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Differential targeting and function of α_{2A} and α_{2C} adrenergic receptor subtypes in cultured sympathetic neurons

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Abstract

Previous research suggested that α_{2A} and α_{2C} adrenergic receptor (AR) subtypes have overlapping but unique physiological roles in neuronal signaling; however, the basis for these dissimilarities is not completely known. To better understand the observed functional differences between these autoreceptors, we investigated targeting and signaling of endogenously expressed α_{2A} and α_{2C} ARs in cultured sympathetic ganglion neurons (SGN). At Days 1 and 4, α_{2A} and α_{2C} ARs could be readily detected in SGN from wild-type mice. By Day 8, α_{2A} ARs were targeted to cell body, as well as axonal and dendritic sites, whereas α_{2C} ARs were primarily localized to an intracellular vesicular pool within the cell body and proximal dendritic projections. Expression of synaptic vesicle marker protein SV2 did not differ at Day 8 nor co-localize with either subtype. By Day 16, however, α_{2C} ARs had relocated to somatodendritic and axonal sites and, unlike α_{2A} ARs, co-localized with SV2 at synaptic contact sites. Consistent with a functional role for α_2 ARs, we also observed that dexmedetomidine stimulation of cultured SGN more efficiently inhibited depolarization-induced calcium entry into older, compared to younger, cultures. These results provide direct evidence of distinct developmental patterns of endogenous α_{2A} and α_{2C} AR targeting and function in a native cell system and that maturation of SGN in culture leads to alterations in neuronal properties required for proper targeting. More importantly, the co-localization at Day 16 of α_{2C} ARs at sites of synaptic contact may partially explain the differential modulation of neurotransmitter release and responsiveness to action potential frequency observed between α_{2A} and α_{2C} ARs in SGN.

Keywords

α_2 adrenergic receptor; Sympathetic ganglion neuron; Trafficking; Dexmedetomidine; SV2

1. Introduction

Release of norepinephrine from sympathetic neurons is modulated by α_{2A} and α_{2C} adrenergic receptors (ARs) located at nerve terminals (i.e. presynaptic autoreceptors) (Hein et al., 1999). Three subtypes of α_2 ARs (α_{2A} , α_{2B} , and α_{2C}) have been identified by molecular cloning. α_{2A} and α_{2C} ARs couple to pertussis toxin-sensitive G_i/G_o signaling pathways to inhibit cAMP production as well as to regulate Ca^{2+} and K^+ channel signaling (MacDonald et al., 1997). Molecular cloning of murine α_{2A} and α_{2C} AR genes led to development of gene knockout (KO) models for in vivo and in vitro physiological research (Altman et al., 1999; Link et al., 1996). By studying mice having gene disruptions for α_{2A} and/or α_{2C} ARs, it was observed that both subtypes were involved in presynaptic regulation of catecholamine release in heart (Hein et al., 1999). However, neurotransmitter release modulation by α_{2A} and α_{2C} ARs were distinguished by their responsiveness to neuronal action potential frequencies; α_{2A} ARs inhibited release faster and at higher stimulation frequencies than α_{2C} ARs, which appeared to operate at lower frequencies.

Both α_{2A} and α_{2C} ARs have overlapping roles in regulating neurotransmitter release. Using a field stimulation assay of [3H]norepinephrine release with various tissues from wild-type (WT), α_{2A} and α_{2C} AR knockout (α_{2A} ARKO and α_{2C} ARKO) mice, it has been shown that α_{2A} ARs are the predominant autoreceptors in most tissues (e.g. vas deferens, hippocampus, cerebral cortex, atria), though α_{2C} ARs play a minor role (Altman et al., 1999; Trendelenburg et al., 1999, 2001a,b). Conflicting information about the role of α_{2A} and α_{2C} ARs as autoreceptors has been revealed following examination of neurotransmitter release from cultured sympathetic ganglion neurons (SGN). For instance, field stimulation assays of SGN cultured from newborn mice suggest that only α_{2A} ARs, but not α_{2C} ARs, function as autoreceptors (Trendelenburg et al., 2001a,b).

Subcellular localization is an important determinant of specialized function between homologous receptors (Xiang and Kobilka, 2003a,b). For example specific PDZ-binding motifs in b_1 and b_2 ARs are responsible for differential membrane trafficking within cardiac myocytes, as well as for opposing effects on cardiac myocyte contraction rates (Xiang and Kobilka, 2003a,b; Xiang et al., 2002). Consistent with functional differences observed above between α_{2A} and α_{2C} ARs, previous research has revealed distinctly different trafficking characteristics in transfected cells. In a variety of cell lines, α_{2C} ARs are retained in the endoplasmic reticulum, whereas α_{2A} ARs are targeted primarily to the plasma membrane (Daunt et al., 1997; Olli-Lahdesmaki et al., 1999; von Zastrow et al., 1993; Wozniak and Limbird, 1996). Recently, it was demonstrated that the efficiency of plasma membrane targeting of α_{2C} ARs was influenced by the cell-type in which it is expressed. Neuroendocrine cell lines such as AtT20 and PC12 cells have efficient plasma membrane targeting of α_{2C} ARs, with little detectable intracellular retention (Hurt et al., 2000).

Based on the conflicting data concerning α_{2A} and α_{2C} AR modulation of neurotransmitter release in heart and cultured SGN, and the observation that plasma membrane localization of these receptors was cell-type specific, we investigated trafficking and function of endogenous α_{2A} and α_{2C} ARs in a native cell population, primary SGN cultures. To differentiate between α_{2A} and α_{2C} ARs, we cultured SGN from WT, α_{2A} ARKO, and

α_{2C} ARKO mice and performed immunocytochemical analysis with subtype specific antisera. We found that endogenously expressed α_{2A} and α_{2C} ARs exhibited differential targeting between cell body, dendritic, and axonal sites of cultured SGN. The temporal differences in neuronal targeting observed between these two autoreceptors correlated with their ability to modulate neuronal function, as measured by regulation of calcium ($[Ca^{2+}]_i$) influx. Consistent with the finding that both α_{2A} and α_{2C} ARs had enhanced targeting to axonal and dendritic sites with increasing culture age, enhanced regulation of $[Ca^{2+}]_i$ influx was detected for older (10–15 days), compared to younger cultures.

Our results provide direct evidence of different developmental patterns of α_{2A} and α_{2C} AR localization and function in cultured SGN, a primary neuron population that endogenously expresses these two adrenergic receptor subtypes. Moreover, they suggest that neuronally expressed α_{2A} AR and α_{2C} ARs are targeted by different mechanisms and that targeting of α_{2C} ARs to the presynaptic membrane may require alterations in neuronal properties, such as expression of a protein(s) not present in immature SGN.

