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# Recombinant bovine neurokinin-2 receptor stably expressed in Chinese hamster ovary cells couples to multiple signal transduction pathways

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**Neurokinins are a family of neuropeptides with widespread distribution mediating a broad spectrum of physiological actions through three distinct receptor subtypes: NK-1, NK-2, and NK-3. We investigated some of the second messenger and cellular processes under control by the recombinant bovine NK-2 receptor stably expressed in Chinese hamster ovary cells. In this system the NK-2 receptor displays its expected pharmacological characteristics, and the physiological agonist neurokinin A stimulates several cellular responses. These include 1) transient inositol 1,4,5-trisphosphate ($IP_3$) formation and $Ca^{2+}$ mobilization, 2) increased out put of arachidonic acid and prostaglandin $E_2$ ($PGE_2$), 3) enhanced cyclic AMP (cAMP) generation, 4) increased de novo DNA synthesis, and 5) an induction of the "immediate early" genes c-*fos* and c-*jun*. Although NK-2 receptor-mediated $IP_3$ formation involves activation of a pertussis toxin-insensitive G-protein, increased cAMP production is largely a secondary response and can be at least partially attributed to autocrine stimulation by endogenously generated eicosanoids, particularly $PGE_2$. This is the first demonstration that a single recombinant neurokinin receptor subtype can regulate, either directly or indirectly, multiple signal transduction pathways and suggests several potential important mediators of neurokinin actions under physiological conditions.**

## Introduction

Neurokinins are a family of closely-related, biologically active neuropeptides including substance P (SP), neurokinin A (NKA), neurokinin B (NKB), neuropeptide K (NPK), and neuropeptide $\gamma$ (NP$\gamma$). These are distributed differentially throughout both the central nervous system (CNS) and peripheral tissues where they regulate a wide variety of physiological processes including smooth muscle contraction, inflammation, perception of pain, secretion, neurotransmission, and proliferation (Nilsson *et al.*, 1985; Grandordy *et al.*, 1988; Nakamura-Craig and Smith, 1989; Rogers *et al.*, 1989; Mayer *et al.*, 1990; Reid *et al.*, 1990). Neurokinin actions are mediated by three pharmacologically distinct cell-surface receptors classified as NK-1, NK-2, and NK-3 that display limited selectivity toward SP, NKA, and NKB, respectively. NK-2 receptors also preferentially bind NPK and NP$\gamma$. Although NK-1 receptors are expressed widely in a variety of tissues including brain and spinal cord, other subtypes appear more selectively localized to either peripheral tissues (NK-2) or specific regions of the CNS (NK-3) (for reviews see Quirion and Dam, 1988; Helke *et al.*, 1990).

cDNA clones for all three neurokinin receptor subtypes have been isolated and primary sequence data indicate that they belong to the super-family of cell surface receptors possessing seven putative membrane-spanning domains (Masu *et al.*, 1987; Yokota *et al.*, 1989; Shigemoto *et al.*, 1990). This class of receptor regulates cellular activities through coupling with heterotrimeric G-proteins, which is consistent with the finding that neurokinin binding can be both modulated by guanine nucleotides and diminished after pretreatment with pertussis toxin (Morishima *et al.*, 1989; Guard *et al.*, 1990). In the past, the majority of evidence suggested that stimulation of neurokinin receptors resulted in activation of a phospholipase C (PLC) to bring about hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate ($PIP_2$) (Hanley *et al.*, 1980; Grandordy *et al.*, 1988). This reaction

generates the second messengers inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol, which mobilize $Ca^{2+}$ from intracellular stores and activate protein kinase C (PKC), respectively (Berridge and Irvine, 1989). Despite this, it is unclear whether $PIP_2$ breakdown is alone responsible for eliciting the wide variety of biological activities identified with this class of neuropeptide. Indeed, neurokinins have limited or no detectable effect on inositol phosphate generation in some tissues, particularly in the CNS (Helke *et al.*, 1990), indicating that other effector and second messenger systems could play a role.

