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Heterooligomerization between Vasotocin and Corticotropin-Releasing Hormone (CRH) Receptors Augments CRH-Stimulated 3′,5′-Cyclic Adenosine Monophosphate Production

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In birds, ACTH release from the anterior pituitary gland during stress is controlled by CRH and arginine vasotocin (AVT). Using 5-wk-old male chicks, simultaneous iv injections of CRH and AVT were found to result in a greater than additive increase in plasma corticosterone levels compared with that obtained with individual administration of either peptide hormone. In order to investigate molecular mechanisms underlying this observation, the chicken CRH receptor (CRHR) and vasotocin VT2 receptor (VT2R) were fused to cyan and yellow fluorescent proteins and expressed in HeLa cells. The resulting CRHR and VT2R fusion proteins were expressed appropriately in the plasma membrane and were found to couple to downstream signal transduction pathways. Quantitative fluorescence resonance energy transfer (FRET) analysis was used to determine whether the CRHR and VT2R formed heterodimers. In the absence of CRH and AVT, the FRET efficiency was 15–18%, and the distance between receptors was 5–6 nm. Treatment of the cells that expressed both cyan fluorescent protein-CRHR and yellow fluorescent protein-VT2R with CRH or AVT alone did not lead to a significant change in the FRET efficiency. However, simultaneous addition of these hormones increased the efficiency of the FRET signal and decreased the distance between the two receptors. In HeLa cells expressing both CRHR and VT2R, treatment with CRH and AVT resulted in a significant increase in cAMP production over that with CRH alone, indicating that heterodimer formation may enhance the ability of the CRHR to activate downstream signal transduction. (*Molecular Endocrinology* 21: 2178–2188, 2007)

STRESS ACTIVATES THE hypothalamic-pituitary-adrenal axis leading to the release of ACTH from the anterior pituitary gland, which subsequently stimulates the release of glucocorticoids from the adrenal cortex (1, 2). ACTH release from anterior pituitary corticotrophs is controlled by hormones produced in the hypothalamus, CRF or CRH, and either arginine vasopressin (AVP) in most mammals or arginine vasotocin (AVT) in nonmammalian vertebrates (3, 4). CRH and AVP/AVT exert their effects through G protein-coupled receptors (GPCRs). In the domestic chicken, the model system used in our laboratory, the GPCRs that mediate the effects of CRH and AVT on ACTH release are the CRH receptor (CRHR) and arginine vasotocin VT2 receptor (VT2R).

In mammals, the receptor for CRH that is expressed by corticotrophs is designated as CRH-R1 and has been cloned from several species including the human (5), monkey (6), rat (7), and mouse (8). An avian CRHR, a 420-amino acid protein that displays 87–88% identity to the CRH-R1 of human, rat, and mouse, has been cloned (9) and shown to be expressed in corticotrophs (10, 11). In mammals and birds, the CRH-R1 and the avian CRHR that are expressed by corticotrophs activate adenylyl cyclase leading to elevation of intracellular cAMP (12, 13). The effect of AVP on ACTH release is mediated by the V1b vasopressin receptor in mammals (14–16), and it is likely that the VT2R has the same role in birds (17). Both the V1b vasopressin receptor and the VT2R activate phospholipase C, leading to the generation of the intracellular second messengers inositol-1,4,5-triphosphate and diacylglycerol and the mobilization of intracellular calcium

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Abbreviations: β_2AR, β_2-Adrenergic receptor; AVP, arginine vasopressin; AVT, arginine vasotocin; BRET, bioluminescence resonance transfer; CFP, cyan fluorescent protein; CORT, corticosterone; CRHR, CRH receptor; FITC, fluorescein isothiocyanate; FRET, fluorescence resonance energy transfer; GPCR, guanine nucleotide protein-coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; POMC, proopiomelanocortin; VT2R; vasotocin VT2 receptor; YFP, yellow fluorescent protein.

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(17, 18). In some species, each hormone alone is able to stimulate ACTH release whereas when both AVP/AVT and CRH are administered together, greater than additive effects on ACTH release and/or adrenal corticosteroid output are observed (19–22). The cellular mechanism underlying the superadditive or synergistic effect of CRH and AVP/AVT on ACTH release is unknown.

Traditionally, GPCRs have been considered to function as monomeric proteins, although this concept has been challenged recently by studies that have demonstrated receptor oligomerization using biophysical methods (reviewed in Ref. 23). Of particular interest is a recent report in which the mammalian V1b vasopressin and the CRH-R1 were found to form heterodimers (24). Increasing evidence suggests that receptor oligomerization may play a role in the modulation of GPCR activity. For example, the formation of heterodimers between type 1 angiotensin II receptors and bradykinin B2 receptors results in increased activation of Gαq and Gαi proteins (25), and dimerization of adenosine A2A and dopamine D3 receptors leads to diminished functional signaling by D3 receptors (26).

In the present study, we examined the possibility that the CRHR and the VT2R form heterodimers. Confocal microscopy and fluorescence resonance energy transfer (FRET) techniques were used to demonstrate colocalization of the receptors and formation of heterotrimeric CRHR and VT2R complexes. The functional consequence of CRHR and VT2R oligomerization was investigated in HeLa cells transiently expressing both receptors and in male chicks administered AVT and CRH iv. Results from these experiments indicated an augmentation of CRH-mediated cAMP accumulation in the presence of AVT and a superadditive effect of CRH and AVT in stimulating corticosterone (CORT) release from the adrenal cortex. These findings, if extended to corticotrophs, would provide insight into the molecular mechanisms underlying the regulation of ACTH release by CRH and AVT.

RESULTS

Localization of Vasotocin VT2R and CRHR in the Anterior Pituitary Gland

To determine cell types within the anterior pituitary gland that expressed VT2R and CRHR, specific antibodies were used to detect the receptors and ACTH in tissue sections. Within the anterior pituitary, immunostaining for VT2R and CRHR was found in the cephalic lobe. Immunoreactivities for both receptors were largely colocalized and concentrated within a narrow cellular compartment outlining perikarya (Fig. 1, A–C), suggesting their likely association with the plasma membrane. Double labeled cells were typically arranged in a form of glandular islets with central sinusoids characteristic for avian corticotrophs. Finally, dual immunofluorescent labeling revealed that virtually all cells labeled with an antibody against proopiomelanocortin (POMC) (marker of corticotrophs) were also labeled with the VT2R antisera (Fig. 1D).