2. Materials and methods

2.1. Generation of α_2 -AR knockout mice

α_{2A} ARKO, α_{2C} ARKO and wild-type C57Bl6/J mice were used to obtain cultured SGN. Homozygous α_{2A} ARKO mice (Altman et al., 1999) and α_{2C} ARKO mice (Link et al., 1995) were bred to wild-type C57Bl6/J mice for five successive generations to produce congenic strains of mice having a uniform, predominantly C57Bl6/J genetic background. Genotyping was confirmed as before by PCR based assays.

2.2. SGN culture

Superior cervical sympathetic ganglia were dissected from 1- to 3-day-old mice and collected in ice-cold Leibovitz's L-15 medium (Life Technologies). Following centrifugation at 1500 rpm for 5 min, ganglia were washed in HBSS buffer (HBSS with 1 mg/ml BSA, Sigma). After removal of L-15 medium, ganglia were incubated for 15 min at 37 °C in 2 ml of HBSS buffer containing 3 mg/ml collagenase IA (Sigma), followed by an additional 30 min incubation at 37 °C with 2 ml HBSS buffer supplemented with 1 mg/ml trypsin. The digestion was quenched by the addition of 6 ml HBSS buffer, and the ganglia centrifuged as above. Next, cells were dissociated by trituration with a fire-polished Pasteur pipette. Cell suspensions were centrifuged (1500 rpm $\times$ 5 min) and the cell pellet was resuspended in SGN culture medium [90% L-15 medium, 10% Nu serum IV (Becton Dickinson), supplemented with 2 mM Glutamax (GibcoBRL), 100 ng/ml nerve growth factor 2.5S (Invitrogen), and 2.5 ml/l ITS liquid media supplement (Sigma)]. Cells were preplated for 1 h at 37 °C in a 10-cm tissue culture dish to remove adherent, non-SGN cells. The final enriched SGN cell population was obtained by gently tapping and aspirating the tissue culture dish. Neurons were collected by centrifugation as above and resuspended in SGN media. Final resuspension volumes (10,000 cells/ml) were chosen to plate ganglion neurons at low-density (2–5 cells/mm²).

Glass coverslips for culturing SGN were prepared by coating twelve millimeter diameter coverslips or Lab-Tek® 8-Chambered Coverglass (Nalge Nunc International) with poly-D-lysine (100 µg/ml in 0.1 M boric acid/NaOH buffer pH 8.4, Sigma) for 24 h at 4 °C. On the following day, coverslips and chambered coverglass were washed with tissue culture quality water, and coated with laminin (10 ng/ml) for 24 h at 37 °C. To culture neurons, laminin solution was aspirated to dryness and SGN were plated onto each coverslip (50 µl volume) and incubated at 37 °C in an atmosphere of 5% CO₂/95% air. After 3 h, 1 ml of fresh media was added to the dish. The next day, cytosine β-D-arabinofuranoside (Sigma) was added to a final concentration of 1 µM to reduce proliferation of non-neuronal cells. On culture Day 2, culture medium was exchanged 1:1 with fresh medium (cytosine β-D-arabinofuranoside-free). After 3 days in culture, SGN were grown in the presence of the α₂AR antagonist RX821002 (100 nM final concentration), in order to prevent potential desensitization and down-regulation of α₂AR and obtain better membrane staining.

2.3. Immunocytochemistry

α_{2A} and α_{2C}AR targeting and membrane distribution was examined using indirect immunocytochemical staining. Neurons were stained with affinity-purified rabbit polyclonal antibody directed against the wild-type α_{2A}AR (1:100 dilution) or α_{2C}AR (1:100 dilution) (Daunt et al., 1997). Analysis of dopamine-β-hydroxylase (DBH) was performed using a rabbit polyclonal anti-DBH antibody (1:2000 dilution; Chemicon). Neuronal somatodendritic regions were labeled with mouse monoclonal anti-microtubule associated protein 2 (MAP2) antibody (1:500 dilution, Sigma), whereas synaptic vesicles were visualized using mouse monoclonal anti-SV2 antibody (1:500 dilution; gift of R. Scheller, Genentech). Primary antibodies were incubated together for the double-labeling experiments; controls showed no cross-reactivity.

Coverslips with plated SGN were fixed in 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS) for 20 min at room temperature. Following fixation, cells were rinsed three times with PBS supplemented with glycine (0.1 M). A blocking agent composed of 0.4% saponin, 50 mM HEPES, 1% bovine serum albumin (BSA, Sigma) and 2% goat serum in PBS was used to reduce nonspecific antibody activity. All antibody applications of fixed specimens were done in the presence of blocking agent for 1 h at room temperature. Primary antibodies were visualized with either Alexa 488 or Alexa 594 fluorescent labeled secondary antibody directed against the species of primary antibody (1:1000 dilution; Molecular Probes, Eugene, OR).

Conventional immunofluorescence microscopy was performed using a Zeiss Axiophot microscope with Zeiss Neofluar 63×/1.25 or Zeiss Neofluar 100×/1.3 objective. Confocal images were acquired using the Zeiss LSM 510 Confocal Laser Scanning Microscope equipped with a Coherent Mira 900 tunable Ti:Sapphire laser for two-photon excitation (Talamasca, Stanford Imaging Facility) (Zeiss, Thornwood, NY) and analyzed by Volocity software by Improvision, Inc. (Lexington, MA, USA).

2.4. Fura-2 Imaging of $[Ca^{2+}]_i$

Fura-2 loading and fluorescence ratio imaging were performed as described previously (Lipscombe et al., 1988). Cultured SGN were loaded with Fura-2 by placing them in a solution of Fura-2AM (Molecular Probes) and Pluronic F-127 (Molecular Probes) at a final concentration of 1–5 μ M of Fura-2AM and 0.05% Pluronic F-127. Neurons were agitated gently in loading solution and incubated for one hour at room temperature, then rinsed twice with PBS and transferred to Fura-2 free DMEM medium without phenol red. For completeness of de-etherification, cells were incubated at 37 °C in an atmosphere of 5% CO₂/95% air for 15 min.

