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Regulation of Prolactin Gene Expression by Vasoactive Intestinal Peptide and Dopamine in the Turkey: Role of Ca^{2+} Signalling

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Abstract

Our recent work has demonstrated that dopamine, acting through D_2 dopamine receptors on pituitary cells, inhibits the stimulatory effects of vasoactive intestinal peptide (VIP) on prolactin release and prolactin gene transcription. It is hypothesised that the stimulatory and inhibitory roles of VIP and dopamine, respectively, on prolactin synthesis and release are mediated by their opposite effects on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in lactotrophs. The present study aimed: (i) to investigate the effect of VIP and dopamine on $[Ca^{2+}]_i$ of cultured turkey anterior pituitary cells and (ii) to examine the role of Ca^{2+} signalling in mediating the regulatory effects of VIP and dopamine on prolactin mRNA levels and prolactin release. Changes in $[Ca^{2+}]_i$ were measured spectrofluorometrically using Fura-2/AM as a fluorescent Ca^{2+} indicator. Semi-quantitative reverse transcription-polymerase chain reaction and radioimmunoassay were used to determine prolactin mRNA levels and prolactin release, respectively. VIP or the L-type Ca^{2+} channel activator, Bay K8644 (Bay) increased $[Ca^{2+}]_i$ in a concentration- and time-dependent fashion, an effect abolished by preincubating the cells with R(–)-propylnorapomorphine HCl, a D_2 dopamine receptor agonist (D_2AG) or Verapamil (VR), a specific L-type Ca^{2+} channel blocker. Similarly, either VR or D_2Ag diminished the VIP/Bay stimulatory effect on prolactin expression and release. On the other hand, pretreatment of pituitary cells with thapsigargin (TG) or neomycin (NEO), to deplete the intracellular Ca^{2+} stores, showed no effect on basal or VIP-stimulated prolactin mRNA levels; although VIP- induced prolactin release was partially inhibited by NEO but not TG. These results suggest that intracellular Ca^{2+} represents a common signal transduction pathway through which VIP and dopamine can exert antagonistic control on prolactin synthesis and release in avian lactotrophs.

Prolactin release and expression are tonically stimulated by vasoactive intestinal peptide (VIP), the avian prolactin-releasing factor in birds (1). Dopamine has been shown to have both stimulatory and inhibitory influences on prolactin secretion (2, 3). Dopamine stimulates prolactin secretion at the hypothalamic level by activating VIPergic neurones in the infundibular nuclear complex via D_1 dopamine receptors (3, 4). In addition, dopamine inhibits prolactin secretion by antagonising the action of VIP at the pituitary level via D_2 dopamine receptors (5, 6). However, the signalling mechanism(s) underlying the interaction between VIP and dopamine in the regulation of prolactin secretion is far from clear. Previous work has implicated the involvement of protein kinase A (7), Ca^{2+} (8) and protein kinase C (PKC) (9) in the stimulatory effects of VIP on pituitary prolactin release.

In mammals, it is well established that dopamine tonically inhibits prolactin secretion and expression via D_2 dopamine receptors (10–12). D_2 dopamine receptors have been shown to be linked, through $G_{\alpha o}$- and $G_{\alpha I}$-containing G proteins, to 'inhibitory' pathways, including inhibition of adenylyl cyclase and L-type Ca^{2+} channels (13, 14). The inhibition of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in lactotrophs is a major mechanism underlying the inhibitory effect of dopamine on prolactin secretion and prolactin expression (11, 15, 16). Pharmacological drugs that increase intracellular Ca^{2+}, $[Ca^{2+}]_i$, including Ca^{2+} ionophores (17, 18), elevated K^+ levels (19) and the Ca^{2+} channel activators, maitotoxin (18) and Bay K8644 (Bay; 20, 21), increase prolactin release from anterior pituitary and clonal growth hormone cells. The prolactin-releasing effects of VIP, thyrotrophin-releasing hormone and high extracellular K^+ are completely abolished in the presence of the voltage-sensitive Ca^{2+} channel inhibitor, verapamil (VR; 22). In addition, agents that selectively enhance or decrease Ca^{2+} entry through voltage-gated channels modulate prolactin gene expression (23, 24).

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Moreover, prolactin-secreting pituitary cells possess both L-type and T-type Ca^{2+} channels (25).

We hypothesise that Ca^{2+} signalling may play a major role in the regulation of prolactin release and prolactin gene expression by VIP and dopamine in avian pituitary cells. In addition, it is hypothesised that the stimulatory role of VIP and the inhibitory role of dopamine on prolactin synthesis are mediated by their opposite effects on $[Ca^{2+}]_i$ in lactotrophs. The present study aimed: (i) to investigate the effect of VIP and dopamine on $[Ca^{2+}]_i$ in cultured turkey anterior pituitary cells and (ii) to examine the role of Ca^{2+} signalling in mediating the regulatory effects of VIP and dopamine on prolactin mRNA levels and prolactin release.

