

Adenosine A₁ Receptor-Mediated Inhibition of Protein Kinase A-Induced Calcitonin Gene-Related Peptide Release from Rat Trigeminal Neurons

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ABSTRACT

Calcitonin gene-related peptide (CGRP), a potent vasodilator, has been implicated in the pathogenesis of migraine. Its release from adult rat trigeminal neurons in culture was shown to be markedly increased by the activation of adenylate cyclase with forskolin. Modulation of this secretion was investigated by a number of agents with known inhibitory effects on cAMP generation mediated via receptor coupling to G_{i/o} proteins. Significantly, forskolin-stimulated CGRP release could be closely correlated with the phosphorylation of the protein kinase A (PKA) substrate cyclic AMP response element-binding protein (CREB). Forskolin-stimulated CGRP release could be potently and effectively inhibited by the adenosine A₁ receptor-selective agonist GR79236X (pIC₅₀ = 7.7 ± 0.1, maximal inhibition 65 ± 2.5% at 300 nM), whereas the A_{2A} (CGS21680) and the A₃ (2-chloro-N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide) receptor-selective agonists were without effect. GR79236X-mediated inhibition

was abolished by the A₁ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine. Immunocytochemical studies and Western analysis revealed the presence of adenosine A₁ receptors on trigeminal neurons. However, despite the additional detection of 5-hydroxytryptamine (5-HT)_{1B} receptors on these cells, the clinically effective antimigraine 5-HT_{1B/1D} agonist sumatriptan did not inhibit forskolin-stimulated CGRP release nor did it show any effect on the concomitant CREB phosphorylation. In contrast, the μ -opioid agonist fentanyl elicited a 74 ± 4% reduction in CGRP levels. Forskolin-stimulated CGRP release and CREB phosphorylation could be mimicked by incubation of the cells with chlorophenylthio-cAMP and blocked by pretreatment with the PKA inhibitor myrPKI₁₄₋₂₂. Taken together, the present data confirm the PKA-dependence of forskolin-stimulated CGRP release and suggest that A₁ adenosine agonists may warrant further investigation in models of migraine and neurogenic inflammation.

The mechanisms involved in the pathogenesis of migraine implicate a major role for calcitonin gene-related peptide (CGRP) through its localization within trigeminal afferents that innervate cerebral and dural vessels (Edvinsson et al., 1987). Activation of these afferents can cause the release of CGRP and Substance P, resulting in vasodilatation, plasma protein extravasation, and mast cell degranulation. CGRP levels have been shown to be elevated in the jugular venous blood of migraineurs coincident with headache pain and these increases are reduced by the clinically effective antimigraine 5-HT_{1B/1D} agonist sumatriptan (Goadsby and Edvinsson, 1993; Humphrey and Goadsby, 1994). Although sumatriptan may act directly to constrict human cerebral arteries via 5-HT_{1B} receptors (Humphrey and Feniuk, 1991;

Nilsson et al., 1999), in experimental animals trigeminal ganglion stimulation leads to a release of CGRP into the cranial circulation that is reduced by sumatriptan (Goadsby and Edvinsson, 1993). Using rat trigeminal ganglion neurons in culture, Durham and Russo (1999) have recently provided evidence that sumatriptan may be able to act directly on 5-HT_{1B/1D} receptors on these neurons to inhibit CGRP release.

In addition to 5-HT, other receptor systems may have the potential to modulate neurogenic inflammation. Depending on the subtype activated, adenosine receptors have been shown to influence pain transmission both centrally and peripherally (Sawynok, 1998). At peripheral nerve terminals in rodents, A₁ receptor activation produces antinociception by decreasing cAMP in the nerve terminal, whereas A₂ receptor activation produces pronociceptive effects by increas-

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ABBREVIATIONS: CGRP, calcitonin gene-related peptide; 5-HT, 5-hydroxytryptamine; DRG, dorsal root ganglion; CREB, cAMP response element-binding protein; PKA, protein kinase A; CMF-Hanks, calcium-, magnesium-, and bicarbonate-free Hanks' balanced salt solution; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TBST, Tris-buffered saline/Tween 20; cpt-cAMP, chlorophenylthio-cAMP; 2-Cl-IB-MECA, 2-chloro-N⁶-(3-iodobenzyl)-adenosine-5'-N-methyluronamide.

ing cAMP levels (Taiwo and Levine, 1991). Adenosine A_3 receptor agonists are thought to produce peripheral nociception indirectly due to the release of 5-HT and histamine from mast cells (Sawynok et al., 1997). Significantly, adenosine A_1 receptors have been previously shown to inhibit CGRP release from capsaicin-sensitive sensory neurons in the spinal cord (Santicioli et al., 1993) and are present on mouse dorsal root ganglion (DRG) neurons in culture (MacDonald et al., 1986).

In the present study, we have used rat cultured trigeminal ganglion neurons as a model to investigate further the mechanisms of CGRP release and its potential modulation by adenosine agonists. Using receptor-selective agonists and antagonists we present evidence that adenosine A_1 receptors are present on trigeminal neurons in culture that can inhibit the release of CGRP from these cells. The release of CGRP in this study was evoked by the adenylate cyclase activator forskolin and one well studied target of the subsequently generated cAMP is the transcription factor cAMP response element-binding protein (CREB). Phosphorylation of serine-133 is a critical event in CREB activation by increasing its transactivation potential enabling the recruitment and binding to coactivators such as CREB-binding protein. Studies have identified protein kinase A (PKA) as the major physiological kinase responsible for serine-133 phosphorylation (Gonzalez and Montminy, 1989). We show here that the inhibition of CGRP release from trigeminal neurons correlates with the ability of an A_1 receptor agonist to attenuate the phosphorylation levels of CREB. This suggests that a possible mechanism for the inhibitory activity of A_1 receptors is via the suppression of cAMP generation and the subsequent decreased activity of PKA, which has been shown to control the fraction of synaptic vesicles available for release (Greengard et al., 1993).