Fura-2 loaded cells were exposed to epi-illumination (mercury arc bulb, Zeiss Axiovert inverted microscope) in conjunction with an $\times$ 40 oil immersion objective (Zeiss). Excitation light passed through 340 or 380 nm excitation filter housed in a computer filter wheel. Images of fluorescence emission at 505 nm long-pass filter, were acquired with a charge-coupled device (ICCD). Fluorescent image intensities were expressed as the 340- to 380-nm ratio to allow quantitative estimates of changes in SGN $[Ca^{2+}]_i$, using Atto-fluor RatioVision™ software for image acquisition (Atto Instruments, Rockville, MD). Cells were depolarized using potassium chloride (KCl, 13 mM), and $[Ca^{2+}]_i$ flux was observed. To study effects of α_2 AR stimulation on $[Ca^{2+}]_i$ following KCl depolarization, cells were challenged with α_2 AR agonist dexmedetomidine (100 nM) prior to depolarization. In order to compare changes in the fluorescence intensity between experimental groups, data were normalized by calculating the fold increase above basal level.

2.5. Statistical analysis for $[Ca^{2+}]_i$ experiments

Data were subject to three-way analysis of variance (ANOVA) with post hoc testing by Fisher's PLSD using SPSS VER. 11.5 (SPSS Inc., Chicago). Dexmedetomidine effects were analyzed using the Levene Test for Equality of Variance and unpaired *t*-test to compare normalized $[Ca^{2+}]_i$ ratios. Statistical significance was considered achieved at a value of *P* 0.05.

3. Results

3.1. Characterization of superior cervical sympathetic ganglion cultures

Sympathetic ganglion neuron cultures were prepared from 1–3-day-old mice. Identification of neurons in these different cultures was determined using a neuronal-specific antibody recognizing MAP2, which specifically recognizes neuronal cell bodies and dendrites (e.g. somatodendritic) (Fig. 1A). The presence of noradrenergic neurons was verified by indirect immunocytochemistry using dopamine b-hydroxylase (DBH), a catecholamine biosynthesis enzyme (Fig. 1B). MAP2 staining was restricted to the neuronal somatodendritic region, whereas DBH staining appeared more diffusely throughout dendrites and axons, with some localized accumulation of enzyme within punctate vesicles.

Affinity purified rabbit polyclonal antisera recognizing the intracellular carboxyl termini of either α_{2A} (C10 antibody) or α_{2C} ARs (C4 antibody) (Daunt et al., 1997) were used to examine endogenous expression and distribution of these receptor subtypes in cultured

SGN. To verify antibody sensitivity and specificity, 4-day-old SGN cultures obtained from WT, α_{2A} ARKO, and α_{2C} ARKO mice were examined (Fig. 2). Neurons were co-labeled with either α_{2A} - or α_{2C} AR and MAP2 antisera.

MAP2 antibody labeled similar intracellular somatodendritic regions of SGN cultured from all three mouse lines, and can be used to distinguish dendrites from axons (Fig. 2A–D). α_{2A} AR antibody labeling of WT SGN revealed predominantly membrane staining of somatodendritic regions and axons (Fig. 2E). In contrast, it did not display specific staining of α_{2A} ARKO mouse SGN (Fig. 2F), consistent with the loss of α_{2A} AR expression in these knockout mice. Immunocytochemical staining with α_{2C} AR specific antisera revealed a different membrane localization pattern. α_{2C} AR antisera labeling of WT SGN revealed restricted intracellular vesicular staining that was confined to the somatodendritic region of the neuron (Fig. 2G). As expected, cultured SGN obtained from α_{2C} ARKO mice did not display specific staining with α_{2C} AR antisera (Fig. 2H). No expression of α_{2A} or α_{2C} ARs was detected in fibroblasts or glial cells (data not shown). These data demonstrate that both C10 and C4 antisera can specifically identify endogenously expressed α_{2A} and α_{2C} ARs, respectively.

3.2. Differential localization of α_{2A} and α_{2C} ARs during maturation of cultured SGN

Since preliminary immunohistochemical staining of endogenous α_{2A} and α_{2C} ARs in cultured SGN suggested differential localization and targeting, we next initiated a more detailed examination during maturation of SGN cultures. Thus, membrane targeting and distribution of α_{2A} and α_{2C} ARs in SGN isolated from WT mice were examined at 1, 4, 8 and 16 days in culture using confocal microscopy (Figs. 3 and 4). To allow for direct comparisons, immunocytochemical staining with α_{2A} and α_{2C} AR antisera and MAP2 antibody was done in parallel SGN cultures, to eliminate possible culture artifacts.

At Day 1, α_{2A} ARs were localized diffusely within somatodendritic regions, with similar labeling of cell body, dendrites and some axonal extensions (Fig. 3A). Somatodendritic regions were delineated by co-staining for the intracellular marker protein MAP2. MAP2 co-staining (Fig. 3B) demonstrated that α_{2A} ARs had limited membrane localization, as seen upon merging the two images (Fig. 3C). At Day 4, α_{2A} ARs begin to appear in more distal axonal extensions (Fig. 3D), with a more predominant membrane localization (note the appearance of green α_{2A} AR fluorescence lateral to the red MAP2 staining within dendrites and axons) (Fig. 3E, F). At Day 8, α_{2A} ARs continued to localize to the cell body and the developing network of axonal projections. They appeared to be targeted to the plasma membrane, as evidenced by the enhanced α_{2A} AR immunofluorescence encircling the MAP2 immunofluorescent signal (Fig. 3G–I). This expression pattern continued to be evident at Day 16 of SGN culture (Fig. 3J–L).

Compared to α_{2A} ARs, α_{2C} AR distribution was restricted to the cell body and appeared to be primarily in an intracellular vesicular compartment at Day 1 and Day 4 (Fig. 4A and D, respectively). This intracellular localization was made more evident by merging MAP2 with α_{2C} AR expression (Fig. 4B,C and 4E,F, respectively). By examining somatodendritic regions visualized by MAP2 at Day 4, it was possible to discern that α_{2A} AR antisera (Fig. 3) labeled a network of axonal and dendritic sites not seen with α_{2C} AR antisera, despite the

presence of a similar network of axons and dendrites as seen by phase-contrast microscopy (data not shown).

This differential pattern of $\alpha_{2C}AR$ targeting continued through Day 8 of culture (Fig. 4G–I). In comparison to $\alpha_{2A}ARs$, $\alpha_{2C}ARs$ continued to reveal a predominant intracellular staining pattern with limited membrane expression; note the absence of membrane enhancement at regions of cell-to-cell contact. Some punctate labeling was noted within axonal extensions (note absence of MAP2 staining in axonal processes). However, the dense network of axonal and dendritic staining seen for $\alpha_{2A}ARs$ was still not apparent with $\alpha_{2C}ARs$ (Fig. 4G). Even at Day 16 of SGN culture, $\alpha_{2C}ARs$ revealed limited membrane expression in the cell body and dendrites (Fig. 4J–L). However, at this later time point, axonal expression was evident as puncta in extensions not stained with MAP2. Membrane localization of these puncta was confirmed by examination of SV2 expression (see below). Despite differential α_{2A} and $\alpha_{2C}AR$ localization, all SGN had similar MAP2 staining (Figs. 3 and 4B, E, H, and K, respectively) and both cultures had similar axonal and dendritic networks as determined by light microscopy (data not shown).