Materials and methods

Experimental animals

Adult large white female Nicholas turkeys (weighing 10–13 kg) were used in these experiments. They were reared and housed on a 15 : 9 h light/dark cycle in temperature-controlled (15–21 °C) rooms with floor pens and food and water continuously available. All hens used were laying eggs. Hens were housed, handled and used in accordance with University of Minnesota Institutional Animal Care and Use Committee Guidelines.

Pituitary tissue collection and cell dispersion

Pituitary glands were collected from decapitated laying turkeys. The posterior lobes of the pituitary glands were removed, and the anterior lobes were dissociated using a modification of the trypsin/neuraminidase procedure, as previously described (26). They were minced and placed in Krebs–Ringer bicarbonate buffer solution (KRB, pH 7.4) supplemented with amino acids (MEM Eagle essential amino acids; BioWhittaker, Walkersville, MD, USA), 0.3 μg/ml glutamine sulphate (Sigma, St Louis, MO, USA), 2.5 mg/ml glucose (Sigma), 3 mg/ml bovine serum albumin (BSA, fraction V; Sigma), and 100 μg/ml gentamycin sulphate (Sigma). Following mechanical shearing, pituitary fragments were digested enzymatically with 1 mg/ml trypsin (Sigma) and 2 μg/ml deoxyribonulease I (DNase I, Sigma) in KRB solution for 15 min at 37 °C in a shaking water bath. The supernatant was removed and replaced by a Ca^{2+}/Mg^{2+}-free supplemented KRB solution containing 2 mM EDTA, disodium salt (Sigma). After 5 min of incubation at 37 °C, the supernatant was removed and replaced by a Ca^{2+}/Mg^{2+}-free supplemented KRB solution containing 1 mM EDTA and 8 μg/ml neuraminidase (Sigma). After 15 min of incubation at 37 °C, the pituitary fragments were mechanically dispersed and the resultant cell suspension was filtered through 60 μm mesh Nitex gauze (Genson Scientific, Middleboro, MA, USA) to remove undigested particles. Following centrifugation at 250–500 ***g*** for 15 min, the supernatant was decanted and the cell pellet was resuspended in tissue culture medium consisting of medium M-199 (Invitrogen, Groningen, the Netherlands) supplemented with 0.35 μg/ml $NaHCO_3$, 3% charcoal-stripped turkey poult serum, 3% fetal calf serum (Sigma), 4.8 μg/ml Hepes (Sigma), 1 μg/ml insulin (Sigma), 50 μg/ml gentamycin sulphate (Sigma), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 5 μg/ml amphotericin B (Sigma). Cell viability (90–95%) was determined using the trypan blue dye exclusion method and cell numbers were counted in a haemocytometer. The dispersed cells were initially incubated for 72 h in siliconised Erlenmeyer flasks at 38.5 °C in humidified atmosphere (95% O_2 and 5% CO_2) before conducting the experiments. After preincubation, cultured pituitary cells were pelleted and resuspended in a serum-free M-199 medium supplemented with 0.1% BSA.

Measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ in primary anterior pituitary cells were measured using a spectrofluorometer and the fluorescent Ca^{2+} indicator Fura-2/AM (Molecular Probes, Eugene, OR, USA). Anterior pituitary cells were loaded with 5 μM of the membrane-permeable acetyl methyl ester form of the Ca^{2+} fluorescent dye Fura-2/AM in Hank's balanced salt solution (HBSS) containing 10 mM glucose and buffered with 10 mM HEPES (pH 7.4). Cells were incubated for 30 min at 37 °C in a 5% CO_2 atmosphere, during which time Fura-2 was trapped intracellulary by esterase cleavage. Cells were then washed twice with fresh HBSS to remove any remaining extracellular ester, and then kept on ice. Fluorescence was measured in a spectrofluorometer (Shimadzu, Kyoto, Japan) with continuous stirring at 37 °C. The fluorescence of Fura-2-loaded cells was detected every 1 s at alternating excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm, and the ratio (F_{340}/F_{380}) was calculated. Ratio values were converted to $[Ca^{2+}]_i$ (nM) according to a Ca^{2+} calibration curve using solutions with known calcium concentrations (Molecular Probes).

