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CO-EXPRESSION OF ALPHA-2A-ADRENERGIC AND DELTA-OPIOID RECEPTORS IN SUBSTANCE P TERMINALS IN RAT DORSAL HORN

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Abstract

Agonists acting at α_2-adrenergic and opioid receptors (α_2ARs and ORs, respectively) inhibit pain transmission in the spinal cord. When co-administered, agonists activating these receptors interact in a synergistic manner. Although the existence of α_2AR/OR synergy has been well characterized, its mechanism remains poorly understood.

The formation of hetero-oligomers has been proposed as a molecular basis for interactions between neuronal G-protein-coupled receptors. The relevance of hetero-oligomer formation to spinal analgesic synergy requires demonstration of the expression of both receptors within the same neuron as well as the localization of both receptors in the same neuronal compartment. We used immunohistochemistry to investigate the spatial relationship between α_2ARs and ORs in the rat spinal cord to determine if co-expression could be demonstrated between these receptors. We observed extensive co-localization between α_{2A}-adrenergic and delta-opioid receptors (DOP) on substance P (SP)-immunoreactive (ir) varicosities in the superficial dorsal horn of the spinal cord and in peripheral nerve terminals in the skin. α_{2A}AR- and DOP-ir elements were co-localized in subcellular structures of 0.5 μm or less in diameter in isolated nerve terminals. Furthermore, co-incubation of isolated synaptosomes with α_2AR and DOP agonists resulted in a greater-than-additive increase in the inhibition of K^+-stimulated neuropeptide release.

These findings suggest that co-expression of the synergistic receptor pair α_{2A}AR-DOP on primary afferent nociceptive fibers may represent an anatomical substrate for analgesic synergy, perhaps due to protein-protein interactions such as hetero-oligomerization.

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INTRODUCTION

Agonists acting at spinal α_2-adrenergic and opioid receptors (α_2ARs and ORs, respectively), share common signal transduction systems mediated through inhibitory G proteins, the activation of which inhibits pain transmission. In addition, agonists acting at α_2ARs and ORs interact in a greater-than-additive (i.e. synergistic) manner when co-administered to the spinal cord (for review see Alguacil and Morales 2004). Synergistic interactions between analgesic agents acting at ORs and α_2ARs have been observed repeatedly in several laboratories after spinal administration using both behavioral (Hylden and Wilcox 1983; Stevens et al. 1988; Monasky et al. 1990; Ossipov et al. 1990a; Ossipov et al. 1990b; Ossipov et al. 1990c; Roerig et al. 1992) and electrophysiological (Sullivan et al. 1987; Wilcox et al. 1987; Omote et al. 1990) methods. Synergistic interactions can result in greater than 100-fold increases in analgesic potency as well as increased maximum efficacy. Synergy is important in clinical pain management as it makes possible production of analgesia at lower doses for each drug, thus reducing undesired side effects and improving treatment outcomes (for review see Walker et al. 2002). Despite the widespread evaluation and characterization of these interactions, the mechanisms underlying analgesic synergy are unclear.

Within the α_2ARs there are three primary subtypes, α_{2A}-, α_{2B}- and α_{2C}AR (for reviews see Aantaa et al. 1995; MacDonald et al. 1997; Philipp et al. 2002). Similarly, there are three primary OR subtypes, delta- (DOP), mu- (MOP) and kappa- (KOP) (for reviews see Kieffer 1999; Law and Loh 1999). Pairs of receptors for which α_2AR-OR synergistic interactions have been documented include DOP and α_{2A}AR (Stone et al. 1997), MOP and α_{2A}AR (Roerig et al. 1992; Stone et al. 1997), and DOP and α_{2C}AR (Fairbanks et al. 2002; Guo et al. 2003).

It is now recognized that G-protein-coupled receptors (GPCRs) can form oligomeric complexes in addition to the traditionally envisaged monomeric species. The demonstration of functional oligomers in transfected cells has led to significant re-evaluation of the mechanisms thought to be involved in GPCR function *in vivo*. These associations can result in novel pharmacological properties distinct from either component receptor, including enhancement of ligand binding affinity, changes in functional coupling and altered receptor trafficking (for reviews see George et al. 2002; Bulenger et al. 2005). The generation of novel properties upon hetero-oligomer formation may represent a molecular mechanism for synergistic interactions between neuronal GPCRs that co-localize *in vivo*. Physical associations suggestive of hetero-oligomer formation have been demonstrated between α_2ARs and ORs in transfected cells *in vitro* (Jordan et al. 2003; Rios et al. 2004; Zhang and Limbird 2004; Vilardaga et al. 2008). The potential relevance of these *in vitro* studies to spinal synergy requires the expression of both receptors within the same neuron as well as the localization of both receptors in the same neuronal compartment.

We examined the expression of DOP, MOP, α_{2A}AR and α_{2C}AR in the superficial dorsal horn of the spinal cord to determine the spatial relationships between these receptors. Co-expression in the spinal cord dorsal horn would provide anatomical evidence to support a role for oligomerization in the phenomena of α_2AR/OR functional synergy. To determine if simultaneous activation of co-localized receptors results in functional synergy, we

challenged depolarization-elicited CGRP release from spinal cord synaptosomes with agonists directed at DOP and α_2AR.

METHODS

Animals

Male Sprague Dawley rats (150-250g; Harlan, WI) were housed in pairs in a temperature- and humidity-controlled environment and maintained on a 12 hr light/dark cycle with free access to food and water. All experiments were approved by either the University of Minnesota's Institutional Animal Care and Use Committee or the McGill University Animal Care and Ethics Committees.

Antisera

The α_{2A}AR antiserum (1:1000) was prepared in rabbit against a synthetic peptide corresponding to $\alpha_{2A}AR_{436-450}$ (AFKKILCRGDRKRIV). This antiserum has been previously characterized and was determined to be specific for α_{2A}AR based on the elimination of staining following pre-adsorption of the antisera with peptide (AFKKILCRGDRKRIV) and the ability of the antisera to label MDCK cells transfected with α_{2A}AR but not those transfected with α_{2B}AR or α_{2C}AR (Stone et al. 1998). This antiserum recognizes a ~50 kDa band by Western blot analysis (Figure 6).

The rabbit- and rat-derived DOP antisera were prepared against synthetic peptides and have been previously characterized. Rabbit anti-DOP$_{3-17}$ (1:1000; LVPSARAELQSSPLV) was previously shown to exhibit staining identical to that of antisera raised against other regions of the receptor, including the rabbit anti-DOP$_{30-46}$ (1:1000) and rat anti-DOP$_{30-46}$ (1:300) antisera used in the current study (AGANASGSPGARSASSL; gift of Dr. Martin W. Wessendorf) (Arvidsson et al. 1995a). Staining with these antisera is eliminated following preadsorption with the corresponding peptides (Dado et al. 1993; Arvidsson et al. 1995a), is reduced following knock-down of DOP with antisense oligonucleotides (Lai et al. 1996) and is virtually eliminated by deletion of the DOP gene in mice (Zhu et al. 1999). The rabbit anti-DOP$_{30-46}$ antiserum recognizes a ~45 kDa band by Western blot analysis (Figure 6).