Experimental Procedures

Cell Culture. Adult rat trigeminal ganglion neuron cultures were prepared as described previously (Eckert et al., 1997). Briefly, adult Wistar rats (150–250 g, either sex) were killed by CO_2 inhalation and decapitation. Trigeminal ganglia were dissected and placed in ice-cold sterile calcium-, magnesium-, and bicarbonate-free Hanks' balanced salt solution (CMF-Hanks'). Ganglia were chopped and incubated at 37°C for 20 min in 3 ml CMF-Hanks' containing 20 U/ml papain and gently mixed after 10 min. After centrifugation at 250g for 3 min and removal of supernatant, the tissue was incubated for a further 20 min in 3 ml CMF-Hanks' containing collagenase (0.3% w/v) and dispase II (0.4% w/v) at 37°C. The tissue was then pelleted at 250g for 3 min and resuspended in 3 ml of CMF-Hanks' plus 2 ml of L-15 media supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 5 mM Na^+ -HEPES, 5 mM D-glucose, 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 3 mg of deoxyribonuclease I. Individual cells were then dissociated by trituration through a graded series of fire-polished Pasteur pipettes. After centrifugation at 250g for 3 min, the resultant pellet was washed in culture media [Ham's F-12 (GlutaMAX-I) containing 10% (v/v) heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μ g/ml streptomycin]. Approximately 200 to 500 neurons were plated per well onto poly(D-lysine)- and laminin-precoated six-well plates (Eckert et al., 1997) in F-12 culture media supplemented with nerve growth factor (mNGF 2.5S, 50 ng/ml). Cells were maintained in a 5% CO_2 , humidified air atmosphere at 37°C. After 24 h, the culture medium was removed and replaced every other day with media containing cytosine- β -D-arabinofuranoside (20 μ M). After treatment of the cells for

4 to 5 days with cytosine- β -D-arabinofuranoside, the cultures were enriched with neurons (estimated to be >80%), based on visual morphological examination and expression of a neuron-specific 160-kDa neurofilament subunit protein.

Release of CGRP from Trigeminal Ganglion Neurons in Culture. After 4 to 6 days in culture, the medium was gently aspirated and the cells washed twice in 2 ml of prewarmed CGRP release buffer (Vasko et al., 1994) [22.5 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 3.3 mM D-glucose, 0.1% (w/v) bovine serum albumin, 0.003% (w/v) bacitracin, and 1 μ M phosphoramidon, pH 7.4 at 37°C]. Cells were incubated in 1 ml of release buffer for 30 min at 37°C in the absence or presence of forskolin alone (3 μ M) or forskolin in combination with test agonist. Vehicle controls (dimethyl sulfoxide, maximal concentration 0.01%) for basal incubations were routinely performed. In some experiments, cultures were preincubated with the adenosine A_1 antagonist DPCPX (100 nM), for 15 min before the forskolin stimulation period. After each incubation, 0.5 ml of release buffer was sampled for CGRP content and stored at -20°C for later analysis. CGRP content was determined using a commercial rat CGRP enzyme immunoassay kit (SPIbio, Massy, France) and quantified photometrically at 405 nm using a microplate reader (Packard SpectraCount). The antibody used in this assay is reported to be 100% cross-reactive between rat CGRP α - and β -forms and <0.01% cross-reactive for Substance P (SPIbio, commercial information) and the minimum assay detection limit was approximately 10 pg/ml. None of the compounds used in this study were found to nonspecifically react with the assay or interfere with CGRP standard values at the concentrations indicated.

Immunocytochemistry. Trigeminal neurons were grown for 4 to 6 days on glass coverslips pretreated with poly(D-lysine) and laminin (Eckert et al., 1997). Cells were washed in 0.1 M phosphate-buffered saline (PBS) and fixed for 15 min in 4% (w/v) paraformaldehyde in 0.1 M PBS. After washing three times with 0.1 M PBS, cells were blocked for 30 min with 10% (v/v) normal goat serum and 0.3% (v/v) Triton X-100 diluted in 0.1 M PBS. Cells were then rinsed with PBS before incubation for 48 h at 4°C with either the anti- A_1 receptor antibody (1:100) or the anti-5-HT_{1B} receptor antibody (1:2000) diluted in 0.1 M PBS containing 0.3% (v/v) Triton X-100 and 10% (v/v) normal goat serum. After three washes in PBS, cells were incubated for 90 min at room temperature with fluorescein isothiocyanate-conjugated anti-rabbit (for A_1 receptor localization) or anti-guinea pig IgG (for 5-HT_{1B}) secondary antibody at a concentration of 1:500 diluted in 0.1 M PBS. Cells were then washed in PBS, rinsed in demineralized water, air dried, and mounted using fluorescence mounting media (DAKO, Bucks, UK) before visualization under a fluorescent microscope (Nikon Optiphot-2). Negative control experiments were carried out in which the primary antibody was omitted.

Western Blotting. At the end of the CGRP release experiments, reactions were terminated by the removal of the remaining media and the addition of 150 μ l of 3 \times strength Laemmli sample buffer. After solubilization, the well contents were transferred to Eppendorf tubes and the wells were washed with 75 μ l of deionized water. Equivalent amounts of protein were electrophoretically resolved on 10% polyacrylamide gels. After electrophoretic transfer onto nitrocellulose (0.22 μ m) using a semidry blotter, the membrane was washed briefly in Tris-buffered saline (TBS) and saturated overnight in TBS supplemented with 0.1% (v/v) Tween 20 and 5% (w/v) dried milk. Antibodies recognizing the rat A_1 adenosine and the 5-HT_{1B} receptors were used at a maximal concentration of 1:100 and 1:25, respectively. For detection of the phosphorylated forms of CREB, the nitrocellulose membrane was incubated with a 1:800 dilution of the anti-phosphospecific antibodies. Antibodies independent of the phosphorylation state of CREB were also used at a 1:1000 dilution. All primary incubations were for 1 h at 22°C in TBS containing 0.1% (v/v) Tween 20 (TBST) followed by washing five times for 10 min each in TBST. Membranes were incubated for 1 h at 22°C with a 1:3000 dilution of the appropriate horseradish peroxidase-conjugated sec-

ondary antibody in TBST containing 5% (w/v) dried milk. Excess antibody was removed by washing as described above and immunocomplexes were visualized using enhanced chemiluminescence detection, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ). To confirm the specificity of the anti-A₁ and the 5-HT_{1B} receptor antibodies, preabsorption experiments were performed, whereby the maximal antibody concentrations were incubated overnight at 4°C in the presence of 200 µg of blocking peptide spotted onto nitrocellulose. The Western blots shown are representative of three separate experiments and each is taken from a single immunoblot.