RNA extraction and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured pituitary cells using Trizol reagent (Invitrogen) as recommended by the manufacturer with minor modifications. To reduce genomic DNA contamination, total RNA was treated with RNase-free DNase I (1 U/μg RNA) for 30 min at 37 °C. After DNA digestion, total RNA was re-extracted using the total RNA isolation protocol (RNeasy Mini Kit system; Qiagen, Valencia, CA, USA) and then RNA concentration was determined using a DU Series 500 spectrophotometer (Beckman, Irvine, CA, USA). Total RNA (250 ng) was reverse-transcripted using SuperScript II reverse transcriptase and Oligo $dT_{12\text{-}18}$ primers (Invitrogen) in a reaction volume of 20 μl containing reverse transcriptase buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.4), 10 mM DTT, 0.5 mM of each dNTP, RNase inhibitor (RNAsin), 2.5 mM Oligo $dT_{12\text{–}18}$, and Superscript II (200 U), and then incubated at 42 °C for 1 h. The reaction was terminated by heating at 70 °C for 15 min. Aliquots (2 μl) of the resultant cDNA were used for the PCR reaction, which was performed in a 25 μl volume containing 200 μM of each dNTP, 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 2 mM $MgCl_2$, 100 ng gene-specific sense and antisense primers, and 2.5 U Ampli Taq DNA polymerase (Perkin Elmer, Norwalk, CT, USA). The PCR profile consisted of an initial denaturation (30 min at 94 °C) followed by cycles of denaturation (1 min at 94 °C), annealing (45 s at 60 °C), extension (1 min at 72 °C), and a final extension for one cycle (10 min at 72 °C). The number of amplification cycles was 20–22 for prolactin and 30 for β-actin, which were within the linear range of amplification. Primer sequences were as follows: prolactin (sense 5′-ACC TCC TTG CCA ATC TGC TCC AGT-3′; antisense 5′-GGA GTC CTC ATC AGC GAG TTG CAG-3′; expected size of the PCR product, 523 bp), and β-actin (sense 5′-ACC AGT AAT TGG TAC CGG CTC CTC-3′; antisense, 5′-TCT GGT GGT ACC ACA ATG TAC CCT-3′; expected size of the PCR product, 450 bp). RT-PCR products were separated in 2% (2 : 1) agarose and NuSieve GTG gel (FMC Bioproducts, Rockland, ME, USA) and visualised with ethidium bromide. The gels were photographed, and the intensities of the PCR products were quantified using NIH Image-J software (developed by Dr Wayne Rasband; ftp.zippy.nimh.nih.gov). To correct for differences in RNA used in RT-PCR reactions, the band intensity for each RT-PCR product of prolactin was normalised to that of β-actin from the same sample.

Prolactin radioimuunoassay (RIA)

Culture media were assayed for prolactin content utilising the homologous RIA, as previously described (27). All samples from the same experiment were assayed simultaneously. All samples from each experiment were assayed in duplicate within a single assay.

Drugs

The drugs used were R(–)-propylnorapomorphine HCl [a D_2 agonist (D_2AG)], (+)-SKF-38393 [a D_1AG], S(–)-eticlopride HCl [a D_2 antagonist (D_2ANT)] (Research Biochemicals International, Natick, MA, USA), VIP (Peninsula Laboratory, San Carlos, CA, USA), VR (Calbiochem, CA, USA), Bay K8644 (Bay), neomycin (NEO), thapsigargin (TG) and actinomycin D (Sigma).

Statistical analysis

The data from $[Ca^{2+}]_i$, RIA and RT-PCR were analysed using the general linear models procedure of the Statistical Analysis System (28). Each data point represents the mean ± SEM of three independent experiments, with

two replicates performed per experiment. Significant differences in mean values of $[Ca^{2+}]_i$, prolactin mRNA or prolactin levels between treatment groups were compared using Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

Results

Effects of VIP and dopamine on $[Ca^{2+}]_i$ of turkey primary anterior pituitary cells

The results of a VIP dose–response study are shown in Fig. 1. VIP caused a significant ($P < 0.05$) concentration- and time-dependent increase in cytosolic calcium levels in primary anterior pituitary cells compared to untreated cells. The increase in $[Ca^{2+}]_i$ induced by VIP was gradual but sustainable, and reached maximum levels after 30 min. VIP caused a maximum increase in $[Ca^{2+}]_i$ at concentration levels of 10^{-7} and 10^{-5} M.