The rabbit- and guinea pig-derived MOP antisera were prepared against a synthetic peptide corresponding to MOP$_{384-398}$ (QLENLEAETAPLP) of the predicted rat MOR1 gene and have been previously characterized (Arvidsson et al. 1995b). The rabbit-derived antiserum was determined to be specific for MOP based on the elimination of staining following pre-adsorption of the antiserum with the cognate peptide (QLENLEAETAPLP), the ability of the antiserum to label COS-7 cells transfected with MOP but not DOP or KOP and the recognition of an immunoreactive band by Western blot analysis of ~60 kDa in rat brain membranes (Arvidsson et al. 1995b). The guinea pig-derived antiserum was generated against the same peptide and produces a staining pattern identical to the previously described rabbit-derived antiserum.

The α_{2C}AR antisera were prepared in both guinea pig and rabbit against a synthetic peptide corresponding to $\alpha_{2C}AR_{446-458}$ (HILFRRRRRGFRQ). These antisera have been previously characterized and were determined to be specific for α_{2C}AR based on the elimination of staining following pre-adsorption of the antisera with the cognate peptide (HILFRRRRRGFRQ), the ability of the antisera to label MDCK cells transfected with α_{2C}AR but not those transfected with α_{2A}AR or α_{2B}AR (Stone et al. 1998) and a reduction in immunoreactivity following knock-down of α_{2C}AR with antisense oligonucleotides (Fairbanks et al. 2002). These antisera produce identical staining patterns and co-localize extensively.

SP antibodies raised in three different species and obtained from three different sources were used in these studies and produced similar results: rat anti-SP (1:1000; Accurate Chemical, NY); rabbit anti-SP (1:1000; gift of Dr. R. Ho to R. Elde); guinea pig anti-SP (1:500; Neuromics Antibodies, Inc. Minneapolis, MN). The rat anti-SP recognizes the COOH terminal of SP and was originally characterized by Cuello et al., (Cuello et al. 1979). This antibody is secreted from hybrid clone line NCI/34 derived from fusion of a mouse NSI/1-Ag 4-1 spleen cell and a spleen cell from a Wistar rat immunized with substance P (CRPKPQQFFGLM) conjugated to BSA. This antibody shows no cross-reactivity to leu- or met-enkephalin, somatostatin or beta-endorphin by radioimmunoassay. Immunofluorescence is blocked by pre-adsorption with synthetic substance P (220 μg/ml) (Cuello et al. 1979). The rabbit-derived SP-antiserum was obtained following immunization with a synthetic SP-thyroglobulin conjugate as originally reported by (Ho and DePalatis 1980). Immunoreactivity was blocked by pre-incubation of this antiserum with synthetic SP (10 μg/ml) but not by pre-treatment with met-enkephalin, neurophysin or somatostatin (Ho and DePalatis 1980). The polyclonal GP-derived antiserum was directed against residues 1-11 of rat SP and is blocked by pre-adsorption with 10 μg/ml of synthetic SP (data not shown). All three antibodies produce similar staining patterns and co-localize extensively.

The antibodies used as loading controls in Western blot analysis were both purchased from commercial sources. The rabbit polyclonal pan-cadherin antiserum (Cat# ab16505, Abcam) was generated against a synthetic peptide conjugated to KLH derived from within residues 850 to the C-terminus of human pan-cadherin. This antiserum recognizes a band of approximately 135 kDa in Western blot analysis that is blocked by pre-adsorption with the cognate peptide (Cat# ab17098, Abcam). A monoclonal antibody to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Millipore (Cat# MAB374, clone 6C5). This antibody was directed against the entire GAPDH protein isolated from rabbit muscle and recognizes a single band in rat spinal cord membranes by Western blot analysis (Figure 6).

Tissue Preparation (Spinal Cord)

Animals were anesthetized with an intramuscular injection of a mixture of 75 mg/kg ketamine, 5 mg/kg xylazine and 1 mg/kg acepromazine and perfused transcardially with 180 mL of oxygenated ice-cold Tyrodes solution (116 mM NaCl, 5 mM KCl, 2 mM $MgCl_2\cdot6H_2O$, 406 μM $MgSO_4\cdot7H_2O$, 2.9 mM glucose, 26 mM $NaHCO_2$). This was followed by 500 mL fixative (4% (w/v) paraformaldehyde, 0.2% (v/v) saturated picric acid solution in 0.1 M phosphate-buffered saline (PBS; 150 mM KH_2PO_4, 170 mM $NaHPO_4\cdot7H_2O$, pH 6.9) and thereafter by 400 mL of 10% sucrose solution (10% (w/v) sucrose in PBS). Spinal cords were dissected and stored overnight in 10% sucrose at 4°C. Tissue sections were prepared using a cryostat at a thickness of 10 or 14 μm, thaw-mounted onto gelatin-coated slides and stored at -20°C.

Immunofluorescence Histochemistry (Spinal Cord)

Cryostat sections were pre-incubated for one hour at room temperature (RT) in diluent containing 1% normal donkey serum, 0.3% Triton X-100, 0.01% sodium azide and 1% bovine serum albumin in PBS. Sections were incubated overnight at 4°C in a humid chamber with primary antisera, rinsed 3 × 10 min with PBS, incubated with fluorescently-tagged species-specific secondary antisera (Jackson Immunoresearch, West Grove, PA) for one hour at RT, rinsed 3 × 10 min with PBS and cover-slipped using either a mixture of glycerol and PBS containing 0.1% p-phenylenediamine or dehydrated, cleared in xylene and mounted with DPX (Fluka). Double- and triple-labeling procedures were adapted from previous studies (Wessendorf and Elde 1985).

Adsorption Controls

To control for cross-reactivity between DOP and α_{2A}AR antisera, immunohistochemistry was performed as above with the exception that the primary antibodies were pre-incubated with 10 μg/mL of either peptide $\alpha_{2A}AR_{436-450}$ (AFKKILCRGDRKRIV) or peptide $DOP1_{30-46}$ (AGANASGSPGARSASSL) (Arvidsson et al. 1995a; Stone et al. 1998). To ensure that secondary antibodies did not cross-react, control experiments were performed in which one or the other primary antisera were omitted and no staining was observed in either of these cases.

Tissue Preparation (Skin)

Animals were anesthetized with 0.4 mL/kg Equithesin (6.5 mg chloral hydrate and 3 mg sodium pentobarbital in a volume of 0.3 mL, i.p., per 100 g body weight) and perfused transcardially with a mixture of 4% paraformaldehyde and 15% (v/v) saturated solution of picric acid in 0.1 M phosphate buffer (PB), pH 7.4, for 30 min. Rat lower lip skin was dissected and post-fixed for 1 hour in the above fixative. The tissue was then incubated in a 30% sucrose solution in PB for at least 12 hours before further processing.