Two additional signal transduction pathways potentially important for neurokinin action are those modulating adenylyl cyclase activity and those mediating the release of arachidonic acid. Interestingly, both neurokinins and treatments raising cyclic AMP (cAMP) levels augment striatal dopamine release (Reid *et al.*, 1990; Santiago and Westerink, 1990), whereas eicosanoids also regulate several cellular functions also controlled by neurokinins (Shimizu and Wolfe, 1990). Moreover, blocking prostaglandin (PG) synthesis can inhibit neurokinin functional effects as indicated by studies on neuromodulation of gastric myenteric acetyl choline release (Mayer *et al.*, 1990) and control of colonic transport activity (Rangachari *et al.*, 1990). Despite this, there has been no clear demonstration of a direct link between stimulation of a defined neurokinin receptor subtype and generation of either cAMP or eicosanoids.

As part of an investigation of signal transduction pathways under control by selected neurokinin receptor subtypes, we have assessed the ability of the recombinant bovine NK-2 receptor stably expressed in Chinese hamster ovary (CHO) cells to regulate a number of effector and second messenger systems. This has overcome problems associated with mixed neurokinin receptor populations expressed endogenously in tissues and has allowed us to demonstrate modulation of multiple cellular responses. In addition to previously described $PIP_2$ hydrolysis and increased de novo DNA synthesis (Nilsson *et al.*, 1985; Grandordy *et al.*, 1988; Soder and Hellstrom, 1989), we report that NK-2 receptor-controlled processes also include $Ca^{2+}$ mobilization, arachidonic acid and eicosanoid release, cAMP generation, as well as induction of "immediate early" genes. These responses could be important for the functional biology of neurokinins under physiological conditions.

## Results

### *Expression of bovine NK-2 receptor in CHO cells*

CHO:K1 cells (plated at densities of $5 \times 10^5/cm^2$) were transfected with mammalian expression vectors encoding bovine NK-2 receptor (pBGL312/SKR) and the bacterial neomycin phosphotransferase gene (pSVtkneo). Recombinants were selected both by resistance to G418 and NK-2 receptor expression as indicated by high affinity [$^{125}$I]NKA binding displaceable by 1 $\mu$M NKA (see Materials and methods). Although neither parental CHO:K1 cells or cells transfected with only selection plasmid (C/MOCK) displayed detectable [$^{125}$I]-NKA binding, one clone (CB/4) expressing 21 300 high-affinity sites/cell was propagated and further characterized pharmacologically by radioligand competition binding. Hence, [$^{125}$I]NKA binding was displaced by NKA with an $IC_{50}$ of 12.6 ± 1.6 nM (n = 3) and by naturally occurring neurokinins with the relative rank order of affinity NKA≫NKB>SP (Figure 1). This rank order of displacement is consistent with radioligand binding to the NK-2 receptor subtype (Grandordy *et al.*, 1988; Regoli *et al.*, 1989).

### *NK-2 receptors mediate increased phospholipase C activity*

To test whether the recombinant NK-2 receptor couples to PLC-dependent hydrolysis of $PIP_2$, $IP_3$ was measured by [$^3$H]$IP_3$ binding displacement assay (see Materials and methods). NKA

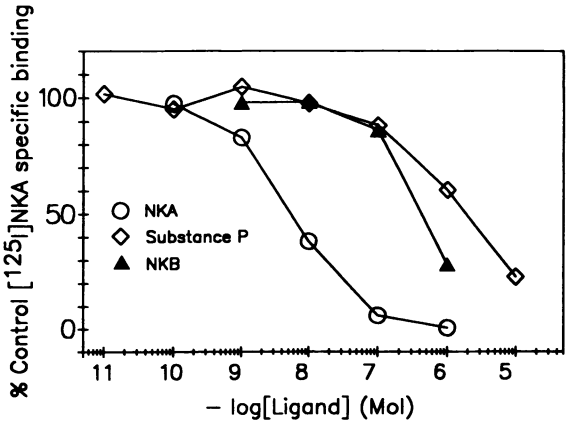


***Figure 1.*** **Binding of neurokinins to intact CB/4 cells.** Concentration-dependent displacement of [$^{125}$I]NKA-binding by different neurokinins. Assays were performed as described in Materials and methods. Data points represent one of three identical experiments performed in triplicate.