Pre-incubating pituitary cells with the D_2AG, R(–)-propylnorapomorphine HCl (10^{-10} M) (5) for 15 min significantly decreased VIP-induced $[Ca^{2+}]_i$ elevation compared to that of cells pretreated with vehicle (Fig. 2A) ($P < 0.05$). The D_2AG had no effect on basal $[Ca^{2+}]_i$. To examine the specificity of the inhibitory effect of the D_2AG on the $[Ca^{2+}]_i$ increase, cells were pretreated with the D_2ANT, S(–)-eticlopride (10^{-10} M) (5) before the addition of the D_2AG and VIP. The D_2ANT completely blocked the inhibitory effect of the D_2AG on VIP-induced $[Ca^{2+}]_i$ increase but had no effect on basal $[Ca^{2+}]_i$ (Fig. 2B). In addition, the D_1AG (+)-SKF-38393 had no effect on basal or VIP-induced $[Ca^{2+}]_i$ elevation (Fig. 2C), which further confirms the specificity of the D_2 dopamine receptors in mediating the inhibitory effect of dopamine on VIP-induced $[Ca^{2+}]_i$ increment of anterior pituitary cells. Taken together, these results suggest a possible involvement of Ca^{2+} in mediating VIP and dopamine effects on prolactin synthesis. Therefore, experiments were designed to examine whether changes in $[Ca^{2+}]_i$ were involved in the interaction between VIP and dopamine in the regulation of prolactin secretion.

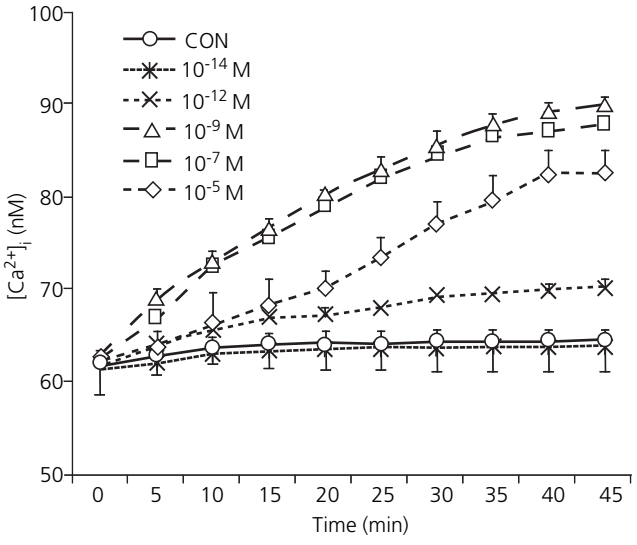

FIG. 1. Concentration-response effect of vasoactive intestinal peptide (VIP) on the $[Ca^{2+}]_i$ of cultured turkey anterior pituitary cells. Pituitary cells (50 000 cells/treatment) in a total volume of 1 ml Hank's balanced salt solution were loaded with 5 μM Fura-2/AM and treated with 5 μl of vehicle or different concentrations of VIP (10^{-14}, 10^{-12}, 10^{-9}, 10^{-7}, 10^{-5} M). The $[Ca^{2+}]_i$ changes was recorded for 60 s to represent the basal level of $[Ca^{2+}]_i$ (0 time), and then every 5 min after the addition of VIP for 45 min. Each point represents the mean of 30 s of continuous reading. The results shown are the mean ± SEM of three independent experiments, with two replicates per experiment. The ratio $[F_{340}/F_{380}]$ values were converted into $[Ca^{2+}]_i$ (nM) according to a prepared Ca^{2+} calibration curve.