Immunofluorescence Histochemistry (Skin)

Tissue sections were trimmed and embedded in an Optical Cutting Temperature (OCT) compound (Tissue-Tek, Sakura Finetek, USA, Torrance, CA, USA). Fifty μm sections were cut on a cryostat (Leica) and collected in PBS containing 0.2% Triton-X 100 (PBS+T). Sections were treated with 50% ethanol for 30 min and then 0.3% hydrogen peroxide (H_2O_2) in PBS for 10 min at RT. Non-specific staining was blocked by incubating sections in 10% normal goat serum and 10% normal donkey serum (NDS) in PBS+T for one hour. Sections were incubated overnight at 4°C in primary antisera, washed with PBS+T, and then incubated for 2 hours with fluorescently-tagged species-specific secondary antisera. These included the highly cross-adsorbed Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400; Molecular Probes, Eugene, OR) and the rhodamine Red-X-conjugated donkey anti-guinea pig IgG (1:500; Jackson Immunoresearch, West Grove, PA). Sections were mounted on gelatin-coated slides and coverslipped with Aquapolymount (Polysciences, Warrington, PA).

Synaptosome Preparation

This preparation is described in greater detail elsewhere (Goracke-Postle et al. 2006). Briefly, spinal cords were collected, homogenized and centrifuged at 800 x g for 10 min at 4°C. The supernatant (S1) was then centrifuged at 15,000 x g for 20 min at 4°C. The resultant pellet (P2) was resuspended and the synaptosomes were further purified by sucrose gradient as previously described (Fried et al. 1989). This preparation has been validated using both functional and anatomical criteria (Goracke-Postle et al. 2006; Goracke-Postle et al. 2007).

Synaptosome Immunofluorescence

The synaptosomes were aliquoted into 4-well Nunc Lab-Tek II CC2 Chamber Slides and incubated at 37°C for 30 min. Slides were then exposed to fixative (see above) for 15 min and washed 3×5 min in PBS and incubated in diluent (see above) for 15 minutes. Slides were then incubated with primary antisera for 30 min at RT in a humid chamber, rinsed 3×5 min with PBS, incubated with fluorescently-tagged species-specific secondary antisera (Jackson Immunoresearch, West Grove, PA) for 30 minutes at RT and rinsed 3×5 min with PBS. The barriers separating the individual wells were removed and the slides were cover-slipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Western Blot Analysis

Proteins from the S1, P2 and synaptosomal fraction obtained from mouse spinal cord were denatured in SDS sample buffer and 20 μg of protein was loaded onto a 10% SDS-PAGE gel and transferred to a PVDF membrane. Membranes were blocked with 5% non-fat dry milk in TBST (Tris-buffered saline with 0.01% Tween, pH 7.4) for 1 hour at RT followed by incubation with either rabbit anti-DOP (1:1000) or anti-α_{2A}AR (1:1000) for 3 hours at RT. After washing 3×10 min with TBST, blots were incubated with a peroxidase-conjugated mouse-anti-rabbit IgG antibody (1:10,000, Jackson Immunoresearch, West Grove, PA) and washed 3×10 min. Membranes were revealed with SuperSignal West Femto Chemiluminescent Substrate (Pierce) and visualized with a digital imaging system equipped with a cooled CCD camera (ChemiGenius, SYNGENE). Membranes were then stripped with Restore Western Blot Stripping Buffer (Pierce) and probed with either a mouse-anti-GAPDH (1:20,000, Chemicon) or a rabbit anti-cadherin antibody (1:20,000, Abcam) for 60 minutes at RT. After washing 3×10 min with TBST, blots were incubated with a peroxidase-conjugated mouse-anti-rabbit IgG or goat-anti-mouse secondary antibody (1:10,000, Jackson Immunoresearch, West Grove, PA). Following the final 3×10 min washes in TBST, membranes were revealed with enhanced chemiluminescence (Pierce) and exposed to light-sensitive film (Clonex Corporation).

Confocal Microscopy

Images of spinal cords and synaptosomes were collected using a BioRad MRC 1000 confocal microscope (Bio-Rad Microscience Division, Cambridge, MA). A 60X, 1.4 NA objective and zoom values ranging from 1 to 5 were used for high magnification. Illumination was supplied by a Kr/Ar-ion laser with emission lines at 488, 568, and 647 nm. The skin samples were examined using a Zeiss LSM 510 confocal scanning laser microscope with argon and helium neon lasers. Micrograghs used in plates were minimally processed (adjusted for sharpness, contrast and brightness) and assembled using Photoshop (Adobe). Semi-quantitative analysis of the images in Figure 1, Column IV was performed in Photoshop (Adobe) by counting yellow (co-localized) pixels and dividing by the total number of red, green and yellow pixels. Similar results were obtained using the colocalization RGB plugin in ImageJ; data are reported for the photoshop method.

Neuropeptide Release Assay

Synaptosomes were prepared as described above, oxygenated for 1 min and allowed to seal during an incubation period of 30 min at 37°C. Samples were centrifuged for 5 min at 21,380 x g and supernatant was removed. After an initial wash in HEPES, a 10 min baseline sample was collected. The synaptosomes were then exposed to either vehicle or test compound (deltorphin II, clonidine or combination) for 10 min and subsequently stimulated with 60mM K^+ (10 min). Samples were centrifuged again for 5 min at 21,380 x g. All supernatants were collected for analysis of immunoreactive calcitonin gene-related peptide (iCGRP) content by a commercial radioimmunoassay (RIA) kit (Phoenix Pharmaceuticals, Burlingame, CA). Data are presented as mean +/- SEM and expressed as the % inhibition of neuropeptide release according to the equation: % Inhibition = [(High K^+ + Drug) -(High K^+)]/[High K^+] x 100.

RESULTS

Relationship between α_{2A}AR-ir, α_{2C}AR-ir, DOP-ir and MOP-ir in the Dorsal Horn of Rat Spinal Cord

The anatomical relationships between α_{2A}AR-ir, α_{2C}AR-ir, DOP-ir and MOP-ir were investigated in rat lumbar spinal cord. We observed extensive co-localization between

α_{2A}AR-ir and DOP-ir. In contrast, co-localization was minimal between α_{2A}AR-ir and MOP-ir, α_{2C}AR-ir and DOP-ir or α_{2C}AR-ir and MOP-ir (Figure 1). Semi-quantitative assessment of co-localization at high magnification ranged from 58% (D, α_{2A}AR-ir + DOP-ir) to 8.2% (H, α_{2A}AR-ir + MOP-ir), 6.1% (L, α_{2C}AR-ir + DOP-ir) and 6.7% (P, α_{2C}AR-ir + MOP-ir).

