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Coupling of I_1-Imidazoline Receptors to Diacylglyceride Accumulation in PC12 Rat Pheochromocytoma Cells

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SUMMARY

The I_1-subtype of imidazoline binding sites has been characterized concerning binding specificity and tissue localization, and several physiological functions have been ascribed to it. However, the signaling pathways coupled to this putative receptor are not known. Pheochromocytoma PC12 cells express I_1-imidazoline binding sites in plasma membrane and lack α_2-adrenergic receptors, which recognize many I_1-imidazoline ligands. In this cellular model, diacylglycerol (DAG), a second messenger, is generated in response to the putative I_1-imidazoline agonist moxonidine. Using radioflux with [^{3}H]myristate and direct measurements of DAG mass, we showed a rapid and transient peak of DAG in undifferentiated PC12 cells within the first 1 min of agonist exposure. In PC12 cells treated with nerve growth factor to initiate differentiation, DAG accumulation at 15 sec was facilitated, and the increase in DAG mass persisted throughout 10 min of agonist treatment. Efaroxan, a putative I_1-antagonist, attenuated the effect of moxonidine on DAG accumulation in nerve growth factor-treated cells, as did D609, an inhibitor of phosphatidylcholine-selective phospholipase C. Phospholipase D did not seem to be involved in generation of DAG in response to I_1-receptor activation, nor was there accumulation of phosphatidic acid. These findings suggest coupling of I_1-imidazoline receptors to a phospholipase C to generate DAG as a second messenger, a process regulated by neuronal differentiation and possibly participating in the physiological responses to I_1-imidazoline receptor activation.

High affinity binding sites for imidazolines distinct from the α-adrenergic subtypes were first characterized in 1987 (1–3). The existence of two major subtypes has been established (4, 5). The I_2-imidazoline binding site has been purified, sequenced, and cloned and shown to be identical to monoamine oxidase (6). The I_1 site can be distinguished from the I_2-subtype by ligand specificity, cellular, and tissue localization (7) and by functional roles. Some of the physiological actions linked to putative I_1-imidazoline receptors are decreased blood pressure mediated within the rostral ventrolateral medulla oblongata (8, 9), decreased ocular pressure (10), increased sodium excretion from the kidney (11), induction in adrenomedullary chromaffin cells of mRNA for phenylethanolamine *N*-methyltransferase, the synthetic enzyme for epinephrine (12), and decreased catecholamine release from chromaffin cells (13). Moreover, an endogenous ligand binding to I_1 sites has been identified as agmatine, a decarboxylation product of arginine (14).

One criterion for a binding site to be recognized as a receptor is identification of signal transduction mechanisms coupled to activation of a putative receptor. Thus far, the evidence obtained concerning the coupling of I_1-imidazoline binding sites to transmembrane signaling pathways is largely negative. In adrenomedullary chromaffin cells and tracheal epithelial cells, clonidine in concentrations of $\leq$0.1 mM had no effect on inositol phosphate accumulation or on basal or forskolin-stimulated cAMP levels (15, 16). Moxonidine and cimetidine were also inactive (16). Clonidine increased cGMP in chromaffin cells, but this effect required a 100 μM concentration, and other I_1 agonists were inactive (15, 17), implicating a nonreceptor-mediated process. Clonidine increases the influx of ^{45}Ca but only after the cells have been preloaded with the isotope and only in concentrations of >10 μM (15). Moreover, clonidine does not affect the levels of intracellular calcium in the absence of nicotine (18). Thus, stimulation of I_1 receptors has no effect on cyclic nucleotides, inositol phosphates, or calcium influx.

Several preliminary reports have suggested that I_1-imidazoline receptors may couple to phospholipid metabolism. DAG accumulates in response to short term exposure to the selective I_1 agonist moxonidine in tracheal epithelial cells (19). Moreover, we used PC12 pheochromocytoma cells, an adrenomedullary tumor cell line, to show that I_1-imidazoline receptors are coupled to prostaglandin E_2 accumulation (7) and arachidonic acid release, the rate-limiting step in pro-

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ABBREVIATIONS: DAG, diacylglycerol; NGF, nerve growth factor; D609, tricyclodecan-9-yl-xanthate potassium salt; PMA, phorbol-12-myristate-13-acetate; TLC, thin layer chromatography; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

duction of prostaglandins (20). Due to their ability to adopt a neuron-like phenotype after treatment with NGF, these cells are used as a cellular model for both adrenomedullary chromaffin cells and catecholaminergic neurons. They lack α_2-adrenergic receptors (7), have I_1-imidazoline binding sites that are localized primarily to plasma membrane fractions (7, 21), and show increased I_1-receptor density when differentiated to a neuronal phenotype by NGF (5, 7).

In the present study, we sought to further characterize plasma membrane I_1-imidazoline binding sites in this cell model and to establish whether DAG, an intracellular second messenger, is generated in response to I_1 receptor activation in these cells.

