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Signal Transduction Mechanisms of Recombinant Bovine Neurokinin-2 Receptor Stably Expressed in Baby Hamster Kidney Cells

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Abstract The bovine neurokinin-2 (NK-2) receptor gene was stably transfected into Baby hamster kidney (BHK-21) fibroblasts and one recombinant clone expressing 17,700 high-affinity [^{125}I]neurokinin A (NKA) binding sites/cell characterized further. [^{125}I]NKA binding was displaced by unlabeled NKA with an IC_{50} of 8.26 ± 2 nM (n = 5) and with the rank order of potency NKA > neurokinin B (NKB) > Substance P (SP) confirming pharmacological characteristics of an NK-2 receptor subtype. Stimulation with NKA resulted in a rapid and dose-dependent increase in inositol 1,4,5-trisphosphate (IP_3) levels (EC_{50} = 32 ± 10 nM; n = 7) which was paralleled by a transient biphasic rise in intracellular free calcium concentration [Ca^{2+}], (EC_{50} = 35 ± 20 nM; n = 3). In addition to phosphoinositide (PI) hydrolysis and Ca^{2+} mobilization, NKA was found to stimulate both cyclicAMP formation (EC_{50} = 1.02 ± 0.26 μM; n = 7) and [^{3}H]arachidonic acid mobilization (EC_{50} = 0.65 ± 0.45 μM; n = 4). Interestingly, cyclicAMP levels also rose after addition of an exogenous arachidonic acid metabolite, prostaglandin E_2 (PGE_2) (EC_{50} = 11.5 ± 2 μM). Similar observations of NKA-induced IP_3 production, Ca^{2+} mobilization, arachidonic acid liberation, and cAMP formation have been made previously following expression of the bovine NK-2 receptor in Chinese hamster ovary (CHO) epithelial cells. The present results suggest that activation of NK-2 receptors leads to characteristic and reproducible intracellular second messenger responses in a subclass of cell types which includes fibroblasts and epithelial cells irrespective of their genetic and phenotypic background.

Key words: neurokinin-2 receptor, IP_3, calcium, cAMP, eicosanoids

The neurokinin-2 receptor is a member of the superfamily of seven transmembrane domain receptors which are functionally coupled to guanine nucleotide binding proteins (G-proteins) [Masu et al., 1987; Hanley and Jackson, 1987]. NK-2 receptors are involved in the regulation of a variety of important physiological responses, including mucus secretion, smooth muscle contraction, and cell proliferation [Rogers et al., 1989; Grandordy et al., 1988; Soder and Hellstrom, 1989]. The molecular mechanisms mediating such cellular responses, however, have not yet been fully characterized. Following the cloning of cDNAs encoding bovine, rat, and human NK-2 receptors [Masu et al., 1987; Sasai and Nakanishi, 1989; Gerard et al., 1990], recombinant expression in suitable recipient cell lines is one potentially important approach to establish signalling events under their control. For example, expression of bovine and rat NK-2 receptors in murine L fibroblasts (subclone B82), C6 glioma cells (subclone 2B), and Chinese hamster ovary (CHO) epithelial cells demonstrated receptor-linked phosphoinositide (PI) hydrolysis, mobilization of intracellular calcium, and modulation of adenylate cyclase (AC) activity [Henderson et al., 1990; DeBernardi et al., 1991; Eistetter et al., 1991; Nakajima et al., 1992]. Interestingly, while NK-2 receptors mediate activation of adenylyl cyclase in CHO cells [Eistetter et al., 1991; Nakajima et al., 1992], C6-2B glioma cells respond with an inhibition of cyclicAMP formation and this effect appears indirect and a consequence of elevated Ca^{2+} levels [DeBernardi et al., 1991]. We have also reported an indirect cyclicAMP response linked to NK-2 receptors which, when expressed in CHO:K1 cells, mediate adenylyl cyclase activation following autocrine stimulation by endogenously synthesized eicosanoids, particularly prostaglandin E_2 [Eistetter

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et al., 1991]. This is an important observation as local eicosanoid generation seems to play a crucial role mediating the physiological actions of neurokinins [Mayer et al., 1990; Rangachari et al., 1990; Yau et al., 1991; Malmberg and Yaksh, 1992].