3.3. Co-localization of α_{2A} and $\alpha_{2C}ARs$ with synaptic proteins in cultured SGN

During maturation of SGN cultures, a visible network of axons and dendritic fields increased in density. One possible explanation for the delayed targeting of $\alpha_{2C}ARs$ to axonal and dendritic sites may be that requirement for synaptic contacts. Thus, we further characterized targeting of α_{2A} and $\alpha_{2C}ARs$ by examining their distribution relative to SV2, a synaptic vesicle protein that localizes to presynaptic sites (Lowe et al., 1988). Parallel SGN cultures were co-stained with both α_{2A} or $\alpha_{2C}AR$ antisera and a monoclonal antibody against SV2. As seen previously, Day 8 cultured SGN expressed α_{2A} and $\alpha_{2C}ARs$ in distinctly different cellular domains. $\alpha_{2A}ARs$ were expressed in the plasma membrane in somatodendritic and axonal regions (Fig. 5A), whereas $\alpha_{2C}ARs$ were present predominantly in an intravesicular compartment and early dendritic regions (Fig. 5C). Staining for SV2 revealed specific labeling of cell body and axonal regions in all neurons examined (Fig. 5B and D). The SV2-labeled axonal projections also stained with $\alpha_{2A}AR$ antisera but not with $\alpha_{2C}AR$ antisera.

By Day 16, further differences in subcellular localization of $\alpha_{2A}AR$ and $\alpha_{2C}AR$ were observed. Expression of $\alpha_{2A}ARs$ continued to be localized to somatodendritic and axonal sites as seen at Day 8, with further development of a dense arborization of axonal and dendritic networks (Fig. 6A and insets 1, 2). However, compared to Day 8, SV2 labeling was more restricted to axons with accumulations at sites of presumed synaptic contact (Fig. 6B and insets 3, 4), with little staining in the cell body. There was little apparent overlap between these SV2 puncta and $\alpha_{2A}ARs$ (Fig. 6C). More interesting was the distribution of $\alpha_{2C}ARs$ within somatodendritic and axonal regions of cultured SGN. Compared to Day 8, $\alpha_{2C}ARs$ were no longer restricted to an intravesicular compartment within the cell body and proximal dendrites. Instead at Day 16, specific labeling of axonal processes, similar to $\alpha_{2A}ARs$, was evident (Fig. 7A). However, one striking difference between α_{2A} and $\alpha_{2C}AR$ expression at Day 16 was the apparent presence of co-localized staining for $\alpha_{2C}ARs$ and SV2 (Fig. 7B). Enlargements of $\alpha_{2C}AR$ and SV2 staining revealed significant areas of

overlap (insets 1,2 and 3,4, respectively), that was more apparent after merging the paired images (Fig. 7C, arrows).

To further define the co-localization of α_{2A} and α_{2C} ARs and SV2 expression, confocal microscopic examination was performed at Day 8 and 16 (Fig. 8). At Day 8, neither α_{2A} (Fig. 8A–C) nor α_{2C} AR (Fig. 8D–F) showed co-localization with SV2 expression. However, co-localization of α_{2C} AR (Fig. 8J) and SV2 (Fig. 8K) expression was noted in axons, as seen upon merging the two images (Fig. 8L). This co-localization, which suggests targeting of α_{2C} ARs to synapses, was not seen with α_{2A} ARs (Fig. 8G–I). Together, the data suggest that there is a temporal alteration in SGN as they mature in culture, corresponding to a change in localization of α_{2A} and α_{2C} ARs, as well as SV2 expression.

3.4. α_{2A} and α_{2C} AR regulation of $[Ca^{2+}]_i$ in cultured SGN

Subcellular targeting of neurotransmitter receptors to specific subcellular domains is critical for their function in vivo. We therefore attempted to determine whether observed temporal and spatial targeting differences between α_{2A} and α_{2C} ARs in cultured SGN influenced their functional properties as autoreceptors. We examined the regulation of $[Ca^{2+}]_i$ by α_{2A} and α_{2C} ARs in cell bodies of cultured SGN using the fluorescent calcium indicator, Fura-2. SGN were obtained from wild-type, α_{2A} ARKO (expressing α_{2C} ARs) and α_{2C} ARKO (expressing α_{2A} ARs) mice and cultured for 7–15 days. The 7-day time point was chosen since it represented the largest contrast between α_{2A} and α_{2C} AR localization and also was the time point used previously for field stimulation assays of cultured SGN ((Trendelenburg et al., 2001a,b). The cultures were morphologically comparable and responded similarly to potassium-induced depolarization with a fast transient increase in $[Ca^{2+}]_i$ in a concentration-dependent manner (data not shown). A concentration of 13 mM KCl was chosen to induce depolarization of the SGN and to assess α_{2A} AR modulation of $[Ca^{2+}]_i$ since it produced a half maximal stimulation of $[Ca^{2+}]_i$ (data not shown).

To correlate $[Ca^{2+}]_i$ with α_{2A} AR expression, similar fluorescent microscope images were obtained from SGN cultured from α_{2A} and α_{2C} AR KO mice at the various time points examined. Similar results were seen for α_{2C} ARs in SGN cultured from α_{2A} ARKO and for α_{2A} ARs in SGN cultured from α_{2C} ARKO mice (Fig. 9). Localization of α_{2A} and α_{2C} ARs in α_{2A} ARKO SGN were similar to that seen previously in WT SGN at Day 1 and 4 (Fig. 9A–D). In addition, Day 7 (Fig. 9E,F) results were similar to that seen at Day 8, with α_{2A} ARs localized to all dendritic and axonal regions, whereas α_{2C} ARs were still predominantly localized to an intracellular compartment.

Similarly, α_{2A} ARs in α_{2C} ARKO SGN showed a similar distribution at Day 15 (Fig. 9G) as seen in WT SGN at Day 16. Interestingly, α_{2C} AR staining in α_{2A} ARKO SGN at Day 10 (Fig. 9H) was similar to that seen at Day 8 in WT SGN, suggesting that the temporal aspects of α_{2C} AR localization to axons occurred at a later time point. MAP2 staining was similar for all neurons (data not shown). Together, these results with α_{2A} ARKO and α_{2C} ARKO SGN suggested that interactions between the two α_2 ARs were not necessary for the differential localization observed, since they have similar localization patterns as those seen in WT SGN.