Experimental Procedures

Materials. RPMI medium and horse serum were obtained from GIBCO (Gaithersburg, MD). Fetal bovine serum, rat tail collagen type I, and mouse 7S NGF were obtained from Upstate Biotechnology (Lake Placid, NY), and bovine serum albumin was obtained from Intergen (Purchase, NY). Moxonidine was kindly provided by Kali-Chemie (Hannover, Germany), BDF-6143 by Beiersdorf-Lilly (Hamburg, Germany), and efaroxan was purchased from Research Biochemicals (Natick, MA). Stock solutions of these imidazolines were made in 0.01 M acetic acid up to 1 week before use. [9,10-^{3}H]Myristic acid (40 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO), and [γ-^{32}P]ATP (4500 Ci/mmol) was from ICN (Irvine, CA). [^{125}I]*p*-Iodoclonidine (2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA), stored at −70° in ethanol, and diluted in water before assays. Octylglucoside was from Boehringer (Mannheim, Germany), and *sn*-1,2-DAG kinase from *Escherichia coli* was obtained from Calbiochem (La Jolla, CA). L-Phosphatidyl-DL-glycerol, phosphatidyl ethanol, L-α-phosphatidic acid (egg monosodium salt), 1,2-dimyristoyl-*sn*-glycero-3-P-ethanolamine, 1,2-dimyristoyl-*sn*-glycero-3-P-choline, and 1,2-dimyristoyl-*sn*-glycerol were from Avanti (Alabaster, AL), and 1,3-dimyristin, monomyristin, and trimyristin were from Nu-Check-Prep (Elysian, MN). D609 was obtained from Biomol (Plymouth Meeting, PA). TLC plates (aluminum sheets of silica gel 60, with concentrating zone and glass plates of silica gel 60, 20 × 20 cm) were from Merck (Darmstadt, Germany). All other chemicals were from Sigma Chemical Co. (St. Louis, MO) or Fisher (Pittsburgh, PA) and were of analytical or the highest available grade.

Cell culture. Rat pheochromocytoma cells (PC12, passages 35–45) were obtained from Dr. Lloyd Greene (Columbia University, New York, NY) and were cultured at 37° with saturated air containing 4.7% CO_2 in RPMI 1640 supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium). Culture flasks (75 cm^2) and six-well plates were coated with rat tail collagen at 2.5 μg/cm^2 to promote cell attachment. Media were changed three times a week, and only three fourths of the medium was exchanged to reduce loss of poorly attached cells. When the cells were near confluency (1 week after plating), they were harvested for passaging by a 2-min exposure to 0.05% trypsin at 37°. Cells to be used in binding assays were exposed to calcium-free Hanks' medium containing 1 mM EGTA at 4°, harvested, centrifuged at 600 × *g*, and flash-frozen. Cells were passaged at a 1:3 or 1:4 ratio. To perform cell counting, PC12 cells were resuspended in 0.1 M citric acid containing 0.1% cresyl violet. After incubation at room temperature for several days, stained nuclei were counted with the use of a hemocytometer.

Plasma membrane isolation. PC12 cells from 12 75-cm^2 flasks were thawed, homogenized by nitrogen cavitation in HEPES-buffered isotonic sucrose, and centrifuged at 4000 × *g* for 5 min at 4° to remove unbroken cells and nuclei. The supernatant was centrifuged at 75,000 × *g* for 24 min, and the resulting P2 pellet was resuspended in 50 mM Tris·HCl buffer, pH 7.7, containing 5 mM EDTA. After recentrifugation at 75,000 × *g* for 24 min, the pellet was resuspended in Tris·HCl alone and centrifuged. Four of these washed crude membrane pellets were resuspended in 0.32 M sucrose and combined in a gradient tube. The P2 membranes were layered first with 0.85 M sucrose and then with 1.2 M sucrose. Gradients were centrifuged at 100,000 × *g* for 90 min, and plasma membranes were collected at the interface between 0.85 M and 1.2 M sucrose.

[^{125}I]*p*-Iodoclonidine binding assays. Radioligand binding assays with [^{125}I]*p*-iodoclonidine were performed as previously described (22–24). Membranes were slowly thawed and resuspended in Tris-HEPES buffer (5.0 mM, pH 7.7, at 25°, containing 0.5 mM EDTA, 0.5 mM EGTA, and 0.5 mM $MgCl_2$) at a concentration of 20 μg protein/ml. Incubations were initiated by the addition of membrane and were carried out for 30 min at 22°. Nonspecific binding was defined in the presence of 10 μM BDF-6143, an imidazoline adrenergic agent. Incubations were stopped by vacuum filtration using a 12-place cell harvester (Brandel) equipped with Teflon tubing to reduce absorption of the radioligand, connected to vacuum pump rated at 120 liter/min (Edwards EM8). Samples were filtered over sheets of glass fiber filter paper (No. 34, Schleicher & Schuell), which were preincubated for 4 hr at 4° in 0.03% polylethyleneimine to reduce nonspecific binding. Each sample well was washed four times with 4 ml ice-cold Tris·HCl, a procedure completed in <12 sec. Individual filters were placed in scintillation vials, covered with 4 ml scintillation cocktail (BioSafe II, Research Products International), and counted at 50% efficiency (Beckman LS5801). Protein was assayed by the bicinchoninic acid method (25).

Cell labeling with [^{3}H]myristate. To assess potential coupling of I_1 receptors to DAG generation, as well as a phospholipid source of DAG, intact PC12 cells were labeled with [^{3}H]myristic acid. This compound has been shown preferentially to incorporate into cellular phosphatidylcholine in different cell types (26). In pilot experiments, cells were labeled with 1.0 μCi/ml [^{3}H]myristate in either complete growth medium or 1% fetal bovine serum in RPMI for 12, 22, or 44 hr. After washing and equilibration in Krebs' buffer, total lipids were extracted and phospholipids were separated by TLC with $CHCl_3$/methanol/acetic acid/water (65:35:1:8). The data showed that isotopic equilibrium was established by 22 hr in low serum but not in complete medium. Of the total label, 69% was incorporated into phosphatidylcholine and 11% into phosphatidylethanolamine.