Co-localization of α_2AR-ir and DOP-ir in the Dorsal Horn of Rat Spinal Cord

The anatomical relationship between α_{2A}AR-ir and DOP-ir was further investigated. In rat lumbar spinal cord, extensive co-localization (white) between α_{2A}AR-ir and DOP-ir was observed in the superficial dorsal horn (Figure 2). This level of co-localization was apparent in confocal images of coronal (Figure 2, A-F) and horizontal (Figure 2, G-O) spinal cord sections. Sections were double-labeled with rabbit-derived anti-α_{2A}AR (Figure 2, 1st column) and rat-derived anti-DOP (Figure 2, 2nd column) and each image represents a single optical section. These pairs of unmerged images illustrate the similarity in staining patterns observed with the different antisera. The results of digital merging revealed extensive co-localization (Figure 2, 3rd column). High magnification microscopy detected α_{2A}AR-ir and DOP-ir co-localization within the same neuronal processes 0.25 μm or less in diameter.

Adjacent sections of rat spinal cord were double-labeled for α_{2A}AR-ir and DOP-ir in the presence or absence of each receptor's cognate peptide (Figure 3). Each cognate peptide binds with high affinity to its corresponding antiserum, thereby inhibiting binding to the tissue and reducing the degree of immunoreactivity observed for that target while leaving the binding of other antiserum unaffected. In the presence of the α_{2A}AR cognate peptide, α_{2A}AR-ir was absent (Figure 3B) whereas DOP-ir (Figure 3E) was unaffected. Similarly, pre-incubation with the cognate peptide for the DOP antiserum blocked DOP-ir (Figure 3F) but not α_{2A}AR-ir (Figure 3C). In parallel experiments, tissue was exposed to one or the other primary antisera and both secondary antibodies or one primary and both secondary antibodies. In these experiments, only immunoreactivity corresponding to the anticipated pair of primary and secondary antibodies was observed (data not shown). Taken together, these studies indicate that antisera used in this study do not cross-react.

Co-localization of α_{2A}AR-ir, DOP-ir and SP-ir in the Dorsal Horn of Rat Spinal Cord

Previous reports have demonstrated that both α_{2A}AR and DOP are expressed in the peptidergic population of primary afferent sensory neurons (Dado et al. 1993; Arvidsson et al. 1995a; Stone et al. 1998; Zhang et al. 1998). Therefore, we double- or triple-labeled spinal cord sections with antibodies directed against α_{2A}AR, DOP and the neuropeptide substance P (SP) (Figure 4). Co-localization of α_{2A}AR-ir and SP-ir was observed with the rabbit-derived α_{2A}AR and both rat- and guinea pig-derived SP antibodies obtained from independent sources (Figure 4A-D, M). Similarly, rabbit-derived anti-DOP labeling co-localized with both the rat- and guinea pig-derived SP antibodies (Figure 4E-H & data not shown). The independent co-localization of α_{2A}AR-ir and DOP-ir with several SP antibodies strongly suggests that the co-localization observed between α_{2A}AR-ir and DOP-ir is not artifactual. Furthermore, DOP-ir and SP-ir co-localization was observed with rabbit- and rat-derived anti-DOP antisera that target different regions of the receptor (Figure 4E-H, N).

Triple-labeled sections of spinal cord are depicted in Figure 4I-U. Images resulting from each antiserum alone (Figure 4I-K) and all possible digitally merged pairs (Figure 4M-O) are shown separately to illustrate the extensive overlap between the antigens. Upon merging the digital images of sections labeled with all three antisera, triple-labeled elements appear white (Figure 4 L,P). Apparent single fibers were identified that showed α_{2A}AR-ir, DOP-ir

and SP-ir (Figure 4Q-T). An enlargement of one such fiber demonstrates co-localization of all three markers along the fiber (Figure 4U).

Co-localization of α$_{2A}$AR-ir and DOP-ir with SP-ir in Rat Peripheral Nerve Terminals

Coincident co-localization of SP-ir was observed with α$_{2A}$AR-ir and DOP-ir in epidermis (Figure 5) and dermis (data not shown) of skin obtained from the rat lip. Although direct co-localization of α$_{2A}$AR-ir and DOP-ir was not possible in peripheral tissues due to technical considerations (the rat-derived DOP antiserum needed for direct α$_{2A}$AR/DOP co-staining is incompatible with peripheral rat tissues), the coincident co-localization of each of the rabbit-derived receptor antisera with SP strongly suggests that α$_{2A}$AR and DOP co-localize in peripheral nerve terminals.

Localization of α$_{2A}$AR-ir and DOP-ir to Isolated Spinal Cord Synaptosomes

To determine if α$_{2A}$AR and DOP are co-localized in nerve terminals, isolated nerve terminals (synaptosomes) obtained from whole rat spinal cords were examined. Co-localization of α$_{2A}$AR-ir, DOP-ir and SP-ir was observed within synaptosomes isolated from rat spinal cord (Figure 6, A-G). At the light level, this triple labeling of structural elements within the synaptosome preparation appeared to be localized in substructures less than 0.5 μm in diameter, consistent with localization on pre-synaptic vesicles.

To confirm the localization of DOP and α$_{2A}$AR to pre-synaptic nerve terminals, we analyzed the composition of the S1, P2 and synaptosomal fractions obtained during synaptosome preparation by Western blot analysis. According to the subcellular fractionation procedure used, S1 should contain total cell extract minus large structures such as nuclei and cellular debris, P2 is a crude membrane preparation including synaptosomes, mitochondria and other organelles, and the synaptosomal fraction is enriched in isolated nerve terminals. We observed a ~50 kDa immunoreactive band when probing with the rabbit anti-α$_{2A}$AR (Figure 6L) and a ~45 kDa immunoreactive band when probing with the rabbit anti-DOP (Figure 6M); both bands increase in intensity as the fractions become enriched in nerve terminals.

Although the same amount of protein from each fraction was loaded in each gel, we could not use a typical loading control strategy since the S1, P2 and synaptosomes fractions have different cytosolic to membrane protein ratios. We therefore used a membrane protein marker, pan-cadherin (Figure 6N), and a cytosolic protein marker, GAPDH (Figure 6O), to demonstrate the simultaneous enrichment in membrane proteins and depletion in cytosolic proteins as the purification progressed. Consistent with our observations with α$_{2A}$AR and DOP, the intensity of the immunoreactive pan-cadherin band increased as the samples became enriched in synaptosomes. On the other hand, the GAPDH immunoreactive band intensity decreased from S1 to P2, indicating that cytosolic proteins were lost at this step. The slight increase in GAPDH intensity between P2 and the synaptosome fraction is expected because membrane-bound synaptosomes containing some cytoplasm are segregated from the total membrane fraction, resulting in a relative increase in cytosolic proteins.

