Signal transduction in the vomeronasal organ of garter snakes: ligand–receptor binding-mediated protein phosphorylation

Jinming Liu b, Ping Chen a, Dalton Wang a,*, Mimi Halpern b

a Department of Biochemistry, SUNY Health Science Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, NY 11203, USA
b Program in Neural and Behavioral Science, SUNY Health Science Center at Brooklyn, Brooklyn, NY 11203, USA

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Abstract

The vomeronasal (VN) system of garter snakes plays an important role in several species-typical behaviors, such as prey recognition and responding to courtship pheromones. We (X.C. Jiang et al., J. Biol. Chem. 265 (1990) 8736–8744 and Y. Luo et al., J. Biol. Chem. 269 (1994) 16867–16877) have demonstrated previously that in the snake VN sensory epithelium, the chemoattractant ES20, a 20-kDa glycoprotein derived from electric shock-induced earthworm secretion, binds to its receptor which is coupled to PTX-sensitive G-proteins. Such binding results in elevated levels of IP3. We now report that ES20–receptor binding regulates the phosphorylation of two membrane-bound proteins with molecular masses of 42- and 44-kDa (p42/44) in both intact and cell-free preparations of the VN sensory epithelium. ES20 and DAG regulate the phosphorylation of p42/44 in a similar manner. ES20–receptor binding-mediated phosphorylation of p42/44 is rapid and transient, reaching a peak value within 40 seconds and decaying thereafter. Phosphorylation of p42/44 appears to be regulated by the countervailing actions of a specific membrane-bound protein kinase and a protein phosphatase. The phosphorylation of these membrane-bound proteins significantly reduces the activity of G-proteins as evidenced by a decrease in GTPase activity, but has little effect on ligand–receptor binding. These findings suggest that p42/44 play a role in modulating the signal transduction induced by ES20 in the vomeronasal system. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Vomeronasal receptor; Signal transduction; Phosphorylation; ES20

1. Introduction

The major nasal sensory systems, including the vomeronasal (VN), olfactory and trigeminal systems, are sensitive to different types of chemical stimuli in the environment. The olfactory system of terrestrial vertebrates is particularly sensitive to volatile odors [3] and the trigeminal system to nasal irritants [4]. The VN system detects complex, non-volatile odors [5] and, in most terrestrial vertebrates, plays an important role in many species-typical behaviors, such as prey recognition by garter snakes and responding to courtship pheromones by many animals. We have previously isolated, purified and characterized several proteins from earthworms that are chemoattractive for garter snakes [1,6–8]. One of
these proteins, ES20, a 20-kDa glycoprotein derived from electric shock-induced earthworm secretion, binds specifically to the membranes of the VN sensory epithelium of garter snakes in a saturable and reversible manner [1]. Irrigation of the VN sensory epithelium with aqueous ES20 leads to an increase in firing rate in mitral cells of the accessory olfactory bulb, the synaptic target of VN sensory neurons [1,9,10]. The chemoattractant ES20 has recently been cloned and functionally expressed in Escherichia coli [11].

A large number of biological systems employ a mechanism for signal transduction involving ligand binding to a cell surface receptor coupled to a guanine nucleotide binding protein (G-protein) [12,13]. We have shown that binding of ES20 to $G_{i/o}$-protein-coupled receptors in the VN sensory epithelium of garter snakes leads to a decrease in cAMP and an increase in inositol trisphosphate (IP$_3$) [2,14]. Electrophysiological experiments have demonstrated that under a whole-cell patch clamp, intracellular dialysis of IP$_3$ into VN bipolar neurons of garter snakes evokes inward currents [15]. IP$_3$ is known to react with its receptor to mobilize intracellularly sequestered calcium [16,17]. In the snake VN epithelium, levels of cAMP are biphasically regulated by calcium ions [2,14]. On the basis of the forementioned several lines of evidence, IP$_3$ appears to be the major second messenger in snake VN signal transduction.