In all subsequent studies, PC12 cells were plated onto collagen in six-well plates and labeled with [^{3}H]myristic acid (1.0 μCi/ml; 40 Ci/mmol) for 22 hr in low serum medium. Duplicate wells were run in each experiment for cell counts. Each well contained an average of 1.8 ± 0.1 × 10^6 cells (20 experiments). After being washed twice in Krebs' (containing 10 mM HEPES, pH 7.4, with 0.1% BSA), cells were preincubated in Krebs' buffer without BSA for 30 min at 37°. After removal of media, different drugs in Krebs' buffer were added for the indicated times.

Treatment of PC12 cells for DAG mass assays. On the day of passage, cells were seeded onto collagen-coated 75-cm^2 flasks and exposed to NGF (50 ng/ml) in RPMI with 1% fetal bovine serum for 36 hr. Cells [1.6 ± 0.4 × 10^6 cells (18 experiments)] were then preincubated with serum-free medium containing 10 ng/ml NGF for 30 min. Incubations took place in fresh medium containing NGF with and without drug treatments. Cells used for measurements of DAG mass that were not NGF treated [2.1 ± 0.1 × 10^6 cells (five experiments)] were grown onto six-well plates in complete medium. Cells were preincubated in Ca^{2+}-free Krebs' medium (10 mM HEPES, pH 7.4) for 30 min and then exposed to moxonidine in complete Krebs' buffer for indicated times.

Lipid extraction and TLC. Lipids were extracted from PC12 cells according to the Bligh and Dyer method. Reactions were stopped with ice-cold methanol (5 ml/4 ml medium), and flasks were put on ice. Cells in the flasks were scraped with rubber policemen,

and the extracts were transferred to 50-ml glass tubes containing 5 ml of cold $CHCl_3$. Washing with methanol (5 ml) was repeated another time. After thorough mixing, another 5 ml of $CHCl_3$ and water was added. After centrifugation and removal of upper phase and interphase, one of the two protocols described below was followed, both including performance of TLC.

In the series of experiments in which [^{3}H]myristic acid was used, an additional wash of the lower phase with $CHCl_3$ was performed. The combined lower phases were evaporated under nitrogen. The dried samples were recovered in $CHCl_3$/methanol (9:1), vortexed, and split in two aliquots that were used for chromatographic separation of neutral and phospholipids. In addition, total extracted lipids were assessed by counting an aliquot for tritium. TLC plates were heat-activated for 1.0 hr at 110°, and the following solvent systems were used for separation of lipids: for phospholipids, $CHCl_3$/methanol/glacial acetic acid (65:15:6, v/v/v); and for neutral lipids, benzene/diethyl ether/ammonia (100:80:0.2, v/v/v). Aliquots of lipid samples were evaporated under nitrogen and reconstituted in 0.050 ml $CHCl_3$/methanol (9:1, v/v). External and internal standards were run on TLC plates as well. Spots were visualized with 2-*p*-toluidinonaphtalane-6-sulfonic acid under UV (365 nm). Values of R_F for 1,2- and 1,3-diacylglycerol were 0.40 and 0.48, respectively. Phosphatidic acid and phosphatidylethanol were separated with R_F values of 0.45 and 0.63, respectively. The latter two lipid species were verified with the use of a second chromatographic system (27). In brief, TLC plates were saturated with 1.3% potassium oxalate dissolved in methanol/water (2:3, v/v) before air-drying and heat-activation. The upper (organic) phase of the following solvent system was used: ethyl acetate/iso-octane/acetic acid/water (13:2:3:10, v/v). Visualization with iodine revealed that phosphatidic acid and phosphatidylethanol could be separated with R_F values of 0.17 and 0.24, respectively.

After the scraping of spots corresponding to desired lipid species, scintillation cocktail was added (10 ml), and samples were counted at 52% efficiency in an LS5801 Beckman liquid scintillation counter.

DAG enzymatic end-point assay and TLC. DAG was determined according to a modified procedure described previously (28). After lipid extraction, an aliquot of the lower phase (1.4 ml) was transferred to a 12 × 75-mm glass tube and mixed with a lipid cocktail containing 6.0 mg/ml octylglucoside and 0.804 mg/ml phosphatidyl glycerol in methanol. This solution was dried under a stream of nitrogen and resuspended in a reaction mixture containing (0.133 ml) 5.0 μmol imidazole buffer, pH 6.6, 2 μmol $MgCl_2$, 0.32 μmol dithiothreitol, and 0.16 μmol EGTA. [Note that imidazole itself does not bind to I_1-imidazoline sites (1).] The reaction was started by the addition of a 0.027 ml solution containing 0.16 μmol ATP, 1.6 μCi [γ-^{32}P]ATP (4500 Ci/mmol), and 1 μg DAG kinase (3.13 units/mg). After mixing, the samples were incubated at room temperature for 1 hr. After the additions of 0.6 ml cold methanol/$CHCl_3$ (2:1), 0.2 ml 1% HCl, and 0.2 ml $CHCl_3$, the sample was vortexed and spun. The upper phase was removed, and an aliquot of the lower phase (0.1 ml) was spotted onto a TLC plate. Plates were developed for 90 min in $CHCl_3$/acetone/methanol/glacial acetic acid (10:4:3:2, v/v/v/v), air-dried, and subjected to autoradiography. The radioactive spots corresponding to phosphatidic acid (R_F = 0.55) were excised and counted.