Deglycosylation of Receptor Protein. *N*-glycosidase F is able to release all common classes of *N*-glycans from the protein backbone by converting asparagine to aspartic acid. A membrane fraction prepared from trigeminal ganglion neuronal cultures was resuspended at a concentration of 3 mg/ml in denaturation buffer (20 mM PBS, 1% SDS, and 1% β-mercaptoethanol, pH 8.6) containing the proteinase inhibitor aminoethylbenzylsulphonyl fluoride (1 mM). The sample was incubated for 3 min at 95°C, and diluted with an equal volume of reaction buffer (20 mM PBS, 10 mM EDTA, and 0.5% *n*-octylglycopyranoside, pH 7.2). The sample was divided into two, and 2.5 U of *N*-glycosidase F per 100 µg of membrane protein was added to one half. Samples were incubated for 2 h at 37°C and the reaction terminated by the addition of Laemmli sample buffer. Samples were analyzed on 10% polyacrylamide gels as described above.

Materials. Cell culture media supplies were purchased from Life Technologies (Paisley, Scotland, UK) and all cell culture plastic ware was from Corning Costar (High Wycombe, UK). Papain and collagenase (type 2) were obtained from Worthington (Reading, UK). Dispase II and recombinant *N*-glycosidase F, tested to be free of contaminating protease, exo- and endoglycosidase activities, were obtained from Roche (Lewes, UK). Poly(D-lysine) (mol. wt. = 150,000–300,000), murine Engelbreth-Holm-Swarm laminin, bovine pancreas crude deoxyribonuclease I, bovine serum albumin (fraction V, protease-free), forskolin, 1,9-dideoxyforskolin, and fentanyl citrate were purchased from RBI/Sigma (Poole, UK). Adenosine 8-(4-chlorophenylthio)-3',5'-cyclic monophosphate (cpt-cAMP) and myristoylated protein kinase A inhibitor 14-22 amide (*myr*PKI₁₄₋₂₂) were from Calbiochem (Nottingham, UK). The anti-rat A₁ adenosine receptor, anti-rat 5-HT_{1B} receptor, and anti-CREB antibodies were obtained from Sigma/RBI, Chemicon (Harrow, UK), and New England Biolabs (Beverly, MA), respectively. The peptides used in the antibody blocking experiments were synthesized by Babraham Technix (Cambridge, UK). Nerve growth factor (mNGF 2.5S) was purchased from Alomone Labs (Botolph Claydon, UK). GR79236X (*N*-[(1*S*,*trans*)-2-hydroxycyclopentyl]adenosine) and sumatriptan (3-[2-dimethylaminoethyl]-*N*-methylindole-5-methanesulfonamide) were synthesized by GlaxoSmithKline Research (Stevenage, UK). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), CGS21680 [2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride], 2-chloro-*N*⁶-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide (2-Cl-IB-MECA), and CP93129 dihydrochloride [1,4-dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5*H*-pyrrolo[3,2-*b*]pyridin-5-one)] were obtained from Tocris Cookson (Bristol, UK). Normal goat serum was obtained from Vector (Peterborough, UK). Stock solutions of 2-Cl-IB-MECA, CGS21680, forskolin, and 1,9-dideoxyforskolin were prepared in dimethyl sulfoxide at 10⁻² M. GR79236X, sumatriptan, fentanyl, and CP93129 were prepared in water at 10⁻³ M and serially diluted in release buffer. DPCPX was prepared in ethanol at 10⁻³ M and diluted directly to 10⁻⁶ M in release buffer.

Data Analysis. CGRP release is expressed in picograms milliliter⁻¹ of release buffer obtained after a 30-min incubation period. In some experiments, to account for the variation in the number of neurons and baseline CGRP levels between individual preparations, agonist effects are expressed as a percentage of forskolin (3 µM)-stimulated CGRP release. Results are given as percentages (means ± S.E.M.) relative to basal (0%) and forskolin (3 µM)-stim-

ulated (100%) CGRP release. The concentration-effect curve was fitted to a sigmoidal model by nonlinear regression using GraphPad Prism 3.0 and a mean pIC₅₀ value generated. Statistical levels of significance were calculated (*P* < 0.05) using Student's *t* test.

Results

Expression of Adenosine A₁ and 5-HT_{1B} Receptors in Cultured Trigeminal Ganglion Neurons. Using immunocytochemical and immunoblotting approaches the expression of adenosine A₁ and 5-HT_{1B} receptors was investigated in 5-day trigeminal neuronal cultures. As shown in Fig. 1A, adenosine A₁ receptor immunoreactivity was detected in a large proportion of cells exhibiting a neuronal morphology. Western analysis of whole cell lysates using this antibody revealed a major immunoreactive product with an apparent molecular mass of 38–40 kDa as well as several other species of greater size (Fig. 1B). Abundant immunoreactivity was also evident in the trigeminal cultures using an antibody directed to the third intracytoplasmic loop of the rat 5-HT_{1B} receptor, which was localized to neuronal cell bodies (Fig. 1C). A concentration-dependent immunoreactive pattern was again obtained after Western analysis of whole cell extracts, with bands detected of apparent molecular masses of 60 and 120 kDa (Fig. 1D). The specificity of the anti-A₁ and 5-HT_{1B} receptor antibodies was established by preincubating with the peptides to which the antibodies had been raised (QPK-PPIDEDLPPEEKAKED for A₁ and VPSESGSPVYVNVQVK for 5-HT_{1B}, single letter code). Preabsorption of the anti-A₁ receptor antibody to the A₁ peptide (200 µg) resulted in an absence of any detectable immunoreactivity. In contrast, incubation of this antibody with the 5-HT_{1B} peptide produced a similar banding pattern to that obtained using the anti-A₁ receptor antibody in the absence of peptide (Fig. 1, B and E). Similarly, the immunoreactivity detected by the anti-5-HT_{1B} receptor antibody was abolished by incubating with the 5-HT_{1B} peptide but unaffected by the presence of the A₁ peptide (Fig. 1, C and E).

The seemingly high molecular masses for some of the immunoreactive products detected by the anti-A₁ and 5-HT_{1B} receptor antibodies, as determined by SDS-polyacrylamide gel electrophoresis, could be a consequence of the retardation of these protein species by the covalent attachment of carbohydrate or phosphate moieties. To substantiate the effect of *N*-linked glycan chains on the electrophoretic mobility of the adenosine A₁ and the 5HT_{1B} receptors, extracts prepared from the trigeminal ganglion neuronal cultures were deglycosylated by incubating with the amidase *N*-glycosidase F. This treatment resulted in a molecular mass shift of the immunoreactive bands obtained with both anti-receptor antibodies to that of a main product of approximately 40 kDa, consistent with the predicted size of the adenosine A₁ and 5-HT_{1B} receptors from their primary structures (Fig. 1E).

