acetate, pH 5.0. PNGase F reaction was supplemented with 20 mM sodium phosphate buffer, pH 8.0, and 10 mM EDTA. Both the PNGase F and endoglycosidase H reactions were done at 37°C for 24 h and terminated by the addition of 4× SDS sample buffer. Samples were loaded and run on 7.5% or 10% SDS-polyacrylamide electrophoresis discontinuous gel. Electrophoresed proteins in the gel were then treated with n-tocitococcosidase (Boehringer). The reacted bands were blotted overnight with 5% dry milk, 2% equine donor serum, 20 mM Tris, pH 7.6, 137 mM sodium chloride, and 0.05% Tween 20. α₂C-adrenoreceptors were labeled with the mouse monoclonal antibody 12CA5 or rabbit polyclonal antibody C4 in blocking solution for 1 h at room temperature. The nitrocellulose membrane was rinsed three times in TBS-Tween and labeled with goat anti-mouse or goat antirabbit (Stress-Gen, Victoria, B.C., Canada), an integral membrane protein. The cis/medial Golgi compartment was labeled with a mouse monoclonal antibody recognizing the resident protein, mannosidase II (BabCo Berkeley Antibody Co.). A rabbit polyclonal antibody (William Brown, Cornell Uni-

**Subcellular Membrane Isolation**—NRK cells stably expressing α₂C-adrenoreceptors were grown in 10-cm dishes to about 80–90% confluency. Cells were washed three times with ice-cold PBS. The cells were scraped off the dish and lysed with 1 ml of ice-cold hypotonic lysis buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 2 mM EGTA, 6 mM magnesium chloride, 1 mM phenylmethylsulfonyl fluoride, 10 µM leupeptin, 10 µM aprotinin, 1 mM benzamidine, 1 µM pepstatin). The collected cell lysate was homogenized with 20 strokes of a tight fitting Dounce followed by two 10-s bursts with a Polytron tissue grinder (Beckman Instruments). Cellular debris and unlysed cells were removed by centrifuging at 1,000 x g for 5 min at 4°C. The supernatant was collected and supplemented with 2.0 µM suroce to achieve a final concentration of 0.2 µM suroce. The discontinuous suroce step gradient was made using the above hypotonic lysis buffer with the addition of suroce at the following molar concentrations: 0.5, 0.9, 1.2, 1.35, 1.5, and 2.0. Each step in the gradient had a final volume of 5 ml. A total of 32 ml of cell lysate, recovered from two 10-cm dishes was applied to the top of the gradient. The samples were centrifuged for 16 h at 27,000 rpm in a Beckman SW28 rotor. The plasma membrane samples were recovered in the sucrose gradient at the interface between 0.5 and 0.9 µM. The endoplasmic reticular membrane samples were recovered at the interface between the 1.35 and 1.5 µM. Samples were diluted with binding buffer and...
centrifuged as outlined above. The membrane pellets obtained for the plasma membrane and endoplasmic reticulum were assessed for functional expression of α2c-adrenoceptors by radioligand binding as outlined earlier. Verification of isolated membranes as either plasma membrane or endoplasmic reticulum was achieved with Western blotting methods as outlined above utilizing rabbit polyclonal antibodies that specifically recognize the α subunit of the Na/K-ATPase pump at dilution of 1:1000 (Kindly donated by W. James Nelson, Stanford University) and calnexin at a dilution of 1:5000, respectively.

RESULTS

Immunolocalization of α2c-Adrenoreceptors—The steady state distribution of HA epitope-tagged α2c-adrenoreceptor was examined in stably transfected NRK cells, AtT20 cells, and PC12 cells by immunocytochemistry using mouse monoclonal antibody 12CA5 recognizing the HA epitope. In panel A, NRK cells expressing α2c-adrenoreceptors display a limited plasma distribution of receptors with a large intracellular pool. In neuroendocrine cell lines (AtT20 cells, panel B, and PC12 cells, panel C) α2c-adrenoreceptors are found predominately in the plasma membrane with a small intracellular perinuclear receptor pool.