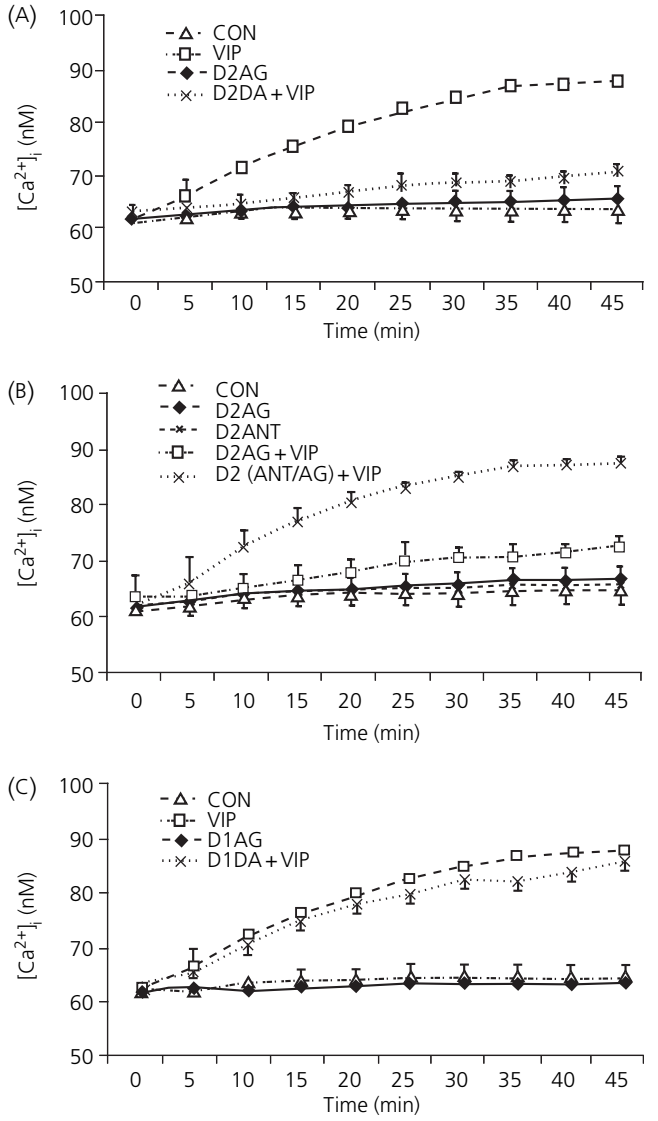

FIG. 2. Effects of D_2AG/D_2ANT or D_1AG on basal and vasoactive intestinal peptide (VIP)-induced $[Ca^{2+}]_i$ elevation of cultured pituitary cells. Cells were pretreated with (A) vehicle/D_2AG (10^{-10} M) for 15 min, and then challenged with VIP (10^{-7} M) at zero time for 45 min. Treatments included vehicle, D_2AG, VIP and D_2AG + VIP and (B) vehicle/D_2ANT (10^{-10} M) for 15 min, and then vehicle/D_2AG (10^{-10} M) were added for an additional 15 min followed by the VIP (10^{-7} M) challenge at zero time. Treatments included vehicle, D_2AG, D_2ANT, D_2AG + VIP, and D_2ANT + D_2AG + VIP, or (C) vehicle/D_1AG (10^{-10} M) for 15 min and were challenged with VIP (10^{-7} M) at zero time. Treatments included vehicle, D_1AG, VIP, or D_1AG + VIP. The $[Ca^{2+}]_i$ changes were recorded, as described in Fig. 1.

Participation of extracellular/intracellular Ca^{2+} in the regulation of prolactin gene expression and prolactin release by VIP and DA

Verapamil (75 μM), an L-type Ca^{2+} channel blocker, significantly inhibited the stimulatory effect of VIP on prolactin mRNA (Fig. 3A,B; $P < 0.01$). By contrast, treatment with Bay (1 μM), an L-type Ca^{2+} channel activator, mimicked the stimulatory effect of VIP on prolactin mRNA levels, suggesting that the influx of extracellular Ca^{2+} through L-type Ca^{2+} channel may play a significant role in mediating prolactin mRNA stimulation by VIP. Similar to VR, D_2AG inhibited VIP-stimulated prolactin mRNA, suggesting that dopamine inhibits the stimulatory effect of VIP on prolactin gene expression, at least in part, by blocking Ca^{2+} influx induced by VIP. Similar to prolactin mRNA, VIP-stimulated prolactin release (20.3 ± 1.3 μg/ml) was diminished by pre-incubating pituitary cells with VR or D_2AG (Fig. 3C; $P < 0.01$). Prolactin release was increased from 2.65 ± 0.02 μg/ml in the control treatment to 16.6 ± 1.8 μg/ml in the 1 μM Bay-treated cells (Fig. 3C; $P < 0.01$). Basal prolactin release was reduced, but not significantly ($P > 0.05$), by VR treatment alone.

Bay caused a concentration-dependent increase in prolactin mRNA levels in cultured anterior pituitary cells (Fig. 4A,B; $P < 0.05$). This stimulatory effect of Bay was inhibited by VR, which suggests that the stimulatory effect of Bay on prolactin mRNA is mediated by Ca^{2+} influx. When cells were incubated with D_2AG for 30 min before the addition of different concentrations of Bay (0.1, 1, 10 μM), prolactin mRNA levels were inhibited in 0.1 μM Bay-treated cells, but not in the cells treated with the higher concentrations (1 μM or 10 μM).

Pre-treatment of pituitary cells with 1 μM TG, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (which depletes intracellular Ca^{2+} stores) did not affect basal or VIP-stimulated prolactin mRNA levels (Fig. 5A,B). Similalrly,