The synaptosome preparation performed here is known to produce a fraction enriched in nerve endings packed with synaptic vesicles containing numerous neurotransmitters, including SP (Fried et al., 1989; Gray and Whittaker, 1962). Our observation that α$_{2A}$AR and DOP are both enriched in synaptosomes, together with the previously demonstrated enrichment of SP, further supports the hypothesis that the receptors may be localized in SP-expressing pre-synaptic nerve terminals in spinal cord dorsal horn.

Greater-Than-Additive Inhibition of Neuropeptide Release by α_{2A}AR and DOP agonists in Spinal Cord Synaptosomes

We tested our synaptosome preparation for evidence of functional α_{2A}AR and DOP by evaluating the ability of agonists acting at these receptors to inhibit K^+-stimulated release of the neuropeptide CGRP. Upon stimulation of spinal cord synaptosomes with 60 mM K^+, immunoreactive CGRP (iCGRP) was increased from 127 pg/ml to 161 pg/ml. This increase was inhibited in a concentration-dependent manner by the non-α_2AR subtype selective agonist clonidine and by the DOP agonist deltorphin II (Figure 7). In order to determine if an interaction exists between the receptors in the synaptosome preparation, samples were incubated with both drugs in combination and the resultant inhibition of K^+-stimulated release was determined. The combination treatment resulted in significant enhancement in both potency and efficacy, suggesting a synergistic interaction exists between the receptors on isolated spinal nerve terminals.

DISCUSSION

The current study revealed extensive overlap between α_{2A}AR-ir and DOP-ir on SP-expressing primary afferent fibers in the dorsal horn of the rat spinal cord, in rat skin obtained from the lip and in isolated nerve terminals (synaptosomes) prepared from whole spinal cord. In contrast, co-localization was not observed between any of the other α_2AR/OR receptor subtype pairs: α_{2A}AR/MOP, α_{2C}AR/MOP or α_{2C}AR/DOP.

Simultaneous activation of α_{2A}AR and DOP resulted in a greater-than-additive interaction in a functional assay measuring inhibition of K^+-stimulated neuropeptide release in synaptosomes. These data indicate that the synergistic interaction observed *in vivo* between spinally administered α_{2A}AR and DOP agonists may be mediated at the level of co-localized receptor pairs within single nerve terminals and has significant implications regarding the mechanism(s) underlying that synergy. Although inter-receptor synergy is not a guaranteed consequence of co-localization, the synergy observed here in isolated synaptic terminals strongly argues for co-localization at the level of primary afferent terminals in a manner not dependent on the specificity of receptor-directed antisera; synergy is unlikely to have occurred if the receptors were localized in separate terminals.

DOP and α_{2A}AR Co-localization in Primary Afferent Nerve Terminals

The extensive overlap between α_{2A}AR-ir and DOP-ir in SP-containing fibers and nerve terminals is consistent with previous reports localizing DOP-ir and α_{2A}AR-ir to peptidergic primary afferent fibers (Dado et al. 1993; Arvidsson et al. 1995a; Stone et al. 1998; Zhang et al. 1998). The presence of SP suggests that these are likely to be C- or A-delta fibers (Lawson et al. 1993). Spinal α_{2A}AR-ir and DOP-ir have both been shown to be decreased following dorsal rhizotomy, suggesting that a major source of these receptors in the spinal cord is the central terminals of primary afferent fibers (Dado et al. 1993; Stone et al. 1998). Similarly, autoradiographic labeling showed that a majority of DOP receptor binding is lost after unilateral dorsal rhizotomy (Besse et al. 1990). The observation in the current study that both DOP-ir and α_{2A}AR-ir co-localize with SP-ir in peripheral nerve terminals in skin strongly suggests that SP-expressing, dorsal root ganglion neurons express DOP and α_{2A}AR and traffic both receptors to both central and peripheral nerve terminals. The examples of co-localization obtained using double- or triple-labeled immunofluorescence histochemistry presented here most likely represent co-localization rather than superposition because the estimated thickness of the high magnification optical sections (<1.0 μm) minimizes the possibility of superposition of terminals with dimensions of 1-2 μm. However, light microscopy lacks the resolution required to a) confirm co-localization within the same neuronal structures and b) to identify the nature of DOP-ir and α_{2A}AR-ir structures (e.g.

axons vs. nerve terminals). Further studies examining the anatomical relationship between DOP-ir and α_{2A}AR-ir at the ultrastructural level within spinal cord are necessary to confirm and extend the present data.

The immunohistochemical demonstrations of co-localization of both receptors and SP-ir in isolated nerve terminals (Figure 6) is augmented by the enrichment of both receptor immunoreactivities shown in the synaptosomal fraction using Western blot and by the supra-additive interaction observed between agonists targeting the two receptors. Although the synaptosomes in this study were isolated from whole spinal cord, the co-localization of α_{2A}AR-ir and DOP-ir with SP-ir in both superficial dorsal horn and in the synaptosome preparation indicates that a subset of the isolated nerve terminals are derived from SP-ir terminals. In addition, the inhibition of CGRP-ir by α_2AR and DOP agonists in the synaptosome preparation suggests that their site of action is the terminals of primary afferent fibers because dorsal root ganglia neurons are thought to be the only source of CGRP within the spinal cord (Chung et al. 1988).

The following observations support the hypothesis that DOP/α_{2A}AR co-localization is not due to cross-reactivity between anti-DOP and α_{2A}AR antisera: 1) α_{2A}AR-ir and DOP-ir co-existed with SP-ir in the presence and absence of antisera to the other receptor. In the case of α_{2A}AR/SP, co-localization was observed using the rabbit-derived anti-α_{2A}AR antiserum and both rat- and guinea pig-derived SP antibodies. In the case of DOP/SP, two DOP antisera, each raised in a different species against a different region of the receptor, co-localized with two different SP antibodies (rabbit-DOP with rat- and guinea pig-SP, rat-DOP with rabbit- and guinea pig-SP); 2) co-localization was abolished by the omission of either the DOP or the α_{2A}AR antisera; 3) co-localization was abolished by pre-adsorption of either the DOP or the α_{2A}AR antisera with the respective cognate peptides.

Functional studies further support the localization patterns of α_{2A}AR and DOP observed in the current study. Activation of both α_2ARs and ORs has been shown to inhibit release of excitatory neurotransmitters (Jessell and Iversen 1977; Kuraishi et al. 1985; Kamisaki et al. 1993; Takano et al. 1993; Zachariou and Goldstein 1996; Li and Eisenach 2001) and to reduce excitatory neurotransmission from primary afferent fibers onto neurons in the superficial dorsal horn (Glaum et al. 1994; Kohno et al. 1999; Kawasaki et al. 2003; Sonohata et al. 2004; Kondo et al. 2005). Spinal analgesic synergy between DOP and α_{2A}AR agonists has been previously documented (Stone et al. 1997). In the current study, we demonstrate inhibition of CGRP release from spinal cord synaptosomes by α_2AR and DOP agonists, consistent with prior reports, and report a greater-than-additive interaction between these agonists. This greater-than-additive interaction in synaptosomes, where no neuronal circuitry remains intact, further supports co-localization at the terminal level because co-activation of receptors localized in separate CGRP-positive synaptosomes could only result in an additive interaction. These data also indicate that synergistic interactions observed *in vivo* between α_{2A}AR and DOP likely occur at the level of co-localized receptor pairs within single nerve terminals.