Calcitonin Gene-Related Peptide Release Induced by Forskolin. In initial experiments, the effect of the adenylate cyclase activator forskolin on the release of CGRP from trigeminal neurons in culture was investigated. Incubation of neurons in release buffer for 30 min resulted in a resting CGRP concentration of 52 ± 7 pg/ml (*n* = 17 independent culture preparations). A concentration-dependent increase in CGRP secretion was observed with forskolin (data not shown) and in all further experiments a forskolin

concentration producing an approximately EC_{90} response ($3 \mu M$) was used. Under these conditions, exposure of the cells to forskolin ($3 \mu M$), elevated CGRP levels to 260 ± 29 pg/ml, representing a $420 \pm 47\%$ increase. The biologically inactive analog of forskolin, 1,9-dideoxyforskolin ($3 \mu M$) (Laurenza et al., 1989), under identical conditions, did not significantly alter CGRP levels (76 ± 12 pg/ml, $n = 3$) compared with basal. Depolarization of the neurons (equimolar substitution

of NaCl for 60 mM KCl in the release buffer) also raised CGRP levels from 36 ± 4 to 398 ± 64 pg/ml ($n = 4$).

Effect of GR79236X on Forskolin-Stimulated CGRP Release. The selective A_1 adenosine agonist GR79236X (Gurden et al., 1993; Knutsen et al., 1999; M. J. Sheehan, unpublished data) potently inhibited forskolin ($3 \mu M$)-stimulated CGRP release from rat trigeminal ganglion neurons in a concentration-dependent manner (Fig. 2). The pIC_{50} value

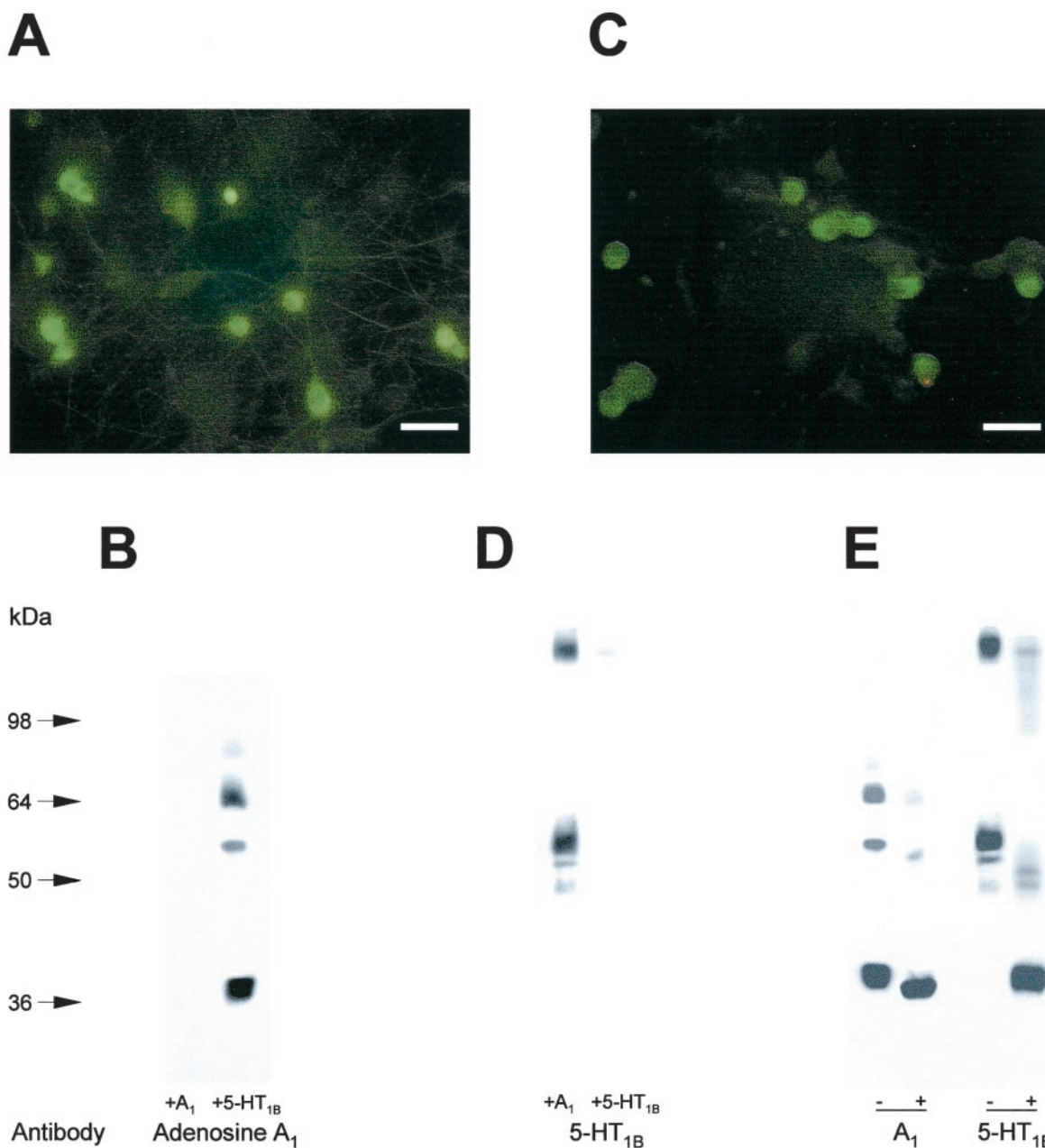


Fig. 1. Detection of adenosine A_1 and 5-HT $_{1B}$ receptor expression in rat trigeminal ganglion neurons. Immunocytochemical localization of the adenosine A_1 receptor using a COOH-terminal directed receptor antibody (1:100 dilution; scale bar, $50 \mu m$) (A) and of the 5-HT $_{1B}$ receptor using an antibody directed to the third intracytoplasmic loop (1:2000 dilution; scale bar, $50 \mu m$) (C). Preparations incubated without primary antibody showed no detectable staining (data not shown). Whole cell protein extracts prepared from trigeminal ganglion neuronal cultures were electrophoretically separated on 10% polyacrylamide gels. After transfer onto nitrocellulose, the membranes were probed either with the anti-adenosine A_1 receptor antibody (1:100 dilution) (B) or with the antibody directed against the 5-HT $_{1B}$ receptor (1:25 dilution) (D). The antibodies had been previously incubated in the presence of the synthetic peptides to which they were raised. The lanes labeled +A $_1$ and +5-HT $_{1B}$ show the immunoreactivity obtained after preincubation of the anti-receptor antibodies with the A $_1$ and 5-HT $_{1B}$ peptides, respectively. E, protein extracts prepared from trigeminal ganglion neuronal cultures were incubated in the presence (+) or absence (-) of the amidase N-glycosidase F, and electrophoretically separated on 10% polyacrylamide gels. The transferred protein was analyzed by the anti-adenosine A_1 receptor antibody (A $_1$, 1:100 dilution) or with the antibody directed against the 5-HT $_{1B}$ receptor (5-HT $_{1B}$, 1:25 dilution). The electrophoretic mobilities of marker proteins are also shown (arrows). The data are representative of at least three separate experiments.