An increase in IP$_3$ is likely to be accompanied by an increase in DAG, a second messenger known to activate protein kinase C [18], which catalyzes the phosphorylation of its target proteins. Protein phosphorylation is known to play a key role in many biological signal transduction systems [19–22], including certain metabolic pathways, hormonal responses, cell division and growth, and olfaction [23]. For example, in the main olfactory system, odorants can significantly enhance the extent of $^{32}$P incorporation in isolated rat olfactory cilia, and specific inhibitors of either PKA or PKC can block the phosphorylation [24]. This phosphorylation is transient and the intensity of labeling decays within 10 s [24]. It has been suggested that phosphorylation of key elements in the olfactory signal transduction cascade plays an important role in desensitization of the odorant responses [24]. However, the role of DAG in both olfactory and vomeronasal chemosignal transduction systems is currently unknown. To investigate the mechanism of signal transduction elicited by ES20–receptor binding and the factors that regulate this signaling process, we examined ES20–receptor-mediated protein phosphorylation in the VN system of garter snakes.

In this communication, we report ligand–receptor binding-mediated protein phosphorylation in the vomeronasal system for the first time. ES20–receptor binding regulated phosphorylation of membrane-bound proteins with molecular masses of 42 and 44 kDa (p42/44). The ES20–receptor binding-induced phosphorylation of p42/44 is rapid and transient and is mediated by the PI-turnover pathway. Phosphorylation of p42/44 reduces the activity of G-proteins as measured by a decrease in GTPase activity. These data suggest that the membrane-bound proteins, p42/44 through their phosphorylation may play an important regulatory role in ES20-induced signal transduction cascade in the VN system of garter snakes.

2. Materials and methods

2.1. Materials

Phorbol-12,13-dibutyrate (PDBu), 1,2-dioctanoyl-sn-glycerol, NF023 and GO 6976 are from Calbiochem. [γ-$^{32}$P]ATP, [γ-$^{33}$P]ATP, and [γ-$^{32}$P]phosphate are from ICN. Protein molecular weight markers are from Bio-Rad. PEI-cellulose TLC plates are from J.T. Baker. Other chemicals are the highest grade commercially available. Garter snakes (Thamnophis sirtalis) were obtained from various suppliers. Earthworms were purchased from Connecticut Valley Biological Supply, South Hampton, MA.

2.2. Preparation of vomeronasal sensory epithelial membranes

VN sensory epithelial membranes were isolated as follows: the dissected VN sensory epithelia were homogenized in cold buffer H (50 mM HEPES, pH 7.4, 2 mM DTT, 1 mM MgAc$_2$, 50 mM NaF, 1 mM Na$_3$VO$_4$, 2.5 mM KCl, 145 mM NaCl, 3 mM glucose, 1 mM PMSF, 1 mM EGTA), and centrifuged.
twice at 500×g for 5 min each to remove debris, and then centrifuged at 30 000×g for 20 min. The resulting supernatant is referred to as the ‘cytosolic fraction’ and the pellet as the ‘membrane fraction’. The entire process was carried out at about 4°C.

2.3. Protein phosphorylation in intact VN sensory epithelium

Each snake has a pair of VN organs. Sensory epithelium from one organ serves as control and that from the other is used for treatment. Two snakes were used in each set of experiments. Dissected VN sensory epithelia were incubated separately in 100 μl of buffer H containing 200 μCi [32P]phosphate for 60 min at 23°C. The samples were centrifuged at 500×g for 5 min at 4°C to remove the free isotope. The two resulting pellets were washed twice with cold buffer H, re-suspended in 100 μl bufer H containing 1 mM free Ca2+, and ES20 was added to one preparation as specified in the figure legends, and no ES20 was added to the other preparation (served as control). Both samples were incubated at 23°C for 10 min. The tissues were recovered after incubation and washed twice with cold buffer H. The washed tissues were transferred into 100 μl SDS-PAGE sample buffer, vortexed vigorously and heated in a boiling water bath for 5 min. Proteins in each sample were resolved by SDS-PAGE.

2.4. Protein phosphorylation of cell-free preparation of VN sensory epithelium

Phosphorylation reactions were conducted as follows: each reaction mixture contained 10–15 μg protein of either the membrane fraction or the homogenate (cytosolic fraction plus membrane fraction) in 20 μl buffer H containing 8 μM free Ca2+ (unless indicated otherwise) and other reagents added at concentrations as specified in each experiment. A reaction was initiated by adding either [γ-32P]ATP or [γ-33P]ATP (10 μCi) as the phosphate donor, and allowed to proceed for 10 min at 23°C unless specified otherwise. The reaction was terminated by adding an equal volume of 20% TCA. Samples were placed on ice for 10 min and then centrifuged at 4°C to recover the precipitated proteins. Proteins in the pellet of each sample were re-dissolved in sample buffer and resolved by means of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% gel under reducing conditions.