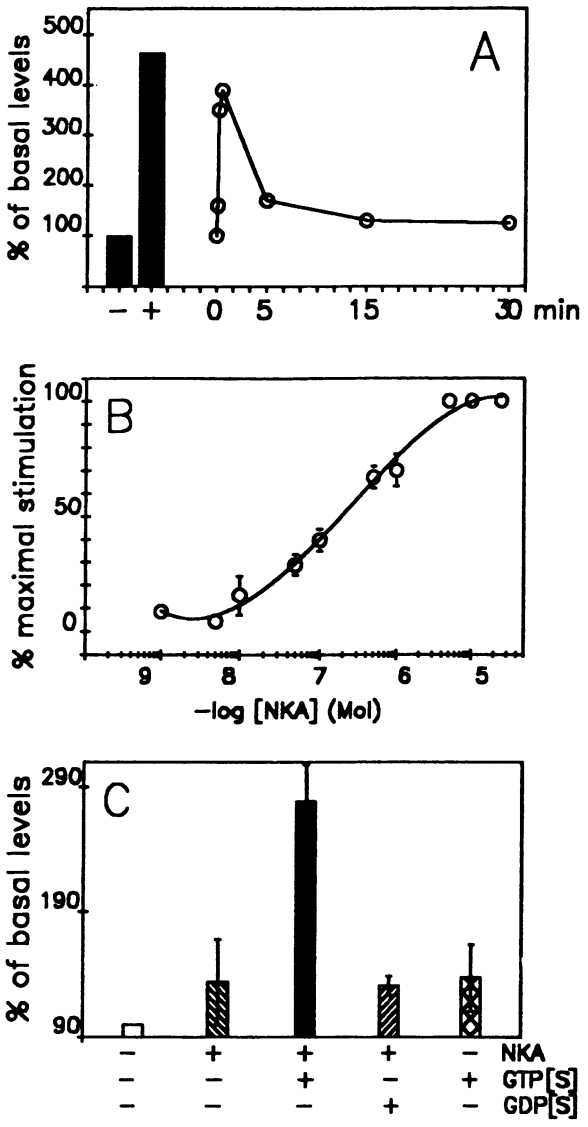


***Figure 2.*** **NKA-stimulated $IP_3$ formation.** (A) Bars show $IP_3$ formation in CB/4 cells incubated in the absence (–) or presence (+) of 1 $\mu$M NKA for 1 min. Time course shows time-dependent changes in $IP_3$ levels after stimulation with 1 $\mu$M NKA. (B) Dose-dependence of NKA-stimulated $IP_3$ formation. $IP_3$ levels were analyzed 1 min after stimulation of cells with increasing concentrations of NKA. (C) Guanine nucleotide-dependence. Streptolysin-O-permeabilized CB/4 cells were incubated either with or without 1 $\mu$M NKA for 1 min in the absence (–) and presence (+) of nonhydrolyzable guanine nucleotides ($10^{-7}$ M) as indicated. Assays were performed as described in Materials and methods. The results depict the means ± SEM of three (C) or six (B) individual experiments each performed in triplicate. Values in (A) are derived from three (bars) or two determinations (time-course) each performed in duplicate. Data are expressed as percentage of either basal levels or the maximal response that have been set to 100% in each case.

at $10^{-6}$ M stimulated a rapid 4.6 ± 0.6-fold (n = 3) increase in $IP_3$ formation that was maximal at 1 min and thereafter declined to basal levels by 15–30 min (Figure 2A). NKA displayed an $EC_{50}$ of 125 ± 11 nM (n = 6) with maximal stimulation observed at 500 nM (Figure 2B). These effects were limited to NK-2 receptor-expressing cells as increased $IP_3$ formation was undetectable in both parental (CHO:K1) and mock-transfected cells (C/MOCK) (data not shown).