Lack of Co-localization between α_{2A}AR/MOP, α_{2C}AR/MOP or α_{2C}AR/DOP in Rat Spinal Cord

Synergy between MOP and α_2AR agonists has been widely reported (Sullivan et al. 1987; Wilcox et al. 1987; Ossipov et al. 1989; Ossipov et al. 1990a; Ossipov et al. 1990b; Ossipov et al. 1990c; Sullivan et al. 1992; Ossipov et al. 1997; Stone et al. 1997; Fairbanks et al. 2000a; Fairbanks et al. 2000b; Fairbanks et al. 2002; Guo et al. 2003). The present study did not detect co-localization of MOP-ir with either α_{2A}AR-ir or α_{2C}AR-ir. One possible interpretation of this finding is that synergy between non-co-localized receptor pairs relies on multicellular mechanisms that have not yet been elucidated. An alternative possibility is

that the antisera used in this study do not recognize their target receptors in all possible cellular environments or in all forms.

The lack of co-expression between MOP-ir and either α_{2A}AR-ir or DOP-ir (or SP, data not shown) is potentially surprising. First, functional studies have shown that incubation with morphine results in receptor-mediated inhibition of neuropeptide release *in vitro* in slices of spinal trigeminal nucleus (Jessell and Iversen 1977) and *in vivo* following intrathecal administration (Yaksh et al. 1980). This inhibition is thought to result from activation of both MOP and DOP (Kondo et al. 2005). Second, anatomical studies clearly localize MOP to the cell bodies and/or central terminals of primary afferent neurons by mRNA (Mansour et al. 1995), autoradiography (Besse et al. 1990) and immunohistochemisty (Arvidsson et al. 1995b). However, careful examination of the relationship between MOP-ir and SP-ir in the spinal cord revealed that co-localization is only occasionally detected (Ding et al. 1995). In fact, the percentage of SP-ir terminals in trigeminal and cervical spinal cord that were also MOP-ir was only 12% and 6%, respectively (Aicher et al. 2000). In contrast, SP-ir terminals are often (>50%) contacted by MOP-ir dendrites (Aicher et al. 2000). The apparent contradiction inherent in the aforementioned studies may be explained by the existence of MOP splice variants. Specifically, in the superficial laminae of the rat spinal cord, immunoreactivity produced by antisera generated against the MOP-1C splice variant differed from that of MOP-1 despite being derived from the same gene (Abbadie et al. 2000); whereas MOP-1 rarely co-localized with SP-ir or CGRP-ir, MOP-1C-ir was often co-localized with CGRP. In the current study, we used an antibody directed against MOP-1 and not MOP-1C. It is therefore possible that DOP and α_{2A}AR will be found in future studies to co-exist with MOP-1C, and this co-localization may provide another anatomical substrate for synergistic receptor interactions.

In addition to MOP, other antisera used in this study may not recognize their target receptors in all possible cellular environments or in all forms. The presence of mRNA encoding α_{2A}AR in spinal cord neurons stands in contrast to the absence of immunohistochemical staining (Shi et al. 1999) and different DOP antibodies differentially label somatodendritic versus axonal compartments (Cahill et al. 2001a). The absence of co-localization between any of the other α_2AR/OP receptor subtype pairs (α_{2A}AR/MOP, α_{2C}AR/MOP and α_{2C}AR/DOP) may therefore be attributable to technical limitations of the antisera used rather than an absence of co-localization. Furthermore, the use of fluorescence for the detection of immunoreactivity may result in under- or over-representation of fiber subtypes.

Regardless, the possibility that our antisera did not recognize all forms of the receptors under evaluation does not detract from the significance of the positive co-localization results; the triple labeling reported in this study is likely to represent expression of both DOP and α_{2A}AR within the same SP-expressing neuronal processes.

Implications of DOP and α_{2A}AR Co-localization

The current results suggest an anatomical substrate for the formation of α_{2A}AR and DOP hetero-oligomers *in vivo* Such associations between GPCRs have been shown to result in novel pharmacological properties distinct from either component receptor, including enhancement of ligand binding affinity, changes in functional coupling and altered receptor trafficking (for reviews, see (George et al. 2002; Bulenger et al. 2005)). Thus, the generation of novel properties upon α_{2A}AR and DOP hetero-oligomer formation may represent a molecular mechanism for the synergistic interactions previously observed between these receptors *in vivo*, and that we now report *in vitro* Physical associations suggestive of hetero-oligomer formation have been demonstrated between α_{2A}ARs and both DOP and MOP in transfected cells *in vitro* (Jordan et al. 2003; Rios et al. 2004; Zhang and Limbird 2004; Vilardaga et al. 2008). The relevance of these *in vitro* studies to spinal α_{2A}AR-DOP synergy

requires the expression of both receptors within the same subcellular structures in native tissues as well as the demonstration of a functional interaction that can be attributed to that cellular localization. The combination of the previous *in vitro* studies and the current findings provide a structural and functional framework for the existence of $\alpha_{2A}AR$ and DOP hetero-oligomers *in vivo*

DOP-ir is associated with large, dense-core vesicles (LDCVs) in axon terminals and ultrastructural evidence exists that these vesicles contain SP (Cheng et al. 1995; Zhang et al. 1998; Bao et al. 2003; Guan et al. 2005). It is thought that DOPs are trafficked to axon terminals in the membranes of these vesicles, where they may be inserted into the plasma membrane in a stimulus-dependent manner (Bao et al. 2003; Guan et al. 2005). DOP availability for binding and activation by extracellular ligands in the terminal is regulated, at least in part, by LDCV release. In addition to direct stimulation, DOP may be translocated to the plasma membrane in response to DOP agonists, chronic morphine exposure, peripheral inflammation, inflammatory mediators and chronic nociceptive stimuli (Cahill et al. 2001b; Bao et al. 2003; Morinville et al. 2003; Morinville et al. 2004a; Morinville et al. 2004b; Guan et al. 2005; Hack et al. 2005; Lucido et al. 2005; Patwardhan et al. 2005; Gendron et al. 2006; Gendron et al. 2007). As a consequence, sensitivity to DOP agonists is increased. For example, Cahill and colleagues demonstrated an increase in both intrathecal DOP agonist-induced analgesia and the number of plasma membrane-associated DOP-ir particles following chronic morphine treatment (Cahill et al. 2001b). This stimulus-triggered exocytosis and consequent surface insertion of DOP is reported to be dependent on the SP domain of preprotachykinin A present in DOP-containing LDCVs (Guan et al. 2005).