for this effect was 7.7 ± 0.1 with a maximal inhibition of $65 \pm 2.5\%$ observed in the presence of 300 nM GR79236X. To test the specificity of the GR79236X response, its inhibitory activity on CGRP release was investigated in the presence of the potent and selective A_1 receptor antagonist DPCPX (Bruns et al., 1987). Incubation of the cultures with DPCPX (100 nM) 15 min before and during the 30-min stimulation period did not alter forskolin (3 μ M)-stimulated CGRP levels but abolished the GR79236X-mediated (100 nM) inhibition of release (Fig. 3A). GR79236X (100 nM) and DPCPX (100 nM) had no significant effect on basal levels of CGRP secretion (data not shown).

Effect of GR79236X on the Phosphorylation of CREB.

The relationship between the cAMP-elevating activity of forskolin and CGRP release was further investigated by monitoring the phosphorylation of the PKA substrate CREB. Using a phospho-specific antibody (Ser-133), changes in the phosphorylation status of CREB could be detected even in the small number of neurons (200–500) used in the CGRP release studies. Over the time course of the experiments there was no detectable change in the expression of CREB protein (data not shown), which remained unaffected by the application of all drug treatments (Fig. 3B). However, forskolin (3 μ M) evoked a marked increase in the phosphorylation of CREB, which was attenuated by GR79236X (100 nM) (Fig. 3B). GR79236X had no observable effect on basal levels of CREB phosphorylation (data not shown). Consistent with parallel observations made in the CGRP release experiments, DPCPX (100 nM) effectively reversed the inhibition of CREB phosphorylation by GR79236X (Fig. 3B), but had no effect on basal levels of phosphorylation or on the increase evoked by forskolin treatment (data not shown).

Effects of CGS21680, 2-CI-IB-MECA, Sumatriptan, and Fentanyl on Forskolin-Stimulated CGRP Release and CREB Phosphorylation. In contrast to GR79236X,

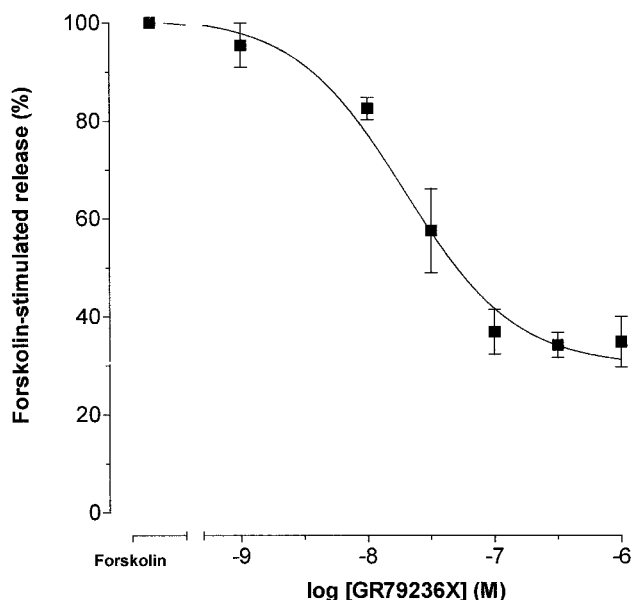


Fig. 2. Concentration dependence of GR79236X-mediated inhibition of forskolin-stimulated CGRP release from adult rat trigeminal ganglion neurons. Cells after 4 to 6 days in culture were incubated in the presence of forskolin (3 μ M) and the indicated concentration of agonist for 30 min. Values are expressed as the percentage of forskolin-induced CGRP release and are means \pm S.E.M. of three to six separate experiments.

the selective A_{2A} (1 μ M) and A_3 (1 μ M) receptor agonists CGS21680 (Hutchison et al., 1990) and 2-CI-IB-MECA (Kim et al., 1994), respectively, had no effect on the CGRP secretion induced by forskolin (Table 1). Moreover, despite the presence of 5-HT_{1B} receptor-like immunoreactivity on trigeminal neurons (Fig. 1B), the 5-HT_{1B/1D} agonist sumatriptan (1 μ M) also failed to significantly modify forskolin-stimulated release (Fig. 4A). Higher concentrations of sumatriptan (50 μ M) similarly had no effect (data not