Effect of AVT and CRH on Plasma CORT Levels in Chicks

To demonstrate a possible synergistic effect of AVT and CRH, male chicks, 5 wk of age, were given iv injections of AVT and CRH that were known to induce modest release of CORT from the adrenal gland. As shown in Fig. 2, the combined administration of AVT (0.25 pmol) and CRH (0.30 pmol) resulted in significantly higher levels of plasma CORT compared with levels achieved after the same dose of each peptide given alone (ANOVA: $F_{1,20} = 18.99$, *post hoc* Bonferroni test: $P \leq 0.001$).

Molecular Cloning and Expression of the Cyan Fluorescent Protein (CFP)- and Yellow Fluorescent Protein (YFP)-Receptor Fusion Proteins

CFP and YFP were fused with the CRH and AVT receptors using the pECFP-Na and pEYFP-N1 vec-

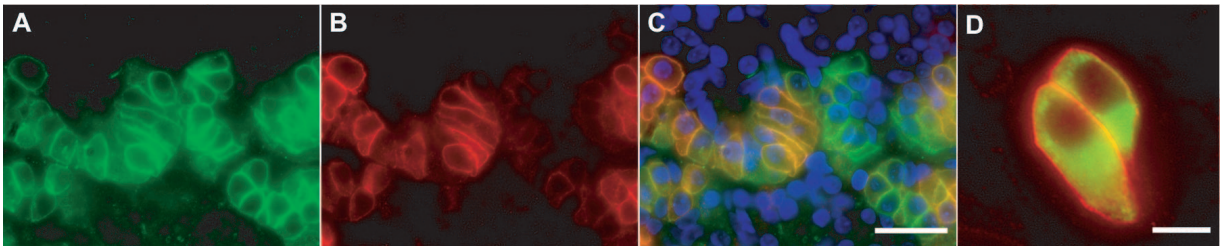

Fig. 1. Cells in the Cephalic Lobe of Chicken Anterior Pituitary Gland Double Immunolabeled for Vasotocin VT2R and CRHR

A, CRHR was detected using goat anti-CRHR primary antibody and biotinylated horse antigoat secondary antibody followed by incubation with streptavidin-FITC conjugate. B, VT2R was detected using rabbit anti-VT2R primary antibody and donkey antirabbit secondary antibody conjugated with Rhodamine Red-X. C, Images from panels A and B were superimposed to demonstrate colocalization of VT2R and CRHR. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole. D, Images obtained using rabbit anti-VT2R primary antibody and donkey antirabbit secondary antibody conjugated with Rhodamine Red-X, and monoclonal mouse antichicken proopiomelanocortin primary antibody and FITC-conjugated goat antimouse secondary antibody were superimposed to demonstrate expression of VT2Rs by corticotrophs. *Scale bars*, A–C, 20 μm; D, 5 μm.

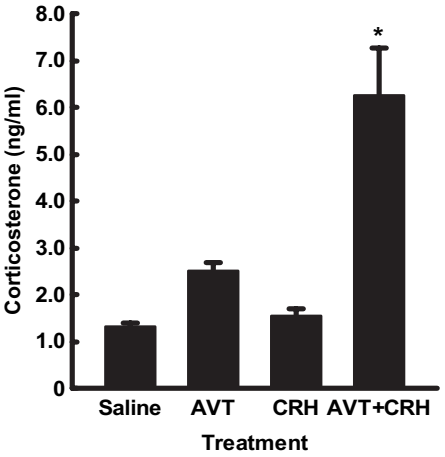

Fig. 2. Plasma CORT Levels

Plasma CORT levels were measured in chicks, 5 wk of age, 30 min after iv administration of either physiological saline, AVT, CRH, or AVT plus CRH (n = 10/treatment). The *asterisk* indicates that the AVT + CRH treatment group displayed significantly higher CORT levels than that observed with the other treatments (ANOVA: $F_{1,20} = 18.99$; *post hoc* Bonferroni Test: $P \leq 0.001$).

tors, respectively. To determine whether the fusion proteins were properly expressed, HeLa cells were transiently transfected with either the CFP-CRHR or YFP-VT2R fusion proteins. Using confocal microscopy we found that CFP-CRHR and YFP-VT2R were expressed on the cell surface (data not shown). Although observations using confocal microscopy confirmed that the CFP- and YFP-receptor fusion proteins were expressed by HeLa cells, these results did not indicate that the fusion proteins were capable of activating signal transduction pathways. Therefore, we conducted experiments to determine whether CFP-CRHR and YFP-VT2R, upon expression in HeLa cells, were able to activate cAMP production and calcium mobilization, respectively. Previously, we had shown that the VT2R, when expressed in fura 2-loaded COS7 cells, was able to mobilize intracellular calcium (17). Therefore, we conducted calcium mobilization experiments in HeLa cells that had been transiently transfected with the YFP-VT2R-encoding an expression plasmid. Nontransfected fura 2-loaded HeLa cells did not respond to the addition of 0.1 μM AVT (Fig. 3A). However, in HeLa cells transfected with either YFP-VT2R or pcDNA3/VT2R there was a rapid rise in intracellular calcium (Fig. 3, B and C). The kinetics of the response was similar with either receptor and was consistent with that observed with other Gq-coupled receptors (27, 28). Upon binding of hormone to CRHRs, intracellular cAMP levels are known to increase in corticotrophs (7, 12). Therefore, we examined cAMP production in HeLa cells transfected with CFP-CRHR treated with CRH. In nontransfected HeLa cells and cells transfected with the pECFP-N1 expression plasmid, CRH did not increase cAMP production (Fig. 4), indicating an absence of CRHRs in HeLa cells. However, in HeLa cells transfected with CFP-CRHR, cAMP levels were significantly ($P < 0.05$) elevated upon the addition of CRH (Fig. 4).