To validate our previous findings in CHO:K1 cells and also to test the concept that recombinant expression technology is useful for analyzing important neurokinin-stimulated signalling events in at least a subset of cell types, we have now stably expressed and functionally characterized the bovine NK-2 receptor in a second, genetically and phenotypically different cell line. This remains an important issue as different functional responses observed not only for the bovine NK-2 receptor [DeBernardi et al., 1991; Eistetter et al., 1991; Nakajima et al., 1992] but also following recombinant expression of the dopamine D_2, M1 muscarinic, and 5HT1A receptors suggest that host cell physiology could be an important determinant of functional activity [Vallar et al., 1990; Stein et al., 1988; Peralta et al., 1988; Liu and Albert, 1991]. In this study we present evidence that in Baby hamster kidney (BHK-21) fibroblast cells the NK-2 receptor is linked to signal transduction responses highly reminiscent of those observed previously in CHO cells including IP_3 formation, mobilization of intracellular Ca^{2+}, arachidonic acid release, and cAMP synthesis. These results suggest that this receptor reproducibly couples to a characteristic set of signalling events common to at least fibroblasts and epithelial cells.

METHODS

Cell Culture, Transfection, and Selection of Recombinant Clones

Parental and transfected BHK-21 cells (American Type Culture Collection, Rockville, MD) were grown as monolayers in Dulbecco's Modified Eagle's/Ham's F12 (1:1) medium (Gibco) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin, and streptomycin (60 μg/ml each; all Gibco) in a humified CO_2 atmosphere (10%) at 37°C. Plasmids pBGL312/SKR [Eistetter et al., 1991] and pSVtkneo [Nicolas and Berg, 1983] were co-transfected (ratio 10:1) into BHK-21 cells using calcium-phosphate precipitation [Wigler et al., 1977]. Transfectants were selected in 600 μg/ml geneticin (G418; Gibco) and tested for their ability to bind [^{125}I]-labeled NKA (Amersham). NK-2 receptor-bearing clones were then propagated for further analyses.

Radioligand Binding to Intact Cells

Cells were grown to confluency in 6-well plates (Costar), rinsed with tissue culture medium, and incubated with gentle agitation for 60 min at 20°C in 0.5 ml of assay medium (serum-free growth medium supplemented with bovine serum albumin (BSA; 50 μg/ml), chymostatin (0.5 μg/ml), leupeptin (1.0 μg/ml), and bacitracin [10.0 μg/ml]) containing [^{125}I]NKA (30–40 pM), $MnCl_2$ (3 mM), and competing neurokinins as indicated. Subsequently, cells were rapidly washed with 2 ml of ice-cold medium and solubilized in NaOH (2 M). Bound [^{125}I]NKA was determined by gamma-counting and non-specific binding determined in the presence of 1 μM unlabeled NKA (Bachem).

Inositol 1,4,5-Trisphosphate (IP_3) Determination

Cells were grown in 6-well plates (1–2 × 10^6/well) and stimulated with different concentrations of NKA in assay medium. Reactions were stopped after 1 min of incubation by addition of 0.2 vol of ice-cold perchloric acid (20%; w/v), cleared by centrifugation, and D-*myo*-IP_3 levels determined using a [^{3}H]-IP_3 competition binding assay (Amersham).

Intracellular [Ca^{2+}] Measurements

Intracellular calcium was measured by using the fluorescent Ca^{2+} chelating compound *fura-2*. Aliquots of *fura-2*–labeled cells (2 × 10^6) were stimulated in suspension with neurokinins as indicated. Fluorescence emission was recorded on an MS-5 spectro-photometer (Perkin Elmer). Loading of cells, fluorescence monitoring, calibration procedures and other experimental details were as described in Capponi et al. [1984].

