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Converging protein kinase pathways mediate adenylyl cyclase superactivation upon chronic δ-opioid agonist treatment

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Running title: Role of protein kinases in AC superactivation

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DPDPE: c[D-Pen^2-D-Pen^5]enkephalin; GW5074: 3-(3,5-dibromo-4-hydroxybenzylidene-5-iodo-1,3-dihydro-indol-2-one; IMDM: Iscove's Modified Dulbecco's Medium; EDTA: ethylenediaminetetraacetic acid; SDS-PAGE: sodium dodecylsulphate-polyacrylamide gel electrophoresis; NTI: naltrindole; SNC 80: (+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxy-benzyl]-N,N-diethyl benzamide

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Abstract

Adenylyl cyclase (AC) superactivation is thought to play an important role in opioid tolerance, dependence and withdrawal. In the present study we investigated the involvement of protein kinases in chronic δ-opioid agonist-mediated AC superactivation in CHO cells stably expressing the human delta opioid receptor (hDOR/CHO). Maximal forskolin-stimulated cAMP formation in hDOR/CHO cells increased by 472±91%, 399±2% and 433±73% after chronic treatment with the δ-opioid agonists SNC 80, DPDPE and deltorphin II, respectively. Concurrently, chronic SNC 80 (1 μM, 4h) treatment augmented [^{32}P]-incorporation into a 200 kDa protein immunoreactive with the ACV/VI antibody by 300±60% in hDOR/CHO cell lysates. The calmodulin antagonist, calmidazolium, significantly attenuated chronic deltorphin II-mediated AC superactivation. Tyrosine kinase- (genistein) and PKC (chelerythrine) inhibitors individually had minimal effect on chronic δ-opioid agonist-mediated AC superactivation. Conversely, simultaneous treatment with both genistein and chelerythrine significantly attenuated AC superactivation. Since we have shown earlier (Varga et al., 2002) that the Raf-1 inhibitor GW5074 attenuates AC superactivation, we hypothesize that parallel, calmidazolium-, chelerythrine-, and genistein-sensitive pathways converge at Raf-1 to mediate AC superactivation by phosphorylating AC VI in hDOR/CHO cells.

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Chronic opioid receptor activation frequently leads to the sensitization of adenylyl cyclase to stimulators after the inhibitory agonist has been removed (AC superactivation). AC superactivation after chronic opioid agonist exposure is thought to contribute to the development of opioid tolerance, dependence and withdrawal (Williams et al., 2001). A better understanding of the molecular mechanisms of chronic δ-opioid agonist treatment-mediated AC superactivation should aid in the development of longer acting analgesics with fewer side effects.

We have reported previously that in Chinese hamster ovary (CHO) cells stably expressing the human δ-opioid receptor (hDOR/CHO), chronic δ-opioid agonist treatment gives rise to AC superactivation (Malatynska et al., 1996). In addition, we also demonstrated that chronic δ-opioid agonist treatment of the hDOR/CHO cells augments [^{32}P]-incorporation into proteins immunoreactive with an AC V/VI specific antibody (Varga et al., 1999). The identity of protein kinase(s) involved in chronic δ-opioid agonist mediated phosphorylation in hDOR/CHO cells, and the role of the phosphorylation in AC superactivation however, has not been previously investigated. In the present work we studied the effect of protein kinase inhibitors on chronic δ-opioid agonist-mediated AC superactivation.

Recent data (Tan et al., 2001) indicates the important role of the protein kinase p74Raf-1 in phosphorylation of adenylyl cyclase VI in transfected HEK293 cells. Moreover, Raf-1-mediated phosphorylation led to the sensitization of AC VI to subsequent stimulation by forskolin or $G_{s\alpha}$. The investigators (Tan et al., 2001) have also demonstrated that a dominant negative Raf-1 construct (NΔRaf) attenuates both phosphorylation and sensitization of AC VI. Interestingly, we found earlier that AC VI is the major adenylyl cyclase isoenzyme in CHO cells, and that a selective inhibitor of Raf-1 (GW5074) significantly attenuates chronic deltorphin II treatment-mediated adenylyl cyclase superactivation in hDOR/CHO cells (Varga et al., 2002).

Raf-1 is the key protein kinase in the p42/44 MAP kinase signal transduction cascade. It has been suggested that δ-opioid receptors activate the p42/44 MAPK cascade using multiple, parallel signal transduction pathways in Chinese hamster ovary cells (Fukuda et al., 1996). In the present work we used selective protein kinase

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inhibitors to demonstrate that similar, redundant signal transduction pathways are involved in the molecular mechanisms of AC superactivation in hDOR/CHO cells.

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Materials and Methods

Chemicals: Calmidazolium was purchased from Calbiochem (San Diego, CA), genistein was from Tocris (Ellisville, MO), chelerythrine and GW5074, (3-(3,5-dibromo-4-hydroxybenzylidene-5-iodo-1,3-dihydro-indol-2-one) were from Sigma (Saint Louis, MO)**.** SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxy-benzyl]-N,N-diethyl benzamide) was synthesized at the National Institute of Health (Bethesda, MD), in the laboratory of Kenner C. Rice. DPDPE (c[D-Pen^2-D-Pen^5]enkephalin) and deltorphin II ([D-Ala^2]deltorphin II) were synthesized at the University of Arizona, in the laboratory of Victor J Hruby. [^{32}P]-orthophosphate (3000 Ci/mmol) was purchased from DuPont NEN (Boston, MA). All other compounds were obtained from commercial sources.

Construction of the hDOR/CHO cell line: The cDNA encoding the human delta opioid receptor (hDOR) was identified and cloned in our laboratory (Knapp et al., 1994). CHO cells were transfected with the cloned hDOR inserted into the pREP10 mammalian expression vector to produce the hygromycin-resistant hDOR/CHO cell line (Malatynska et al., 1995). Radioligand binding experiments were used to characterize the cell line. The K_d value for [^{3}H]NTI was 139 pM with a B_{max} value of 968 fmol/mg protein (Malatynska et al., 1996). The selective δ-opioid agonist SNC 80 inhibited forskolin-stimulated cAMP formation in hDOR/CHO cells with an EC_{50} value of 1.3±0.4 nM (n=9) (E_{max}: 6±2% of control) (Rubenzik et al., 2001).

Drug pretreatment: hDOR/CHO cells were incubated (4-24h, 37^{o}C) in the presence or absence of SNC 80 (10 nM-1 μM) or deltorphin II (100 nM). The kinase inhibitors chelerythrine (5 μM), calmidazolium (0.5 μM), or genistein (100 μM)) were added 30-60min before starting the agonist treatment. The cells were washed in IMDM 3 times (15min, 37^{o}C) before forskolin-stimulated cAMP formation-, or [^{32}P]-incorporation into AC VI were determined.