To test whether recombinant NK-2 receptors interact with a G-protein when mediating $PIP_2$ breakdown, NKA-stimulated $IP_3$ formation was measured in streptolysin-O permeabilized CB/4 cells. In permeabilized cells G-protein-dependent agonist-stimulated phosphoinositide responses have been shown to require addition of exogenous GTP or an analogue (Fain, 1990). Indeed, whereas neither $10^{-6}$ M NKA nor the hydrolysis-resistant guanosine 5′-[$\gamma$-thio]triphosphate (GTP$\gamma$S; $10^{-7}$ M) increased $PIP_2$ hydrolysis when acting alone, together these agents stimulated $IP_3$ formation by 2.8 ± 0.2-fold (n = 3) (Figure 2C). This NKA response was not observed in the presence of the GDP analogue guanosine 5′-[$\beta$-thio]diphosphate at $10^{-7}$ M (Figure 2C). To test whether the putative G-protein involved is sensitive to modification by pertussis toxin, CB/4 cells were preincubated with 1000 ng/ml of islet-activating protein for 6 h. Such treatment failed to suppress NKA-stimulated $IP_3$ formation (data not shown). This preincubation appears maximally effective at ADP-ribosylating endogenous G-proteins as it blocks totally subsequent labeling of a 41K band obtained on treating membrane fractions with preactivated pertussis toxin in the presence of [$^{32}$P]NAD (Figure 3).

### *NK-2 receptor-dependent $Ca^{2+}$ mobilization*

NKA at 1 $\mu$M stimulated an immediate (<2 s) but transient biphasic increase in cytosolic $Ca^{2+}$ concentration with maximal levels detected within 30 s and basal values re-established after ~3–4 min (Figure 4A). NKA-stimulated $Ca^{2+}$ mobilization was also observed after chelating extra-cellular $Ca^{2+}$ in the presence of 2 mM ethylene glycol-bis ($\beta$-aminoethyl ethyl)-*N,N,N′,N′*-tetraacetic acid (EGTA), although under these conditions the early peak response was reduced to ~25% of the levels observed in the absence of EGTA. In addition, the transient rise was followed by a much more rapid return to resting levels within 40 s (Figure 4B). This time course is similar to that observed for NKA-stimulated

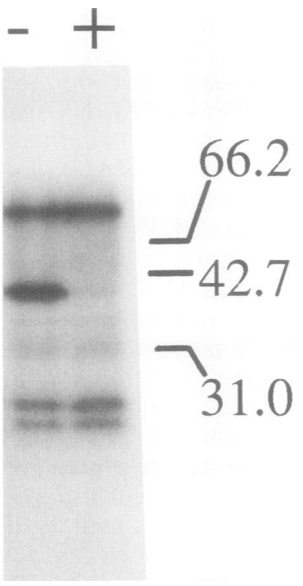


***Figure 3.*** **Pertussis toxin ADP-ribosylation of CB/4 membranes.** Membranes obtained from control (−) and pertussis toxin pretreated (1000 ng/ml) CB/4 cells (+) were ADP-ribosylated by incubation with activated pertussis toxin (40 μg/ml) in the presence of [$^{32}$P]NAD as described in Materials and methods. Samples were separated on a 12% SDS polyacrylamide gel that was dried and subjected to autoradiography. Positions of mw markers are indicated.

$IP_3$ generation (Figure 2A) and is consistent with the role of this second messenger in mobilizing intracellular $Ca^{2+}$ stores (Berridge and Irvine, 1989). Also, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8) is an inhibitor of intracellular $Ca^{2+}$ release (Felder *et al.*, 1989), which at 100 μM diminished the NKA response by 50–75% (data not shown). Neurokinin-stimulated $Ca^{2+}$ mobilization demonstrated the same rank order of agonist potency observed for displacement of [$^{125}$I]NKA binding (see above) with NKA, NKB, and SP displaying $EC_{50s}$ of 0.05 ± 0.02 μM (n = 6), 1.0 ± 0.10 μM (n = 3), and 2.6 ± 0.45 μM (n = 3), respectively (Figure 4C). In contrast to observations with clone CB/4, NKA failed to elevate cytosolic $Ca^{2+}$ levels in cells devoid of NK-2 receptors (CHO:K1 and C/MOCK) (data not shown). Altogether, these findings imply that the NKA-stimulated $Ca^{2+}$ response is mediated by NK-2 receptors and involves both influx from outside the cell and mobilization of intracellular stores.