Measurements of cAMP Levels

Cells (2.5–5 × 10^6) were incubated at 37°C in 200 μl assay medium containing 0.5 mM IBMX (3-isobutyl-1-methylxanthine), together with NKA or PGE_2 as indicated. After 6 min incubations were terminated by addition of 50 μl of ice-cold 25% (w/v) trichloroacetic acid followed by immediate freezing in dry ice/methanol. Samples were then extracted three times in 5 vol of water-saturated ether, freeze dried, and assayed for cAMP using a [^{3}H]cAMP competition bind-

ing kit (Amersham) according to the manufacturer's guidelines.

[^{3}H]Arachidonic Acid Mobilization

Cells were cultured in 6-well dishes (1–2 × 10^6/well) and labeled for 2 h by incubation in serum-free medium containing [^{3}H]arachidonic acid (1 μCi/ml) and fatty acid-free BSA (FAF-BSA) (2 mg/ml). Subsequently, cells were washed three times and stimulated with various concentrations of NKA (20 min, 37°C) in 1 ml of medium containing FAF-BSA. Finally, 800 μl aliquots were transferred to ice-cold eppendorf tubes, centrifuged at 500*g* (5 min), and supernatants counted directly for arachidonic acid release.

RESULTS

Expression of Recombinant NK-2 Receptor

Transfected BHK-21 cells were selected by resistance to geniticin (G418) and NK-2 receptor expression was assessed by specific [^{125}I]NKA binding. Neither parental nor mock-transfected cells displayed any detectable binding. One clone (BB/7) expressing 17,700 high-affinity receptor sites/cell was propagated and characterized further using radioligand competition binding experiments. [^{125}I]NKA binding was displaced by unlabeled NKA with an IC_{50} of 8.26 ± 2.0 nM (n = 5) and by naturally occuring neurokinins with the relative rank order of affinity NKA > NKB > SP (Table I). This rank order of displacement is consistent with radioligand binding to the NK-2 receptor subtype previously observed both in tissues [Buck et al., 1986; Regoli et al., 1989] and in recombinant CHO:K1 cells [Eistetter et al., 1991]. Interestingly, however, [^{125}I]NKA binding was found to be significantly more sensitive to inhibition by NKB and SP in BHK-21 (IC_{50} values of 32.22 and 330 nM, respectively; see Table I) compared to CHO:K1 cells (IC_{50} values ≫ 600 nM for both neuropeptides [Eistetter et al., 1991]). This could suggest subtle differences in receptor conformation or neuropeptide breakdown in these two cell types.

PI Turnover and Ca^{2+} Mobilization

NKA at 1 μM stimulated a rapid 3.5 ± 0.9–fold (n = 7) increase in IP_3 formation (basal levels of 1.05 ± 0.55 pmol/10^6 cells). This effect was dose dependent and displayed an EC_{50} of 32 ± 10 nM (n = 7) (Fig. 1). These effects were specific to NK-2 receptor-bearing BB/7 cells as NKA-mediated IP_3 formation could not be detected in either parental or mock-transfected cells (data not shown). NKA at 1 μM also stimulated an immediate (< 2 s) but transient biphasic increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Fig. 2A). Maximum $[Ca^{2+}]_i$ levels were observed within 20 s of stimulation with basal levels (about 350 nM) re-established after 5–10 min. This response was still observed after chelating extracellular Ca^{2+} by addition of 2 mM ethylene glycol-bis (β-aminoethyl ethyl)-N,N,N′,-N′-tetraacetic acid (EGTA), although peak levels were diminished by approximately 70% and basal levels were re-established within 40 s (Fig. 2A). Together these observations indicate that the NK-2 receptor-linked Ca^{2+} response in BB/7 cells reflects both increased influx and mobiliza-

TABLE I. Comparison of Potencies of Tachykinins as Inhibitors of Specific [^{125}I]Neurokinin A Binding to Intact BB/7 Cells*

Peptide	IC_{50} value (nM)	n
Neurokinin A (NKA)	8.26 ± 2.00	5
Neurokinin B (NKB)	32.22	2
Substance P (SP)	330	2