In 7-day-old cultured SGN obtained from WT mice, potassium-induced depolarization produced a significant increase in $[Ca^{2+}]_i$ to 1.5-fold above basal levels (Fig. 10A). Preincubation of WT SGN with the α_2 AR agonist dexmedetomidine (100 nM) for 90 s had no effect on basal $[Ca^{2+}]_i$, but attenuated the potassium-induced increase in $[Ca^{2+}]_i$ by 6%. In SGN obtained from α_{2A} ARKO and α_{2C} ARKO, the baseline potassium-induced increase in $[Ca^{2+}]_i$ did not differ from those of wild-type SGN (data not shown). However, 7-day-old SGN obtained from α_{2A} ARKO and α_{2C} ARKO mice showed only a 2% and 3% attenuation of potassium-induced increase in $[Ca^{2+}]_i$ respectively, following dexmedetomidine pretreatment (Fig. 10A), suggesting that both α_{2A} and α_{2C} ARs are involved with the reduction seen in WT SGN. However, dexmedetomidine effects of $[Ca^{2+}]_i$ was only statistically significant for WT SGN ($P < 0.02$).

To determine whether age-dependent changes in cultured SGN affected the ability of α_2 ARs to modulate potassium-induced increases in $[Ca^{2+}]_i$, SGN were studied at 15 days in culture for WT and α_{2C} ARKO mice, and 10 days for α_{2A} ARKO mice. Culturing of α_{2A} ARKO SGN beyond 10 days was not possible due to increased cell death, despite the presence of the competitive α_2 AR antagonist RX821002 (data not shown). At 15 days in culture, SGN obtained from WT mice showed a greater potassium-induced increase in $[Ca^{2+}]_i$, to 3-fold above basal levels suggesting that maturation of the cultures led to alterations in neuronal properties (Fig. 10B). This increase in $[Ca^{2+}]_i$ transients was also reflected in the effects of the dexmedetomidine pretreatment. Specifically, pretreatment with dexmedetomidine to activate α_{2A} and α_{2C} ARs found on wild-type SGN, led to an 18% inhibition of the potassium-induced increase in $[Ca^{2+}]_i$ ($P < 0.02$). Dexmedetomidine pretreatment of 15-day-old cultured SGN obtained from α_{2C} ARKO mice and 10-day-old cultured SGN from α_{2A} ARKO mice caused a 14% and 6% decrease in potassium-induced $[Ca^{2+}]_i$, respectively; however, dexmedetomidine effects were only significant for α_{2C} ARKO mice ($P < 0.005$). Thus maturation of cultured SGN from 7 to 15 days led to an enhanced dexmedetomidine effect on potassium-induced $[Ca^{2+}]_i$, suggesting an important role for localization in α_{2A} AR function as a target for dexmedetomidine effects. Analysis between time points revealed that the enhancement of dexmedetomidine effects at Day 15 compared to Day 7 for both WT and α_{2C} ARKO SGN was significant ($P < 0.05$), suggesting a more important role for α_{2A} ARs as a target for dexmedetomidine effects.

4. Discussion

Previous work has demonstrated that both α_{2A} and α_{2C} ARs act as presynaptic autoreceptors in cardiac tissue and that coupling of receptor stimulation to inhibition of neurotransmitter release is faster for α_{2A} ARs than α_{2C} ARs (Hein et al., 1999). Both α_{2A} and α_{2C} ARs appear to be physiologically important modulators of noradrenaline release on sympathetic nerve terminals, since mice lacking both α_{2A} and α_{2C} ARs demonstrate sympathetic hyperactivity and develop heart failure at 4 months of age (Brum et al., 2002; Hein et al., 1999). Interestingly, mice lacking only single α_{2A} or α_{2C} AR subtypes do not develop symptoms of heart failure, suggesting a functional overlap (Altman et al., 1999; Link et al., 1995). Though α_{2A} ARs are the predominant autoreceptors in the central and peripheral nervous system, α_{2C} ARs may play an important role in modulating neurotransmitter release in heart

and appear to be the major subtype regulating catecholamine release from adrenal gland (Brede et al., 2003; Trendelenburg et al., 1999, 2001a,b).

Trafficking and localization of receptors and ion channels in neurons and other cells may be important determinants of their functional roles (Tan et al., 2004; Wenthold et al., 2003). The studies presented here demonstrate clear differences in α_{2A} and α_{2C} AR targeting to plasma membranes and axonal projections in cultured SGN. At early stages of culture (Days 1 to 4), α_{2A} ARs were localized predominantly to somatodendritic regions; however, α_{2C} ARs were predominantly found in a small intracellular vesicular compartment in the cell body. As the culture matured to Day 8, α_{2A} ARs remained at somatodendritic regions; however, they also began to localize to axons. Despite the trafficking of α_{2A} ARs to axonal process during this time period, α_{2C} ARs remained predominantly within an intracellular vesicular pool. Only at Day 16 was specific expression of α_{2C} ARs at axonal sites evident. In vivo analysis of α_{2A} and α_{2C} AR expression patterns has not been performed in sympathetic ganglia. However, immunocytochemical analysis has revealed prominent post-synaptic localization of α_{2C} ARs in catecholaminergic locus ceruleus neurons, consistent with our results demonstrating dendritic and cell body staining (Lee et al., 1998). Localization of α_{2A} and α_{2C} ARs to pre- or post-synaptic sites was not possible with our current experimental system.

In an initial attempt to investigate α_{2A} and α_{2C} AR signaling and the role of localization in this process, we measured neuronal cell body $[Ca^{2+}]_i$ transients induced by potassium depolarization. Pretreatment of SGN with the α_{2A} and α_{2C} AR agonist dexmedetomidine led to decreased $[Ca^{2+}]_i$ transients, possibly due to inhibition of N-type Ca^{2+} channels or activation of G protein-coupled inwardly rectifying potassium channels (Dolezal et al., 1994; Koh and Hille, 1997). Potassium-induced depolarization of Day 7 SGN cultures led to an increased $[Ca^{2+}]_i$ over basal levels; this increase was reduced by pretreatment with dexmedetomidine in WT SGN. The magnitude of $[Ca^{2+}]_i$ transients due to potassium-induced depolarization was higher in older SGN cultures, suggesting that maturation of the cultures did lead to alterations in neuronal properties. In addition, increasing the age of SGN cultured from WT and α_{2C} ARKO mice demonstrated enhanced effects of α_{2A} AR agonist pretreatment on the reduction of $[Ca^{2+}]_i$. Unfortunately it was not possible to study 15-day-old α_{2A} ARKO SGN, because a significant increase in cell death was noted beyond 10 days in culture, as compared to SGN obtained from WT and α_{2C} ARKO mice (data not shown), suggesting other functional differences between α_{2A} and α_{2C} ARs. It is possible that the loss of α_{2A} AR inhibition of norepinephrine release may have led to toxic neurotransmitter levels and hence SGN apoptosis or degeneration (Zilkha-Falb et al., 1997). Together, these data suggest that maturation of SGN cultures leads not only to an alteration in α_{2A} and α_{2C} AR localization, but that it also affects their functional properties as autoreceptors as measured by $[Ca^{2+}]_i$. Similar to previous work with $[^3H]$ norepinephrine overflow assays, the results obtained with SGN from WT and α_{2C} ARKO mice suggest that α_{2A} ARs appear to have a more prominent functional role in older SGN cultures.