Data analysis. Radioligand binding data were analyzed by nonlinear curve-fitting with InPlot (GraphPAD Software, San Diego, CA). Data were derived from at least three separate experiments and are shown as mean percentage change from unstimulated controls ± standard error. Data were analyzed with the use of paired *t* test or one-way analysis of variance with Dunnett's multiple-comparisons test.

Results

Binding properties of I_1-imidazoline sites in plasma membrane fractions from PC12 cells. Saturation experiments (eight ligand concentrations ranging from 40 pM to 2 nM) indicated that [^{125}I]*p*-iodoclonidine binding to PC12 cell plasma membranes was of high affinity (K_d = 0.19 ± 0.04 nM by LIGAND analysis of four experiments in triplicate) and saturable (B_{max} = 560 ± 120 fmol/mg protein). At the K_d concentration, nonspecific binding was approximately one half of the total (378 ± 32 dpm total and 196 ± 8 nonspecific, of which ~100 dpm represented filter binding).

Specific [^{125}I]*p*-iodoclonidine binding sites were also present in crude particulate membranes fractions but in much lower density (B_{max} = 40 ± 8 fmol/mg protein).

Competition binding experiments (Fig. 1) showed that [^{125}I]*p*-iodoclonidine sites had a high affinity for the I_1-antagonist efaroxan (pK_i = 8.75 ± 0.06) and the I_1-agonist moxonidine (pK_i = 8.11 ± 0.05). In contrast, the nonimidazoline α_2-adrenergic antagonist SK&F 86466 was much less potent (pK_i = 5.59 ± 0.02). These affinity values are in close agreement with those obtained at I_1-imidazoline sites in bovine rostral ventrolateral medulla oblongata (7). (−)-Epinephrine at a concentration of 0.1 mM did not affect [^{125}I]*p*-iodoclonidine binding (specific binding = 104 ± 10% of vehicle control, six experiments). The α_2-adrenergic antagonist rauwolscine was likewise inactive at 10 μM (103 ± 4% of control, eight experiments). The low affinity of potent α_2-adrenergic agents for specific [^{125}I]*p*-iodoclonidine binding sites confirmed that PC12 cells did not express α_2-adrenergic receptors.

Effect of moxonidine on the accumulation of [^{3}H]1,2-DAG. Incubation of PC12 cells with I_1-receptor agonist moxonidine (1 μM) led to a time-dependent increase in [^{3}H]1,2-DAG (Fig. 2). The effect of I_1-imidazoline receptor activation seemed transient as there was no significant increase in DAG after 1 min. The peak responses of [^{3}H]1,2-DAG to 1 μM moxonidine varied in time between cell samples and was observed at 15 sec (11 of 19 experiments), 30 sec (1 of 19 experiments), or 1 min (7 of 19 experiments). Unstimulated

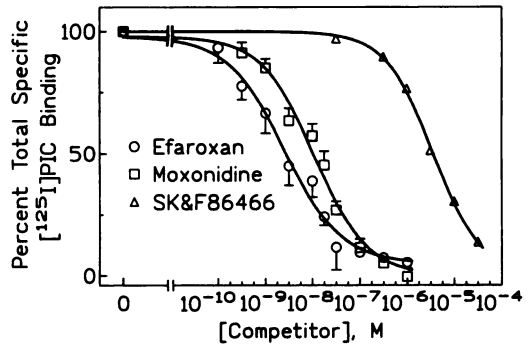

Fig. 1. Dose-dependent inhibition of [^{125}I]*p*-iodoclonidine binding to PC12 cell plasma membranes by increasing concentrations of I_1-imidazoline and α-adrenergic agents. Data represent the mean ± standard error of six (efaroxan and moxonidine) or two (SK&F 86466) experiments and are expressed as percentage of total specific [^{125}I]*p*-iodoclonidine binding as defined by 10 μM BDF-6143. Curves were generated by nonlinear curve-fitting to a logistic equation. The I_1-imidazoline antagonist efaroxan and agonist moxonidine showed nanomolar affinity for [^{125}I]*p*-iodoclonidine binding sites, whereas the nonimidazoline α_2-adrenergic antagonist SK&F 86466 was >2 orders of magnitude less potent.