FRET Analysis

The potential to form heterodimers between CRHR and VT2R was studied in transfected HeLa cells using FRET analysis. This method detects the transfer of excitation energy from a donor fluorophore to a nearby acceptor that can occur only over a distance of about 3–7 nm (29–31). We used the acceptor photobleaching technique that entails measuring an increase in donor fluorescence after photobleaching of the acceptor. The dequenching donor emission after acceptor photobleaching provides a direct measure of FRET efficiency (30–33). Results from a typical experiment are shown in Fig. 5. Images of the acceptor (YFP-VT2R,

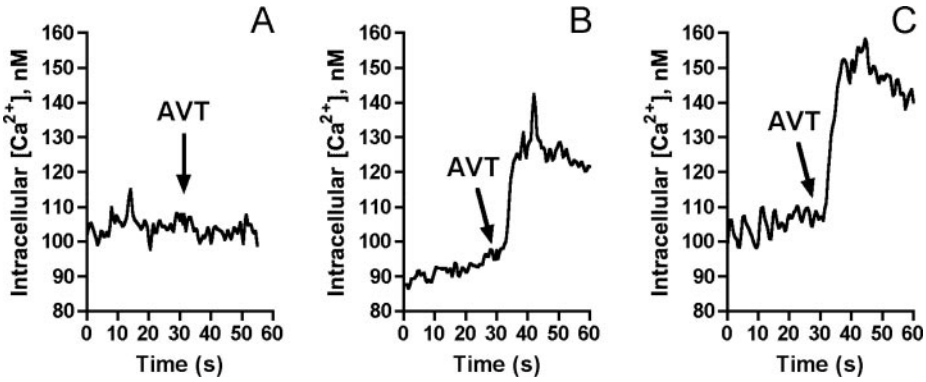

Fig. 3. AVT-Stimulated Calcium Mobilization in VT2R-Expressing HeLa Cells

HeLa cells were either untransfected, transfected with pcDNA3/VTR or pYFP-VT2R, and subsequently treated with 0.1 μM AVT. Changes in fura 2 fluorescence were used to monitor intracellular calcium levels. A, Representative tracing from control HeLa cells that demonstrates unresponsiveness to AVT. B, Representative tracing from HeLa cells that had been transfected with pcDNA3/VT2R 24 h before fura 2 loading and treatment with AVT. C, Representative tracing from HeLa cells that had been transfected with pYFP-VT2R 24 h before fura 2 loading and treatment with AVT. s, Seconds.

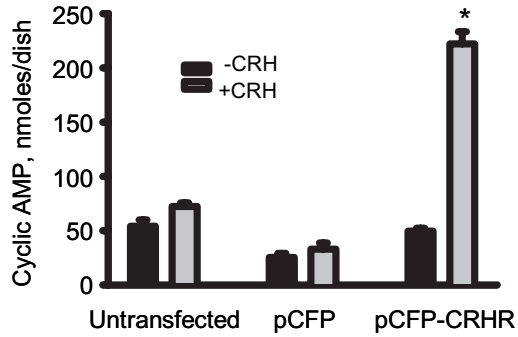

Fig. 4. CRH-Stimulated cAMP Production in CRHR-Expressing HeLa Cells

HeLa cells were either not transfected, transfected with pCFP or pCFP-CRHR, and 24 h later were treated with 0.1 μM CRH in the presence of 1 mM IBMX, a phosphodiesterase inhibitor. Cells were treated with either vehicle or CRH for 30 min, followed by cell lysis and determination of intracellular cAMP content by enzyme immunoassay. Values are expressed as the mean ± SEM (n = 4). *, Significantly different ($P < 0.001$) from all other groups.

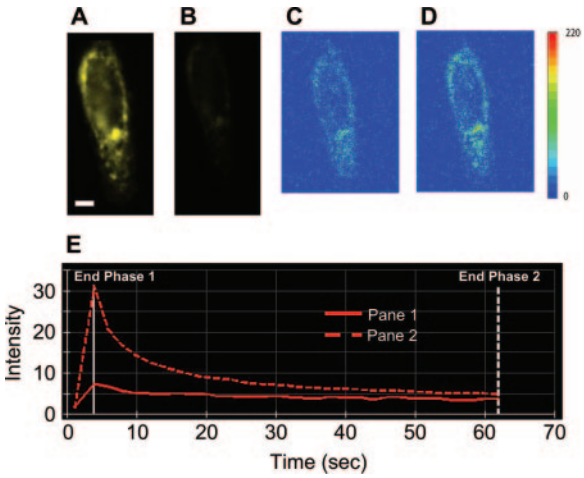

Fig. 5. Confocal FRET Microscopy with Photobleaching

HeLa cells were transfected with pCFP-CRHR and pYFP-VT2R, and 24 h later were treated with 0.1 μM CRH and 0.1 μM AVT. FRET measurements were taken 30 min after hormone addition. A, Image of a HeLa cell taken with the acceptor (YFP) excitation wavelength (514 nm) and observed at 545/40 nm (acceptor channel). B, Image of a HeLa cell after YFP photobleaching. C, Image of a HeLa cell taken with the donor (CFP) excitation wavelength (457 nm) and collected with an emission wavelength of 485/30 nm (donor channel). D, Image of the same HeLa cell after photobleaching of the acceptor (YFP) with the same excitation and emission wavelengths used in panel C. E, Intensity of YFP fluorescence during photobleaching at a power setting of 960 μW. A color scale with the equivalent numerical values for the FRET intensity is displayed. *Red* represents a high FRET signal, and *blue* represents a low FRET signal. *Scale bar*, 20 μm.

Fig. 5A) and donor (CFP-CRHR, Fig. 5C) are shown before photobleaching. Corresponding images of the acceptor and donor are shown after photobleaching of the acceptor (Fig. 5, B and D). The increase in donor fluorescence (CFP-CRFR) after acceptor (YFP-VT2R) photobleaching was observed in all experiments. The acceptor (YFP-VT2R) fluorescence was decreased by 98% after photobleaching (Fig. 5E). To obtain a quantitative measure of FRET efficiency and estimate the distance between donor and acceptor molecules from the same cell, image data were processed using PFRET software obtained from the W. K. Keck Center for Cellular Imaging (University of Virginia, Charlottesville, VA) (34, 35). Quantitative FRET analysis (Fig. 6) demonstrated that CFP-CRHR and YFP-VT2R formed heterodimers with a calculated distance of 5–6 nm between molecules. Based on our calculation, the FRET efficiency was 15–36%. A low-resonance energy transfer signal was obtained in untransfected HeLa cells (Fig. 6). Cotransfection of HeLa cells with CFP-CRH-R and YFP-VT2R resulted in a constitutive resonance energy transfer between the fluorescence donor and acceptor (Fig. 6). Addition of either CRH or AVT separately resulted in a small, statistically nonsignificant, augmentation of FRET efficiency, whereas simultaneous addition of the two hormones resulted in a significant ($P < 0.05$) increase in FRET efficiency (Fig. 7).