We report here that both DOP-ir and $\alpha_{2A}AR$-ir are highly co-localized with SP-ir at the light microscope level in primary afferent fiber terminals in both skin and spinal cord. This three-way co-localization raises two possibilities: a) the $\alpha_{2A}AR$ is trafficked and stored within the same vesicles that contain SP and DOP (Zhang et al., 1998), and b) that the availability of $\alpha_{2A}AR$ on the plasma membrane is controlled by the same regulatory factors that control DOP availability. Evaluation of these possibilities will require further anatomical studies by electron microscopy. We hypothesize that DOP agonist-mediated translocation of DOP will also result in translocation and subsequent enhanced availability of $\alpha_{2A}AR$. The presence of a small amount of the DOP agonist deltorphin-II would potentially result in a dramatic increase in $\alpha_{2A}AR$ agonist efficacy as a result of vesicle fusion and receptor translocation. This scenario would similarly apply if $\alpha_{2A}AR$ agonists cause vesicle fusion and receptor translocation in a manner similar to that already demonstrated for DOP agonists, resulting in mutual potentiation between DOP and $\alpha_{2A}AR$. This proposed model for DOP-$\alpha_{2A}AR$ synergy is based entirely on co-operative receptor trafficking and would predict a greater-than-additive interaction between DOP-$\alpha_{2A}AR$ in isolated synaptosomes as we currently demonstrate and represents an alternative hypothesis regarding the mechanism(s) underlying synergy that is supported by the current findings.

Conclusions

This study provides definitive evidence of extensive co-localization between $\alpha_{2A}AR$ and DOP in SP-containing peripheral and central nerve terminals. $\alpha_{2A}AR$/DOP co-localization can be observed in axonal terminals and isolated nerve terminals. Agonists acting at α_2AR and DOP inhibited K^+-induced neuropeptide release from synaptosomes and interacted in a greater-than-additive manner, indicating a synergistic interaction. This supra-additive interaction in isolated nerve terminals provides a functional implication to the immunohistochemically demonstrated receptor co-localization. Taken together, these data raise the possibility that DOP and $\alpha_{2A}AR$ hetero-oligomeric receptor complexes could exist *in vivo* and may represent a molecular substrate for the analgesic synergy commonly observed between agonists acting at these receptors.

Acknowledgments

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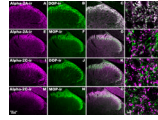
Figure 1. Relationship between α₂ₐAR-ir, α₂cAR-ir, DOP-ir and MOP-ir in Rat Spinal Cord Dorsal Horn