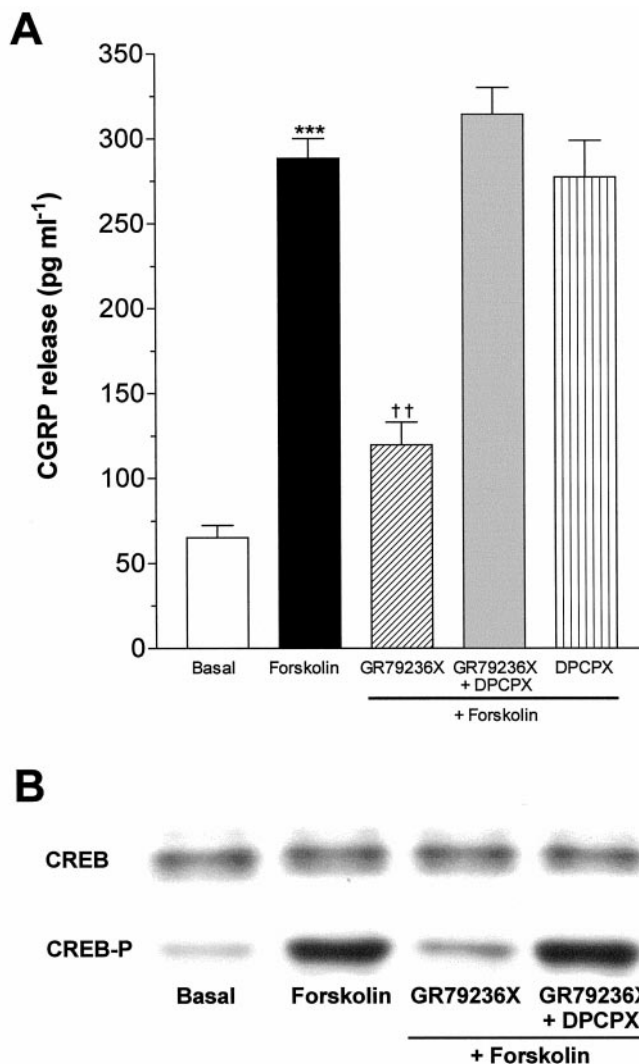


Fig. 3. Effect of the A_1 receptor agonist GR79236X and antagonist DPCPX on forskolin-stimulated CGRP release and CREB phosphorylation. **A**, CGRP release (pg/ml) was determined for rat trigeminal ganglion neurons (4–6 days in culture) over a 30-min incubation period in release buffer (basal; □), forskolin (3 μ M; ■) or forskolin in the presence of GR79236X (100 nM; ▨). Neuronal cultures were also preincubated with DPCPX (100 nM) for 15 min before stimulation with forskolin (▩) as well as in the presence of GR79236X (▨). Data are means \pm S.E.M. of three to four independent culture preparations. ***, significantly different from basal ($P < 0.001$); **, significantly different from the release observed in the presence of forskolin alone ($P < 0.01$). **B**, Western analysis was performed using samples prepared from whole cell extracts of the trigeminal cultures incubated for 30 min in release buffer (basal), forskolin (3 μ M), or forskolin in the presence of GR79236X (100 nM) with and without a 15-min preincubation with DPCPX (100 nM). Detection was made with an anti-CREB antibody (CREB) to substantiate consistency of protein loading and with a phospho-specific CREB antibody (CREB-P). The Western blot shown is taken from a single immunoblot and is representative of three separate experiments.

shown). In marked contrast, however, the μ -opioid-selective agonist fentanyl (100 nM) produced a $74 \pm 4\%$ reduction in the forskolin-stimulated CGRP levels (Fig. 4A; Table 1). None of the drugs had any effect on basal CGRP secretion (data not shown). The effects on CGRP release were closely mirrored by concomitant changes in the phosphorylation of CREB (Fig. 4B). Thus, with the notable exception of fentanyl, CGS21680, 2-CI-IB-MECA, and sumatriptan had no effect on forskolin-induced CREB phosphorylation. None of the treatments had any effect on the expression levels of CREB (Fig. 4B) or on basal levels of CREB phosphorylation (data not shown).

Effect of the Myristoylated PKI₁₄₋₂₂ Peptide on Forskolin-Stimulated CGRP Release and the Phosphorylation of CREB. A 1-h pretreatment of cultures with the myristoylated peptide *myr*PKI₁₄₋₂₂ (100 μ M; Harris et al., 1997) effectively blocked forskolin (3 μ M)-stimulated CGRP release (Fig. 5A) and CREB phosphorylation (Fig. 5B). Furthermore, incubation of the cells with the cell-permeable cAMP analog cpt-cAMP (1 mM; Xu et al., 1989) significantly increased CGRP secretion and CREB phosphorylation (Fig. 5, A and B). These treatments had no effect on the expression levels of CREB (Fig. 5B) at the time point investigated.

Discussion

Despite considerable debate on the pathophysiology of migraine, there is general agreement that the trigeminal innervation of the cerebral circulation forms the basis of not only the afferent neuronal pathway but also of an efferent neurogenic component. In the present study, adult rat trigeminal ganglion neurons in culture were used to compare a number of pharmacological agents on CGRP release without the complicating influences of other neuronal and non-neuronal factors. Using immunocytochemical, immunoblotting, and functional approaches, we have demonstrated that adenosine A₁ receptors are present in cultured rat trigeminal neurons, in accord with the recently demonstrated localization of A₁ receptors in human trigeminal ganglia (Schindler et al., 2001). In addition, a large number of 5-HT_{1B}-like immunoreactive neurons was identified in trigeminal ganglion cultures in agreement with previous studies conducted in human, guinea pig, and rat trigeminal preparations (Longmore et al., 1997; Bonaventure et al., 1998; Wotherspoon and Priestley, 1999). The presence of these receptors is also similar to that

reported in cultured rat DRG neurons where multiple 5-HT receptor subtypes were identified by radioligand binding with 5-HT_{1B} receptors representing 60% of the total binding sites (Chen et al., 1998).

An important finding of this study is that activation of the cAMP transduction pathway can stimulate the release of CGRP from these neurons. In the absence of phosphodiesterase inhibition, forskolin but not 1,9-dideoxyforskolin, stimulated CGRP release, suggesting that its effects are due to its ability to elevate intracellular cAMP (Laurenza et al., 1989). Furthermore, forskolin-stimulated CGRP release was associated with a marked phosphorylation of CREB, a transcription factor that is phosphorylated by the catalytic subunit of PKA (Gonzalez and Montminy, 1989). The effect of forskolin on CGRP release and CREB phosphorylation was mimicked by the cell permeable cAMP analog cpt-cAMP and impor-