Fig. 1. Steady state distribution of HA epitope-tagged α2c-adrenoreceptors in stably transfected cell lines. Cells were grown on glass coverslips and processed for indirect immunofluorescence microscopy, as outlined under “Experimental Procedures.” The mouse monoclonal antibody, 12CA5 (recognizing the HA epitope), was used with detergent (0.2% Nonidet P-40) to allow labeling of both plasma membrane and intracellular distributions of α2c-adrenoreceptors. In panel A, NRK cells expressing α2c-adrenoreceptors display a limited plasma distribution of receptors with a large intracellular pool. In neuroendocrine cell lines (AtT20 cells, panel B, and PC12 cells, panel C) α2c-adrenoreceptors are found predominately in the plasma membrane with a small intracellular perinuclear receptor pool.

The labeling of α2c-adrenoreceptors that reside in the plasma membrane is compared with the labeling of total cellular α2c-adrenoreceptors in NRK, AtT20, and PC12 cells (as described under “Experimental Procedures”). Briefly, viable cells were exposed to the HA epitope antibody 12CA5 at 4 °C to label the surface α2c-adrenoreceptors in the plasma membrane (panels A, C, and E labeled Nonpermeabilized). The cell preparations were rinsed three times with PBS, fixed, and permeabilized prior to labeling the combined plasma membrane and intracellular α2c-adrenoreceptors with the carboxyl-terminal rabbit polyclonal antibody (panels B, D, and F labeled Permeabilized). In NRK cells expressing α2c-adrenoreceptors, only a small fraction of receptors are found on the cell surface (panel A) compared with the large intracellular pool of receptors (panel B). Conversely, in the two neuroendocrine cell lines, AtT20 cells (panels C and D) and PC12 cells (panels E and F), there is only a small amount of α2c-adrenoreceptors in a perinuclear vesicular compartment. α2c-Adrenoreceptor are primarily found in the plasma membrane for both PC12 cells and AtT20 cells.

Subcellular Distribution of α2c-Adrenoreceptors—To identify the intracellular compartment containing α2c-adrenoreceptors...
in the cell lines studied, we used indirect immunocytochemical methods to compare the distribution of these receptors with markers for the following intracellular compartments: the mannose 6-phosphate receptor, which cycles between the plasma membrane and the trans-Golgi through endosomes; lysosomal glycoprotein 120, a lysosomal membrane protein; BiP, a luminal protein of the endoplasmic reticulum; calnexin, an integral membrane protein of the endoplasmic reticulum; and mannosidase II, a resident cis/medial Golgi marker. In both NRK and PC12 cells, there is no consistent co-localization of the α2C-adrenoceptor with the lysosomal marker, lysosomal glycoprotein 120 (Figs. 3, A and B, and 4, A and B, respectively). The trans-Golgi/endosomal marker, the mannose 6-phosphate receptor, lacked any significant overlap of staining with the α2C-adrenoceptors in NRK cells (Fig. 3, G-H). However, in PC12 cells there is considerable similarity in the staining of a central perinuclear vesicular pool by α2C-adrenoceptors and mannose 6-phosphate receptors (Fig. 4, G-H). The subcellular distribution of the α2C-adrenoceptors in NRK cells most closely resembles the distribution of BiP (Fig. 3, C and D) and mannosidase II (Fig. 3, E-F), indicating that most of the intracellular α2C-adrenoceptors in NRK cells resides within the endoplasmic reticulum and cis/medial Golgi compartments of cells. In addition, we found that calnexin, an integral membrane protein of the endoplasmic reticulum, only co-localized with the centrally staining α2C-adrenoceptors in NRK cells. The more peripheral fine reticular endoplasmic reticulum structure did not colocalize with α2C-adrenoceptors (data not shown). In PC12 cells most of the α2C-adrenoceptor is in the plasma membrane with focal concentration in plasma membrane extensions but with no significant colocalization with the ER marker BiP (Fig. 4, C-D). In PC12 cells, there is a small perinuclear pool of α2C-adrenoceptor that localizes with the mannosidase II and mannose 6-phosphate receptor for Golgi complex and endosomal compartment (Fig. 4, E-F and G-H, respectively).