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Measurement of forskolin-stimulated cAMP formation: Following chronic drug treatment, the cells were washed three times (15min each) with fresh IMDM. The IMDM was then aspirated and replaced with 5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma Chemical, St. Louis, MO) in IMDM. Adenylyl cyclase was stimulated with 100 μM water-soluble forskolin (7-deacetyl-7-(O-N-methylpiperazino)-γ-butyryl, diHCl) (Calbiochem, San Diego, CA). The cells were incubated in a humidified incubator at 37°C (5% CO_2), for 20min. The reaction was terminated by replacing the medium with ice-cold Tris/EDTA buffer (50 mM Tris HCl , 4 mM EDTA, pH=7.5). The cells were lysed by boiling (10min) and centrifuged. 50 μl of the supernatant was incubated with 4 nM [^{3}H]cAMP (Perkin Elmer Life Sciences, Boston, MA) and 30 μg/ml PKA (Sigma Chemical, St. Louis, MO). Serial dilutions of cAMP were run in parallel to obtain a cAMP standard curve. After a 2h incubation at 4°C, activated charcoal (26 mg/ml) (Norit, The Netherlands) was added to adsorb free cAMP. The mixture was then centrifuged and 200 μl of the supernatant was counted in EcoLite (ICN Pharmaceuticals, Costa Mesa, CA) scintillation fluid.

Phosphorylation of adenylyl cyclase VI in the hDOR/CHO cells after chronic δ opioid agonist (SNC 80) treatment: A previously described (Varga et al., 1999) metabolic labeling/immunoprecipitation method was used to measure [^{32}P]-incorporation into the protein band immunoreactive with an ACV/VI-specific antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). Briefly, hDOR/CHO cells were phosphate-starved and labeled with [^{32}P]-orthophosphate. In experiments where specific protein kinase inhibitors were used, the inhibitors were added together with the [^{32}P]-orthophosphate. After a 1h preincubation, SNC 80 (10 nM-1 μM) was added and the incubation continued for 1-24h. After the agonist treatment, the cells were thoroughly washed and homogenized in solubilization buffer (50 mM Tris, 250 mM sucrose, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaF, 10 mM Na-pyrophosphate and 10 μl/ml protease inhibitor cocktail, 0.2 mM Na-orthovanadate and 100 nM okadaic acid, pH 7.4). The lysate was centrifuged, precleared by incubating in the presence of 1μg preimmune rabbit IgG and 10μl protein A-agarose for 1h. After cenrifugation, the precleared lysate was incubated with the ACV/VI specific antibody and Protein A-agarose beads for 4h. After thorough

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washes in solubilization buffer, with detergent concentrations reduced to 0.075% Triton X-100, 0.05% Igepal CA-630 and 0.1% digitonin (4^{o}C), the immunocomplexes were eluted in glycine-Cl buffer (pH=2.3), neutralized, solubilized in Laemmli-buffer and resolved on 7.5% SDS-PAGE. The gel was silver-stained, dried and subjected to autoradiography. The amounts of immunoprecipitated protein-, and the extent of [^{32}P]-incorporation, were quantified by scanning densitometry of the silver stained gel and the autoradiography film, respectively.

Data analysis: Radioligand binding data were analyzed by nonlinear regression, using the Prism^{R} (version 3.0) computer program. Data are represented as mean±SEM, unless otherwise indicated. Standard statistical tests, such as one-way ANOVA followed by Dunnett's multiple comparison tests; were used to determine statistical differences between treatment groups (*$p<0.05$, **$p<0.01$, and ***$p<0.001$).

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Results

The effect of chronic δ-opioid agonist treatment on forskolin-stimulated cAMP formation in hDOR/CHO cells: We have demonstrated previously (Malatynska et al., 1996; Rubenzik et al., 2001) that chronic (4-24h) SNC 80 (100-1000 nM) treatment of CHO cells stably transfected with the human δ-opioid receptor (hDOR/CHO) significantly increases forskolin-stimulated cAMP formation after the removal of the agonist. In the present work we tested the ability of other, structurally different δ-opioid agonists to produce adenylyl cyclase superactivation upon chronic treatment in hDOR/CHO cells. As seen in Fig. 1, chronic (4h) treatment of the hDOR/CHO cells with 100 nM SNC 80; DPDPE, or deltorphin II augmented forskolin (100 μM) stimulated cAMP formation to 472±91% (n=10); 399±2% (n=4) and 433±73 % (n=13) of the IMDM-treated control, respectively. Basal cAMP formation was not significantly different between IMDM-treated and agonist-treated hDOR/CHO cells (5.8±4.4 (n=24) and 6.3±14 (n=22) pmol/million cells, respectively, $p>0.5$). The selective δ-opioid antagonist, naltrindole (1 μM) attenuated deltorphin-mediated adenylyl cyclase superactivation (forskolin-stimulated cAMP formation: 123±2% of control, $P>0.5$). Statistical significances ($p<0.001$) were determined using using One-Way ANOVA followed by Dunnett's Multiple Comparison test.

Phosphorylation of adenylyl cyclase VI in the hDOR/CHO cells upon chronic δ opioid agonist (SNC 80) treatment: It was shown previously that chronic opioid treatment-mediated AC superactivation is isoenzyme specific (Avidor-Reiss et al., 1997). AC VI was one of the few AC isoenzymes that became sensitized to forskolin stimulation upon chronic opioid receptor stimulation (Avidor-Reiss et al., 1997). We have previously determined that AC VI is a major native isoenzyme in CHO cells (Varga et al., 1998). Thus, we hypothesized that AC VI is that major isoenzyme involved in forskolin-stimulated cAMP overshoot upon chronic δ-opioid agonist treatment in the hDOR/CHO cells.