In some instances, dimers can form between two EFP molecules regardless of the existence of any other dimerization of the proteins fused to these fluorescent proteins (36). A single point mutation, A207K, in the fluorescent part of the fusion protein has been shown to prevent the formation of nonspecific dimerization (36). To demonstrate that a FRET signal results from a specific interaction between CRHR and VT2R rather than due to a nonspecific interaction occurring between the fluorophores, the site-directed mutation of Ala ^{207} to Lys was performed in the fluorescent parts of the cyan fluorescent protein (CFP)-CRHR, CFP-VT2R, and YFP-VT2R fusion proteins, as well as the YFP-β_2-adrenergic receptor fusion protein (YFP-β_2AR), which was used as a negative control. The mutated variants of the fusion proteins were expressed in the same cellular compartments as wild-type receptors (data not shown). FRET analysis of different combinations using mutant (m) and wild-type proteins showed that dimerization of CRHR and VT2R occurred between the proteins themselves and not through the fluorescent parts of the fusion proteins (Fig. 8). Our results also showed that neither the CRHR nor the VT2R was able to dimerize with the β_2-adrenergic receptor (Fig. 8). This observation further demonstrated the specificity of the interaction between the CFP-CRHR and the YFP-VT2R.

Molecular Signaling in HeLa Cells Coexpressing CFP-CRHR and YFP-VT2R

To obtain functional data on the possible interaction between the hormones and/or their respective receptors, CRHR and VT2R were transiently coexpressed in HeLa cells and tested for their abilities to mediate stimulation of intracellular calcium mobilization (Fig. 9)

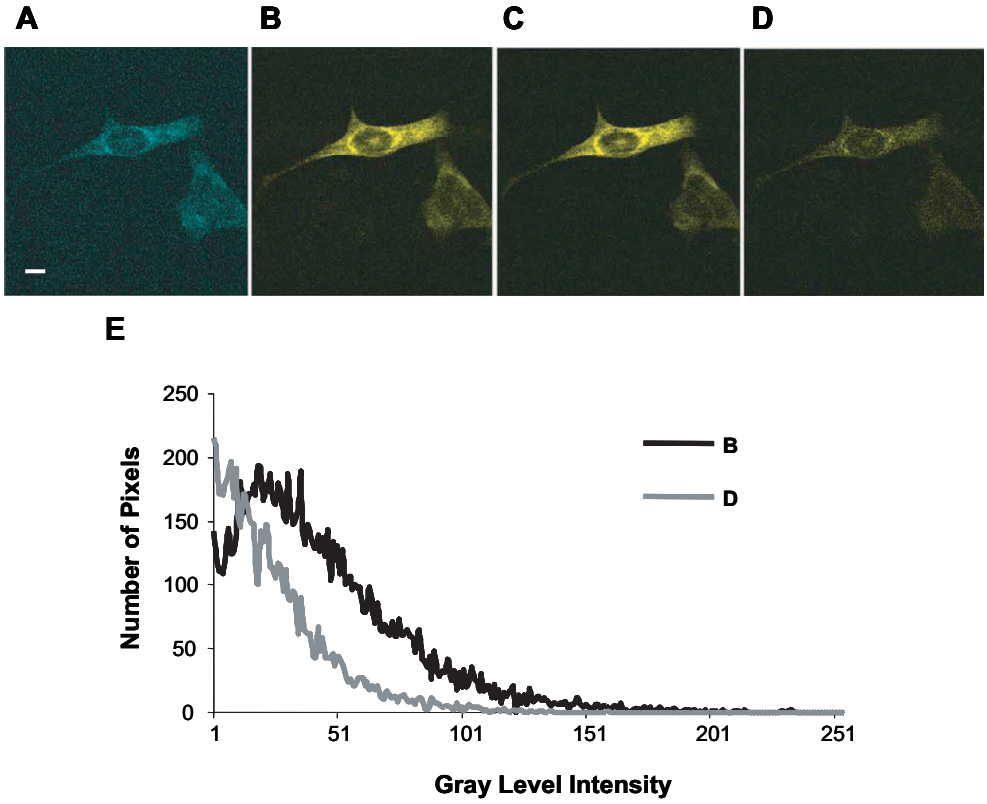

Fig. 6. Images for Algorithm FRET Method

Laser scanning confocal images of CRHR and VT2R in HeLa cells. HeLa cells were labeled with CFP (donor, CRHR) and YFP (acceptor, VT2R). To set the parameters for quantitative FRET, the images of HeLa cells expressed only CFP-CRFR, and only YFP-AVTR have been taken with donors and acceptors filter sets (images are not shown). Using Bio-Rad Radiance 2100 confocal microscopy, confocal processed FRET (PFRET) fluorescence signals (nonradioactive transfer of energy from CRHR donor fluorophore to VT2R acceptor fluorophore) were collected in the same double-labeled cells. Images are representative of six experiments. Images collected during panel A: double-labeled donor excitation, donor channel revealing the distribution of CRHR; panel B: double-labeled, donor excitation, acceptor channel; panel C: double-labeled acceptor excitation, acceptor channel revealing the distribution of VT2R. D, PFRET calculated with algorithm methods; E, respective histograms. *Scale bar*, 20 μm.

and cAMP production (Fig. 10). When added alone, AVT, but not CRH, stimulated [Ca^{2+}] mobilization in fura 2-loaded HeLa cells, whereas CRH was much more effective than AVT in stimulating cAMP production. There was no additive or synergistic effect of the two hormones in [Ca^{2+}] mobilization experiments. The level of calcium mobilization in HeLa cells expressing both the CRHR and VT2R obtained with the addition of AVT was not changed with the addition of CRH (Fig. 9). Significantly, in HeLa cells expressing both the CRHR and VT2R, cAMP production in the presence of both CRH and AVT was approximately 3-fold higher compared with cAMP levels achieved with CRH administration alone (Fig. 10).