Functional Receptor Expression—The large intracellular pool of α2C-adrenoceptors found in NRK cells may or may not be functional since misfolded proteins tend to accumulate in the ER. To determine the functional state of the total plasma membrane and intracellular α2C-adrenoceptors in NRK cells and PC12 cells, we compared receptor antigenic expression and receptor ligand binding in crude membrane preparations from these two cell lines. By Western blot analysis, it was found that...
cells and NRK cells express comparable levels of calnexin. Conversely, the membrane fraction 2 was enriched for endo-ATPase, and depleted of endoplasmic reticular membrane protein. Membrane fraction 1 was enriched for plasma membrane protein, Na/K-receptors located in the endoplasmic reticulum of NRK cells, we used cell fractionation to separate plasma membranes from endoplasmic reticulum membranes. Using discontinuous sucrose gradient, we were able to obtain membranes highly enriched for either plasma membrane or endoplasmic reticulum. Plasma membranes were isolated from the gradient at the interface between 0.5 and 0.9 M sucrose (fraction 1) while endoplasmic reticulum membranes were isolated at the interface between 1.35 and 1.5 M sucrose (fraction 2). The upper panel of Fig. 5B shows the Western blot of the two fractions of pooled membranes from several experiments probed with rabbit polyclonal antibody for Na/K-ATPase, a plasma membrane marker. This blot shows that fraction 1 isolated from NRK cells stably expressing α2c-adrenoceptors is enriched for Na/K-ATPase while no detectable Na/K-ATPase is detected in an equivalent amount of membrane protein from fraction 2. The blot was then stripped and reprobed with rabbit polyclonal antibody for the protein, calnexin, an endoplasmic reticular membrane marker (Fig. 5B, lower panel). Fraction 2 is enriched in calnexin, while no detectable calnexin is detected in an equivalent amount of membrane protein from fraction 1. Both plasma membrane and endoplasmic reticular membrane fractions isolated from NRK cells stably expressing α2c-adrenoceptors possessed functional receptors as determined by radioligand binding, 0.89 ± 0.080 and 0.136 ± 0.036 pmol/mg protein, respectively.

α2C-Adrenoceptors expressed in both NRK and PC12 cells functionally couple to G-proteins as assessed by the effect of α2C-adrenoceptor activation on forskolin-induced cellular cAMP production. In both NRK and PC12 cells expressing α2C-adrenoceptors the nonselective α2-adrenoceptor agonist dexmedetomidine inhibited cAMP production by 90% (IC50 ~ 0.039 nM) and 55% (IC50 ~ 0.161 nM), respectively (Fig. 5, C and D).

Expression and coupling of α2c-adrenoceptors in NRK and PC12 cells. A, antigenic expression of α2c-adrenoceptors in stably transfected NRK cells and PC12 cells. Membrane preparations of NRK and PC12 cells were subjected to Western blot analysis with the mouse monoclonal antibody, 12CA5. Increasing amounts of membrane protein from NRK cells expressing α2c-adrenoceptors were run along with a fixed amount of membrane protein from PC12 cells expressing α2c-adrenoceptors. NRK and PC12 cells express α2c-adrenoceptor protein at comparable levels. Similar results were obtained in three independent experiments with both the mouse monoclonal antibody 12C4 and affinity purified rabbit polyclonal antibody C4. B, isolation and purification of endoplasmic reticular and plasma membranes. Membrane fractions were collected from interfaces between 0.5 and 0.9 M sucrose (Fraction 1) and 1.35 and 1.5 M sucrose (Fraction 2). Membrane fraction 1 was enriched for plasma membrane protein, Na/K-ATPase, and depleted of endoplasmic reticular membrane protein, calnexin. Conversely, the membrane fraction 2 was enriched for endoplasmic reticular membrane protein, calnexin, and depleted of plasma membrane protein, a-Na-K-ATPase. C and D, inhibition of forskolin stimulated adenyl cyclase by α2c receptor in NRK cells (panel C) and in PC12 cells (panel D). Dex is the non-subtype selective α2 agonist dexmedetomidine.

80 μg of NRK cell membrane and 80 μg of PC12 cell membrane contained approximately equivalent amounts of receptor antigen (Fig. 5A). Similar results were obtained with the affinity purified rabbit polyclonal antibody C4 (data not shown). Saturation binding with the α2c-adrenoceptor radioligand, RX821002, revealed identical Kd values and similar Bmax values (in picomole per milligram of membrane protein): AtT20 cells, 1.357 ± 0.065; NRK cells, 1.085 ± 0.043; PC12 cells, 0.962 ± 0.17. Taken together these results show that PC12 cells and NRK cells express comparable levels of α2c-adrenoceptor protein and have comparable numbers of α2c-adrenoceptor-binding sites, suggesting that the large pool of the α2c-adrenoceptor resides within the ER in NRK cells includes functional receptor.