Since the catalytic activity of adenylyl cyclase isoenzymes is frequently regulated by protein kinases (Ishikawa, 1998), we tested the effect of chronic δ-opioid agonist treatment on the phosphorylation state of AC

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VI in hDOR/CHO cells. A metabolic labeling/immunoprecipitation method, followed by SDS/PAGE, was used to measure [^{32}P]-incorporation into AC VI upon chronic SNC 80 treatment, as previously described (Varga et al., 1999). Two bands, with apparent molecular weights of about 130 and 200 kDa, were routinely obtained on both the silver stained gel and the autoradiography film. Figure 2 shows a representative autoradiography film obtained after SNC 80 treatment of hDOR/CHO cells for 4h with increasing doses of the drug. The 200 kDa band is presumably the glycosylated form of the 130 kDa protein, since only the 130 kDa band was apparent on the gel when after the immunoprecipitate was pretreated with N-aminoglycosidase F (data not shown). Immunoprecipitation was prevented by saturation of the antibody with a blocking peptide (Santa Cruz Biotechnologies, Santa Cruz, CA). The optical densities of the bands in the silver-stained gel (protein content), and in the autoradiography film ([^{32}P]-incorporation) were measured by scanning densitometry. Since a similar protein/phosphate ratio was measured for the 130 kDa- and the 200 kDa bands in initial experiments, we routinely used the 200 kDa band to quantitate phosphorylation. Data in Figs 2 and 3 show the density ratios ([^{32}P]-incorporation/protein stain) calculated as the percent of the IMDM-treated control. Chronic (4h) SNC 80-treatment-mediated phosphorylation of AC VI was at a maximal level (557% of untreated control, range: 400-714, n=2) at 100 nM SNC 80 concentrations (Fig. 2). Fig. 3 shows a time course for SNC 80- (1 μM) mediated [^{32}P]-incorporation into the 200kDa band. Phosphorylation was maximal (303±61% of control, $P<0.001$, n=7) after 4h treatment. Phosphorylation of AC VI remained at similar levels upon longer (up to 24h, 204±29%, $P<0.01$, n=3) treatment.

The effect of protein kinase inhibitors on chronic SNC 80-mediated AC superactivation in hDOR/CHO cells: In the next set of experiments we investigated the effect of selective protein kinase inhibitors on SNC 80-mediated adenylyl cyclase superactivation. The calmodulin antagonist, calmidazolium completely attenuated chronic SNC 80 -mediated AC superactivation (Fig. 4). Forskolin-stimulated cAMP formation in calmidazolium (0.5 μM) pretreated hDOR/CHO cells was similar after IMDM- or chronic SNC 80 treatment (108±3% of IMDM-treated control, n=3). It should be noted however, that there was a considerable difference

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in maximal forskolin-stimulated cAMP formation in the control IMDM- and calmidazolium-treated hDOR/CHO cells (669 ± 140 and 1821 ± 109 pmol per million cells, respectively, $P<0.001$) in the absence of SNC 80. The PKC inhibitor, chelerythrine on the other hand, had only a minimal effect on cAMP overshoot in δ-opioid agonist treated hDOR/CHO cells (Fig.5). Forskolin-stimulated cAMP formation was 375±87% ($P>0.1$, $n=7$).

Phosphorylation and sensitization of AC VI by the Raf-1 protein kinase has been recently demonstrated (Tan et al., 2001). Therefore earlier we have tested the effect of a selective Raf-1 inhibitor (GW5074) on chronic δ-opioid agonist-mediated AC superactivation in hDOR/CHO cells. We found that pretreatment (30min, 37^{o}C) of hDOR/CHO cells with GW5074 (10 μM) attenuated chronic deltorphin II-mediated AC superactivation by 40% (**$p<0.01$) (Varga et al., 2002). Since the catalytic activity of Raf-1 is modulated by multiple independent mechanisms (Belcheva and Coscia, 2001), in order to achieve complete inhibition it is frequently necessary to inhibit multiple pathways simultaneously (Fukuda et al., 1996). Accordingly, chronic deltorphin II treatment-mediated cAMP overshoot (355±65 % of control) was not notably attenuated in hDOR/CHO cells pretreated individually with the PKC inhibitor chelerythrine (375±87%, 91±7% of control, $P>0.05$), or the tyrosine kinase inhibitor genistein (213±34, 78±9% of control, $P>0.05$). Simultaneous application of the two inhibitors however, led to a significant blockade (cAMP overshoot: 143±19% of deltorphin II-treated control, $P<0.05$, $n=7$) of AC superactivation in hDOR/CHO cells (Fig. 5). Statistical significances (∗=$p<0.05$; ∗∗=$p<0.01$, ∗∗∗=$p<0.001$) were determined using One-Way ANOVA followed by Dunnett's Multiple Comparison tests.

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Discussion

Chronic stimulation of $G_{i/o}$ protein-coupled receptors often leads to a compensatory increase in the response of adenylyl cyclase to stimulators (AC superactivation) after the removal of the inhibitory agonist. AC superactivation was first described for the opioid receptors in NG108-15 cells (Sharma et al., 1975). Although AC superactivation is thought to play an important role in opioid tolerance, dependence and withdrawal, its molecular mechanism is still not completely understood (Williams et al., 2001). Down-regulation of the opioid receptors is probably not involved in AC superactivation since increased cAMP levels (cAMP overshoot) become evident after agonist withdrawal. Down-regulation of Gi/o proteins (vanVliet et al., 1991), subcellular translocation of Gi- (Bayewitch et al., 2000) and Gs- (Ammer and Schulz, 1997) proteins, attenuation of phosphodiesterase activity (Law and Loh, 1993) and switching from Gi/o-mediated signaling to Gs-mediated signaling (Crain and Shen, 1998) have been suggested as possible explanations for cAMP overshoot.

AC superactivation has been observed in brain areas involved in opiate addiction, but it can also be demonstrated in recombinant cell lines heterologously expressing opioid receptors. We (Malatynska et al., 1996), and others (Avidor-Reiss et al., 1995) demonstrated that chronic opioid-treatment of CHO cells expressing δ–, or μ-opioid receptors leads to AC superactivation. It has also been shown previously that adenylyl cyclase superactivation is isoenzyme specific (Thomas and Hoffman, 1996, Avidor-Reiss et al., 1997, Cumbay and Watts, 2001). Using an RT-PCR method we previously found that AC VI and AC VII are the major AC isoenzymes expressed in CHO cells (Varga et al., 1998). It was demonstrated earlier that AC VI is one of the few AC isoenzymes that becomes sensitized to forskolin after chronic opioid receptor stimulation and subsequent withdrawal (Avidor-Reiss et al., 1997). Therefore, we hypothesized that the AC VI isoenzyme has an important role in augmentation of forskolin-stimulated cAMP formation by chronic δ-opioid agonist treatment in hDOR/CHO cells.

Compensatory feedback regulation of the concentration or/and catalytic activity of adenylyl cyclase is an attractive hypothesis to account for cAMP overshoot. Thus, it was shown earlier that chronic opioid

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treatment increases the mRNA levels of AC isoenzymes in the nucleus accumbens (Nestler and Aghajanian, 1997) and AC VII in the ileum longitudinal muscle myenteric plexus (LMMP)(Rivera and Gintzler, 1998). The relatively rapid kinetics and cycloheximide-insensitivity of AC superactivation in hDOR/CHO cells (Avidor-Reiss et al., 1995, Rubenzik, 2002), however suggests that new protein synthesis is not the sole mechanism involved in this process. It was also demonstrated previously that G protein βγ–subunits, released upon agonist-stimulation of the transiently transfected μ-opioid recetor, have a major role in AC superactivation in COS-7 cells (Avidor-Reiss et al., 1996). Earlier we also found that overexpression of a free βγ-subunit scavenger (the α-subunit of rod transducin (αt1)) completely attenuates SNC 80-mediated AC superactivation in hDOR/CHO cells (Rubenzik et al., 2001).