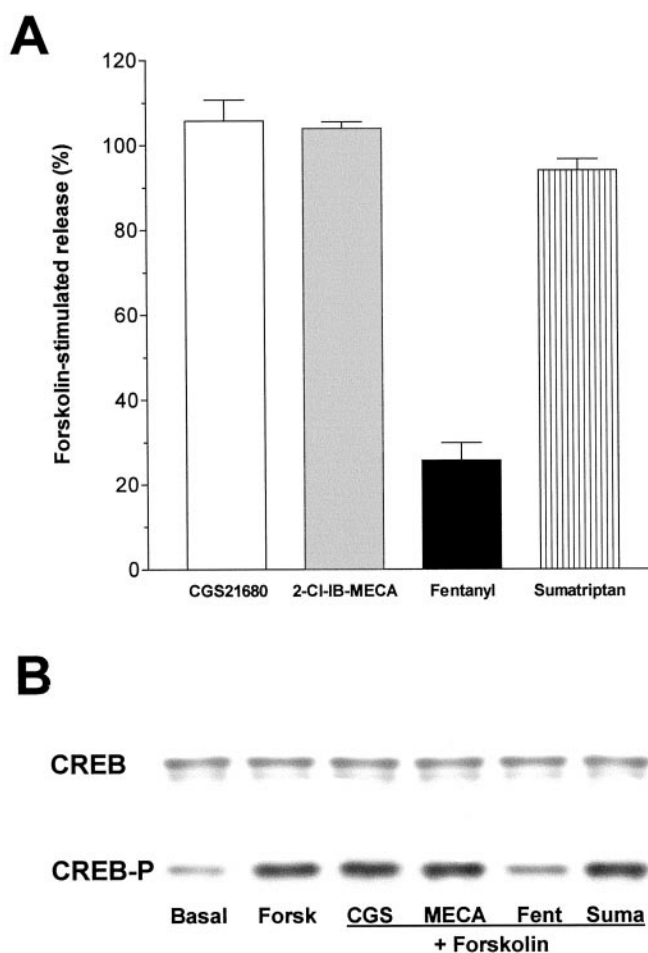


Fig. 4. Effect of CGS21680, 2-CI-IB-MECA, sumatriptan, and fentanyl on forskolin-stimulated CGRP release and CREB phosphorylation. A, rat trigeminal ganglion neurons (4–6 days in culture) were incubated with CGS21680 (1 μ M), 2-CI-IB-MECA (1 μ M), fentanyl (100 nM), or sumatriptan (1 μ M) in the presence of forskolin (3 μ M) for 30 min. Data are means \pm S.E.M. of three to four independent culture preparations and expressed as a percentage of the forskolin-stimulated release. B, Western analysis was performed using samples prepared from whole cell extracts of the trigeminal cultures at the end of the CGRP release experiments. The levels detected after incubation with release buffer (basal) or forskolin (3 μ M; Forsk) for 30 min are also shown. Detection was made with an anti-CREB antibody (CREB) to substantiate consistency of protein loading and with a phospho-specific CREB antibody (CREB-P). The Western blot shown is taken from a single immunoblot and is representative of three separate experiments.

TABLE 1

Modulation of CGRP-release from adult rat trigeminal ganglion neurons

The CGRP concentration (mean \pm S.E.M.; pg ml⁻¹) in the culture medium of trigeminal ganglion neurons was determined after a 30-min incubation period in the presence of forskolin (3 μ M) with or without the following agents: GR79236X (100 nM), CGS21680 (1 μ M), 2-CI-IB-MECA (1 μ M), sumatriptan (1 μ M), or fentanyl (100 nM). Basal values represent the CGRP secretion obtained after treatment with the appropriate vehicle control. None of the treatments had any significant effect on basal levels of CGRP secretion (data not shown).

Treatment	Basal	Forskolin	Forskolin + Treatment	n
GR79236X	65 \pm 7	288 \pm 12 ^a	139 \pm 23 ^b	4
CGS21680	53 \pm 12	232 \pm 18 ^a	238 \pm 14	3
2-CI-IB-MECA	37 \pm 10	188 \pm 35 ^a	195 \pm 37	4
Sumatriptan	37 \pm 10	193 \pm 33 ^a	185 \pm 34	4
Fentanyl	45 \pm 10	219 \pm 36 ^a	91 \pm 18 ^b	4

^a For every preparation of trigeminal primary cultures, forskolin induced a significant increase in CGRP release over basal ($P < 0.001$).

^b Significantly different from that incubated with forskolin alone ($P < 0.01$).

tantly, the forskolin-mediated events could be inhibited by pretreatment of the cultures with the selective and cell-permeant PKA inhibitor *myr*-PKI₁₄₋₂₂. These data suggest that forskolin-stimulated CGRP release is largely PKA-dependent. These observations differ slightly from studies made in rat DRG cultures in which forskolin was shown to increase intracellular cAMP levels but had no effect on resting CGRP or Substance P release, although subsequent responses to bradykinin and capsaicin were sensitized by cAMP-elevating agents (Hingtgen et al., 1995). However, Hingtgen and Vasko (1994) have reported that prostacyclin receptor agonists can stimulate resting CGRP and Substance P release from cultured DRG neurons, although it is not clear from this study whether these increases were mediated by raised cAMP levels (Smith et al., 1998). Clearly, further work is required to test whether these differences are inherent to

trigeminal ganglion neurons compared with DRG neuronal cultures and whether the source of the tissue (i.e., embryonic, neonatal, or adult) has any bearing on the functional outcome.

With regard to this and previous work, elevated PKA activity could increase resting or augment evoked release of neuropeptides in a number of ways. PKA activity has been shown to modulate multiple ion channels (e.g., potassium currents), to enhance excitability of sensory neurons (Evans et al., 1999), and also to phosphorylate synaptic proteins such as synapsin I, which controls the fraction of synaptic vesicles available for release, ultimately increasing secretion (Greengard et al., 1993). In this report we have made use of the phosphorylation status of CREB to determine changes in the activity of PKA. Phosphorylation of CREB at Ser-133, a key regulatory site controlling its transcriptional activity (Gonzalez and Montminy, 1989), has been shown to be a major substrate for the catalytic subunits of PKA. In addition, phosphorylation at Ser-133 has also been shown to occur via the p44 and p42 forms of mitogen-activated protein kinase. It is unlikely that the mitogen-activated protein kinases are having any effective contribution to the observed changes in CREB phosphorylation determined in this study, because the phosphorylation of these kinase themselves remained unaffected by the application of forskolin (L. A. Sellers, unpublished observations). Although the cellular mechanisms mediating the facilitation of transmitter release from mammalian sensory neurons have not been elucidated, increasing evidence suggests that protein phosphorylation plays an important role (Greengard et al., 1993). In addition to PKA-mediated CGRP release, stimulation of protein kinase C has also been shown to induce the release of neuroactive peptides. Because the resting levels of CGRP release from the trigeminal neurons were unaffected by the application of the PKA inhibitor, an alternative mechanism such as protein kinase C activation is presumably responsible for basal secretion.

Another intriguing finding of this study is that GR79236X potently and effectively inhibited forskolin-stimulated CGRP release from trigeminal ganglion neurons, suggesting that A₁ receptors are present on a large proportion of CGRP-positive neurons. GR79236X is a highly selective A₁ agonist (A₁ versus A₂ selectivity ~1400-fold; Gurden et al., 1993; human A₁ versus A_{2A}, A_{2B}, and A₃ receptors >116-fold, M. J. Sheehan, unpublished observations) and its effects in this study seem to be exclusively mediated through the A₁ receptor as evidenced by the DPCPX blockade and the lack of effect of the A_{2A} and A₃ agonists CGS21680 and 2-Cl-IB-MECA, respectively. Further evidence that A₁ receptors can mediate release was shown by the ability of N⁶-cyclopentyladenosine to inhibit CGRP secretion (53% at 300 nM, *n* = 2). DPCPX did not alter responses to forskolin, suggesting that endogenous adenosine release contributes little, if at all, to resting or forskolin-stimulated CGRP levels measured under these experimental conditions. Furthermore, the effects of GR79236X on CGRP release could also be closely correlated with CREB phosphorylation, consistent with an interaction of GR79236X with adenosine A₁ receptors coupled negatively to adenylate cyclase activity.