To further examine the functional status of the α2c-adrenoceptors located in the endoplasmic reticulum of NRK cells, we
the \( \alpha_{2C} \)-adrenoceptors present at steady state are resistant to endoglycosidase H digestion. PNGase F was capable of deglycosylating the \( \alpha_{2C} \)-adrenoceptor expressed in all of the cell lines (Fig. 6).

**Development of \( \alpha_{2C} \)-Adrenoceptor Microdomains in PC12 Cells**—The treatment of PC12 cells with NGF resulted in neuronal induction of cells and the development of neurite extensions having focal accumulations of the synaptic vesicle marker SV2 in the terminal regions of these extensions (Data not shown). We labeled the cell surface and total cellular receptor distributions in PC12 after NGF treatment for 12 (Fig. 7, A and B) or 48 h (Fig. 7, C and D). The induction of a neuronal phenotype in PC12 cells did not alter the targeting to the plasma membrane of \( \alpha_{2C} \)-adrenoceptors. However, the plasma membrane distribution of \( \alpha_{2C} \)-adrenoceptors in PC12 cells became more heterogeneous with focal accumulations of receptors in the plasma membrane concentrated at peripheral margins and membrane extensions of cells following exposure to NGF for 12 h (Fig. 7). Prolonged treatment with NGF for 48 h resulted in the development of neurite extensions having focal accumulations of \( \alpha_{2C} \)-adrenoceptors at the tips (Fig. 7, C and D). These focal accumulations of \( \alpha_{2C} \)-adrenoceptors in the plasma membrane have not been observed in any other cell line studied. Interestingly, PC12 cells displaying focal accumulations of \( \alpha_{2C} \)-adrenoceptors in the plasma membrane have not been observed in any other cell line studied. Interestingly, PC12 cells displaying focal accumulations of \( \alpha_{2C} \)-adrenoceptors in the plasma membrane have not been observed in any other cell line studied. Interestingly, PC12 cells displaying focal accumulations of \( \alpha_{2C} \)-adrenoceptors in the plasma membrane have not been observed in any other cell line studied. Interestingly, PC12 cells displaying focal accumulations of \( \alpha_{2C} \)-adrenoceptors in the plasma membrane have not been observed in any other cell line studied. 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The \( \alpha_{2B} \)-adrenoceptor subtype is found in vascular smooth muscle where their activation leads to elevation of systemic blood pressure (20). The tissue distribution of \( \alpha_{2A} \)-adrenoceptors is primarily confined to the central nervous system (21, 22). Recent evidence from transgenic and knockout mouse models show that the \( \alpha_{2C} \)-adrenoceptors modulate several aspects of behavior (5, 14, 15). Both \( \alpha_{2A} \) and \( \alpha_{2C} \) receptors have been shown to regulate catecholamine release from sympathetic nerve terminals (16, 17). These studies showed that both the \( \alpha_{2A} \) and \( \alpha_{2C} \) subtypes are required for normal presynaptic control of transmitter release from sympathetic nerves in the heart and from central noradrenergic neurons. \( \alpha_{2A} \) receptors inhibit transmitter release at high stimulation frequencies

![Fig. 6. The post-translational processing of HA epitope-tagged \( \alpha_{2C} \)-adrenoceptors in AtT20, COS7, NRK, and PC12 cells was examined by Western blotting.](image)

Membrane preparations of cells expressing \( \alpha_{2C} \)-adrenoceptor were run on a 9 or 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The blots were probed for \( \alpha_{2C} \)-adrenoceptors with the HA epitope antibody 12CA5. Membrane preparations were treated with either endoglycosidase H or PNGase F to remove asparagine (N) linked glycosidic residues. In panel A, COS7 cells expressing \( \alpha_{2C} \)-adrenoceptors show a predominant band of receptor and multiple minor bands possibly due to different levels of glycosylation or receptor aggregation. The \( \alpha_{2C} \)-adrenoceptors made by COS7 cells are sensitive to both endoglycosidase H and PNGase F digestion as seen by the shift in size of the predominant band. In contrast, endoglycosidase H digestion did not significantly change the mobility of the majority of \( \alpha_{2C} \)-adrenoceptors expressed AtT20 cells, NRK cells or PC12 cells (panels B-D).