Since phosphorylation regulates the catalytic activity of adenylyl cyclase isoenzymes (Ishikawa, 1998) previously we have tested whether chronic δ-opioid agonist treatment leads to phosphorylation of the AC VI isoenzyme in hDOR/CHO cells. We found that concurrent to AC superactivation, chronic SNC 80 treatment augments [^{32}P]-incorporation into a 130-, and a 200 kDa protein band, immunoprecipitated by an AC V/VI specific antibody from hDOR/CHO cells. Since only the 130 kDa band was apparent after aminoglycosidase F treatment of the hDOR/CHO cell extracts, the 200 kDa band presumably corresponds to the glycosylated form of the 130 kDa protein. Chronic SNC 80-mediated [^{32}P]-incorporation in the hDOR/CHO cells was attenuated by naltrindole, a selective δ opioid receptor antagonist (Varga et al., 1999) and was SNC 80 dose- and treatment-time dependent (present work). Importantly, the time course of the onset of SNC 80-mediated AC superactivation (Rubenzik, 2002) and AC phosphorylation (present work) are remarkably similar. Interestingly, Chakrabarti et al. (1998) have simultaneously observed that chronic morphine treatment augments AC phosphorylation in LMMP preparations.

In order to test the involvement SNC 80-mediated phosphorylation in AC superactivation we examined the effect of protein kinase inhibitors on chronic δ-opioid agonist-mediated cAMP overshoot in hDOR/CHO cells. We found that the calmodulin antagonist, calmidazolium completely attenuates chronic SNC 80-mediated AC superactivation, while the PKC inhibitor chelerythrine had only minimal effect. In preliminary experiments

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we also tested the protein kinase inhibitor sensitivity of SNC 80 -mediated phosphorylation of AC VI in hDOR/CHO cells. The protein kinase inhibitor H-89 and the calmodulin antagonist, calmidazolium completely attenuated SNC 80 induced [^{32}P]-incorporation into the immunoreactive bands.

Chakrabarti el al. (1998) have previously demonstrated that chronic morphine treatment-mediated phosphorylation of adenylyl cyclase in guinea pig LMMP preparation was attenuated by chelerythrine pretreatment, indicating the involvement of PKC in this process. Agonist-activated opioid receptors in recombinant CHO cells have been shown to stimulate IP_1 formation (Rubenzik et al., 2001), arachidonate release and as PI-3 kinase (Fukuda et al., 1996). Either of these pathways may lead to the activation of PKC isoenzymes in hDOR/CHO cells. However, since we found that the PKC inhibitor, chelerythrine, has only a very moderate effect on AC superactivation, PKC-mediated phosphorylation of the adenylyl cyclase isoenzymes is unlikely to play a major role in chronic δ-opioid agonist-mediated AC superactivation in hDOR/CHO cells.

Recently, phosphorylation of AC VI by the Raf-1 protein kinase has been demonstrated (Tan et al., 2001). Interestingly, similar to chronic opioid treatment-mediated AC superactivation, Raf-1-mediated phosphorylation was shown to sensitize the AC VI to stimulators, such as forskolin and Gs_α. Therefore, we tested the involvement of Raf-1 in chronic δ-opioid agonist -mediated AC superactivation in hDOR/CHO cells. We found that pretreatment of hDOR/CHO cells with the selective Raf-1 inhibitor GW5074 significantly attenuates chronic δ-opioid agonist-mediated AC superactivation (Varga et al., 2002).

Raf-1 is a serine/threonine kinase that acts downstream of activated tyrosine kinases in the p42/44 mitogen activated protein kinase (MAPK) signal transduction cascade (Belcheva and Coscia, 2002). The catalytic activity of Raf-1 is modulated by multiple, independent mechanisms. Thus, in CHO cells only simultaneous inhibition of both tyrosine kinases and PKC attenuated DPDPE-mediated MAPK phosphorylation completely (Fukuda et al., 1996). Our present data demonstrates that similarly, inhibition of tyrosine kinases or PKC individually has only minimal effect on AC superactivation, while simultaneous pretreatment with both genistein and chelerythrine obliterates chronic δ-opioid agonist-mediated AC superactivation.

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Earlier it was concluded that the MAP kinase pathway is not involved in AC superactivation, since neither overexpression of dominant negative Ras, nor the PI-3-kinase inhibitor wortmannin, were able to attenuate AC superactivation in COS-7 cells co-transfected with the μ opioid receptor and AC V (Avidor-Reiss et al., 1996). However, since the catalytic activity of Raf-1 is modulated by multiple mechanisms, blocking a single pathway may only shunt the signal to alternative, parallel pathways. Thus, simultaneous inhibition of both ras-dependent-, and ras-independent pathways may be necessary to achieve complete inhibition of AC superactivation, as we indeed found in hDOR/CHO cells.

We also found that both AC superactivation and AC VI phosphorylation can be completely attenuated by pretreatment with calmidazolium. Calmidazolium is a calmodulin antagonist that competes with calmodulin-sensitive intracellular effectors in low micromolar concentrations (Gietzen et al, 1981). Interestingly, the involvement of a calmodulin-sensitive step, upstream of opioid-mediated transactivation of receptor- and non-receptor tyrosine kinases and the small G protein Ras, was earlier demonstrated in opioid receptor-mediated MAPK activation (Belcheva et al., 2001). In addition, recent data indicate that calmidazolium also inhibits Raf-1 directly, by antagonizing calmodulin binding to the enzyme (Egea et al., 2000). It should be noted however, that maximal values for forskolin-stimulated cAMP formation were also different in IMDM-, and calmidazolium-treated hDOR/CHO cells. Since earlier we found that CHO cells do not express calmodulin-, or calmodulin kinase-sensitive AC isoforms, the reason for increased basal cAMP formation in calmidazolium treated cells is presently not clear. Calmidazolium however is a nonselective calmodulin antagonist that interferes with a number of other intracellular enzymes, such as calmodulin-dependent protein phosphatases and -phosphodiesterases. This may change the basal phosphorylation of every AC isoenzyme in CHO cells and also may affect cellular cAMP degradation rate, contributing to increased basal cAMP formation in calmidazolium treated cells.