### *NK-2 receptor stimulation mobilizes arachidonic acid and generates $PGE_2$*

The rate-limiting step for eicosanoid synthesis is mobilization of arachidonic acid from membrane phospholipids. To test for NK-2 receptor coupling to this response, we measured release of [$^3$H]arachidonic acid from prelabeled CB/4 cells. NKA stimulated a rapid 160% increase in [$^3$H]arachidonic acid mobilization that was maximal by 20 min and displayed an $EC_{50}$ of 44.2 ± 37.4 nM (n = 6) (Figure 5A). Liberated arachidonic acid can be metabolized by cyclooxygenase to form several PGs (Shimizu and Wolfe, 1990) and, although $PGD_2$, $PGI_2$, $PGF_{2\alpha}$, and thromboxane $A_2$ formations were undetectable by radioimmunoassay (RIA) (data not shown), NKA stimulated a rapid 8.7-fold increase in $PGE_2$ release with an $EC_{50}$ of 28.1 ± 26.4 nM (n = 6) (Figure 5B).

### *NKA and $PGE_2$ stimulate cAMP formation in CB/4 cells*

To test whether the recombinant NK-2 receptor can modulate adenylyl cyclase activity, cAMP was measured in CB/4 cells exposed to increasing concentrations of NKA, which raised endogenous levels by up to sixfold with an $EC_{50}$ of 1.1 ± 0.26 μM (n = 8) (Figure 6A). This cAMP response is likely to reflect stimulation of adenylyl cyclase as experiments were performed in the presence of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) to inhibit phosphodiesterase activity. Furthermore, stimulation is dependent on NK-2 receptor expression as NKA is ineffective in either parental CHO:K1 or mock-transfected C/MOCK cells (data not shown). In addition to NKA, exogenous $PGE_2$ increased cAMP formation by up to 3.9-fold above basal levels with an $EC_{50}$ = 62.5 ± 9.6 nM (n = 5) (Figure 6A). This action appears specific for $PGE_2$ in sofar as $PGI_2$ was ineffective (data not shown).

Measurements of adenylyl cyclase activity were also performed in washed membrane fractions (see Materials and methods) where enzyme regulation by a stimulatory G-protein ($G_S$) can be demonstrated with GTPγS, which acting alone augments cAMP generation by up to 4.3 ± 0.12-fold (n = 3) with an $EC_{50}$ of 1.0 ± 0.1 μM (n = 3). For measuring agonist-stimulated adenylyl cyclase activity, GTPγS was employed routinely at $10^{-7}$ M (see Materials and methods), and under these conditions $PGE_2$ stimulated a 100% increase with an $EC_{50}$ of 67.6 ± 30.4 nM (n = 4) (Figure 6B), a potency identical to that measured for this eicosanoid in intact cells (see above). Interestingly, in contrast to this and observations in intact cells, NKA-stimulated adenylyl cyclase activation in membrane