DISCUSSION

Peripheral administration of both AVT and CRH to chicks demonstrated that the two peptides or neurohormones acted synergistically to augment the release of CORT in the plasma (Fig. 2). Our dual-labeled immunocytochemistry studies showed that the VT2R and CRHR were located on the same pituitary cell (Fig. 1C) and that the VT2R was located predominantly in corticotrophs (Fig. 1D). We therefore designed a set of experiments to test the hypothesis that AVT and CRH form heterodimers on the membrane surface, thereby playing a potential role in enhancing the cell signaling pathways responsible for the ultimate, augmented release of CORT from the adrenal gland. The following discussion includes information from past studies and current data supporting our hypothesis.

Over the past several years, resonance energy transfer approaches, namely fluorescence resonance energy transfer (FRET) and bioluminescence resonance transfer (BRET), have been widely used to monitor interactions between proteins in living cells (37). Our results using FRET demonstrate that, when transiently expressed in HeLa cells, the avian CRHR and VT2R may form heterodimers. Thus, our results confirm a recently reported finding in which a mammalian

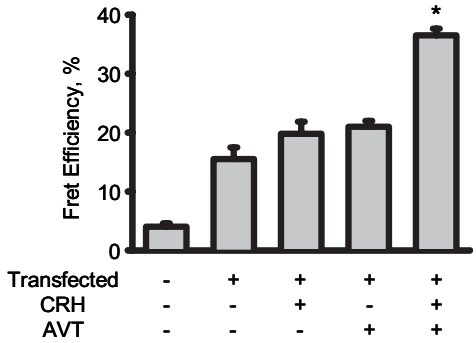

Fig. 7. FRET Efficiency after Treatment with CRH and AVT

HeLa cells were either not transfected or transfected with pCFP-CRHR and pYFP-VT2R and 24 h later were treated with either vehicle, 0.1 μM CRH, 0.1 μM AVT, or 0.1 μM CRH and 0.1 μM AVT together. FRET measurements were taken 30 min after hormone addition. Data are the means ± SEM (n = 6). *, Significantly different ($P < 0.05$) from all other groups.

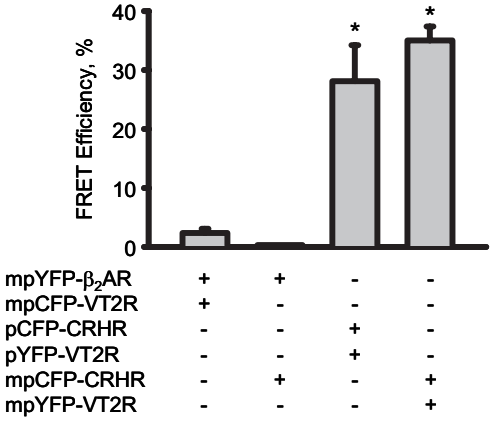

Fig. 8. Specificity of the FRET Signal

HeLa cells were transfected with various expression plasmids that resulted in expression of fluorophore-receptor fusion proteins and 24 h later were treated with 0.1 μM CRH and 0.1 μM AVT. FRET measurements were taken 30 min after hormone addition. Data are the means ± SEM (n = 6). *, Significantly different ($P < 0.05$) compared with HeLa cells expressing either mutated YFP-β2AR and mutated CFP-VT2R or mutated CFP-CRHR and mutated YFP-VT2R.

V1b vasopressin receptor and the CRH-R1 were observed to form heterodimers (24) Interestingly, we found that the formation of CRHR-VT2R heterodimers is augmented in the presence of endogenous ligands for the CRHR and VT2R, CRH and AVT, respectively. Neither hormone when administered alone was capable of stimulating heterodimer formation. Our findings with the VT2R and CRHR are different than those of Young *et al.* (24), who found no effect of AVP and CRH on receptor oligomerization.

To date, studies of oligomerization of G protein-coupled receptors have been performed in transfected immortalized cell lines, rather than in primary cell culture. In part, this is because many immortalized cell lines are readily transfected with fluorescently tagged receptor protein-expressing plasmids. A concern that is sometimes raised in studies of GPCR oligomerization in transiently transfected cell lines is the possibility that FRET or BRET signals might be artifacts that arise from high level expression of introduced receptor genes. However, in the study of homodimerization of neuropeptide Y receptors, the FRET effect was shown to be independent of the amount of receptor that was expressed by baby hamster kidney and Chinese hamster ovary cells (38). Moreover, heterodimerization of β_2- and β_3-adrenergic receptors has been shown in BRET studies to be independent over a wide range of receptor protein expressed in human embryonic kidney 293 cells (39). In our studies, although we were unable to measure directly the level of receptor expression because radioligands are not available for the avian CRHR and VT2R, transfection conditions were set to ensure that receptor levels achieved were not excessive.

We selected HeLa cells for our studies because they readily expressed both the fluorescently tagged CRHR and VT2R and were easily manipulated in the FRET studies. Importantly, we determined that both fluorescently tagged receptors were appropriately expressed in the plasma membrane of transfected HeLa cells with some expression evident in the endoplasmic reticulum. Both fluorescently tagged receptors retained their respective abilities to activate downstream signal transduction pathways. In experiments with HeLa cells transiently expressing the CFP-CRHR fusion protein, the addition of CRH resulted in a rapid increase in intracellular cAMP. Previous studies have shown that the avian CRHR, when activated by CRH, is coupled to cAMP generation (40). Similarly, in HeLa cells transiently expressing YFP-VT2R and loaded with fura 2, the addition of AVT resulted in a rapid rise in intracellular calcium levels, consistent with previously demonstrated effects of AVT on VT2R function (17).

Importantly, in an extension of the experiments to determine whether fluorescently tagged CRHR and VT2R retained their abilities to activate their respective downstream signal transduction pathways, we determined that in the presence of AVT, cAMP production was greatly enhanced in HeLa cells expressing both receptors that were treated with CRH. Interestingly, in parallel experiments using fura 2-loaded HeLa cells, there was no effect of CRH on AVT-mediated calcium mobilization. These findings suggest that heterodimerization of CRHR and VT2R, rather than having generalized effects on signal transduction pathways, instead has a specific effect on the cAMP pathway. Although speculative, one possible explanation is that compared with CRHR monomers, CRHR-VT2R heterodimers couple more efficiently to the G protein Gs, which couples the CRHR to adenylyl cyclase.