2.5. Ligand binding in both control and phosphorylated VN membrane

VN membrane containing 15 μg of protein was first phosphorylated in the presence of 8 μM free Ca2+ and 0.5 μM ATP at 23°C for 10 min, then the membrane was recovered by centrifuging at 30 000×g for 20 min and re-suspended in 10 μl buffer H. Control samples were treated identically except that no ATP was added. 125I-labeling of ES20 was performed according to the procedures described by Jiang et al. [1]. The total binding was determined as follows: 10 μl of VN membrane (control or phosphorylated) was mixed with buffer H containing 0.75 μg 125I-ES20 (8.9×10⁵ cpm) in a total volume of 20 μl. CaCl2 was added to a final free Ca2+ concentration of 8 μM. The reaction mixtures were incubated at 23°C for 2 h. Following centrifugation at 30 000×g for 20 min, the pellets (receptor–ligand complex) were recovered and washed three times with 100 μl of buffer H. The level of radioactivity in the pellets was determined by liquid scintillation counting. The non-specific binding was assayed in the presence of 100-fold excess of unlabeled ES20. The rest of the procedures were the same as described for total binding. Specific binding = total binding – non-specific binding.

2.6. GTPase assay

The GTPase assay was performed according to the procedures described by Wagner et al. [25] with slight modification. VN homogenate (3 μg of protein) was mixed with 20 μl buffer H containing 40 nM free Ca2+, 1 mM ouabain, 1 mM AMP-PNP and various concentration of ES20 as specified in the figure legends. Ouabain and AMP-PNP was used to reduce non-specific GTP hydrolysis. [γ-32P]GTP (1.5 μCi, 3000 Ci/mmol) was added to start the reaction. Samples were incubated for 20 min at 23°C. An aliquot of 2 μl reaction mixture was taken out immediately after adding GTP (t₀) and at the end of 20 min of incubation (t₂₀), and mixed with 8 μl stopping solution (0.1 M EDTA, pH 8.0, 0.2% SDS) to terminate reaction mixtures.
the reaction. An aliquot of 2 μl was taken out from the mixture and spotted on a piece of PEI-cellulose TLC plate (20×10 cm). The TLC plate was developed in an ascending manner with a solvent containing 1 M LiCl and 1 M formic acid. The developed plate was dried in a fume hood for 60 min. The separation of GTP and GDP was visualized and analyzed by using PhosphoImager. The percentage of GTP hydrolysis = (radioactivity of GDP)/(radioactivity of GDP+radioactivity of GTP). The percentage of GTP hydrolysis at $t_0$ is considered as background for GTP hydrolysis. The percentage of GTP hydrolysis at $t_{20}$ is considered as total GTP hydrolysis. Net GTP hydrolysis = total GTP hydrolysis−background of GTP hydrolysis.

2.7. Effect of phosphorylation on GTPase activity

VN homogenate (30 μl) was first phosphorylated in buffer H containing 8 μM free Ca$^{2+}$ and 50 μM ATP at 23°C for 10 min, and centrifuged at 30,000×g for 20 min to recover the membrane. The membrane fraction was washed three times with buffer H and re-suspended in 30 μl fresh cytosolic fraction. Control samples were treated identically except that no ATP was added. GTPase assays were carried out as described above.

2.8. Other methods

The chemoattractant ES20 was isolated and purified from electric shock-induced earthworm secretion according to the procedures described earlier [1]. The concentration of ES20 or proteins in the VN preparations was estimated with BSA as the standard using the BCA protein kit from Pierce. Free calcium concentration in the reaction buffer was calculated by using the program 'EqCal' from Biosoft. Proteins in each sample were separated by means of SDS-polyacrylamide gel electrophoresis in 10% acrylamide gel under denaturing and reducing conditions. Gels with resolved proteins were dried and processed for autoradiography. Quantitation of the extent of protein phosphorylation was made by PhosphorImager or by analyzing the autoradiograph with KODAK 1D image analysis software. All values are means ± S.E.