The absence of α_{2C} ARs from axons at Day 8 may explain the inability of Trendelenburg et al. to detect any inhibition of neurotransmitter release in cultured SGN from α_{2A} ARKO mice (which only express α_{2C} ARs) at this early time point (Trendelenburg et al., 2001a,b).

Their analysis of field-stimulated [^3H]norepinephrine release was performed with Day 7 cultured SGN. In addition, the delayed expression of $\alpha_2\text{C}$ ARs in cultured SGN seen in this study correlated with the delayed functional development of $\alpha_2\text{C}$ ARs as autoreceptors, as seen in field stimulation assays (Schelb et al., 2001). In this later study, $\alpha_2\text{A}$ AR regulation of field-stimulated [^3H]norepinephrine release from atria, cortical brain slices, and vas deferens was evident in tissues taken from post-natal Day 1 mouse pups and adult animals, whereas similar regulation by $\alpha_2\text{C}$ ARs was only evident in tissues taken from adult animals, suggesting a role for postnatal development of $\alpha_2\text{C}$ AR function.

The alteration in SV2 expression in cultured SGN from Day 8 to Day 16 (Figs. 6–8) suggested that synaptic activity or synaptic vesicle proteins may play a role in the expression and localization of $\alpha_2\text{C}$ ARs. Examination of synaptic vesicle protein expression in developing hippocampal neuron cultures has revealed that synaptic machinery proteins, including SV2, are transported in packets down the axon (Ahmari et al., 2000). Thus it is possible that $\alpha_2\text{C}$ ARs also are being transported in similar packets, thus accounting for their late expression patterns within cultured SGN axons, following development of synaptic contact sites. Interestingly, this hypothesis could explain the presence of the punctate $\alpha_2\text{C}$ AR staining seen at Day 8 within axonal projections (data not shown). The lack of SV2 co-localization with $\alpha_2\text{A}$ ARs suggests that they may be present at extrasynaptic sites, whereas $\alpha_2\text{C}$ ARs accumulate at synaptic contacts, thus further differentiating their physiological functions. Such differential localization could explain in part their responsiveness to neuronal action potential frequencies (Hein et al., 1999), with $\alpha_2\text{C}$ ARs being more sensitive to low frequency stimulation due to their proximity to sites of catecholamine release.

The relative scarcity of $\alpha_2\text{C}$ ARs in the plasma membrane of sympathetic neurons at early culture time points was intriguing. We have previously reported the presence of a large intracellular pool of $\alpha_2\text{C}$ ARs in stably transfected epithelial and fibroblast cell lines (Daunt et al., 1997; von Zastrow et al., 1993). However, stably transfected neuroendocrine cell lines, such as AtT20 and PC12 cells, express $\alpha_2\text{C}$ ARs predominately in the plasma membrane (Hurt et al., 2000). Based on these results we would expect that endogenously expressed $\alpha_2\text{C}$ ARs in cultured SGN would target to the plasma membrane, as seen with neuroendocrine cell lines.

Several potential mechanisms could account for the predominant presence of $\alpha_2\text{C}$ ARs in an intracellular compartment in young cultured SGN. First, many G protein-coupled receptors undergo agonist-induced internalization and downregulation, including $\alpha_2\text{C}$ ARs, though $\alpha_2\text{A}$ ARs do not appear to do so (Daunt et al., 1997; Hurt et al., 2000). Therefore, one might expect that the high levels of norepinephrine in SGN cultures could contribute to the large $\alpha_2\text{C}$ AR intracellular pool, by stimulating agonist-induced internalization. However, SGN were cultured in the presence of an $\alpha_2\text{AR}$ antagonist (RX821002) to prevent agonist-induced internalization. Second, plasma membrane trafficking of newly synthesized $\alpha_2\text{C}$ ARs may require the presence of an associated protein(s) that functions as a chaperone and/or forms a functional complex with the receptor. Third, maturation of cultured SGN may involve alterations in neuronal properties, such as synaptic activity or neurotransmitter phenotype (e.g. cholinergic vs. adrenergic).

The temporal aspect of α_{2A} and α_{2C} AR localization in cultured SGN may relate to interplay of various forces, including the effects of neuron activity and target cells. For instance, depolarization of PC12 cells was shown to regulate trafficking of α opiate receptors from intracellular pools to the membrane and this trafficking was due to a carboxyl terminal signal (Kim and von Zastrow, 2003). Therefore, trafficking of α_{2C} AR to axons in cultured SGN may be dependent on factors expressed by SGN themselves, other cell types (e.g. glial) and/or resultant neuronal activity. During SGN culture, the development of a dense arborization of axons and dendrites was evident, correlating with SV2 staining. It may be that α_{2C} AR, and not α_{2A} AR, expression and trafficking requires synaptic contact and resultant neuronal activity.

Differences in membrane targeting and subcellular localization have been reported for other G protein-coupled receptors and ion channels (Bernard et al., 1999; Dournaud et al., 1998; Hubert et al., 2001; Tan et al., 2004; Wenthold et al., 2003). Though much is known about membrane trafficking, specific protein motifs that regulate differential trafficking of α_{2A} and α_{2C} ARs in various cell types have not been delineated. Unique binding partners may play a role, though no direct evidence has been found to date. Potential partners could include spinophilin I, arrestin-3, and the zeta isoform of the 14-3-3 scaffold protein, all of which have been shown to bind to the third intracellular loop of α_{2A} and α_{2C} ARs (Prezeau et al., 1999; Richman et al., 2001). These previous bio-chemical and pharmacological investigations were performed in non-neuronal cell lines, where the endogenous physiological role of α_{2A} and α_{2C} ARs as autoreceptors could not be examined. However, the lack of differential binding to any of these candidate proteins suggests that they are not directly responsible for the differences noted in localization in cultured SGN. Alternatively, an endoplasmic retention signal that prevents surface trafficking until assembly with other yet unknown proteins may be present in α_{2C} ARs. Such retention signals are found in other GPCRs, including metabotropic glutamate and GABA α_B receptors (Francesconi and Duvoisin, 2002; Pagano et al., 2001).