*Cells were assayed for displacement of specific [^{125}I]NKA binding to intact cells as described in Methods IC_{50} values were calculated as means of two to five experiments each performed in triplicate

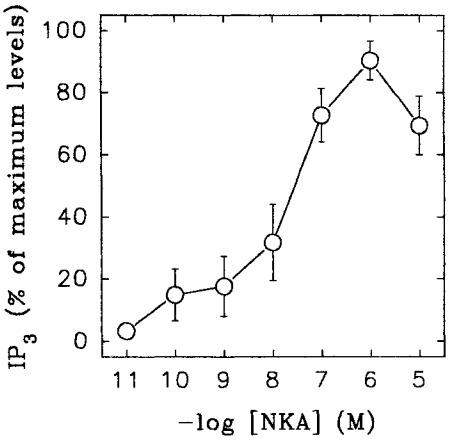

Fig. 1. Dose-dependence of NKA-stimulated IP_3 formation in BB/7 cells IP_3 levels were assayed 1 min after stimulation of cells with varying concentrations of NKA The results depict the means ± SEM of seven individual experiments performed in duplicate Data are expressed as percentage of the maximum response

tion from intracellular stores and NKA-stimulated IP_3 generation is likely to be an important mediator of the latter [Berridge and Irvine, 1989]. As observed for displacement of [^{125}I]NKA binding (Table I), neurokinin-mediated Ca^{2+} mobilization displayed the agonist rank order NKA > NKB > SP (EC_{50} values NKA, 35 ± 20 nM (n = 3); NKB, 295 ± 155 nM (n = 3); and SP, 5.2 ± 2.3 μM [n = 3]) (Fig. 2B) which is identical to that observed for various functional responses in tissues [Bristow et al., 1987; Grandordy et al., 1988]. Neurokinin-mediated Ca^{2+} mobilization was not found in parental or mock-transfected BHK-21 cells.

The coupling of the bovine NK-2 receptor to phospholipase C (PLC) via a pertussis toxin (Ptx)-insensitive G-protein such as Gq [Taylor et al., 1991] in CHO:K1 cells has been described [Eistetter et al., 1991]. The same mechanism is also suggested in BHK-21 cells, as pretreatment of BB/7 cells with Ptx (1,000 ng/ml, for 6 h) did not significantly affect either NKA-mediated IP_3 formation or Ca^{2+} mobilization (data not shown).

Arachidonic Acid Mobilization and CyclicAMP Formation

Recombinant bovine NK-2 receptors were found to be linked to arachidonic acid mobilization and cAMP production in CHO:K1 cells [Eistetter et al., 1991]. Consistent with this, in BB/7 cells NKA induced both an increased liberation of [^{3}H]arachidonic acid (2.36 ± 0.28–fold; EC_{50} = 1.02 ± 0.26 μM; n = 4) (Fig. 3A) and a significant rise in cAMP levels (3.8 ± 1.4–fold; EC_{50} = 0.65 ± 0.45 μM; n = 7) (Fig. 3B). This cAMP response is likely to reflect adenylyl cyclase (AC) stimulation as experiments were performed in the presence of 0.5 mM IBMX to inhibit phosphodiesterase activity. For both the arachidonic acid and cAMP responses maximum stimulatory NKA concentrations were observed at approximately 10 μM, although at higher levels a dramatic decrease in cAMP formation was observed. Neither parental nor mock-transfected BHK-21 cells displayed NKA-mediated arachidonic acid release or cAMP production (data not shown). Surprisingly, half-maximal stimulation of arachidonic acid release in CHO:K1 cells (EC_{50} = 44.2 ± 37.4 nM [Eistetter et al., 1991]) occurs at approximately twenty-fold lower NKA concentrations than in BHK-21 cells (EC_{50} = 1.02 ± 0.26 mM) (see Fig. 3A).