In summary, we have demonstrated that chronic SNC 80 treatment augments [^{32}P]-incorporation into two protein bands immunoreactive with the ACV/VI specific antibody. Both chronic SNC 80-mediated [^{32}P] incorporation and AC superactivation are naltrindole-sensitive and exhibit similar SNC 80 dose-, and time-dependence. Calmidazolium and a selective Raf-1 inhibitor (GW5074) significantly attenuated chronic δ-opioid

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agonist-mediated adenylyl cyclase superactivation in hDOR/CHO cells. Tyrosine kinase- (genistein) and PKC (chelerythrine) inhibitors individually had a minimal effect. However, simultaneous treatment with both genistein and chelerythrine abolished AC superactivation. Based on our experimental data we suggest that multiple redundant pathways contribute to δ-opioid receptor-mediated activation of Raf-1, that in turn leads to the phosphorylation and sensitization of AC VI in hDOR/CHO cells. Fig. 6 shows a putative molecular model to interpret the role of chelerythrine-, genistein-, and calmidazolium-sensitive signal transduction pathways in Raf-1 activation and AC VI phosphorylation. Adenylyl cyclase superactivation is an important molecular mechanism contributing to the development of tolerance and dependence to chronic opioid treatment. Better understanding of this important cellular compensatory mechanism should ultimately lead to the development of longer acting analgesic drugs with fewer side effects.

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Footnotes

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Figure legends

Figure 1. Maximal forskolin-stimulated cAMP formation in hDOR/CHO cells after chronic treatment with different δ-opioid agonists and/or naltrindole. Pretreatment (4h) of hDOR/CHO cells with 100 nM SNC 80, DPDPE, or deltorphin II augmented forskolin (100 μM) stimulated cAMP formation by 472±91% (n=10), 399±2% (n=4) and 433±73% (n=13) of IMDM-treated control, respectively. Maximal forskolin-stimulated cAMP formation after pretreatment of hDOR/CHO cells with deltorphin II (100 nM) in the presence of naltrindole (1 μM) on the other hand, was not significantly different from buffer-treated control cells (123±2 % of IMDM-treated control).

Figure 2. Dose-response of chronic (4h) SNC 80-mediated phosphorylation of AC VI: hDOR/CHO cells were metabolically labeled with 200 μCi/ml [^{32}P]-orthophosphate and treated with increasing concentrations of SNC 80 for 4h. Cell lysates were immunoprecipitated by an AC V/VI specific antibody and Protein A-agarose. The immunoprecipitated proteins were resolved on 7.5% SDS-PAGE. The gels were silver-stained and subjected to autoradiography. The figure shows a representative autoradiogram. The locations of the molecular weight- standards on the corresponding silver-stained gel are indicated by arrows. Optical densities in the silver-stained gel (protein content) and in the autoradiography film ([^{32}P]-incorporation) were measured for the 200 kDa band using scanning densitometry. [^{32}P]-incorporation was normalized for the amount of protein by calculating a ratio between the measured optical densities. The values underneath each SNC 80 dose show the range and the mean of the normalized [^{32}P]-incorporation, calculated as percent of IMDM-treated control (n=2).

Figure 3. Time course of SNC 80-mediated phosphorylation of AC VI: hDOR/CHO cells were metabolically labeled with 200 μCi/ml [^{32}P]-orthophosphate prior to treatment with SNC 80 (1 μM) for different time intervals. The cell lysates were immunoprecipitated, and the immunoreactive proteins were measured as described in the legend for Fig. 2. The density ratio ([^{32}P]-incorporation/protein) increased to

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303±61% of IMDM-treated control (n=7) after 4h SNC 80 pretreatment, and remained at a similar level upon longer (up to 24h, 204±29%, n=3) treatment.

Figure 4. The effect of calmidazolium pretreatment on forskolin-stimulated cAMP formation in SNC 80-treated hDOR/CHO cells: Forskolin -stimulated cAMP formation after chronic treatment with SNC80 (100 nM, 4h) was 180 ± 11% of IMDM-treated control hDOR/CHO cells at maximal (100 μM) forskolin concentration (∗=P<0.05, n=3). Conversely, after pretreatment (30min) in the presence of 0.5 μM calmidazolium (CMZ), chronic SNC 80 treatment did not lead to a cAMP overshoot in hDOR/CHO cells. Forskolin-stimulated cAMP formation in calmidazolium-treated cells was 108 ± 3% of IMDM-treated control (P>0.1, n=3). The data are normalized as percentage of maximal stimulation in IMDM-treated control cells in the absence or presence of calmidazolium, respectively. Raw values of maximal forskolin-stimulated cAMP formation in the absence of SNC 80 were 669 ± 140 and 1821 ± 109 pmol per million cells in IMDM- and calmidazolium-treated control hDOR/CHO cells, respectively (P<0.001).

Figure 5. Simultaneous inhibition of PKC- and Tyr-kinases attenuates chronic deltorphin II treatment-mediated cAMP overshoot: hDOR/CHO cells were pretreated (0.5-1h) in the absence or presence of 5 μM chelerythrine or 100 μM genistein individually or with both inhibitors simultaneously. The treatment continued for a further 4h in the presence or absence of 100 nM deltorphin II. The cells were throroughly washed and forskolin (100 μM) stimulated cAMP formation was determined. Data are represented as percent (mean±SEM) of maximal forskolin-stimulated cAMP formation in IMDM-treated control cells (n=7). Chronic deltorphin II-mediated cAMP overshoot was 355±65 % of control in IMDM-treated cells. cAMP overshoot was not significantly different in hDOR/CHO cells pretreated with genistein (213±34, P>0.05) or chelerythrine (375±87%, P>0.1), individually. Conversely, simultaneous application of the two inhibitors led to a significant blockade of deltorphin II-mediated AC superactivation (cAMP overshoot: 143±19% of deltorphin II-treated control, ∗=P<0.05) in hDOR/CHO cells. Basal cAMP formation was 4.3±0.6; 4.6±0.6; 7.0±1.3; and 4.7±0.5 pmol/million cells in untreated-, genistein-, chelerythrine-, and genistein+chelerythrine-treated cells,

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respectively. Maximal forskolin-stimulated cAMP formation was 163±21; 305±37; 96±70 and 217±27 pmol/million cells, in untreated-, genistein-, chelerythrine-, and genistein+chelerythrine-treated cells, respectively.