The lack of effect of sumatriptan on CGRP release was surprising. In the present study, neither sumatriptan nor the potent and selective 5-HT_{1B} agonist CP93129 (Martin and

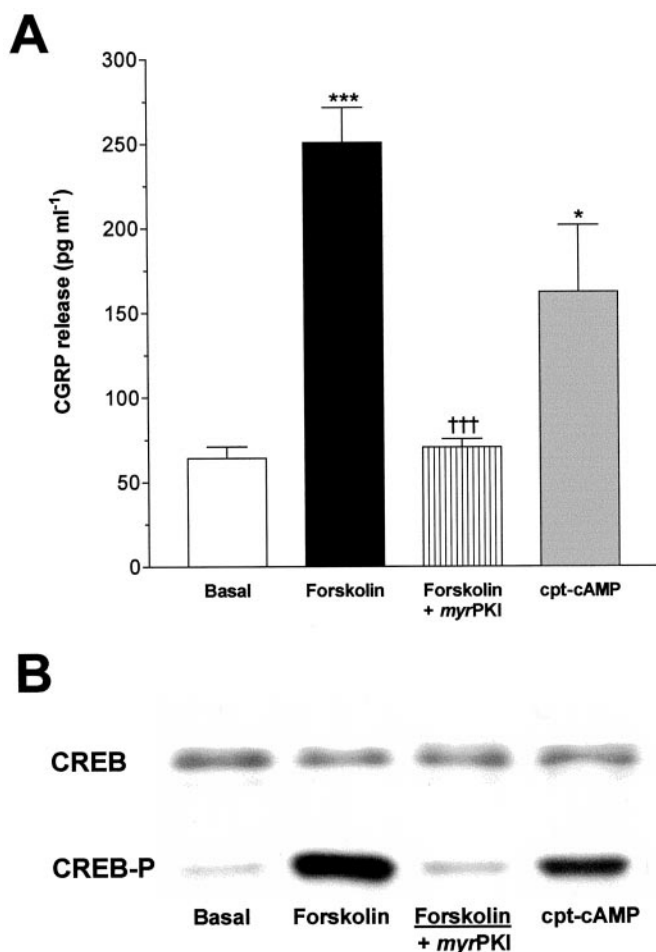


Fig. 5. Effect of the myristolated peptide PKA inhibitor *myr*-PKI₁₄₋₂₂ on forskolin-stimulated CGRP release and CREB phosphorylation. **A**, rat trigeminal ganglion neurons (4–6 days in culture) were incubated for 30 min in release buffer (basal; □), forskolin (3 μ M; ■), or cpt-cAMP (1 mM; ▤). Neurons were also preincubated for 1 h with *myr*-PKI₁₄₋₂₂ (100 μ M; ▨) before forskolin stimulation. Data are means \pm S.E.M. of three to four independent culture preparations. ***, significantly different from basal ($P < 0.001$); * $P < 0.05$; †††, significantly different from the release observed in the presence of forskolin alone ($P < 0.001$). **B**, Western analysis was performed using samples prepared from whole cell extracts of the trigeminal cultures at the end of the CGRP release experiments. Detection was made with an anti-CREB antibody (CREB) to substantiate consistency of protein loading and with a phospho-specific CREB antibody (CREB-P). The Western blot shown is taken from a single immunoblot and is representative of three separate experiments.

Humphrey, 1994) reduced forskolin-stimulated CGRP release, despite the evidence of 5-HT_{1B}-like immunoreactivity on these neurons. In addition, sumatriptan did not inhibit forskolin-induced increases in the phosphorylation status of CREB, suggesting that 5-HT_{1B} receptors do not mediate a decrease in cAMP levels in these cells, a function normally associated with this and other G_{i/o} protein-linked receptors. Recent data also show that the 5-HT_{1B} receptor can mediate a weak, pertussis toxin-resistant stimulation of inositol phosphate, which is markedly amplified on coexpression of recombinant G_{α15} subunits (Wurch and Pauwels, 2000), suggesting this receptor can couple to multiple G proteins. Recently, Durham and Russo (1999) demonstrated that sumatriptan could inhibit depolarization (60 mM K⁺)-induced CGRP release by approximately 70% from rat neonatal trigeminal neurons. Consistent with the present study, sumatriptan was found not to reduce forskolin-stimulated cAMP levels, but rather mediated a slow and prolonged increase in intracellular calcium, hypothesized to inhibit CGRP release by the activation of calcium-dependent phosphatases (Durham and Russo, 1999). To date, however, we have found no evidence of a sumatriptan-mediated inhibition of depolarization-evoked release in trigeminal ganglion cultures derived from both neonatal and adult animals. In this regard, it bears emphasis that agonists that are effective against forskolin-stimulated CGRP release; i.e., GR79236X and fentanyl seem to be ineffective against depolarization (60 mM K⁺)-evoked release (our unpublished observations).

The effectiveness of fentanyl to inhibit forskolin-stimulated CGRP release confirms the presence of functional μ-opioid receptors on CGRP-positive neurons. In addition, the fact that opioid receptors mediate their effects via G_{i/o} proteins is consistent with our contention that CGRP release from the trigeminal neuronal cultures is linked to increases in cAMP levels. Our findings are also consistent with previous observations that low concentrations of morphine have been shown to inhibit the release of Substance P from rat trigeminal nucleus slices (Suarez-Roca et al., 1992). Furthermore, equilibrium binding studies of [³H]diprenorphine on membranes from rat DRG neurons grown in culture for 13 to 15 days have demonstrated high levels ($B_{\max} = \sim 1300$ fmol mg of protein⁻¹) of binding sites, where μ-, κ-, and δ-opioid receptor subtypes represented 70, 10, and 5%, respectively, of specific binding (Chen et al., 1997).

In conclusion, we have shown that activation of PKA by cAMP-elevating agents results in CGRP release from trigeminal neurons, an observation that may suggest a role for PKA activity in migraine and persistent pain states in general (Aley and Levine, 1999). Significantly, we have identified functional A₁ adenosine receptors on CGRP-positive neurons that can inhibit this release. Adenosine receptors represent significant potential for development as therapeutic targets for pain (Kaiser and Quinn, 1999), so these observations warrant further investigation in animal models relevant to migraine and humans. Indeed, preliminary observations have demonstrated that GR79236X can inhibit evoked firing of the trigeminal nucleus caudalis neurons in the anesthetized rat (Bland-Ward et al., 2000) and reduce neurogenic CGRP release in vivo (Honey et al., 2000), suggesting that A₁ receptors may have potential utility in migraine and neurogenic inflammation in general.

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