**Fig. 7. Focal clustering of \( \alpha_{2C} \)-adrenoceptors in the plasma membrane of PC12 cells treated with NGF.** PC12 cells were treated with NGF for 12 h (panels A and B) or 48 h (panels C and D). The cell surface \( \alpha_{2C} \)-adrenoceptors of viable cells were selectively labeled with the mouse monoclonal antibody, 12CA5, at 4 °C (panels A and C). The preparations were rinsed, fixed, permeabilized with 0.2% Nonidet P-40 prior to labeling the combined surface and intracellular \( \alpha_{2C} \)-adrenoceptor distributions with the rabbit polyclonal antibody, C4 (panels B and D). In PC12 cells treated with NGF for 12 h (panels A and B) there is an accumulation of \( \alpha_{2C} \)-adrenoceptors in very restricted regions of the plasma membrane. After 48 h of treatment, accumulation of \( \alpha_{2C} \)-adrenoceptors are observed in regions where membrane extensions are present.

**DISCUSSION**

Given the overall high structural and functional similarity among three \( \alpha \)-adrenergic receptor subtypes, it is very interesting that there are distinctly different cellular targeting characteristics, physiologic functions, and tissue expression patterns for each of the subtypes (for reviews, see Ref. 5). Unlike the \( \alpha_{2C} \)-adrenoceptor, both the \( \alpha_{2A} \) and \( \alpha_{2B} \)-adrenergic receptors target efficiently to the plasma and lack an intracellular ER distribution (1, 2, 6). The \( \alpha_{2A} \)-adrenoceptors are found in many tissues including platelets where they modulate platelet aggregation and in the central nervous system where they influence pain perception and blood pressure (18, 19). The \( \alpha_{2B} \)-adrenoceptor subtype is found in vascular smooth muscle where their activation leads to elevation of systemic blood pressure (20). The tissue distribution of \( \alpha_{2A} \)-adrenoceptors is primarily confined to the central nervous system (21, 22). Recent evidence from transgenic and knockout mouse models show that the \( \alpha_{2C} \)-adrenoceptors modulate several aspects of behavior (5, 14, 15). Both \( \alpha_{2A} \) and \( \alpha_{2C} \) receptors have been shown to regulate catecholamine release from sympathetic nerve terminals (16, 17). These studies showed that both the \( \alpha_{2A} \) and \( \alpha_{2C} \) subtypes are required for normal presynaptic control of transmitter release from sympathetic nerves in the heart and from central noradrenergic neurons. \( \alpha_{2A} \) receptors inhibit transmitter release at high stimulation frequencies

![Fig. 7. Focal clustering of \( \alpha_{2C} \)-adrenoceptors in the plasma membrane of PC12 cells treated with NGF.](image)
whereas the α2c subtype modulates neurotransmission at lower levels of nerve activity. Both low and high frequency regulation appear to be physiologically important as mice lacking both receptor subtypes have elevated plasma norepinephrine levels and develop cardiac hypertrophy with decreased left ventricular contractility by 4 months of age (16). Since the expression of α2c-adrenoreceptors is primarily in neurons it is possible that neuronal cells may possess the appropriate factors/proteins that not only allow efficient delivery and targeting of receptors to the plasma membrane but more importantly to specific pre-synaptic and post-synaptic domains in their plasma membrane.