Fig. 6. Putative molecular mechanism of δ-opioid agonist-mediated phosphorylation of adenylyl cyclase VI in hDOR/CHO cells. Stimulation of the human δ-opioid receptor (hDOR) by an opioid agonist (✸) in CHO cells liberates G protein βγ subunits. Free G protein βγ subunits interact with multiple effectors, leading to the activation of protein kinase C (PKC) isoforms; recruitment of β-arrestin (β-arr) and the non-receptor tyrosine kinase Src; and transactivation of tyrosine kinase receptors (RTK), such as the platelet-derived growth factor receptor. Activation of PKC and Src on the other hand results in Raf-1 activation through Ras-independent-, or Ras-dependent pathways, respectively. Activated Raf-1 phosphorylates and sensitizes adenylyl cyclase VI (AC VI), leading to a cAMP overshoot. The putative molecular targets of the inhibitors used in the present work are indicated in the figure. Calmidazolium is a calmodulin antagonist, genistein is a tyrosine kinase inhibitor, while chelerythrine non-selectively inhibits PKC isoenzymes. GW5074 is a selective Raf-1 inhibitor. Other abbreviations: MAPK – mitogen activated protein kinase, MEK – MAP kinase kinase.

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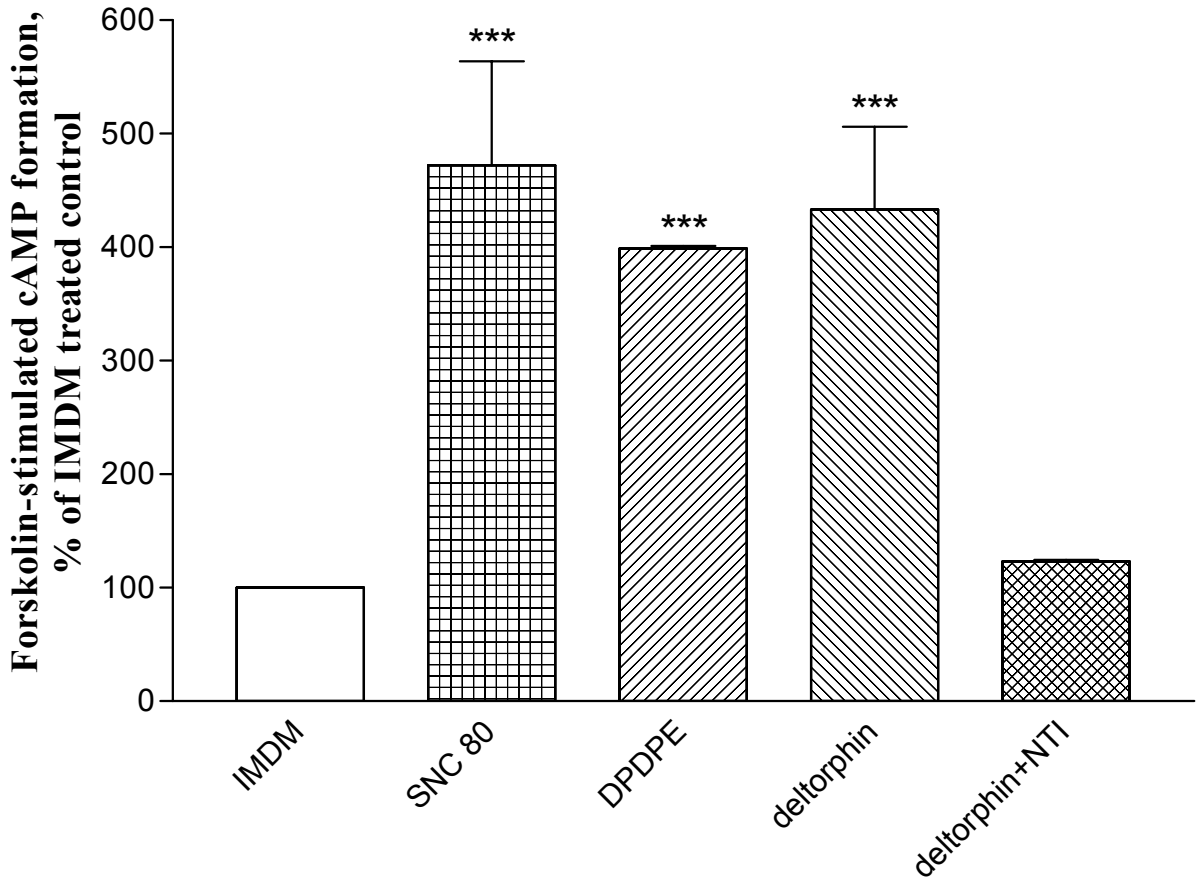

Figure 1.

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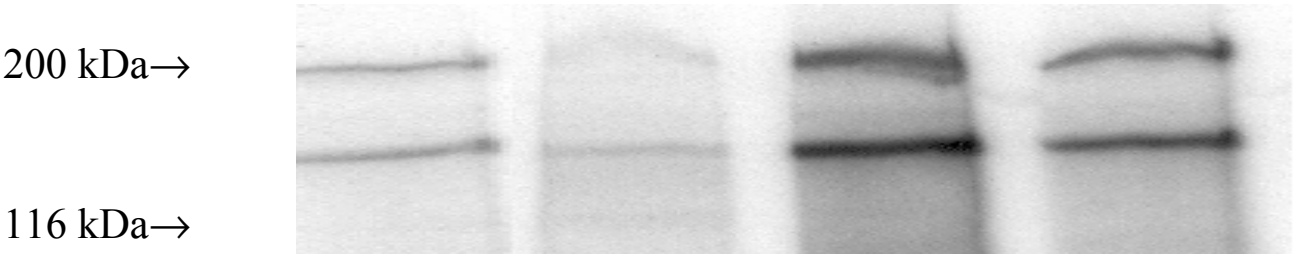

[SNC 80], nM	0	10	100	1000
[^{32}P]/[Protein], Mean, %	100	109	557	431
Range, %	-	57-162	400-714	263-600

Figure 2.