Our findings place the CRHR and VT2R in a growing group of G protein-coupled receptors that have been shown, at some point in their life cycle, to form oligomers either as homodimers or as heteromers with

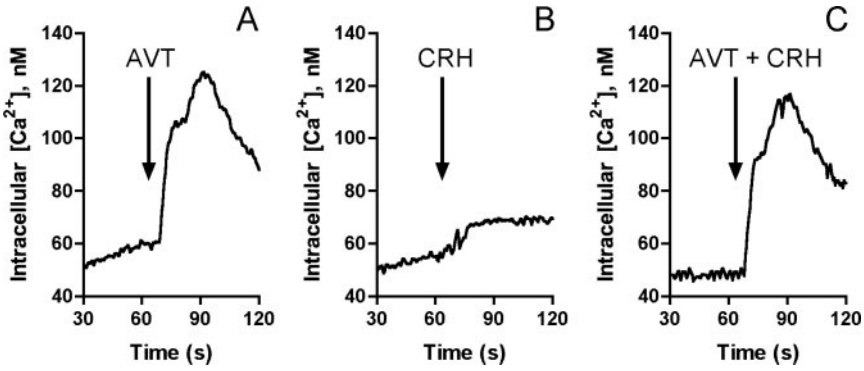

Fig. 9. Calcium Mobilization in YFP-CRHR- and YFP-VT2R-Expressing HeLa Cells

HeLa cells were transfected with pYFP-CRHR and pYFP-VT2R and 24 h later treated with either AVT, CRH, or AVT plus CRH. Changes in fura 2 fluorescence were used to monitor intracellular calcium levels. A, Transfected HeLa cells were treated with 0.1 μM AVT. B, Transfected HeLa cells were treated with 0.1 μM CRH. C, Transfected HeLa cells were treated with 0.1 μM AVT and 0.1 μM CRH. s, Seconds.

other G protein-coupled receptors (23, 24, 41–43). We believe that the ability of the CRHR and VT2R to form heterodimers, especially in the presence of CRH and AVT, may underlie the well-known greater than additive effect and, in some cases, synergistic effect that is observed in the release of ACTH in the presence of both CRH and AVP in mammalian systems (19, 21, 22), and in the presence of CRH and AVT in at least one nonmammalian vertebrate system (20). Our findings indicate that when administered peripherally, simultaneous delivery of CRH and AVT leads to greater circulating CORT levels than with administration of either hormone alone. Thus, the domestic chicken can be added to the list of organisms in which CRH and either AVP or AVT have been shown to synergistically activate the hypothalamic-pituitary-adrenal axis. Given the important role that the corticotroph plays in mediating systemic responses to stress, one might imagine that CRHR-VT2R dimerization, in the case of nonmammalian vertebrates, and CRH-R1-V1b-vasopressin receptor dimerization, in the case of mammalian species including humans, may be altered under different stress conditions. This, in turn, could alter responsiveness of the hypothalamic-pituitary-adrenal axis and ultimately the ability of the organism to adapt to stress.

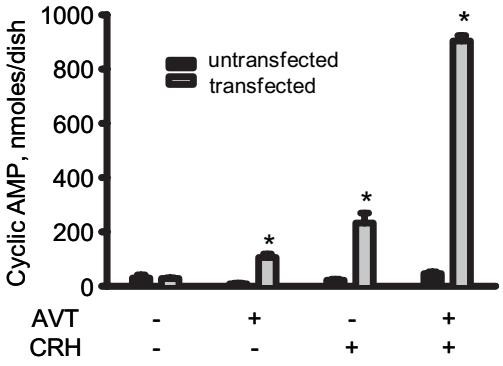

Fig. 10. cAMP Production in YFP-CRHR- and YFP-VT2R-Expressing HeLa Cells

HeLa cells were either not transfected or transfected with pCFP-CRHR and pYFP-VT2R and 24 h later were treated with CRH, AVT, or CRH plus AVT in the presence of 1 mM IBMX, a phosphodiesterase inhibitor. Cells were treated with hormones for 30 min, followed by cell lysis and determination of intracellular cAMP content by enzyme immunoassay. Values are expressed as the mean ± SEM (n = 4). *, Significantly different ($P < 0.001$) from untransfected cells.

In conclusion, our work demonstrates that the CRHR and VT2R proteins are coexpressed in corticotrophs of the chicken pituitary gland. Moreover, simultaneous iv injections of CRH and AVT in male chicks were found to result in a greater than additive increase in plasma CORT levels compared with that obtained with individual administration of either peptide hormone. When expressed in HeLa cells, CRHR and VT2R were found to form heterodimers in the presence of CRH and AVT. A correlate to heterodimer formation was observed in HeLa cells expressing both CRHR and VT2R. We found that the combination of CRH and AVT significantly augments cAMP production when compared with the administration of CRH alone. Our studies carried out in a heterologous expression system have allowed us to make predictions regarding the functional consequences of heterodimerization in cells that normally express CRHR and VT2R. Future studies using corticotrophs should help determine the physiological significance of CRHR-VT2R heterodimerization during stress.

MATERIALS AND METHODS

Dual Immunofluorescence Labeling

Two male and two female 6- to 9-wk-old broiler chickens were deeply anesthetized with sodium pentobarbital (35 mg/kg, iv) and perfused through the carotid arteries with 150 ml heparinized 0.1 M sodium phosphate buffer (pH 7.4)