3. Results

3.1. ES20−receptor binding-mediated protein phosphorylation in vomeronasal sensory epithelium

To investigate ES20−receptor binding-mediated phosphorylation of proteins, intact tissues were used first. Since each snake possesses a pair of VN organs, simple comparative experiments can be performed in intact tissue preparations, with one organ serving as a control and the other being used to assess changes induced by experimental manipulation. As illustrated in Fig. 1A, when intact VN sensory epithelium, preloaded with $[^{32}P]$phosphate, was treated with 2.8 μM ES20, the phosphorylation of
several proteins was augmented. Among these phosphorylated proteins, the phosphorylation of a protein with a molecular mass of 44 kDa was consistently augmented by ES20–receptor binding (control: ES20 treated = 100:155.7 ± 24.2, n = 4). This protein is the only prominent membrane-bound phosphorylated protein (Fig. 1B). The relatively large variation in the effect of ES20 may be due to biological variation between snakes.

The 44-kDa protein appeared as a single radioactive band when [$\gamma$-32P]ATP was used as a phosphate donor, and as two distinct bands, with molecular masses of 42 and 44 kDa, when low energy-emitting [$\gamma$-33P]ATP was used (Fig. 1B). Subsequently, these proteins will be referred to as p42/44. The proteins, p42/44 were further resolved into three distinct entities by means of two-dimensional electrophoresis as shown in Fig. 2. Since their identities are not known, the possibility that they may be the same polypeptide, but with different degrees of phosphorylation cannot be excluded.

Experiments carried out with cell-free preparations revealed that p42/44 were the only prominent phosphorylated proteins in the membrane fraction of VN sensory epithelium and that they were phosphorylated without the participation of cytosolic fraction (Fig. 1B). In contrast, no phosphorylated p42/44 could be detected in reactions carried out with cytosolic fraction only (data not shown). These results suggest that p42/44 are membrane-bound proteins, that they are phosphorylated by a membrane-bound protein kinase(s) and the phosphorylation of p42/44 appears to be closely associated with the ES20–receptor binding. Thus, we focused our investigation on p42/44.

3.2. Involvement of protein phosphatase and kinetics of ES20–receptor-mediated p42/44 phosphorylation

Reversible protein phosphorylation catalyzed by protein kinases and phosphatases is an important aspect in the regulation of signal transduction [26]. In previous experiments, reaction buffer contained the protein phosphatase inhibitors, Na3VO4 and NaF, to prevent dephosphorylation. To investigate the effect of these phosphatase inhibitors on the phosphorylation of p42/44 and how rapidly p42/44 proteins are phosphorylated following binding of ES20 to its receptors, the kinetics of p42/44 phosphorylation were examined in the presence and absence of these phosphatase inhibitors. As illustrated in Fig. 3, the phosphorylation of p42/44 elicited by ES20–receptor binding in the absence of phosphatase inhibitors occurred rapidly and attained a peak value within 40 s. The phosphorylation decreased at longer incubation times, suggesting that protein phosphatase is involved in the dephosphorylation of the phosphorylated p42/44. In contrast, in the presence of the phosphatase inhibitors Na3VO4 and NaF, there was no decrease in p42/44 phosphorylation, but rather a continued increase as a function of incubation time. The level of p42/44 phosphorylation in the presence of Na3VO4 and NaF is much higher than that in the absence of these inhibitors (please note the y-axes are different in Fig. 3B and C) (Fig. 3C). These results suggest that these inhibitors effectively inhibit VN protein phosphatase activity. Further experiments carried out in the presence of phosphatase inhibitors revealed that the phosphorylation of p42/44 reached a maximal value in 10 min and levelled off thereafter (the ratio of extent of p42/44 phosphorylated at 4, 10 and 20 min is 100:120:123.) These results suggest that the phosphorylation of p42/44 mediated by ES20–receptor binding is transient in nature, and the extent of their phosphorylation is dually regulated by a protein kinase and a specific protein phosphatase in the VN sensory epithelium.

Two phosphatase inhibitors were used in the reactions. These two inhibitors are known to function on different protein phosphatases. NaF is specific for
Serine/threonine phosphatase and Na$_3$VO$_4$ is specific for tyrosine phosphatase [27]. So it is important to distinguish whether NaF or Na$_3$VO$_4$ is included. Samples were phosphorylated in the absence of 2.8 μM ES20 and 8 μM free Ca$^{2+}$. NaF (50 mM) and Na$_3$VO$_4$ (1 mM) were added as indicated. Reactions were terminated at indicated time intervals. (A) Autoradiograph of phosphorylated p42/44 resolved on SDS-PAGE gel (a representative of three similar experiments). (B) Quantitation of the extent of p42/44 phosphorylated in the absence of protein phosphatase inhibitors, NaF and Na$_3$VO$_4$. (C) Quantitation of the extent of p42/44 phosphorylated in the presence of protein phosphatase inhibitors, NaF and Na$_3$VO$_4$. Each value represents an average of samples from three separate experiments.