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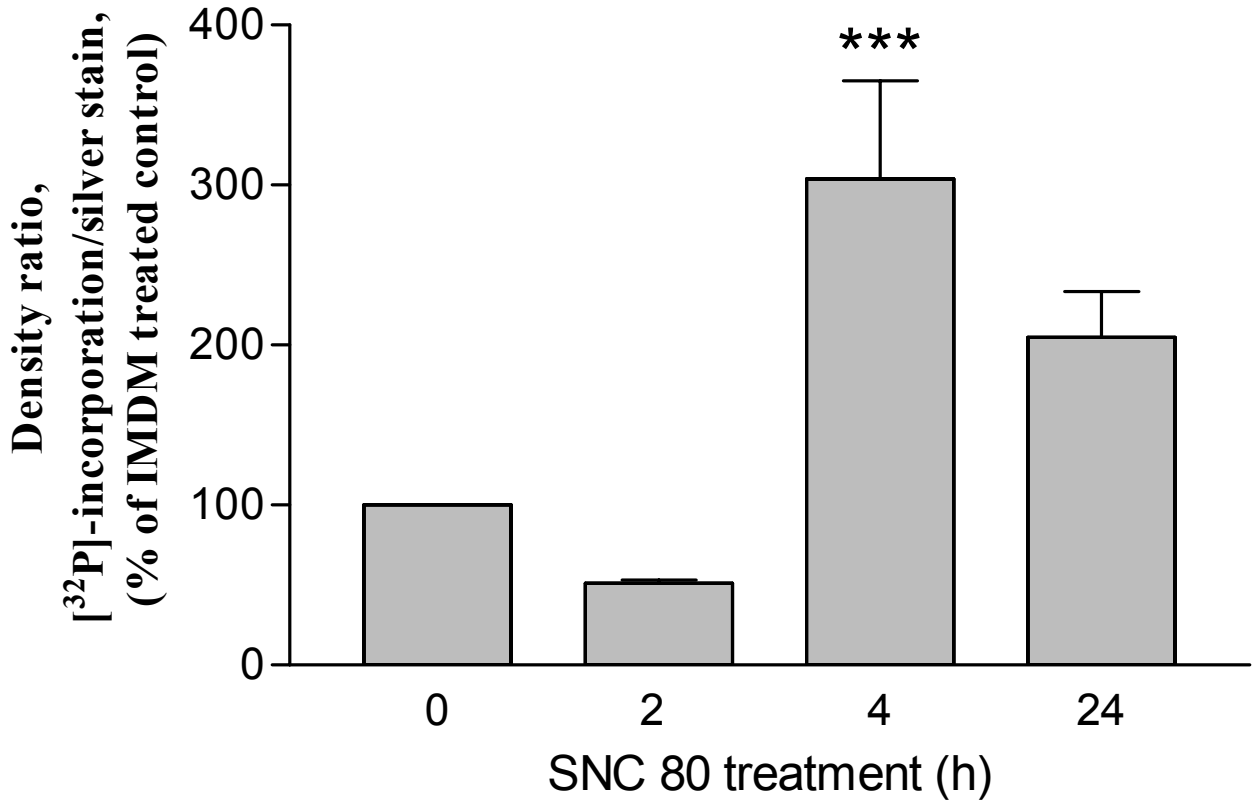

Figure 3.

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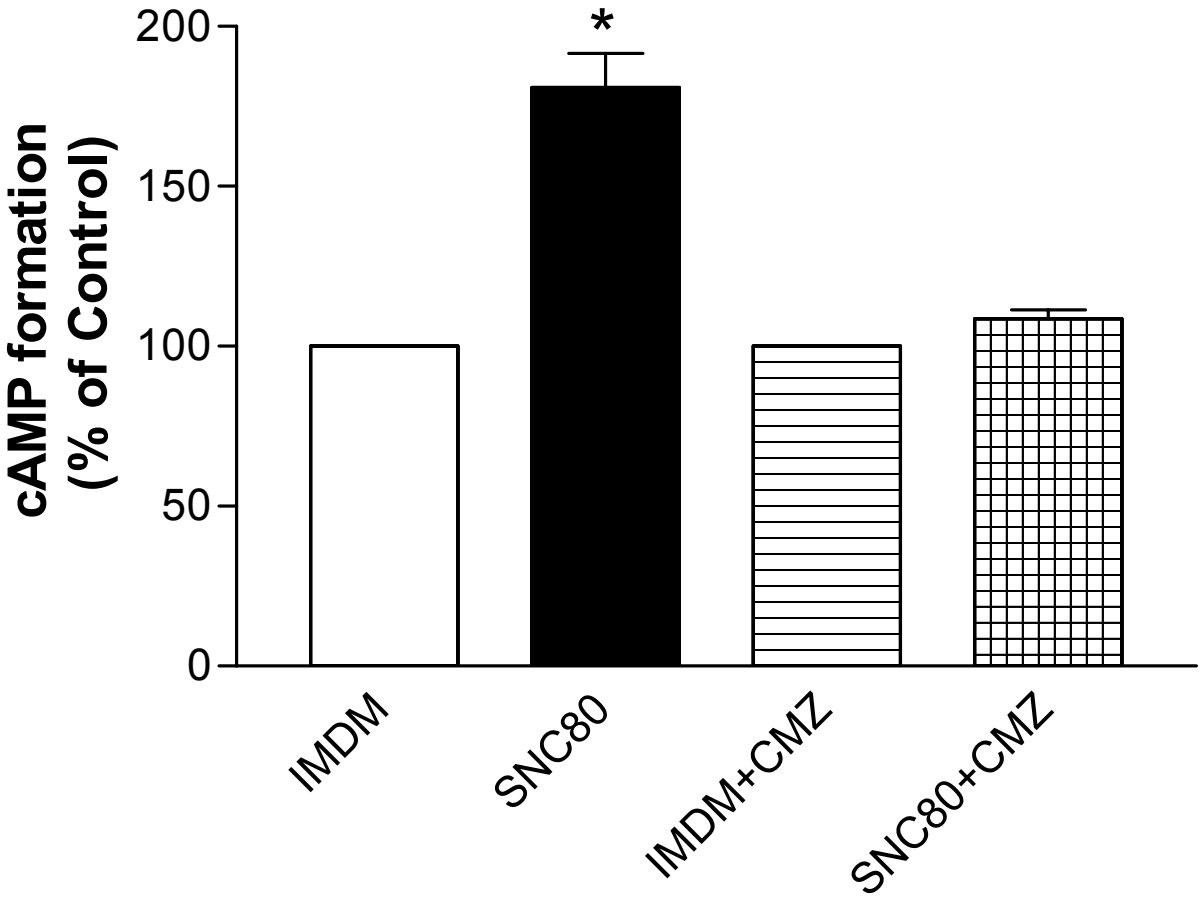