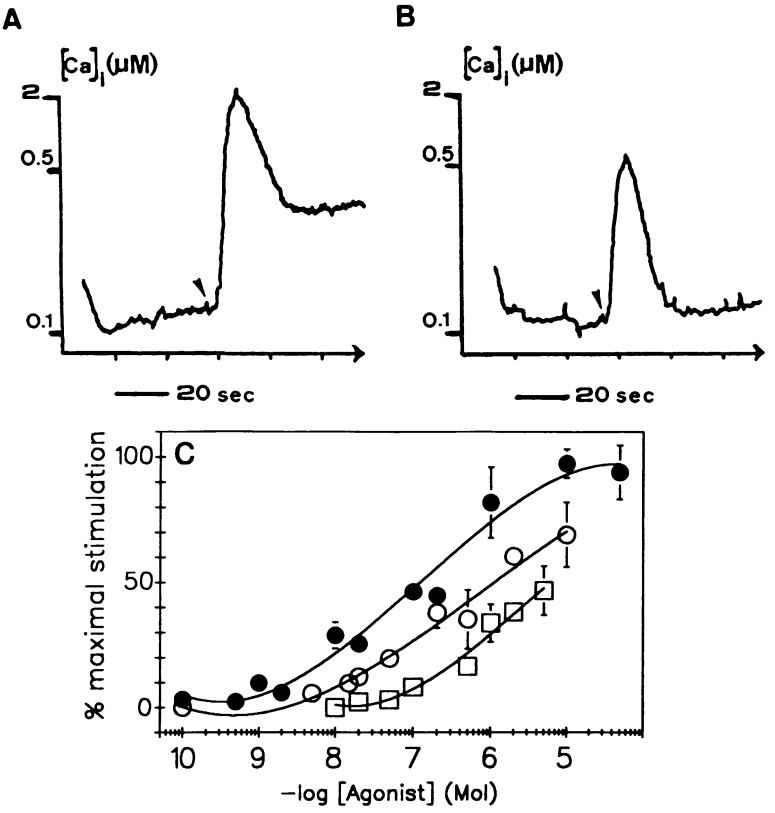


***Figure 4.*** **NK-2 receptor-mediated $Ca^{2+}$ responses.** Time courses of NKA-induced rises in intracellular calcium concentrations $[Ca^{2+}]_i$ in *fura-2*-loaded CB/4 cells in the presence (A) and absence (B) of extracellular calcium (1.2 mM). Two millimolar of EGTA was included in (B). Arrowheads indicate addition of NKA (1$\mu$M). The fluorimetric recording of one typical experiment is shown in each case. (C) Pharmacological characterization of calcium-mobilization in CB/4 cells as a function of increasing concentrations NKA (●), NKB (○), and SP (□) in the presence of 2 mM EGTA. Data points are the means ± SEM of three to six individual experiments. Results are presented as percentage of maximal stimulation (= 100%).

fractions was substantially weaker than detected with $PGE_2$ and limited to 20% above basal levels (Figure 6B). These results demonstrate that although adenylyl cyclase can be regulated through interaction with a stimulatory G-protein, NK-2 receptors appear unable to activate this pathway directly, suggesting that NKA-stimulated cAMP formation in intact cells is indirect.

One such potential indirect mechanism for enhanced cAMP generation involves calmodulin-sensitive adenylyl cyclase stimulated in the presence of elevated $Ca^{2+}$ levels. However, in CB/4 membranes no such activity was detectable as the rate of cAMP formation was insensitive to changes in $Ca^{2+}$ concentration between $10^{-8}$M to $10^{-4}$M and was inhibited by 42% (n = 2) at $10^{-3}$M (data not shown).

### *Inhibition of eicosanoid generation suppresses NK-2 receptor-dependent cAMP formation*

$PGE_2$ stimulates adenylyl cyclase in a wide range of cell and tissue types and this eicosanoid represents a further key mediator potentially important for NKA actions on cAMP in intact CB/4 cells. This was tested with a number of agents suppressing mobilization and metabolism of arachidonic acid. Several phospholipases responsible for generating eicosanoids are activated by $Ca^{2+}$ (Exton, 1990), and 50–75% inhibition of intracellular $Ca^{2+}$ mobilization by 100 $\mu$M TMB-8 (see above) not only decreases NKA-stimulated formation of [$^3$H]-arachidonic acid and $PGE_2$ but also suppresses similarly the cAMP response (Figure 7). In addition, the release of [$^3$H]arachidonic acid and $PGE_2$ were diminished after chelation of extracellular $Ca^{2+}$ with EGTA (2 mM), and this treatment likewise suppressed NKA-stimulated cAMP formation (Figure 7). Moreover, the calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalene- sulfonamide at 100 $\mu$M (W7) (Felder *et al.*, 1989) also caused parallel suppression of NKA-stimulated [$^3$H]arachidonic acid, $PGE_2$, and cAMP generation (Figure 7). Another important regulator of phospholipases associated with arachidonic acid generation is PKC (Exton, 1990), and inhibition with staurosporine (1 $\mu$M) suppressed substantially NKA-stimulated [$^3$H]arachidonic acid and $PGE_2$ output and this was accompanied by a similarly