Fig. 3. The kinetics of p42/44 phosphorylation induced by ES20 in VN sensory epithelial homogenate. Homogenate of VN sensory epithelium was prepared in buffer N, which is identical to buffer H except that no NaF or Na$_3$VO$_4$ is included. Samples were phosphorylated in the presence of 2.8 μM ES20 and 8 μM free Ca$^{2+}$. NaF (50 mM) and Na$_3$VO$_4$ (1 mM) were added as indicated. Reactions were terminated at indicated time intervals. (A) Autoradiograph of phosphorylated p42/44 resolved on SDS-PAGE gel (a representative of three similar experiments). (B) Quantitation of the extent of p42/44 phosphorylated in the absence of protein phosphatase inhibitors, NaF and Na$_3$VO$_4$. (C) Quantitation of the extent of p42/44 phosphorylated in the presence of protein phosphatase inhibitors, NaF and Na$_3$VO$_4$. Each value represents an average of samples from three separate experiments.

Serine/threonine phosphatase and Na$_3$VO$_4$ is specific for tyrosine phosphatase [27]. So it is important to distinguish whether Na$_3$VO$_4$ or NaF alone is sufficient to decrease phosphatase activity. In the absence of Na$_3$VO$_4$ and NaF, little phosphorylated p42/44 was observed, only NaF inhibited the activity of protein phosphatase (Fig. 4). There was little or no significant change in the phosphorylation of other proteins in the presence and absence of NaF, suggesting that the protein phosphatase inhibited by NaF is rather specific for p42/44 phosphoproteins. Furthermore, the protein phosphatase that dephosphorylates p42/44 appears to be a membrane-bound enzyme as evidenced by the results from experiments with membrane fractions alone (data not shown). Thus, there is a membrane-bound phosphatase that appears to be specific for p42/44 in the snake vomeronasal epithelium. For the purpose of consistency, both inhibitors were used in all other experiments, although this led to a high background phosphorylation of p42/44.

3.3. Effect of ES20–receptor binding on VN protein phosphorylation

To further characterize ligand–receptor binding-mediated protein phosphorylation, ES20 concentration titration was carried out in cell-free preparations of VN sensory epithelium. The binding of ES20 to its receptors elevated the levels of phosphorylation of several proteins as shown in Fig. 5A. In the homogenate, phosphorylation of proteins with molecular
masses of 99 (p99), 55 (p55) and 35 (p35) kDa increased as a function of ES20 concentration in the reaction solution. In previous experiments, we determined that these proteins are cytosolic (data not shown) and p42/44 are the only prominent phosphorylated membrane proteins. The phosphorylation of p42/44 was regulated by ES20 biphasically. The extent of phosphorylation of p42/44 increased as a function of ES20 concentration up to 11 μM and decreased above that concentration. These results suggest that the extent of p42/44 phosphorylation is rather sensitive to ES20. The mechanism of inhibition of p42/44 phosphorylation by high concentration ES20 remains to be elucidated.

3.4. Involvement of G\textsubscript{i/o} proteins in ES20-induced p42/44 phosphorylation

The ES20 receptors are coupled to PTX-sensitive G\textsubscript{i/o}-proteins [2]. It is possible that inhibition of G\textsubscript{i/o}-proteins with a specific inhibitor would reduce the ES20–receptor binding-mediated phosphorylation of p42/44. A selective G-protein inhibitor, NF023, which has been shown to inhibit G\textsubscript{i/o}-proteins by preventing the formation of receptor–G-protein complex [28], was used for this purpose. The ability of NF023 to inhibit G-proteins was verified by GTPase assay. NF023 inhibits the GTPase activity of G-proteins by 38.2 ± 2.6% (n = 3). As predicted, NF023 reduced ES20-induced p42/44 phosphorylation (control: NF023 treated = 100; 68.02 ± 0.98, n = 4), suggesting that ES20–receptor binding induced phosphorylation of these membrane-bound proteins is through the activation of G\textsubscript{i/o}-proteins.