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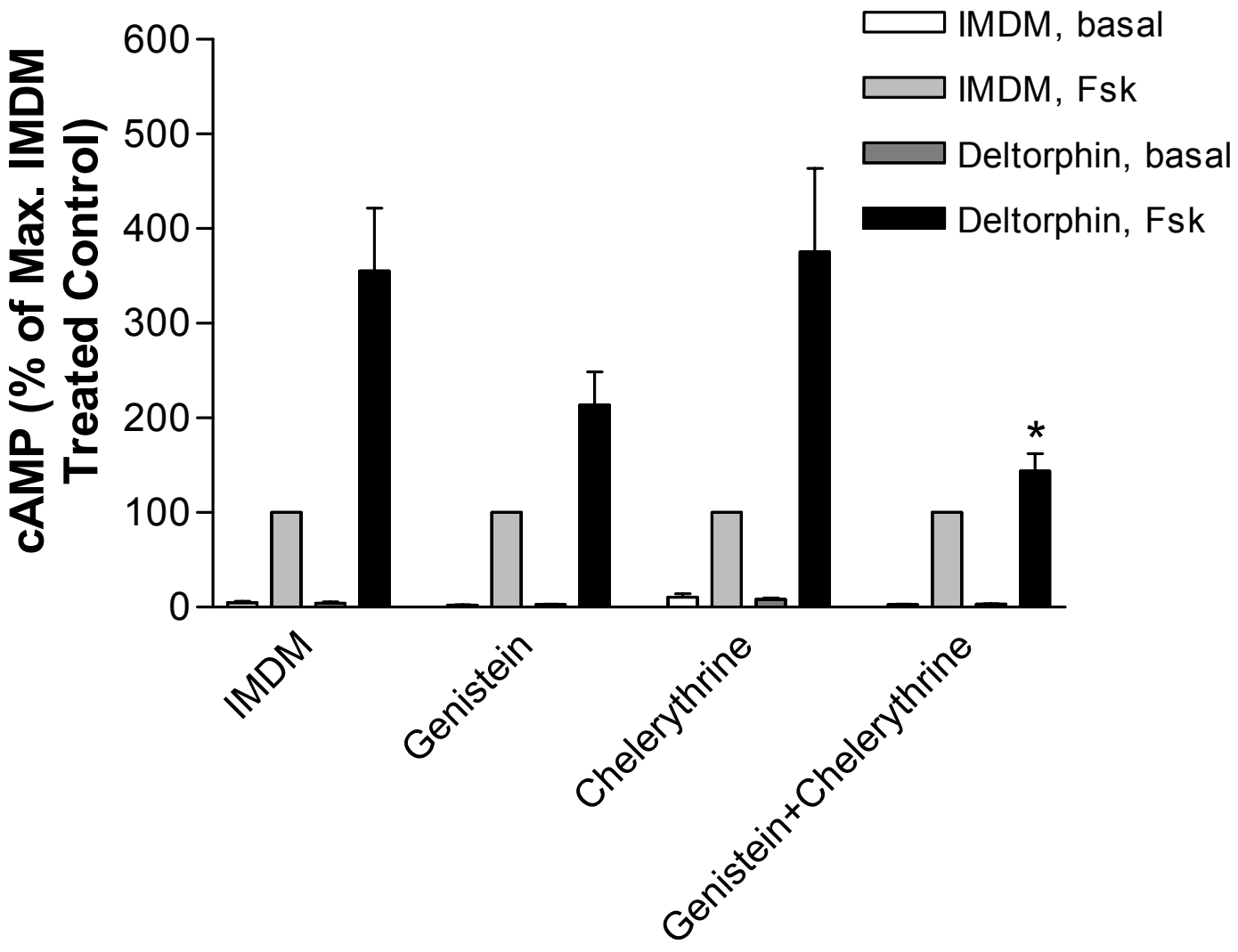

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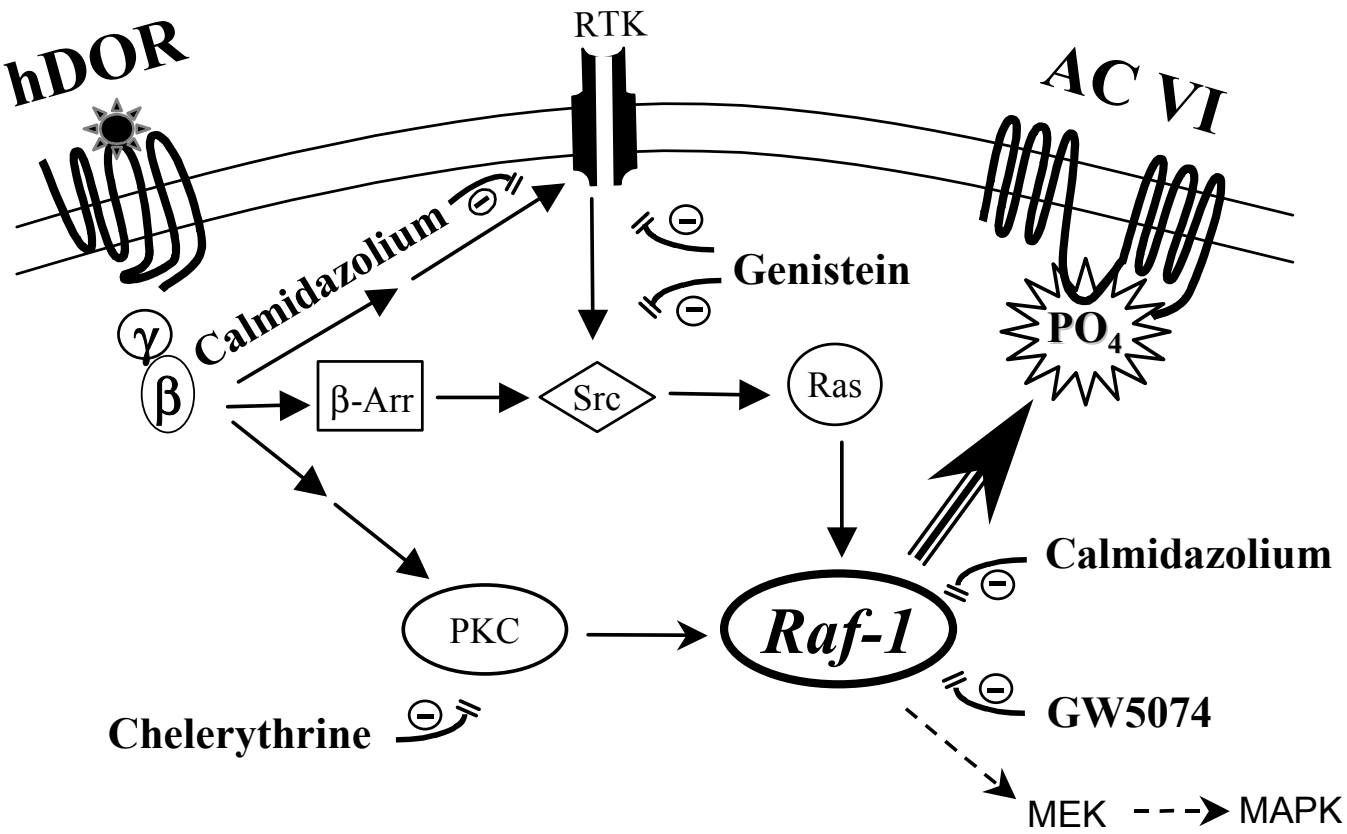

Figure 6.

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