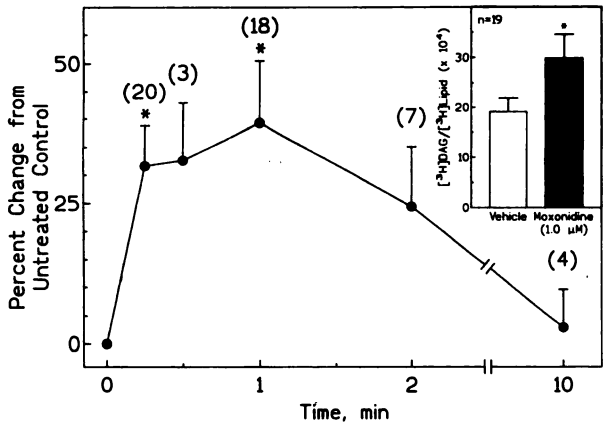

Fig. 2. Effect of moxonidine on the accumulation of [^{3}H]1,2-DAG. PC12 cells were labeled with [^{3}H]myristic acid for 22 hr, washed twice with 0.1% BSA in Krebs' buffer, and preincubated in BSA-free Krebs' for 30 min at 37°. Fresh media containing moxonidine (1 μM) was then added for indicated times. Lipids were extracted and separated by TLC as described in Experimental Procedures. The results were calculated as [^{3}H]]DAG radioactivity (in dpm)/[^{3}H]total lipid radioactivity (in dpm) and are expressed as percentage ± standard error change from matched untreated controls. In one typical experiment, 3072 dpm were recovered as [^{3}H]1,2 DAG and 1,610,900 dpm were recovered in the total lipid extract. The number of independent measurements is given in parentheses. *, Significantly different change; $p < 0.05$, one-way analysis of variance and Dunnett's multiple-comparisons test. *Inset*, peak responses of [^{3}H]1,2 DAG to 1 μM moxonidine expressed as the ratio of [^{3}H]1,2-DAG radioactivity (in dpm) to the total lipid radioactivity (in dpm) × 10^4. *, Significantly different from untreated controls; $p < 0.05$, paired *t* test.

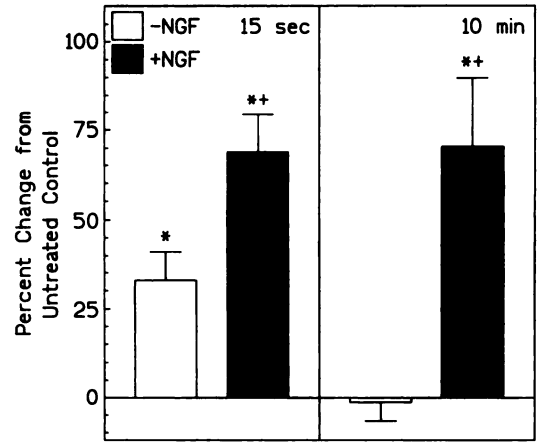

Fig. 3. Effect of moxonidine on DAG mass accumulation in PC12 cells with and without NGF treatment. To initiate differentiation, PC12 cells were treated with NGF (50 ng/ml) for 36 hr. After 30-min preincubations, differentiated and undifferentiated PC12 cells were incubated in the presence of moxonidine (1 μM) for 15 sec or for 10 min, with and without NGF (10 ng/ml). DAG mass was determined with enzymatic end point assay as described in Experimental Procedures. Levels of DAG in unstimulated control cells not treated with NGF were 722 ± 37 pmol/dish (13 experiments) and 767 ± 78 pmol/dish (five experiments) at 15 sec and 10 min, respectively, whereas basal DAG levels in NGF-treated PC12 cells were 660 ± 130 pmol/dish (14 experiments) and 642 ± 75 pmol/dish (30 experiments) at the same respective time points. The data were obtained from at least five separate cell cultures and are shown as percentage ± standard error change from matched untreated controls. *, Significantly different from vehicle-treated controls; $p < 0.05$, paired *t* test. †, Significantly different from cells not treated with NGF; $p < 0.05$, paired *t* test.

levels of DAG were 19.1 ± 2.8 dpm/total lipid radioactivity (in dpm) × 10^4 (19 experiments). Moxonidine elicited an average peak increase over unstimulated control levels of [^{3}H]1,2-DAG of 59 ± 9% (Fig. 2, *inset*). These results suggested that I_1-receptor activation was linked to DAG accumulation.

Effect of moxonidine on DAG mass accumulation and the influence of differentiation with NGF. To test whether the elevation in [^{3}H]DAG reflected an actual change in the total mass of DAG in PC12 cells, a DAG kinase enzymatic end point assay was used. Similar to what was observed in [^{3}H]DAG experiments, there was a significant increase in DAG mass at 15 sec, an effect that disappeared by 10 min (Fig. 3). Moreover, there may be a much larger increase in DAG production in NGF-treated cells at 15 sec than in undifferentiated PC12. This effect was maintained at 10 min in cells differentiated with NGF.

Dose-dependency of DAG mass accumulation in response to moxonidine. To test the relationship of agonist concentration to the magnitude of the DAG response, PC12 cells treated with NGF were exposed to increasing concentrations of moxonidine (Fig. 4). The accumulation of DAG in response to moxonidine was dose dependent up through 1.0 μM, as suggested with the use of a nonparametric analysis of variance by dose (Kruskal-Wallis statistic = 10.2, $p = 0.036$). The highest concentration (10 μM) elicited an attenuated response, indicating that this dose is supramaximal. The total cellular DAG in PC12 cells treated with 0.1 μM, 1.0 μM, and 10 μM moxonidine was significantly greater than that in untreated controls run in parallel ($p < 0.05$, paired *t* test).