followed by 350 ml of Zamboni's fixative (pH 7.4). Pituitary glands were dissected, postfixed for 5–8 h in the same fixative, and cryoprotected in 30% sucrose in phosphate buffer at 4 C, frozen in dry ice, and stored at −85 C until sectioned at 10 μm in a horizontal plane using a cryostat (Leica CM 3050 S). Sections were mounted on gelatin-coated glass slides and stored at −85 C in sealed boxes until processed for immunohistochemistry. Sections were washed in three changes (10 min each) of 0.02 M PBS (pH 7.4), treated with 0.4% Triton X-100 in PBS for 20 min, and blocked for 30 min with 5% BSA in PBS containing 0.2% Triton X-100. Sections were then incubated in a mixture of rabbit antibody against chicken VT2R (1:6000) and goat antibody against a 20-amino acids peptide matching a domain at the carboxy terminus of the human CRH-R1/2 receptor precursor (sc-1757, 1:3000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). According to the manufacturer, the amino acid sequence of the peptide used to produce the latter antibody shares 95% identity with chicken CRH-R1 and 85% identity with chicken CRH-R2. Primary antibodies were diluted in 5% BSA in PBS containing 0.2% Triton X-100 with added 0.2% BSA and were applied to the sections in a humidified chamber for 40 h at 4 C. After three 10-min washes in PBS, sections were incubated for 90 min with a mixture of donkey antirabbit antibody conjugated with Rhodamine Red-X (1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and biotinylated horse antigoat antibody (1:400; Vector Laboratories, Inc., Burlingame, CA). Sections were then washed 3 × 10 min in PBS, incubated with streptavidin-fluorescein isothiocyanate (FITC) conjugate (1:300, Jackson ImmunoResearch) for 2 h, washed 2 × 10 min in PBS, briefly rinsed with distilled water, and coverslipped with Vectashield mounting medium (Vector Laboratories). To identify the endocrine phenotype of VT2-immunoreactive pituitary cells, sections of the pituitary gland were treated with a mixture of antibody against the chicken VT2R diluted 1:6000 and mouse monoclonal antibody 16D9 against chicken proopiomelanocortin (POMC, marker of corticotrophs, provided by Dr. L. R. Berghman) diluted 1:5000. Labeling was visualized by incubating sections sequentially in goat antimouse antibody conjugated with FITC (1:400, Jackson ImmunoResearch) and donkey anti-rabbit antibody conjugated with Rhodamine Red-X (1:400). Specificity of the antiserum to chicken VT2R was extensively tested elsewhere (44). Additional controls of immunolabeling specificity included omission of one of the primary antibodies from the mixture. It resulted in a complete loss of fluorescent labeling used to visualize a specific antigen. Pituitary sections were viewed using a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Thornwood, NY) equipped with epifluorescent attachment. Images were acquired using a Hamamatsu Orca_ER camera and Simple PCI software (Compix, Inc., Sewickley, PA). All procedures using animals in this study had been previously approved by the University of Arkansas Institutional Animal Care and Use Committee.

CORT Analysis in Plasma

Forty chicks, 5 wk of age, were used in the study and divided into four treatment groups: saline, AVT, CRH, and AVT plus CRH (n = 10/treatment). Birds were administered, via the brachial vein, either the carrier (physiological saline), AVT (0.25 pmol), CRH (0.30 pmol) or combination of AVT (0.25 pmol) and CRH (0.30 pmol). Thirty minutes later, a blood sample (2.0 ml) was taken and plasma analyzed for CORT by RIA as previously described (45).

Molecular Cloning of CFP-CRHR and YFP-CRHR Fusion Proteins

CRHR was amplified by PCR from a pcDNA1-CRHR plasmid that we obtained from Dr. Abou-Samra (Massachusetts General Hospital, Boston, MA) using sense and antisense primers (5′-GGCCGAATTCATATGGTGCCCGGCCCGCG-3′ and 5′-TCAACGTGGATCCACTGCTGAGGACTGCT-3′, respectively) containing sites for *Eco*RI and *Bam*HI restriction enzymes, respectively. TheVT2R was amplified by PCR from a pcDNA3-AVTR construct (17) with sense and antisense primers (sense, 5′-GGCCGAATTCATATGGAGCCGGGATGGAGCT-3′, and antisense, 5′-TCAACGTGGATCCAGCGTGCCCGATTCAGT-3′) containing sites for *Eco*RI and *Bam*HI restriction enzymes, respectively. The PCR products were initially cloned into the pGEM-T vector (Promega Corp., Madison, WI). The identity of the insert was confirmed by sequencing, and then the insert was excised using *Eco*RI and *Bam*HI restriction enzymes and recloned in pECFP-N1 or pEYFP-N1 expression vectors (CLONTECH Laboratories, Inc., Mountain View, CA) using the same restriction enzyme sites to obtain pECFP-CRHR, pEYFP-CRHR, pECFP-VT2R, and pEYFP-VT2R constructs.

Molecular Cloning of Human β_2-Adrenergic Receptor (YFP-β_2AR)

The β_2AR gene was amplified from human genomic DNA (CLONTECH) by PCR with the primers 5′-CCGGAATTC-CAGTGCGCTTACCTGCCAGA-3′ and 5′-CAGCAGTGAGT-CATTTGTACTA CAATTC-3′. The PCR product was digested with *Eco*RI, and the 1274-bp fragment was purified from an agarose gel slice using the gel extraction kit from QIAGEN (Valencia, CA). The *Eco*RI-digested PCR product was ligated into the multiple cloning site of pEYFP-N1 (CLONTECH).

DNA Sequencing

The orientation and sequence of all inserts were confirmed using an Applied Biosystems 377 DNA sequencer and a Dye Terminator DNA sequencing kit with AmpliTaq DNA polymerase FS (PE Applied Biosystems, Warrington, UK). For DNA sequence analysis, the GenBank database and the MacVector computer program were used.

Transient Transfection and Fusion Protein Expression

HeLa cells (ATCC no. 30–2003) were cultured in MEM, supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM glutamine, 100 U/ml penicillin G, and 100 U/ml streptomycin in a humidified incubator with 95% air and 5% CO_2 at 37 C. Transient transfections were done in six-well culture plates at a density of 4×10^5 cells per well (70–80% confluency). A solution containing 1 μg of the appropriate plasmid in 375 μl serum-free MEM were mixed with another solution containing 12 μl Lipofectamine (Invitrogen) in 375 μl serum-free MEM, mixed gently, and incubated at room temperature for 20 min as described in the manufacturer's protocol. The DNA ratios from 1:1 to 1:1.2 of the two expression vectors were adjusted to obtain approximately equal amounts of the pair of CFP and YFP fusion proteins. Cells were washed twice with 2 ml serum-free MEM. For each transfection, 750 μl of serum-free MEM were added to each tube containing the lipid-DNA complexes. The diluted complex solution was mixed and gently overlaid onto the washed cells. Cells were then incubated for 5 h at 37 C in a CO_2 incubator. Another 1.5 ml MEM with 10% FBS was added without removing the transfection mixture. The medium was replaced 18–24 h after start of transfection. Cells were used for assays 24–72 h after transfection. The efficiency of transient expression detectable by fluorescence of fusion proteins was analyzed under a fluorescent microscope.