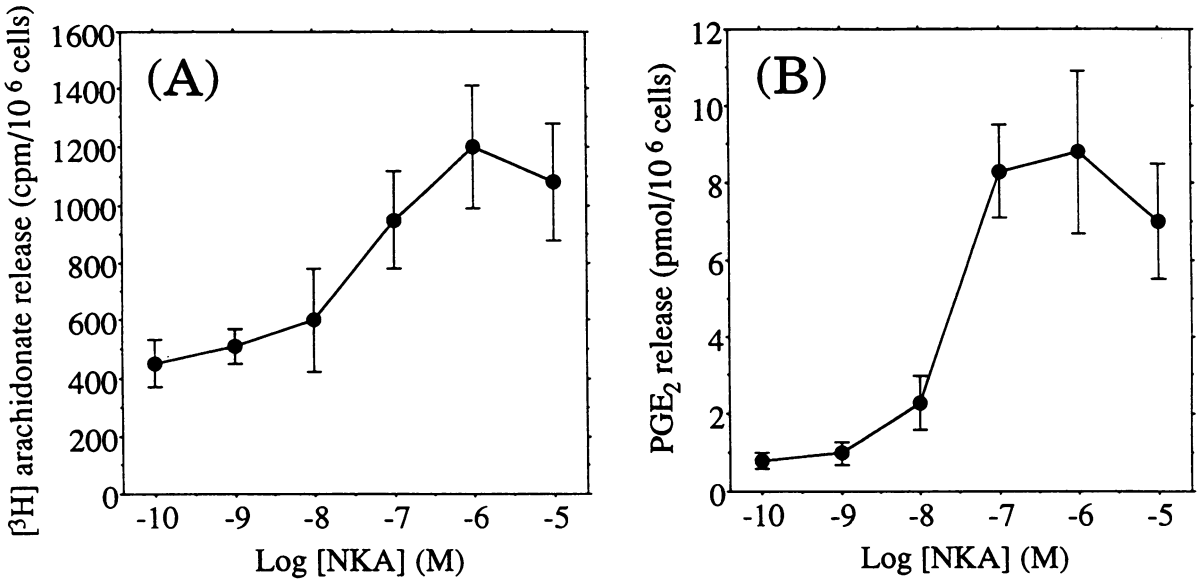


***Figure 5.*** **NKA-stimulated arachidonic acid and $PGE_2$ release.** (A) Release of [$^3$H]arachidonic acid in the presence of increasing concentrations of NKA. Stimulation was for 20 min and all procedures were performed as described in Materials and methods. Values are the mean ± SEM of six experiments performed in triplicate. Samples from (A) were assayed by $^{125}$I-RIA to give dose-dependent formation of $PGE_2$ shown in (B). Values represent the means ± SEM of six experiments performed in triplicate.

diminished cAMP generation (Figure 7). Inhibition of phospholipase $A_2$ by quinacrine (100 $\mu$M) also caused a large parallel inhibition of all three responses (Figure 7). Moreover, inhibition of cyclooxygenase-dependent arachidonic acid metabolism by 10 $\mu$M indomethacin reduced $PGE_2$ and cAMP formation by 97.5 ± 3.5% (n = 4) and 45.6 ± 37.3% (n = 4), respectively. Together, these observations demonstrate that a variety of agents depressing arachidonic acid and $PGE_2$ release also cause similar blockade of NK-2 receptor-dependent cAMP formation. This could reflect a causative relationship between these responses.