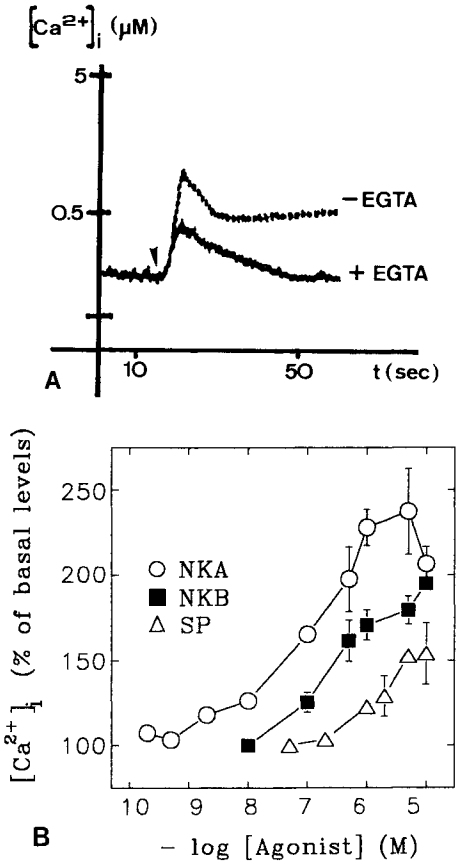

Fig. 2. NK-2 receptor-mediated calcium response. **A**: Time courses of NKA-induced rises in intracellular calcium concentrations $[Ca^{2+}]_i$ in *fura-2*–loaded BB/7 cells in the presence (−EGTA) and absence (+EGTA) of extracellular calcium (1.2 mM). The arrowhead indicates the time of addition of 1 μM NKA; the fluorimetric recordings of two typical experiments are superimposed. **B**: Pharmacological characterization of Ca^{2+} mobilization in BB/7 cells as a function of increasing concentrations of NKA, NKB, and SP. Data points are the means ± SEM of three to five individual experiments. Results are presented as increases in percent of basal levels. EGTA (2 mM) was present in all experiments.

Although currently this phenomenon cannot be explained on a molecular level, it is likely to reflect variations in the degree of signal amplification between receptor stimulation and the functional response being measured.

Liberated arachidonic acid can be metabolized by cyclooxgygenase to form various prostaglandins (PGs) [Shimizu and Wolfe, 1990]. Thus, in CHO:K1 cells transfected with the bovine NK-2 receptor gene NKA induces the synthesis and release of PGE_2, which was shown to stimulate adenylate cyclase in an autocrine manner [Eistetter et al., 1991]. Interestingly, as observed in this system, exogenous PGE_2 stimulated mark-

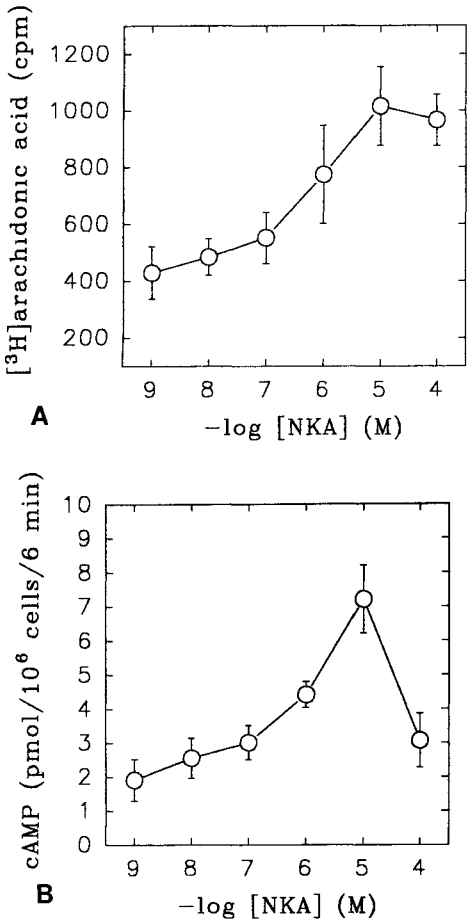

Fig. 3. NKA-induced cAMP formation and arachidonic acid mobilization Intact BB/7 cells were stimulated with increasing concentrations of NKA **A** [^{3}H]arachidonate release (in cpm) from prelabeled BB/7 cells was measured after 20 min **B** cAMP levels (pmoles/6 min/10^6 cells) were determined by [^{3}H]cAMP binding competition assay as described in Methods Values represent the means ± SEM of four (A) and seven (B) experiments performed in triplicate