The large intracellular pool of α2c-adrenoreceptors in NRK cells may be incorrectly folded and non-functional and thereby retained in the endoplasmic reticulum. It has been shown that some misfolded proteins tend to be retained by BiP, an endoplasmic reticulum chaperone protein, and retrieved from early Golgi regions of cells by BiP as well (23). Interestingly, by indirect immunolocalization we have found that most of the intracellular α2c-adrenoreceptors are restricted to a domain of the ER where BiP is localized. These observations would suggest that the intracellular α2c-adrenoreceptors in NRK cells are non-functional. However, we have determined that the combined intracellular and plasma membrane fractions of NRK cells and PC12 cells are comparable in expression by Western blotting and in function by saturation binding experiments. Furthermore, we were able to isolate plasma membrane and endoplasmic reticulum membrane fractions from NRK cells stably expressing α2c-adrenoreceptors and show functional expression of receptors by radioligand binding in both fractions. We found the density (in picomole/milligram) of functional expression of receptors by radioligand binding in the plasma membrane fraction of α2c-adrenoreceptors expressed in NRK cells is is slow processing of receptors with a concurrent short plasma membrane half-life. Transfection of cells with a powerful promoter such as the cytomegalovirus promoter, present in pCDNA3, can potentially raise protein expression by several orders of magnitude over normal physiologic or endogenous expression. This may overwhelm the cells biosynthetic pathways with the result being a large accumulation of intracellular protein. Given the large intracellular pool of α2c-adrenoreceptors present in NRK, we expected to find that a large fraction of receptors would be sensitive to endoglycosidase H digestion. Analysis of the asparagine-linked glycosylation of α2c-adrenoreceptors in the cell lines studied was able to detect immature endoglycosidase H-sensitive receptor in COS7 cells, but did not reveal significant amounts of endoglycosidase H-sensitive receptor in AtT20 cells, PC12 cells, or NRK cells. This would indicate that most of the intracellular α2c-adrenoreceptors have been processed in the cis/medial Golgi. Furthermore, it suggests that the α2c-adrenoreceptors expressed in NRK cells are not static within the ER but are actively retrieved from the early Golgi regions and returned to the ER of the cell possibly by a chaperone protein such as BiP. Thus, differences in the rate of protein processing cannot account for the observed differences in plasma membrane targeting.

We have previously demonstrated that the plasma membrane fraction of α2c-adrenoreceptors expressed in NRK cells is stable for several hours with minimal agonist-induced internalization (2). In addition, Wozniak and Limbird (6) reported that the expression of α2c-adrenoreceptors in Madin-Darby canine kidney cells to be limited to the basolateral domains of the plasma membrane intracellular pool of receptor (6). The more efficient delivery of the α2c-adrenoreceptors to the plasma membrane and the lack of a large intracellular pool of receptors in the neuroendocrine cell lines, PC12 cells and AtT20 cells, suggests that these cells express a factor(s) that facilitate either the proper processing and/or targeting to the plasma membrane. Alternatively, these cells may lack a factor(s) that retains the α2c receptor in the biosynthetic pathway.

PC12 cells undergo neuronal differentiation in response to NGF treatment with the accumulation of synaptic vesicles in peripheral neurite extensions. The neuronal induction of PC12 cells did not affect the targeting of α2c-adrenoreceptor to the plasma membrane. However, after short periods of NGF treatment, the α2c-adrenoreceptor distribution in the plasma membrane displayed clustering in regions where membrane extensions were present. These regions of the membrane extensions also display increased concentration of F-actin as labeled by fluorescein isothiocyanate-conjugated phalloidin D. We have occasionally observed focal accumulations of α2c receptors in PC12 cells that have not been treated with NGF. This may be due to the fact that some PC12 cell may be partially differentiated without NGF treatment. These focal accumulations of α2c-adrenoreceptors were never observed in any of the none...
neuronal cell lines examined. The localization of cytoskeletal proteins, such as F-actin and microtubules, with receptors has been shown to occur in post-synaptic regions of the plasma membrane in neurons (24, 25) and in neuromuscular junctions (26) where the receptors are anchored to the cytoskeleton via linker/clustering proteins such as gephyrin, α-actinin-2, PSD-95, and rapsyn/43k proteins. Recent studies have found α2C-adrenergic receptors in both pre-synaptic (21) and post-synaptic densities (21) depending on the location and type of neurons in the central nervous system. Since the neuroendocrine cell line, PC12 cells, possess some functional characteristics of neurons, it is likely that they not only express factors/proteins that facilitate the efficient targeting of α2C-adrenergoreceptor to the plasma membrane but more importantly the development of specialized receptor targeting and signaling domains in the plasma membrane.

It has been suggested that G protein-coupled receptors are located in plasma membrane microdomains along with specific G-proteins and effector molecules (27). Thus, by directing the receptor to a specific microdomain, the cell may more efficiently control the effector systems modulated by that receptor. In PC12 cells, α2C-adrenergoreceptors are found concentrated at the developing neurite extensions having accumulations of F-actin. The targeting of receptors in neurons in these locations has been shown to have a role in directional chemotaxis of developing neurite extensions (28). Further comparison of other signal transduction components and regulation of α2C-adrenergoreceptor trafficking in PC12 cells and other cells such as NRK cells may provide clues to the functional importance of the cell-specific trafficking of G protein-coupled receptors.

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