Single confocal optical sections of lumbar rat spinal cord were double-labeled with combinations of guinea pig, rat or rabbit-derived antisera. The **1st column** depicts immunoreactivity of antisera directed against α_{2A}AR (A,E) and α_{2C}AR (I,M). The **2nd column** represents the same sections double-labeled with either DOP (B,J) or MOP (G,O) antisera. In the **3rd column** the results of digitally merging images from the first two columns are shown (C,G,K,O). The **4th column** contains higher magnification images of these combinations (D,H,L,P). In merged images, the appearance of white indicates probable co-localization. Antisera combinations were as follows: **A-D:** Rabbit-derived anti-α_{2A}AR (A) with rat-derived anti-DOP (B), highly co-localized (C,D). **E-H:** Rabbit-derived anti-α_{2A}AR (E) with guinea pig-derived anti-MOP (F), rarely co-localized (G,H). **I-L:** Guinea pig-derived anti-α_{2C}AR (I) with rabbit-derived anti-DOP (J), rarely co-localized (K,L). **M-P:** Rabbit-derived anti-α_{2C}AR (M) with guinea pig-derived anti-MOP (N), rarely co-localized (O,P). The lack of overlap between α_{2C}AR and MOP was further supported by identical results obtained using rabbit-derived MOP paired with guinea pig-derived α_{2C}AR (data not shown).
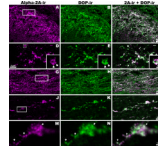
Figure 2. Close Association of DOP-ir and α₂ₐAR-ir in Rat Spinal Cord Dorsal Horn
Single confocal optical sections of rat spinal cord dorsal horn double-labeled with rabbit-derived α_{2A}AR and rat-derived DOP antisera. The **1ˢᵗ column** depicts α_{2A}AR-ir (A,D,G,J,M) and the **2ⁿᵈ column** represents DOP-ir (B,E,H,K,N) in the same sections. Pairs of unmerged images illustrate the similarity in expression patterns of α_{2A} AR and DOP. When the images in the 1ˢᵗ and 2ⁿᵈ columns are digitally merged (C,F,I,L,O), the large proportion of white suggests extensive co-localization. **A-F:** Coronal sections of rat dorsal horn at low (A-C) and higher (D-F) magnification. The insets in D-F are enlargements of the boxed area marked in D. **G-I**: A horizontal section at moderate magnification. **J-L:** Higher magnification images of the boxed area shown in G. **M-O:** Enlargements of the boxed region in J. Images in D-F (insets) and M-O demonstrate close associations of α_{2A}AR-ir and DOP-ir in the same sub-cellular regions 0.25 μm or less in diameter (arrows: possible vesicles or clusters of vesicles).
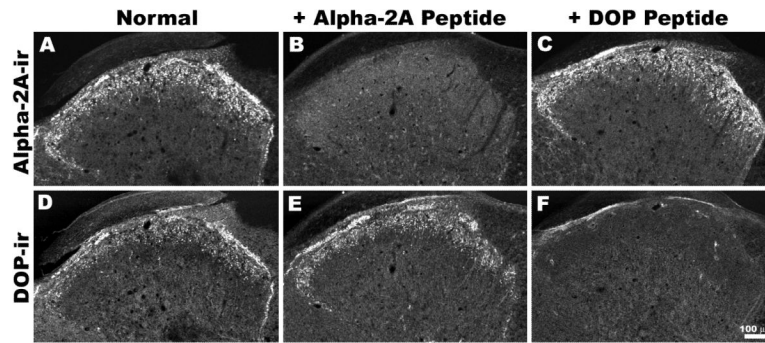
Figure 3. Cross-reactivity Controls for DOP-ir and α$_{2A}$AR-ir Co-localization
Single confocal optical sections show the results of double labeling in the presence or absence of cognate peptide absorption controls in three adjacent sections. Under normal conditions, α$_{2A}$AR-ir (A) and DOP-ir (D) exhibit similar patterns of expression. In the presence of its cognate peptide, labeling for α$_{2A}$AR is absent (B) whereas DOP-ir (E) is unaffected. Similarly, pre-incubation with the cognate peptide for the DOP antisera blocks DOP-ir (F), but not α$_{2A}$AR-ir (C).
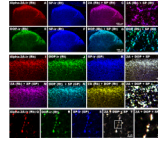
Figure 4. Triple Labeling of α₂ₐAR-ir, DOP-ir and SP-ir in Rat Spinal Cord Dorsal Horn
A-D: Representative section of rat spinal cord double-labeled with rabbit-derived α₂ₐAR (A, Red) and rat-derived SP (B, Blue) antibodies. When images A & B are digitally merged (C, D), instances of co-localization appears as fushia. **E-H:** Representative section of rat spinal cord double-labeled with rabbit-derived DOP (E, Green) and rat-derived SP (F, Blue) antisera. When images E & F are digitally merged (G, H), instances of co-localization appears as turquoise. **I-P:** A single confocal optical section of rat spinal cord triple-labeled with rabbit-derived α₂ₐAR (I, Red), rat-derived DOP (J, Green), and guinea pig-derived SP (K, Blue) antisera. Each of the possible digital pairings of these images is shown where M = α₂ₐAR-ir + SP-ir (co-localization = fuchsia); N = DOP-ir + SP-ir (co-localization = turquoise) and O = α₂ₐAR-ir + DOP-ir (co-localization = yellow). Image L is the result of digital combination of Images I, J & K in which triple-labeled elements appear white. Enlargement of an area from L is shown in P. **Q-U**: Example of a triple-labeled single fiber for α₂ₐAR-ir (Q, Red), DOP-ir (R, Green) and SP-ir (S, Blue). Image T is the result of digital combination of Images Q, R & S in which triple-labeled elements appear white. Image U is an enlargement the area indicated in T. The close association of all three markers along a single fiber suggests that α₂ₐAR and DOP may be associated with SP-containing pre-synaptic vesicles.
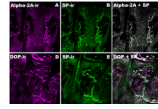
Figure 5. Co-localization of α$_{2A}$AR and DOP with SP in rat skin
A-C: Representative images of rat lower lip skin double-labeled with rabbit-derived α$_{2A}$AR (A, Magenta) and rat-derived SP (B, Green) antisera. When images A & B are digitally merged (C), instances of co-localization appears as white. **D-F:** Representative section of rat spinal cord double-labeled with rabbit-derived DOP (D, Magenta) and guinea pig-derived SP (E, Green) antisera. When images D & E are digitally merged (F), instances of co-localization appears as white. The extensive co-localization observed between both α$_{2A}$AR and DOP with SP suggests that α$_{2A}$AR and DOP co-localize on SP-containing fibers in the periphery.
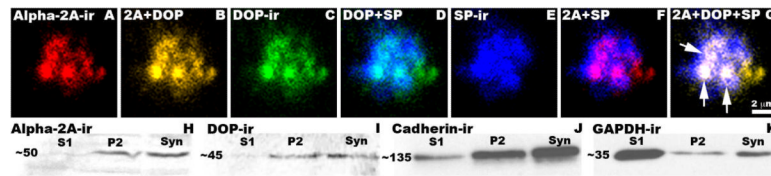
Figure 6. Labeling of α₂AAR-ir, DOP-ir and SP-ir in Spinal Cord Synaptosome(s)
A-G: Nerve terminals were isolated from rat spinal cord and triple-labeled with rabbit-derived α_{2A}AR (A, Red), rat-derived DOP (C, Green), and guinea pig-derived SP (E, Blue) antisera. Each of the possible digital pairings of these images is shown where B = α_{2A}AR-ir + DOP-ir (co-localization = yellow); D = DOP-ir + SP-ir (co-localization = turquoise); F = α_{2A}AR-ir + SP-ir (co-localization = fuchsia). Image G is the result of digital combination of Images A, C & E in which triple-labeled elements appear white. Arrows in G indicate several structural elements within the synaptosome that are triple-labeled. **H-K:** Enrichment of α_{2A}AR-ir and DOP-ir in isolated nerve terminals. Subcellular fractions S1 (total protein), P2 (membrane fraction) and Syn (synaptosomes) were analyzed by Western blot. Immunoreactive bands for anti-α_{2A}AR (~50 kDa, L), anti-DOP (~45 kDa, M) and the membrane marker pan-cadherin (~135 kDa, N) were all enriched in the purified membrane and synaptosome fractions. The cytosolic marker GAPDH was most abundant in the S1 fraction (~35 kDa, O).
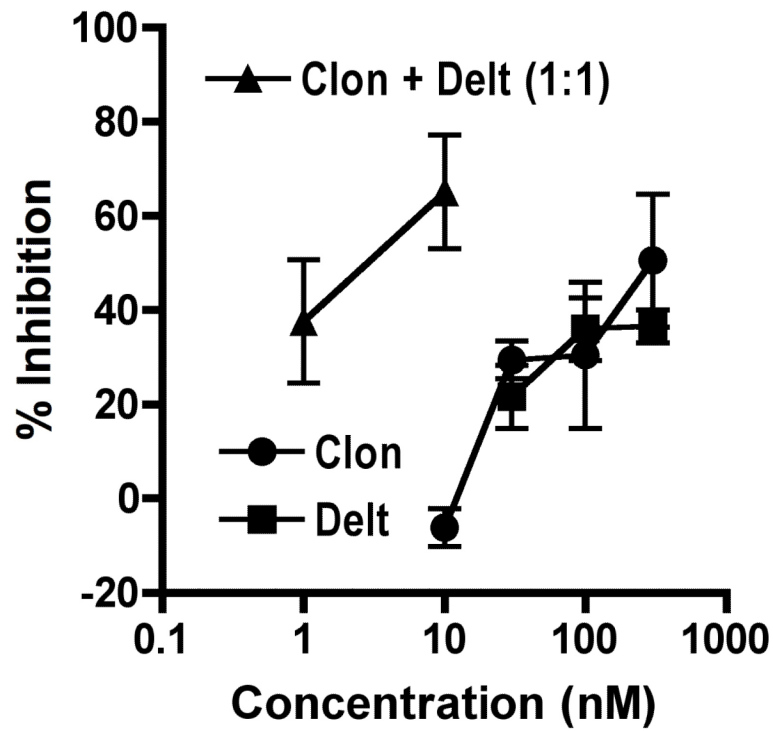
Figure 7. Inhibition of neuropeptide release by α_2AR and DOR agonists in spinal cord synaptosomes

Synaptosomes were exposed to vehicle, the α_2AR agonist clonidine (circles), the DOP agonist deltorphin II (squares) or the combination of clonidine + deltorphin II (triangles) and stimulated with 60 mM K^+. Clonidine and deltorphin II inhibited calcitonin gene-related peptide (CGRP) release in a concentration-dependent manner. Co-incubation with both agonists together resulted in enhanced effectiveness over either agonist alone. Error bars represent ±SEM for each concentration ($n = 3$ replicates/concentration).