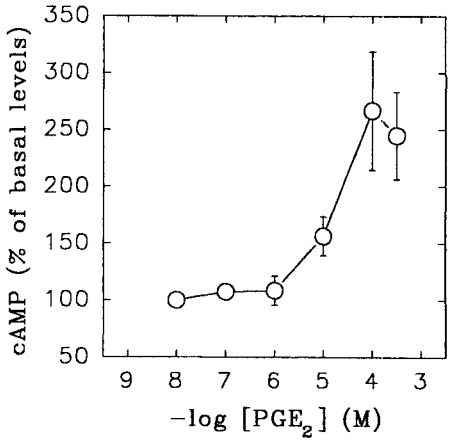

Fig. 4. PGE_2-stimulated adenylyl cyclase Dose dependence of prostaglandin-stimulated cAMP formation Intact BB/7 cells were stimulated for 6 min at 37°C with increasing concentrations of PGE_2 cAMP levels were measured as described in Methods Values represent the means ± SEM of three experiments performed in triplicate

edly cAMP formation in BB/7 cells (2.7 ± 1.0–fold; EC_{50} = 11.5 ± 2 μM; n = 3) (Fig. 4).

DISCUSSION

Recombinant expression of cloned 7-TM receptor genes in mammalian cell lines has become a powerful approach for studying agonist-stimulated signal transduction events. However, a single molecularly defined receptor subtype may elicit various signalling responses depending upon the host-cell employed. Thus, the M1 muscarinic receptor expressed in RAT-1 fibroblasts links into PI hydrolysis and inhibition of AC [Stein et al., 1988], while the same receptor expressed in human embryonic kidney (293) cells stimulates both PI hydrolysis and cAMP production [Peralta et al., 1988]. Similarly, the dopamine D_2 receptor expressed in mouse L(tk$^-$) fibroblasts has been found to activate PI turnover, mobilize Ca^{2+}, and inhibit cAMP formation, while in a rat somatomammotrophic epithelial cell, GH_4C_1, Ca^{2+} levels were suppressed and PI hydrolysis could not be observed [Vallar et al., 1990]. Moreover, the 5HT1A receptor has been demonstrated to both stimulate PI turnover and inhibit AC in L(tk$^-$) cells, while in GH_4C_1 cells only the inhibition of AC was found [Liu and Albert, 1991].

Consistent with these observations, stimulation of recombinant bovine NK-2 receptors in C6-2B rat glioma cells leads to inhibition of agonist-induced AC activity [DeBernardi et al., 1991], while in CHO cells increased cyclicAMP generation can be readily observed [Eistetter et al., 1991; Nakajima et al., 1992]. In the present study we have confirmed the latter findings in a second, genetically and phenotypically different cell line, BHK-21 fibroblasts. In these cells the bovine NK-2 receptor appears to couple to 1) stimulation of PI hydrolysis and Ca^{2+} mobilization via a Ptx-insensitive mechanism; 2) arachidonic acid liberation; and 3) activation of adenylate cyclase. All of these effects can be attributed to the action of the recombinant receptor as both parental and mock-transfected BHK-21 cells do not respond to NKA. Moreover, the NK-2 receptor-selective agonist [Lys^3, Gly^8-R-γ-

lactam-Leu^9]NKA(3-10) (GR64349 [Hagan et al., 1991]) induced [^{3}H]arachidonic acid release and cAMP formation with potencies similar to those observed for NKA (data not shown). This study has therefore demonstrated a clear similarity of NK-2 receptor-linked primary second messenger events in BHK-21 and CHO:K1 cells [Eistetter et al., 1991]. This is an important observation and suggests that in a subclass of cell types which include fibroblasts and epithelial cells, recombinant expression is a useful approach for studying molecular mechanisms underlying physiological actions of neurokinins. Such a notion is consistent with observations that stimulation of endogenous receptors elicits a similar increase in phosphoinositide hydrolysis [Bristow et al., 1987; Grandordy et al., 1988; Suman-Chauhan et al., 1990] and arachidonic acid mobilization (see below) in a number of cell and tissue types including the central nervous system, smooth muscle, endothelial cells, and astrocytes.