Fig. 4. Effect of protein phosphatase inhibitor on the phosphorylation of VN sensory epithelial membrane-bound p42/44 proteins. Experiments were performed in a manner similar to that described in Fig. 4 except that no ES20 was added. Homogenate containing 10 μg of protein was phosphorylated in buffer N with 8 μM free Ca\textsuperscript{2+}. Reactions were terminated at 10 min. Samples are in duplicates. Lanes 1 and 2, control (no inhibitors); lanes 3 and 4, treated with 50 mM NaF; lanes 5 and 6, treated with 1 mM Na\textsubscript{3}VO\textsubscript{4}.

Fig. 5. The effect of ES20 on protein phosphorylation in homogenate of VN sensory epithelium of garter snakes. (A) Autoradiograph of phosphorylated proteins resolved by SDS-PAGE (a representative of five similar experiments). (B) Quantification of the relative extent of p42/44 phosphorylation. The basal level of p42/44 phosphorylation was considered as 100.
3.5. Effect of Ca\(^{2+}\) on the phosphorylation of p42/44

We have previously shown that ES20–receptor binding activates G\(_{i/o}\) proteins and results in elevated levels of IP\(_3\) [2] and presumably also DAG, suggesting that the signal generated by this chemoattractant is transduced through the PI-turnover pathway. IP\(_3\) is known to mobilize intracellularly sequestered calcium and there is evidence for this role of IP\(_3\) in the VN system of garter snakes [14]. Since calcium has been considered as a second messenger in many biological systems [29] and a number of protein kinases require Ca\(^{2+}\)/CaM for activation [30], the effect of calcium ions on the ES20-mediated phosphorylation of p42/44 was investigated.

In membrane preparations, the phosphorylation of p42/44 was inhibited by calcium ions in a concentration-dependent manner (Fig. 6), suggesting that the membrane-bound protein kinase at least is not the conventional type of PKC that requires Ca\(^{2+}\)/CaM for activation [30]. This interpretation is consistent with the observation that this membrane-bound protein kinase was neither sensitive to the Ca\(^{2+}\)-dependent PKC inhibitor GÖ6976 (data not shown) nor could its activity be augmented by phorbol ester, PDBu (data not shown).

3.6. Effect of DAG on the phosphorylation of p42/44

We have evidence for the role of IP\(_3\) in the VN system of garter snakes [2,14], but the role of DAG, the second messenger co-generated with IP\(_3\), remains to be determined. Therefore, the effect of the DAG, 1,2-diocatanoyl-sn-glycerol, on protein phosphorylation in VN sensory epithelium was examined. In membrane fractions of VN sensory epithelium, exogenously supplied DAG produced an effect on phosphorylation of p42/44 similar to that of ES20 (Fig. 7). At low concentrations, DAG stimulated phosphorylation and at high concentrations, it inhibited phosphorylation, although the degree of inhibition is weaker than that induced by ES20 (Fig. 5). These results are consistent with the idea that ES20-mediated phosphorylation of p42/44 is through the PI-turnover pathway. The mechanism of inhibition of p42/44 phosphorylation induced by high concentrations of ES20 and DAG is not known and remains to be determined.

3.7. Role of p42/44 phosphorylation in VN signal transduction

Protein phosphorylation plays a key role in desensitization of signal transduction in many systems, such as adrenergic receptor and olfactory systems. In a signal transduction pathway, several steps are sensitive to protein phosphorylation, including li-
Ligand binding has been used extensively as a major criterion in establishing the functional integrity of receptors. ES20 binds specifically to its VN receptor [2]. In order to assess the effect of p42/44 phosphorylation on VN ligand–receptor binding, experiments comparing binding of ES20 to its receptors in control preparation and VN membrane containing phosphorylated p42/44 were performed. The results of these experiments revealed that phosphorylation of p42/44 had little effect on specific binding of ES20 to VN sensory membrane. The ratio of ES20 binding in control vs. phosphorylated membrane is 100:106.7 ± 3.3 (n = 3). These results suggest that phosphorylation of p42/44 does not significantly affect the ligand receptor binding.