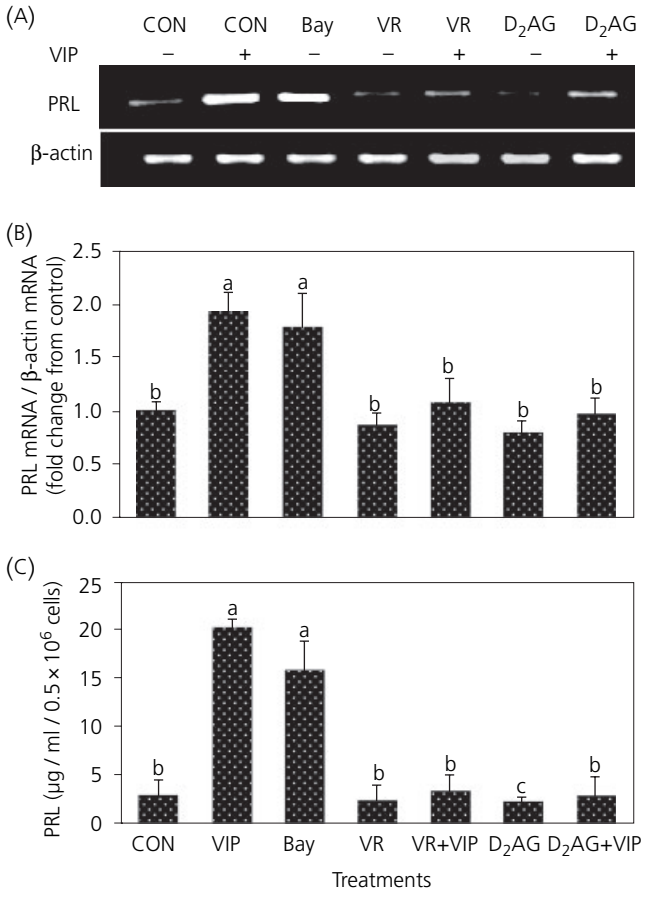

FIG. 3. Extracellular Ca^{2+} influx mediates vasoactive intestinal peptide (VIP) effects on prolactin gene expression and prolactin release in cultured turkey primary pituitary cells. Cultured pituitary cells (0.5×10^6 cells/treatment) were treated with: vehicle (CON), VIP (10^{-7} M), Bay (1 μM), VR (75 μM), VR + VIP, D_2AG (10^{-10} M), or D_2AG + VIP for 3.5 h. (A) A representative micrograph of reverse transcriptase-polymerase chain reaction products separated by agarose gel electrophoresis and ethidium bromide-stained of prolactin and β-actin. (B) Quantification of prolactin mRNA levels normalised to those of the β-actin mRNA internal control. Data (mean ± SEM) are expressed as fold change from vehicle-treated control (basal) value, which was assigned a value of 1.0. (C) Prolactin released into the media. There were three experiments with two replicates each. Significant differences ($P < 0.05$) are identified by different letters above the columns.

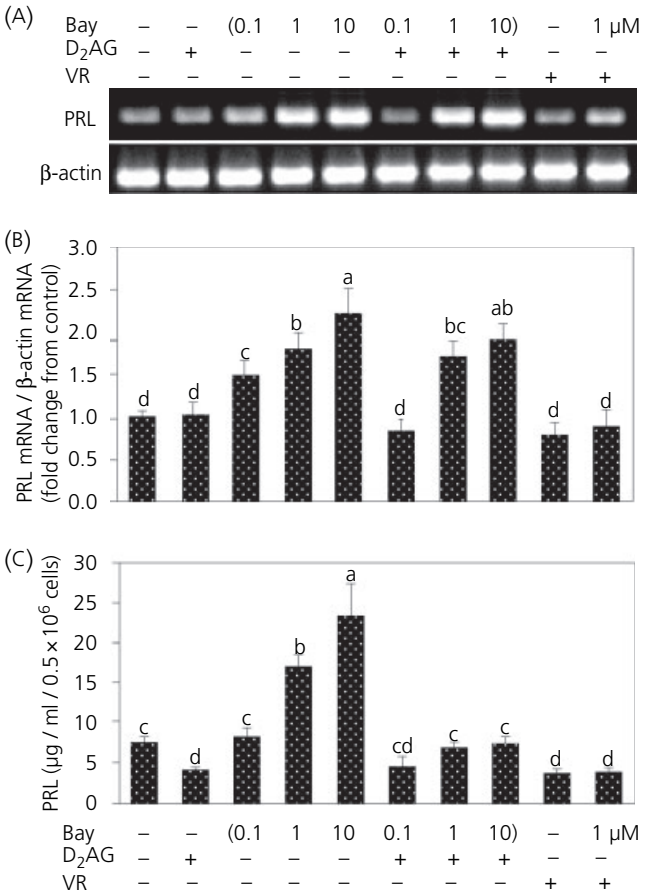

FIG. 4. Extracellular Ca^{2+} influx mediates the inhibitory effect of D_2AG on prolactin gene expression and prolactin release in cultured turkey primary pituitary cells. Cultured pituitary cells (0.5×10^6 cells/treatment) were incubated with either vehicle (control) or D_2AG (10^{-10} M) for 30 min, followed by a subsequent incubation for 3 h in the absence or presence of different concentrations of Bay (0.1, 1, 10 μM). Further details are provided in Fig. 3.