Increased arachidonic acid release is the rate limiting step in the synthesis of eicosanoids [Shimizu and Wolfe, 1990] and has been observed following recombinant expression of both the NK-2 receptor in CHO:K1 and BHK-21 cells [Eistetter et al., 1991; this study] as well as the NK-3 receptor in CHO cells [Buell et al., 1992]. Endogenous neurokinin receptors also mediate eicosanoid production in a variety of cell types such as endothelial cells [Marceau et al., 1989], macrophages [Hartung et al., 1986], and astrocytes [Marriot et al., 1991]. Moreover, blocking prostaglandin (PG) synthesis inhibits neurokinin-dependent control of myenteric acetylcholine release, colonic transport activity, and spinal hyperalgesia [Mayer et al., 1990; Rangachari et al., 1990; Yau et al., 1991; Malmberg and Yaksh, 1992] while exogenous eicosanoids mimic neurokinin actions to generate mechanical hyperalgesia [Ferreira and Nakamura, 1979; Nakamura-Craig and Smith, 1989], provide gastric mucosal protection [Whittle et al., 1990], enhance secretion of atrial natriuretic peptide from cardiomyocytes [Gardner and Schultz, 1990], and increase airway mucus secretion [Rogers et al., 1989]. Together, these observations indicate that neurokinin receptors are linked to increased eicosanoid generation in a range of cell and tissue types and that local actions of these modulators play a key role mediating the biological effects of neurokinins. Thus, recombinant expression of a defined neurokinin receptor subtype in cells such as CHO:K1 or BHK-21 is likely to facilitate the identification of crucial molecular interactions and signalling events underlying these particular physiological properties of this class of neuropeptide.

Local eicosanoid generation could also mediate secondary functional responses following stimulation of the NK-2 receptor recombinantly expressed in BHK-21 cells. These cells could be similar to CHO:K1 where NK-2 receptor-linked arachidonic acid mobilization and subsequent autocrine stimulation by eicosanoids represents an important mechanism for neurokinin-stimulated cyclicAMP generation [Eistetter et al., 1991]. However, although exogenous PGE_2 mimicks NKA action to increase cyclicAMP in BHK-21 cells (Fig. 4), it remains to be tested whether a similar autocrine stimulation accounts also for adenylyl cyclase stimulation in this system. Alternative mechanisms leading to AC activation in BHK-21 cells might include direct NK-2 receptor coupling through the stimulatory G-protein Gs [Nakajima et al., 1992] as well as indirect stimulation following Ca^{2+} mobilization or PKC activation as suggested for recombinantly expressed α1 adrenergic and M1 muscarinic receptors [Felder et al., 1989; Cotecchia et al., 1990].

In contrast to early second messenger signal transduction events it remains to be elucidated whether the expression of bovine NK-2 receptor in BHK-21 cells affects other, more long-term cellular responses such as proliferation. Surprisingly, NKA stimulation of serum-starved BB/7 cells did not result in an increase in de novo DNA synthesis (data not shown), although such an effect had been observed both in recombinant CHO:K1 cells [Eistetter et al., 1991] and primary thymocytes [Soder and Hellstrom, 1989]. While this result is currently lacking a molecular explanation, it could reflect divergence of signalling pathways beyond the primary second messenger responses in fibroblasts and epithelial cells.

In summary, the data presented in this report show that recombinant bovine NK-2 receptors expressed in BHK-21 cells retain their pharmacological characteristics and reproducibly modulate specific primary second messenger systems common to fibroblasts and epithelial cells.

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