The next step in the VN signal transduction pathway is G-protein activation. The G-protein α-subunit has intrinsic GTPase activity and activated receptor can stimulate the GTPase activity of G-proteins [32]. This is also true in the VN system, since binding of ES20 to its receptor can stimulate GTPase activity up to 80% (Fig. 8). When VN membrane was phosphorylated, the ES20-induced GTPase activity was reduced by more than 18% (in the presence of 20 μM ES20, control vs. phosphorylated membrane = 100:81.73 ± 3.0, n = 5). Although almost all known G-proteins have been identified in the VNO, including Gα, Gβ, Gγ, ES20 receptor appears to be coupled only to Gβ and/or Gγ protein [2]. Therefore, the reduction in GTPase activity is particularly striking because it could be related to the modulation of Gβ/γ proteins. A similar extent (24%) of inhibition of GTPase activity by phosphorylation has been reported in the β-adrenergic system by Benovic et al. [33] in which purified receptor and G-protein were used. Together these results suggest that phosphorylation of p42/44 could regulate the VN signal transduction mainly through the down-regulation of G-protein activity.

4. Discussion

In this study, we have demonstrated, for the first time, the phosphorylation of several proteins in cell-free preparations and intact tissues of VN sensory epithelium of garter snakes upon ligand–receptor binding. Among these ligand-induced phosphorylated proteins, the membrane-bound p42/44 proteins are particularly sensitive to this chemoattractant. Furthermore, in cell-free VN sensory epithelium of garter snakes, p42/44 are the only prominent phosphorylated membrane-bound proteins. The ES20-elicited phosphorylation of these membrane-bound proteins appears to be mediated by components of the PI-turnover cascade. This conclusion is based on the following observations: (1) The ES20 receptor is coupled to PTX-sensitive G-proteins [2]; (2) ES20–receptor binding elicits elevated levels of IP3 and presumably also DAG [2]; (3) the second messenger, DAG, mimics the effect of ES20–receptor binding on phosphorylation of p42/44, i.e. lower concentrations of ES20 and DAG stimulate whereas higher concen-
trations of ES20 and DAG inhibit p42/44 phosphorylation; and (4) the selective G\textsubscript{i/o} protein inhibitor, NF023 can block the ES20-induced p42/44 phosphorylation.

Desensitization refers to the waning of responses to continuous or repeated stimulation. This process occurs within minutes of agonist exposure and involves protein phosphorylation (for review, see [34]). The binding of ES20 to its receptors elicits a rapid and transient phosphorylation of membrane-bound p42/44 proteins. Such phosphorylation of p42/44 attains a peak value within 40 s and then is quickly dephosphorylated by a membrane-bound specific phosphatase. This phenomenon is analogous to desensitization of agonist-elicited signals in other biological signal transduction systems. For instance, the desensitization of the β2AR/AC system (for review, see [35]) and of odorant-induced 32P-incorporation of the receptors by protein kinases. It is possible, therefore, that the ES20-mediated phosphorylation of p42/44 may be involved in the process of desensitization of the ES20 receptor. The desensitization process of ES20 signal transduction pathway in the VN system may also involve mechanisms other than protein phosphorylation, since ES20 and DAG both inhibited p42/44 phosphorylation at high concentrations.

Phosphorylation of membrane-bound p42/44 proteins may play an important role in desensitization of ES20-elicited signals. However, this role does not appear to involve interference with ES20–receptor binding. On the other hand, phosphorylation of p42/44 does reduce the activity of G-proteins as reflected by lower activity of GTPase.

Since phosphorylation of p42/44 reduces the activity of G-protein, p42/44 should logically be located up-stream of the G-protein in the signal transduction pathway. One can speculate that p42/44 may be subtypes of the VN ES20 receptor, since they have different molecular masses. All cloned vomeronasal odorant receptors contain putative seven-transmembrane domains [36–38]. p42/44 fall in the same molecular weight range.

Since DAG shows a biphasic effect on the phosphorylation of p42/44, it appears that this second messenger may play a dual role in regulating the membrane-bound protein kinase: at low concentrations it stimulates kinase activity whereas at higher concentrations it inhibits kinase activity. The latter phenomenon, to our knowledge, has not been reported in the literature. The mechanism of action and its functional significance are not known and remain to be investigated, although this phenomenon resembles the action of Ca\textsuperscript{2+} on VN adenylate cyclase type VI [14,39,40]. If DAG biphasically regulates the membrane-bound kinase in the VN sensory epithelium, this protein kinase may serve as a switch and the phosphorylation of p42/44 by this kinase may be turned-on or turned-off by DAG. However, the possibility that an additional factor requires high concentrations of DAG for activation before it can negatively regulate the protein kinase, cannot be excluded.

In conclusion, our results demonstrate that the signal generated by ES20–receptor binding is transduced through the PI turnover pathway and such signal appears to be negatively modulated by the phosphorylation of the membrane-bound proteins, p42/44.

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References