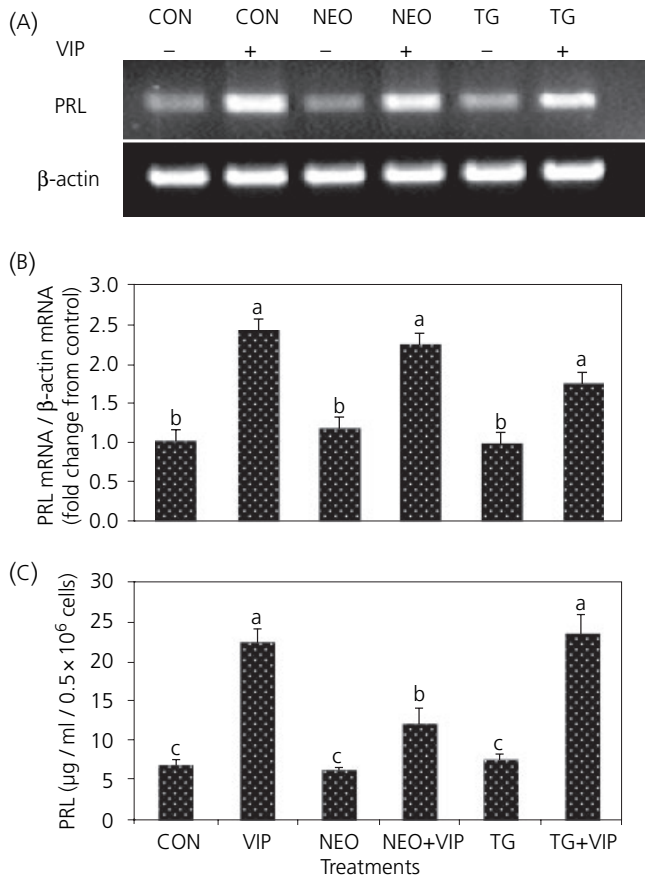

FIG. 5. Intracellular Ca^{2+} and the regulation of prolactin gene expression and prolactin release by vasoactive intestinal peptide (VIP) in cultured turkey primary pituitary cells. Cultured pituitary cells (0.5×10^6 cells/treatment) were treated with: vehicle (control), VIP (10^{-7} M), neomycin (NEO) (1 μM), NEO + VIP, thapsigargin (TG) (1 μM), or TG + VIP for 3.5 h. Further details are provided in Fig. 3.

preincubation of pituitary cells with 1 μM NEO, a nonspecific inhibitor of phospholipase C (PLC), had no effect on basal or VIP-stimulated prolactin mRNA levels. These results suggest that intracellular Ca^{2+} stores are not involved in VIP-induced prolactin gene expression. However, VIP-induced prolactin release was reduced by approximately 50% in NEO-treated cells (12.5 ± 2.1 μg/ml versus 23 ± 1.3 μg/ml, $P < 0.05$) (Fig. 5C), but not in TG-treated cells. Neither NEO nor TG affected basal prolactin release.

Discussion

The present results demonstrate the involvement of Ca^{2+} signalling in the regulation of prolactin mRNA expression and prolactin release by VIP and dopamine. Vasoactive intestinal peptide has been shown, for the first time in an avian species, to increase $[Ca^{2+}]_i$ of cultured anterior pituitary cells, an effect that can be inhibited by a D_2AG. The data reported here suggest that the Ca^{2+} signalling activity prompted by VIP and dopamine is dependent on voltage-gated Ca^{2+} influx but not on Ca^{2+} release from intracellular stores. The results further suggest that Ca^{2+} signalling represents a signal transduction pathway that VIP and dopamine can alter to antagonise each other to regulate prolactin synthesis and release in avian anterior pituitary cells.

Mounting evidence indicates that VIP stimulates prolactin release and expression *in vivo* and *in vitro* in several avian species (29–32), and dopamine, through D_2 receptors, antagonises this effect at the pituitary level (5, 6, 33). Little is known about the intracellular signal transduction mechanisms mediating the effects of VIP and dopamine. In the present study, VIP significantly increased $[Ca^{2+}]_i$ of cultured anterior pituitary cells in a concentration- and time-dependent manner (Fig. 1). The D_2AG, but not the D_1AG (Fig. 2B,C) inhibited this effect. These results, taken together with the finding that the inhibitory effect of a D_2AG on VIP-induced increase in $[Ca^{2+}]_i$ was abolished by preincubating the cells with a D_2ANT (Fig. 2B), suggest the involvement of the Ca^{2+} signalling pathway in the stimulatory and inhibitory influences exerted by VIP and dopamine, respectively, on prolactin release and expression. It also suggests that dopamine may antagonise the stimulatory effect of VIP on prolactin release and synthesis, which we have previously reported (5, 6, 33), by modulating the VIP effect on $[Ca^{2+}]_i$. The dopaminergic inhibitory effect on $[Ca^{2+}]_i$ in lactotrophs has been reported in mammals (11, 15, 16). Several reports have indicated that the inhibition of Ca^{2+} influx is the main mechanism mediating the dopaminergic tonic inhibitory effect on prolactin release and transcription in mammalian species (11, 34, 35).