Effect of efaroxan on DAG mass in NGF-treated PC12 cells. Efaroxan has been shown to antagonize actions

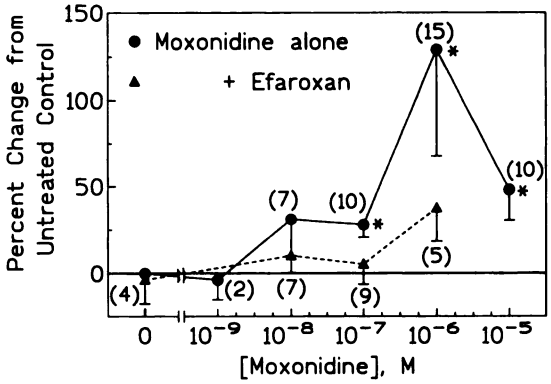

Fig. 4. Effect of efaroxan on the accumulation of DAG mass in response to moxonidine in NGF-treated PC12 cells. Cells were treated with NGF (50 ng/ml) for 36 hr. After 20-min preincubations, cells were further preincubated with or without efaroxan (1 μM) for 10 min. Incubations were carried out in control buffer with NGF (10 ng/ml) and the indicated concentration of moxonidine with and without 1.0 μM efaroxan. The results were determined from the number of separate cell cultures shown in parentheses and are calculated as percentage ± standard error change from matched untreated controls. Untreated control levels for DAG are given in the legend for Fig. 3. *, Significantly different from vehicle treated controls; $p < 0.05$, paired *t* test.

of moxonidine that are independent of α_2-adrenergic receptors, such as centrally mediated falls in blood pressure (29) and ocular pressure (10) and the induction of arachidonic acid release (20). To test whether the increase in DAG accumulation elicited by moxonidine was a receptor-mediated event, PC12 cells were pretreated with efaroxan (1 μM) for 10 min before exposure to increasing concentrations of mox-

onidine (Fig. 4). These experiments were run in parallel to those described in the preceding section with increasing doses of moxonidine in the absence of efaroxan. The antagonist did not affect resting levels of DAG when given alone. The effect of moxonidine was significantly reduced by treatment with efaroxan. None of the responses to moxonidine reached statistical significance, although the response to 1.0 μM moxonidine was nearly significant ($p = 0.10$, paired t test). Therefore, it seemed that observed increases in DAG by moxonidine were blocked by the antagonist efaroxan in a manner consistent with competitive antagonism, suggesting the involvement of specific I_1-receptors in this process.

To test the specificity of the blocking action of efaroxan, in a single representative experiment PC12 cells were incubated with $10^{-6.5}$ M moxonidine with or without pretreatment with the α_2-adrenergic antagonist SK&F 86466 at a concentration of 10 μM, which is nearly 1000-fold higher than its K_i at α_{2A}-adrenergic receptors (23). Moxonidine at $10^{-6.5}$ M increased DAG mass by 77% (from 19.9 to 35.3 pmol/10^6 cells), which is consistent with the concentration-response relationship shown in Fig. 4. In the presence of SK&F 86466, DAG mass was increased by 58% (31.5 pmol/10^6 cells), suggesting that SK&F 86466 was a weak antagonist at best, consistent with the lack of α_2-adrenergic receptors in PC12 cells and the low affinity of SK&F 86466 for I_1-imidazoline receptors (Fig. 1).

Tests of the coupling of phospholipase D and phosphatidylcholine-selective phospholipase C to I_1-receptor activation. One pathway leading to the accumulation of DAG within cells is through receptor-mediated stimulation of phospholipase D, leading to production of phosphatidic acid, which can be dephosphorylated by phosphatidic acid phosphohydrolase to generate DAG. To test the hypothesis that I_1-imidazoline receptors generates DAG through this pathway, we exploited the property of phospholipase D to transfer the phosphatidyl group of phosphatidylcholine to ethanol to form phosphatidylethanol. If I_1-receptors were coupled to phospholipase D, then phosphatidic acid accumulation should occur, and the addition of ethanol would elicit accumulation of phosphatidylethanol at the expense of phosphatidic acid formation. Phosphatidylethanol levels were not affected by the additions of moxonidine and ethanol at either 15 sec or 10 min (Fig. 5A). However, the protein kinase C activator PMA significantly increased the accumulation of phosphatidylethanol over 10 min of incubation. Similarly, ATP and carbachol, agonists for purinergic and muscarinic receptors, respectively, also increased the accumulation of this product of phospholipase D activity.

Although DAG was increased in the presence of moxonidine at 15 sec and not at 10 min (Fig. 2), phosphatidic acid levels were elevated at neither 15 sec nor 10 min, regardless of the presence or absence of ethanol (2%) (Fig. 5). In contrast, the three agonists shown to activate phospholipase D all increased phosphatidic acid despite the presence of ethanol. Presumably, the effects of PMA, ATP, and carbachol on phosphatidic acid accumulation would have been more substantial in the absence of ethanol, but this was not tested in the current study. These data imply that phospholipase D is not activated under conditions leading to accumulation of DAG in response to I_1-imidazoline receptor stimulation in PC12 cells, even though under the same conditions phospholipase D can be activated by several other agonists in these cells.