Hormonal Treatment

AVT and CRH were diluted into modified receptor buffer (10 mM KCl; 10 mM $MgCl_2$; 2 mM EGTA; 20 mM HEPES; 120 mM NaCl; 1 mg/ml BSA, pH 7.4) as previously described (46). HeLa cells transiently transfected with CRHR-, VT2R-, and β_2AR-fusion protein constructs were incubated with 100 nM human CRH and 100 nM (Arg8)-vasotocin for 20 min at 37 C.

cAMP Determination

Intracellular cAMP content was measured with the cAMP Biotrack Enzyme Immunoassay (EIA) System (Amersham Biosciences, Piscataway, NJ). Cells were seeded in 96-well plates at a density of approximately 1–10 $\times$ 10^5 cells per well in 200 μl media and allowed to grow for 24 h at 37 C. On the day of an experiment, the medium was replaced with the fresh media containing 1 mM 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cyclic nucleotide phosphodiesterase, 30 min before the addition of the hormones to prevent the breakdown of accumulated cAMP. The hormones were added and the incubation continued at 37 C for 30 min, at which time 100 μl lysis reagent was added to each well, and plate was shaken on the microplate shaker for 10 min. From each unknown sample, 100 μl from the cell culture plate were transferred into the appropriate well of the immunoassay microtiter plate. cAMP was measured using the manufacturer's nonacetylation protocol (Amersham Biosciences). Calculations were performed using Multiskan Ascent software (Thermo Fisher Scientific, Inc., Waltham, MA).

Measurement of [Ca^{2+}]

Intracellular $[Ca^{2+}]_i$ concentration was measured in stirred suspensions of cells harvested using trypsin (0.25%) and EDTA (2.21 mM). Harvested cells were washed and resuspended in a modified Geys buffer containing 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose, 1 mM probenecid, 10 mM HEPES, pH 7.4. Cells were loaded with the intracellular fluorescent calcium dye fura 2-acetoxymethyl ester for 20 min at 25 C. Cells were then washed twice (200 $\times$ *g* for 2 min) in modified Krebs' buffer and resuspended in the same buffer to a concentration of approximately 1.5 $\times$ 10^6 cells/ml. Aliquots (1.5 ml) were allowed to warm to 37 C for 3 min before the measurement of fura 2 fluorescence while inside a 1-cm^2 cuvette with continuous stirring using a Hitachi F2000 spectrofluorometer (Hitachi Instruments, Danbury, CT) equipped with a thermostatic cell holder and magnetic stirrer. At the end of this equilibration period, a 30-sec baseline reading was made before the addition of the CRH or AVT. Measurements of the 340:380 nm emission ratio were made using an excitation wavelength of 510 nm. The emission was measured at each excitation wavelength every 0.5 sec. Maximum fluorescence was determined by the addition of 0.2% Triton X-100 (final concentration). Minimum fluorescence was obtained by the subsequent addition of 10 mM EGTA (final concentration). Intracellular free calcium $[Ca^{2+}]_i$ has been calculated using the following formula: $[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R)$, where R is the 340:380 fluorescence ratio and dissociation constant (K_d) = 224 nM (47). Each set of experiments was completed within 30–60 min after loading with fura 2.

Site-Directed Mutagenesis

Mutations of Ala 207 to Lys in the fluorescent part of pECFP-N1 and pEYFP-N1 expression vectors were made by using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA), and *PfuTurbo* DNA polymerase (Stratagene). The 50 μl reaction mixtures each contained approximately 40 ng plasmid template, 125 ng each oligonucleotide primer sense A206K top: 5′-CAG TCC AAG CTG AGC AAA GAC CCC AAC GAG AAG CGC GAT CAC-3′; and antisense A206K bottom: 5′-GTG ATC GCG CTT CTC GTT GGG GTC TTT GCT CAG CTT GGA CTG-3′ (36) (Integrated DNA Technologies, Coralville, IA), each deoxynucleoside triphosphate (Stratagene) to a final concentration of 0.05 mM, and 2.5 U *PfuTurbo* DNA polymerase. After a 1-min hot start at 95 C, 16 cycles of the following program were run: denaturation for 30 sec at 95 C, primer annealing for 1 min at 55 C, and polymerization for 7 min at 68 C. The PCR product was digested for 1 h at 37 C with restriction enzyme *Dpn*I (Stratagene) to eliminate the original template and thereby increase mutation efficiency. The DNA sequences of the mutated plasmids were verified by sequencing as described above.

FRET Analysis

HeLa cells were grown as described above on cover slips placed on the bottom of six-well plates for 24 h. Before transfection with CFP/YFP constructs, the cover slips were moved to new six-well plates containing fresh media. The fluorescent fusion proteins were visualized in intact cells 24 after transfection. We used SimplePCI software (Compix, Cranberry Township, PA), and images were collected by using Bio-Rad Radiance 2100 laser scanning confocal microscope coupled to Nikon TE-300 epifluorescence microscope with a $\times$60 water immersion objective. CFP was excited at 457 nm (power 118 μW) observed from 485/30 nm. YFP was excited at 514 nm (power 29 μW), observed from 545/40 nm. Images from all single-labeled (CFP or YFP fusion proteins samples) or double-labeled (CFP and YFP fusion proteins) of specimens were taken under exactly the same conditions (PCM 1024 $\times$ 1024 color, $\times$2.3 zoom, no processing). There are two contaminants in the FRET signal: donor cross talk and acceptor bleed through. We used the previously developed algorithm (34), which removes these contaminants pixel-by-pixel on the basis of matched fluorescence levels between the double-label specimen and a single-label reference specimen, using seven images: two single-label donor reference images (donor excitation/donor channel and acceptor channel); two single-label acceptor reference images (donor and acceptor excitation, both in the acceptor channel); and three double-label images (acceptor excitation/acceptor channel, and donor excitation/donor and acceptor channels. This algorithm allows the capture of a pure FRET image with following E% analysis and distance calculation (36). Grayscale images with no saturated pixels were taken in sequence without changing any parameters.

Statistics

Statistical evaluation of the data was carried out using either ANOVA followed by a *post hoc* test, or the Student's *t* test. $P < 0.05$ was considered to be significant.

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