The increase in $[Ca^{2+}]_i$ in response to VIP may result from inositol triphosphate-induced release of intracellular stored Ca^{2+} and/or from the influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels. The present results show that blocking the influx of extracellular Ca^{2+} with VR, an L-type Ca^{2+} channel blocker, mimicked the inhibitory effect of D_2AG on prolactin mRNA levels and prolactin release from turkey anterior pituitary cells (Fig. 4). On the other hand, increasing the influx of extracellular Ca^{2+} with Bay, an L-type Ca^{2+} channel activator, mimicked the stimulatory effect of VIP on prolactin mRNA levels and release (Fig. 4). Moreover, depleting the intracellular Ca^{2+} stores with TG had no effect on basal or VIP-stimulated prolactin mRNA levels and release. These results implicate the involvement of extracellular, but not intracellular Ca^{2+}, in the regulation of prolactin mRNA expression and release by VIP and dopamine in cultured avian anterior pituitary cells. In addition, these results suggest that intracellular Ca^{2+} represents a common signal transduction pathway through which VIP and dopamine can exert antagonistic control on prolactin synthesis and release in avian lactotrophs. This is the first indication in avian species that the modulation of Ca^{2+} influx can influence prolactin mRNA level and prolactin release. These findings are consistent with previous studies on mammalian lactotrophs demonstrating that prolactin expression can be dramatically changed by modulating Ca^{2+} influx through L-type Ca^{2+} channels using dihydropyridine, a Ca^{2+} channel modulator (23, 24). In growth hormone clonal cells, VIP-stimulated prolactin release (22) was abolished in the presence of the L-type Ca^{2+} channel blocker, VR. In contrast, increasing Ca^{2+} influx by Bay or by high extracellular K^+ activated prolactin mRNA expression (36) and prolactin release (22).

The mechanism(s) by which VIP-induced $[Ca^{2+}]_i$ elevation stimulates prolactin expression remain largely unexplored. However, previous studies demonstrate that the signal transduction cascades initiated by increases in intracellular Ca^{2+} concentrations control the transcription rate of inducible genes by modifying transcription factors that interact with specific *cis*-acting DNA regulatory elements in the promoter of the gene. We propose two possible signal transduction pathways by which Ca^{2+} activates specific transcription factor(s) involved in the transcriptional regulation of prolactin. The first possible pathway involves the activation of the c-*fos* transcription factor gene, a strongly calcium-inducible gene (37). Two control regions in the c-*fos* promoter, the cAMP response element and the serum response element, have been identified as calcium-responsive enhancer elements (38–40). Much evidence indicates the involvement of c-*fos* in the stimulation of prolactin gene transcription (41). The other possible pathway that Ca^{2+} may activate prolactin gene expression is through the PKC signalling pathway (42, 43). PKC, in turn, induces prolactin transcription by activating the mitogenic activated protein kinase (43), which leads to phosphorylation of an Ets transcription factor and activation of prolactin gene transcription (43). Previous work from our laboratory has demonstrated the involvement of the PKC pathway in mediating the VIP stimulatory effect on prolactin mRNA expression in cultured turkey anterior pituitary cells (9).

In the present study, we used two different approaches to examine the involvement of Ca^{2+} released from intracellular stores on VIP-induced stimulation of prolactin expression and release. The use of TG to deplete the intracellular Ca^{2+} stores had no effect on basal or VIP-stimulated prolactin expression and release. Even though the use of NEO to inhibit PLC had no effect on VIP-stimulated prolactin mRNA content, it resulted in a 50% reduction in VIP-stimulated prolactin release (Fig. 5). The inhibitory effect of NEO on prolactin release induced by VIP is difficult to interpret. However, the possibility remains that the PKC pathway, which can be blocked by PLC inhibition with NEO, may explain the NEO inhibition of VIP-stimulated prolactin release. This is supported by the findings that PKC desensitisation decreases VIP-stimulated prolactin release from turkey anterior pituitary cells (9).

In conclusion, the present results show, for the first time in birds, that VIP increases $[Ca^{2+}]_i$ by the activation of the Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels, an effect that is abolished by dopamine via D_2 dopamine receptors. In addition, the results demonstrate the involvement of Ca^{2+} signalling pathway in the regulation of prolactin gene expression and prolactin release by VIP and dopamine in cultured avian (turkey) anterior pituitary cells. This is the first report to link the actions of VIP, the prolactin-releasing factor in avian species, and dopamine, the prolactin release-inhibiting factor in mammals, through a common intracellular signalling mechanism governing prolactin release and gene expression.

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