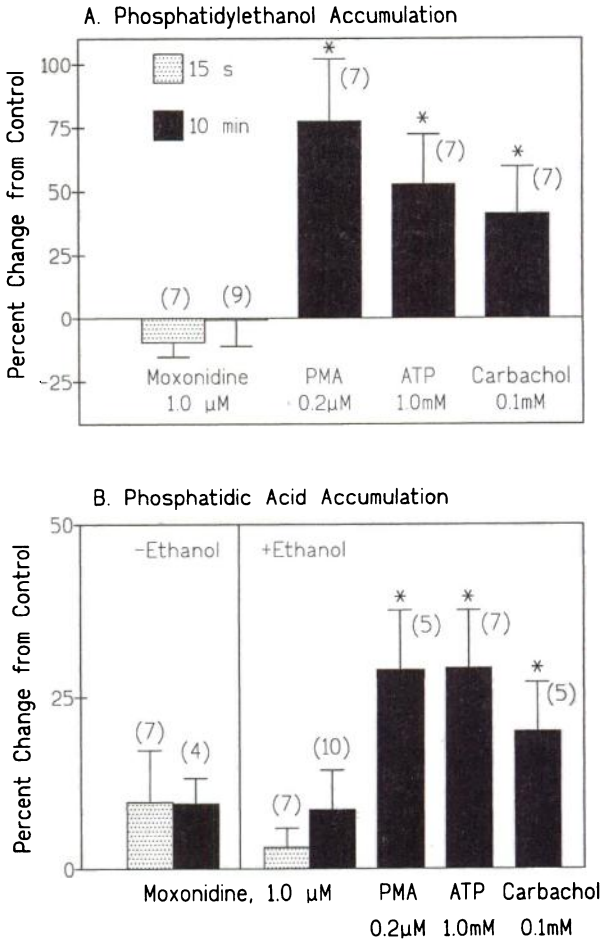

Fig. 5. Effect of moxonidine on phospholipase D activity. After 22-hr labeling with [^{3}H]myristate, PC12 cells were washed twice in Krebs' containing 0.1% BSA and then preincubated for 30 min in BSA-free Krebs'. For moxonidine experiments, incubations were carried out in Krebs' with and without ethanol (2%) and moxonidine (1.0 μM) for 15 sec and 10 min. For positive control experiments with PMA (0.2 μM), ATP (1.0 mM), and carbachol (0.1 mM), ethanol was included, and incubations were carried out for 10 min. Lipids were extracted and analyzed with the use of TLC as described in Experimental Procedures. Data were quantified as radioactivity incorporated either into phosphatidylethanol (A) or phosphatidic acid (B) as a proportion of incorporation into total lipids. Unstimulated control levels of phosphatidylethanol for 10 min of incubation were 35.6 ± 10.6 dpm/total lipid radioactivity (in dpm) × 10^4 (12 experiments). In a typical experiment, 2810 dpm would be recovered as phosphatidylethanol and 1,127,380 dpm would be recovered in the total lipid extract. Control levels of phosphatidic acid were 123 ± 18 (17 experiments) and 119 ± 26 (12 experiments) dpm/total lipid radioactivity (in dpm) × 10^4 in the absence and presence of ethanol, respectively. In a typical experiment, 12,176 dpm would be found in the phosphatidic acid fraction compared with 987,867 dpm in total lipids. *Bars*, mean values from at least five separate experiments; expressed as percentage ± standard error change from matched untreated controls. Data in the absence of ethanol are expressed relative to untreated control, and data in the presence of ethanol are expressed compared with cultures treated with ethanol alone.

In one series of experiments, we tested the effect of a putative inhibitor of phosphatidylcholine-selective phospholipase C, D609 (30), on the accumulation of DAG in response to moxonidine in PC12 cells treated with NGF (50 ng/ml for 36 hr). Pretreatment of PC12 cells with 100 μM D609 for 10 min had no significant effect on resting levels of cellular DAG mass (941 ± 40 pmol/dish, two experiments) relative to con-

trols (1037 ± 136 pmol/dish, eight experiments). Moxonidine treatment (1.0 μM for 10 min) increased DAG mass in cells pretreated with vehicle (1360 ± 161 pmol/dish, seven experiments) but not in cells pretreated with D609 (1060 ± 71 pmol/dish, seven experiments). These data suggest that the response to moxonidine can be prevented by treatment with D609.

Discussion

In this study, we demonstrated that DAG is produced in response to the I_1-imidazoline agonist moxonidine in PC12 cells. This response was blocked by the selective I_1-imidazoline antagonist efaroxan, suggesting that it was probably a receptor-mediated response. We used two different experimental strategies to test for potential coupling of I_1-receptors to generation of DAG. First, we measured the flux of [^{3}H]myristate, which was incorporated primarily into phosphatidylcholine and phosphatidylethanolamine, into DAG. Second, we determined the total cellular mass of DAG by using a DAG kinase enzymatic end point assay. Stimulation of DAG accumulation was observed in two different phenotypes of PC12: undifferentiated chromaffin-like cells and NGF-differentiated neuron-like cells. Both NGF-treated neuron-like cells and untreated chromaffin-like cells showed elevated levels of DAG in response to moxonidine, but their responses differ in time courses and magnitude of DAG produced, with the differentiated cells showing larger and more sustained responses to I_1-imidazoline receptor stimulation. The increase in cellular DAG content was dose-related up through a 1.0 μM concentration of moxonidine and was antagonized in an apparently competitive fashion by the putative I_1-imidazoline blocker efaroxan. Phospholipase D seemed not to participate in the generation of DAG in response to stimulation of this receptor, suggesting that DAG may be produced directly by a phospholipase C. The current study is one of the first to provide positive evidence in support of the coupling of these novel receptors to signaling pathways.

Indeed, investigations of signaling pathways coupled to I_1-imidazoline receptors have yielded mainly negative data thus far. Phosphatidylinositol-specific phospholipase C may not be linked to activation of I_1-receptors by clonidine in adrenomedullary chromaffin cells (15), adrenal slices (17), brain slices (31), or tracheal epithelial cells (16). Similarly, cAMP and cGMP levels are not affected by I_1-imidazoline receptor stimulation (15–17, 31). Calcium influx and/or efflux in adrenomedullary chromaffin cells seems to be affected by clonidine (15), but this effect might be downstream from other signaling events. Direct effects of clonidine on intracellular calcium in PC12 cells have not been observed, except an attenuation of the calcium influx elicited by nicotine (18).