Cultured SGN have proven to be a valid model system for investigating the role of various growth factors and cytokines (e.g. NGF, NT-3, LIF) in sympathetic neuron apoptosis and neurotransmitter phenotype switching. In fact, direct correlation between in vivo and in vitro results has been possible (Francis and Landis, 1999). However, the embryological developmental time course of sympathetic neurons and its correlation with sympathetic neuron maturation in culture have not been discerned. Thus, direct comparisons between in vivo and in vitro maturation and development of SGN, and specifically α_{2A} and α_{2C} ARs, cannot be made. It is plausible that the time course of α_{2A} and α_{2C} AR trafficking seen in cultured SGN reflects recovery from the trauma of dissection and/or the development of new synaptic contacts necessary for their localization; it may not reflect the in vivo time course or developmental pattern.

Though we did not characterize the neuronal connections formed in our cultured SGN during the 16-day time course of our studies, it seems reasonable to assume that they represent functional synapses, as shown by others. Sympathetic synapse development in vitro has revealed that SGN develop cholinergic synapses between axons, dendrites and cell bodies, complete with co-localization of nicotinic acetylcholine receptors and agrin, a

scaffold protein commonly found at nicotinic synapses (Gingras and Ferns, 2001). Moreover, it has been shown that development of such synapses in vitro was similar functionally and biochemically to synapses found in vivo in mouse embryos (Gingras et al., 2002).

It is possible that the delayed trafficking of α_{2C} ARs may reflect the need for cultured SGN to recover from their dissection from the ganglia, the need to develop synaptic contacts, or both. Either way, our data suggest that α_{2A} and α_{2C} ARs show a unique temporal localization pattern to somatodendritic and axonal sites in cultured SGN, and that this pattern correlated with regulation of $[Ca^{2+}]_i$ transients. The dissimilarities in targeting seen for these two autoreceptors may underlie some of the previously observed functional differences observed in vivo and in vitro.

The localization at Day 16 of α_{2C} ARs, but not α_{2A} ARs, at sites of synaptic contact in SGN (e.g. SV2) is consistent with the observation that α_{2C} ARs appear to be more sensitive to low-frequency sympathetic stimulation (Hein et al., 1999). The greater affinity of α_{2C} ARs for norepinephrine together with a synaptic localization would make them more sensitive to low levels of neurotransmitter release. In addition, the extrasynaptic localization of α_{2A} ARs would be consistent with their sensitivity to high frequency sympathetic stimulation. Future experiments utilizing amperometric techniques (Koh and Hille, 1997) may make it possible to discern receptor localization and function at a subcellular level and to further investigate the role of neuronal activation in α_{2A} and α_{2C} AR expression and trafficking in a native cell population.

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Fig. 1.

Characterization of superior cervical sympathetic ganglion cultures by indirect immunofluorescence. SGN were obtained from wild-type mice and cultured on glass coverslips for 7 days and analyzed by indirect immunofluorescence microscopy as outlined in Section 2. (A) SGN labeled with an antibody recognizing the somatodendritic protein, MAP2; note cell body and dendritic staining. (B) Same SGN labeled with DBH antibody, demonstrating extensive intracellular staining with discrete punctate accumulations in neurite extensions. Similar exposure times were used for all images (magnification 630 $\times$).

Fig. 2.

Antibody specificity for endogenously expressed α_{2A} and α_{2C} ARs in SGN. SGN were obtained from WT (A, C, E, G), α_{2A} ARKO (B, F), and α_{2C} ARKO (D, H) mice. SGN were cultured for 4 days and analyzed by indirect immunofluorescence microscopy as outlined in Section 2. SGN were labeled with mouse monoclonal antibody MAP2 and co-stained with either affinity purified rabbit polyclonal antibody C10, recognizing the carboxyl terminal of α_{2A} AR or C4, recognizing the carboxyl terminal of α_{2C} AR. MAP2 antibody labeling of SGN displayed intracellular somatodendritic staining in WT (A and C), α_{2A} ARKO (B), α_{2C} ARKO (D) SGN, while C10 antibody displayed extensive plasma membrane staining with limited intracellular vesicular staining in WT (E) but not α_{2A} ARKO (F) SGN. Similarly, affinity purified rabbit polyclonal antibody C4

revealed extensive staining of an intracellular vesicular compartment in WT (G) but not α_2C ARKO (H) SGN. Similar exposure times were used for all images (magnification 630 $\times$).

Fig. 3.

Time course of differential localization of α_{2A} ARs in cultured SGN. SGN were obtained from WT mice and cultured for up to 16 days on glass coverslips and analyzed by confocal fluorescence microscopy using α_{2A} (C10) antisera as outlined in Section 2.

SGN were co-stained for MAP2 expression to demarcate cell body and dendritic regions (e.g. somatodendritic). MAP2 is an intracellular protein and thus was used delineate the region beneath the cell membrane. α_{2A} AR(left), MAP2 (middle), and merged (right) images are shown for each time point. Images were taken at Day 1 (A–C), Day 4 (D–F), Day 8 (G–I), and Day 16 (J–L). Multiple confocal planes were taken through each SGN and the confocal image plane that best revealed cell body and

axonal processes is shown, to delineate membrane localization. MAP2 staining was similar for all SGN, revealing a similar distribution of dendrites. Similar exposure times were used for all images (magnification 650×).

Fig. 4.

Time course of differential localization of α_{2C} ARs in cultured SGN. SGN were obtained from WT mice and cultured for up to 16 days on glass coverslips and analyzed by confocal fluorescence microscopy using α_{2C} (C4) antisera as outlined in Section 2. SGN were co-stained for MAP2 expression to demarcate cell body and dendritic regions (e.g. somatodendritic). MAP2 is an intracellular protein and thus was used delineate the region beneath the cell membrane. α_{2C} AR(left), MAP2 (middle), and merged (right) images are shown for each time point. Images were taken at Day 1 (A–C), Day 4 (D–F), Day 8 (G–I), and Day 16 (J–L). Multiple confocal planes were taken through each SGN and the confocal image plane that best revealed cell body and

axonal processes is shown, to delineate membrane localization. MAP2 staining was similar for all SGN, revealing a similar distribution of dendrites. Similar exposure times were used for all images (magnification 650×).

Fig. 5.

Co-localization of SV2 with α_{2A} and α_{2C} ARs in cultured SGN at Day 8. SGN were obtained from WT mice and cultured for 8 days on glass coverslips and analyzed by indirect immunofluorescence microscopy as outlined in Section 2. Parallel cultures were labeled with antibodies C10 and C4 against α_{2A} and α_{2C} ARs (A and C, respectively) and co-stained with a mouse monoclonal antibody against the synaptic vesicle protein SV2 (B and D). Similar to Fig. 3, α_{2A} AR expression was predominantly in somatodendritic regions, whereas α_{2C} ARs were located in an intracellular vesicular compartment. No difference was noted for SV2 antibody labeling of cell body and axons. Note the developing axonal network present in both

microscopic fields, despite the differential localization of α_{2A} and α_{2C} ARs. Similar exposure times were used for all images (magnification 630 $\times$).

Fig. 6.

Co-localization of SV2 with α_{2A} ARs in cultured SGN at Day 16. SGN were obtained from WT mice, cultured for 16 days on glass coverslips, and analyzed by indirect immunofluorescence microscopy as outlined in Section 2. Two representative neuronal fields are shown for each condition, separated by a dotted white line. Cultures were co-stained for expression of α_{2A} ARs and synaptic vesicle protein SV2 (A and B, respectively). Enlargements of selected fields for α_{2A} AR (insets 1 and 2) and the corresponding SV2 images (insets 3 and 4) show no significant overlap. Merging of the paired, enlarged images further demonstrates the lack of overlap between the expression of these two proteins (C). Similar exposure times were used for all images (magnification 630 $\times$).

Fig. 7.

Co-localization of SV2 with α_2C ARs in cultured SGN at Day 16. SGN were obtained from WT mice, cultured for 16 days on glass coverslips, and analyzed by indirect immunofluorescence microscopy as outlined in Section 2. Two representative neuronal fields are shown for each condition, separated by a dotted white line. Cultures were co-stained for expression of α_2C ARs and synaptic vesicle protein SV2 (A and B, respectively). Enlargements of selected fields for α_2C AR (insets 1 and 2) and the corresponding SV2 images (insets 3 and 4) revealed co-localization (white arrows) of α_2C AR and SV2 staining. Merging of the paired, enlarged images further demonstrates the degree of overlap between the expression of these two proteins at many synaptic sites (C). Similar exposure times were used for all images (magnification 630 $\times$).

Fig. 8.

Confocal microscopic examination of α_2A or α_2C AR and SV2 co-localization in cultured SGN. SGN were obtained from WT mice and cultured for up to 16 days on glass coverslips and analyzed by confocal fluorescence microscopy using α_2A or α_2C AR specific antisera as outlined in Section 2. SGN were co-stained for SV2 expression to demarcate areas of synaptic contact. α_2A AR (left), SV2 (middle), and merged (right) images are shown for each time point. α_2A AR images were taken at Day 8 (A–C) and Day 16 (G–I). Similar images were taken at Day 8 (D–F), and Day 16 (J–L) for α_2C AR expression. Multiple confocal planes were taken through each SGN and the confocal image plane that best revealed cell body and axonal processes is shown, to

delineate points of synaptic contact. Areas of co-localization of α_2 AR and SV2 expression are visible as yellow in the merged images (right column). Similar exposure times were used for all images (magnification 650 $\times$).

Fig. 9.

Time course of differential localization of α_{2A} and α_{2C} ARs in cultured ARKO SGN. SGN were obtained from α_{2A} ARKO and α_{2C} ARKO mice and cultured in parallel from 1 to 15 days on glass coverslips. α_{2A} ARs were stained in α_{2C} ARKO SGN, while α_{2C} ARs were stained in α_{2A} ARKO SGN; SGN were labeled with α_{2A} (C10) and α_{2C} AR (C4) antibodies and analyzed by indirect immunofluorescence microscopy as outlined in Section 2. Time points were chosen to correspond with $[Ca^{2+}]_i$ (Fig. 8). Each SGN was co-stained for MAP2 expression to delineate cell body and dendritic regions (data not shown). α_{2A} AR plasma membrane staining of α_{2C} ARKO SGN at Day 1 and 4 was similar to that seen in WT SGN (A and C); similarly α_{2C} AR expression in α_{2A} ARKO SGN was predominantly restricted to intracellular vesicular compartment staining over a similar time

course (B and D, respectively). Day 7 staining for both α_{2A} AR and α_{2C} ARs (E and F, respectively) showed no differences compared to expression in WT SGN at Day 8. α_{2A} ARs were studied at Day 15 in α_{2C} ARKO SGN (G) and had a similar expression pattern as that seen in WT SGN at Day 16. Note that at Day 10, α_{2C} AR expression in α_{2A} ARKO SGN was more similar to the Day 8 time point than the Day 16 time point (H). Similar exposure times were used for all images (magnification 630 $\times$).

Fig. 10.

α_{2A} and α_{2C} AR regulation of $[Ca^{2+}]_i$ in cultured SGN. Cytosolic $[Ca^{2+}]_i$ transients in SGN were measured using the fluorescent calcium indicator Fura-2, following potassium-induced depolarization, as outlined in Section 2. Regulation of $[Ca^{2+}]_i$ by endogenous α_2 ARs in SGN was examined by comparing $[Ca^{2+}]_i$ transients triggered by potassium-induced depolarization in control and dexmedetomidine pretreated neurons. Data are expressed as fold change in $[Ca^{2+}]_i$ from base line as determined by F340/F380 ratio at baseline and peak responses. Data is expressed as the mean standard error of the mean. The number of cells studied for each condition is given in the bar graph. Results were analyzed using an unpaired *t*-test to compare control and pretreated samples, as well as to compare dexmedetomidine effects between paired SGN cultures at different time points. (A) SGN obtained from WT, α_{2A} ARKO and α_{2C} ARKO mice cultured for 7 days. Dexmedetomidine (100 nM) pretreatment was found to significantly inhibit $[Ca^{2+}]_i$ elevations, following potassium-induced depolarization for WT, but not α_{2A} ARKO and α_{2C} ARKO SGN. (B) SGN obtained from WT (15 days), α_{2A} ARKO (10 days) and α_{2C} ARKO (15 days). SGN obtained from WT and α_{2C} ARKO mice cultured for 15 days and SGN from α_{2A} ARKO mice cultured for 10 days produced significantly greater overall inhibition of potassium-induced elevations in $[Ca^{2+}]_i$ than corresponding 7-day-old cultures. The enhancement of dexmedetomidine effects between older and younger cultures was only significant for WT and α_{2C} ARKO SGN ($P < 0.05$).