Contrary to these largely negative data, our results confirmed and extended a preliminary report showing a rapid and transient increase in DAG levels in tracheal epithelial cells in response to moxonidine (19). Although these cells express both α_2-adrenergic and I_1-imidazoline binding sites (16), selective stimulation of α_2-adrenergic receptors increased DAG to a lesser degree and with a different time course compared with I_1 receptor activation (19). Therefore, these findings are consistent with nonadrenergic action of imidazolines on DAG accumulation. The current study provides unambiguous support for coupling of I_1-imidazoline receptors to DAG accumulation because the PC12 cells used in these studies do not express α_2-adrenergic receptors, the response was dose related and could be blocked by an antagonist, and DAG generation could be documented by two independent methods.

The rise in DAG at 15 sec, observed in both differentiated and undifferentiated PC12 cells in response to moxonidine, is not without precedent. For example, a short term elevation of DAG levels in response to receptor activation, with a peak occurring within 30 sec, has been described in many cell types (32–35), including PC12 cells (36). A source of this transiently increased DAG is thought to be inositol phospholipids (33, 37), although not exclusively (32–34). In response to a variety of stimuli, a peak in DAG generation is observed at 15 sec in two different cell lines (32). This DAG is thought to originate from phospholipase C hydrolysis of phosphatidylcholine, based on the accumulation of phosphocholine. Similarly, in response to endothelial growth factor, fibroblasts show DAG accumulation with a peak at 25 sec and returning to the base-line by 5 min (34). After ruling out involvement of inositol phosphates and phospholipase D, the authors suggested that this early rise in DAG is probably due to phosphatidylcholine hydrolysis by phospholipase C.

We did not test in the current study whether the short term generation of DAG was mediated by phosphatidylinositol-specific phospholipase C. However, two lines of evidence weigh against involvement of inositol phospholipid breakdown. First, inositol phosphates have not been identified in response to I_1-receptor activation in either adrenal chromaffin cells (15, 17) or PC12 cells.[1] Second, [^{3}H]myristic acid labeled primarily phosphatidylcholine and some phosphatidylethanolamine, a finding that suggests that phosphatidylcholine or phosphatidylethanolamine rather than phosphatidylinositol, was the source of I_1-receptor-stimulated DAG. In an interesting precedent for this phenomenon, a 5-sec stimulated peak in DAG in alveolar type II cells has been ascribed to phosphatidylcholine/phospholipase C action based on mass balance analysis of net changes in DAG, phosphatidylinositol pools, and phosphocholine accumulation (35).

The increase in DAG accumulation was maintained in NGF-treated cells for ≥10 min. This time course parallels that obtained in a number of stimulated cells from activation of either phosphatidylcholine/phospholipase C or phospholipase D (27, 33, 36, 38, 39). Although we do not know whether NGF-differentiated cells indeed had a typical biphasic response in DAG production, they certainly show a different time course from undifferentiated cells and a greater magnitude of response to I_1-receptor stimulation. These findings could be explained by an increased receptor density in NGF-treated cells (5) and possibly by the change to more neuron-like phenotype.

In some cell types, the phospholipase D/phosphatidic acid phosphohydrolase pathway is a major mechanism of DAG generation in response to receptor stimulation (38). When these signaling systems are activated, formation of phosphatidic acid is elevated before DAG and transphosphatidylation products accumulate in the presence of alcohols (33, 36, 40). Receptor-stimulated phospholipase D has been documented in PC12 cells (27, 36). We therefore explored the potential

[1] D. Separovic, M. Kester, and P. Ernsberger, unpublished observations.

coupling of I_1 receptors to phospholipase D as an indirect route of generating DAG. However, we failed to demonstrate any phospholipase D activation in response to the I_1 agonist moxonidine, although this pathway was active in the PC12 cells used in these experiments as shown by the stimulation of phospholipase D activity by protein kinase C activation with phorbol ester and by the purine agonist ATP and the cholinergic agonist carbachol. Furthermore, there was no accumulation of phosphatidic acid at either short or long exposures to moxonidine. These data, together with prior results consistent with a lack of inositol phosphate accumulation and the selective labeling of the phosphatidylcholine pool with [^{3}H]myristate, argue in favor of the hypothesis that DAG is generated by a direct route, such as a phospholipase C acting on phosphatidylcholine or phosphatidylethanolamine. Furthermore, a putative inhibitor of phosphatidylcholine-selective phospholipase C (30) blocked the action of moxonidine on DAG accumulation.

We conclude that I_1-imidazoline binding sites expressed in the plasma membrane compartment of PC12 cells are functional receptors coupled to the generation of DAG. The pathway or pathways responsible for the generation of DAG in response to I_1-imidazoline receptor stimulation remain to be established, but involvement of either phospholipase D or a phosphatidylinositol-specific phospholipase C is unlikely. Thus, we speculate that phosphatidylcholine-selective phospholipase C is coupled to I_1-imidazoline receptors. Generation of DAG in response to I_1-receptor agonists may contribute to some of the physiological responses attributed to this proposed class of receptor.

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