### *Immediate early genes and de novo DNA synthesis are induced by activated NK-2 receptors*

To test whether the recombinant NK-2 receptor can initiate de novo DNA synthesis, the effect of NKA on [$^3$H]thymidine incorporation was analyzed under conditions of CHO cell growth arrest (Ashkenazi *et al.*, 1989a). NKA stimulated de novo DNA synthesis by nearly 100% with half-maximal effect observed at 1.4 ± 0.6 nM (n = 6) (Figure 8A). As cells were serum-depleted and assays performed in serum-free medium (see Materials and methods) this growth-stimulating activity was not dependent on other mitogens. Indeed, parental CHO:K1 and mock-transfected C/MOCK cells were unresponsive to NKA further supporting involvement of NK-2 receptors (data not shown).

As activation of immediate early genes frequently accompanies mitogen-induced proliferation (Schonthal, 1990), we tested whether NK-2 receptor-mediated de novo DNA synthesis is associated with increased expression of c-*fos* and/or c-*jun*. Incubation of CB/4 cells with 1 $\mu$M NKA resulted in a rapid but transient stimulation of c-*fos* transcription as observed by Northern blot analysis (Figure 8B). c-*fos* mRNA, which was not detected in unstimulated cells, was found within 5 min of receptor stimulation. Transcript levels decreased to basal again after 15–30 min. In contrast to this NKA effect, treatment with phorbol ester ($10^{-7}$ M phorbol 12-myristate 13-acetate [PMA]) for 15 min to activate PKC failed to induce c-*fos* mRNA. Transcription of c-*jun* was also found to be enhanced after NK-2 receptor stimulation. Maximum levels were observed within 15–30 min of challenge with 1 $\mu$M NKA, whereas basal levels were restored after ~4 h (data not shown). Interestingly, in contrast to observations with c-*fos*, identical incubation with phorbol ester did stimulate expression of c-*jun*.

## Discussion

To investigate the range of effector and second messenger systems under control by a single

neurokinin receptor subtype, we have expressed recombinant bovine NK-2 receptor in CHO cells. The displacement of bound [$^{125}$I]-NKA, as well as neurokinin-stimulated $Ca^{2+}$ mobilization, displays the rank order of potency NKA≫NKB>SP, which is identical to that reported for NK-2 receptor-dependent binding and functional responses in several tissues (Grandordy *et al.*, 1988; Regoli *et al.*, 1989). Moreover, NKA-stimulated $IP_3$ generation in the CB/4 clone is consistent with similar observations in guinea pig trachea (Grandordy *et al.*, 1988), whereas NK-2 receptor-mediated increases in de novo DNA synthesis has also been reported previously in thymocytes, smooth muscle cells, and skin fibroblasts (Nilsson *et al.*, 1985; Soder and Hellstrom, 1989). Together, these observations indicate that the recombinant NK-2 receptor expressed in clone CB/4 displays pharmacological and functional characteristics similar to endogenously expressed NK-2 receptors. The major finding of our studies is that in addition to increasing phosphoinositide hydrolysis and DNA synthesis, recombinant NK-2 receptors are also linked simultaneously to pathways mediating increased eicosanoid release and cAMP formation as well as transcription of the immediate early genes c-*fos* and c-*jun*.

Signal transduction pathways dependent on G-proteins require addition of exogenous guanine nucleotides when activated in membrane fractions and permeabilized cells (Fain, 1990). Because NKA-stimulated $IP_3$ formation demonstrates an absolute dependency for GTPγS in streptolysin-O-permeabilized CB/4 cells, this response is likely to involve direct interaction between recombinant NK-2 receptor and a G-protein. Although we have not characterized this G-protein in detail, it is insensitive to pertussis toxin pretreatment and may be the same component mediating increased phosphoinositide hydrolysis in response to stimulation of cholecystokinin and recombinant muscarinic M1 and M3 receptors in CHO cells (Ashkenazi *et al.*, 1989b). This modulator could be the pertussis toxin insensitive 42K G-protein activating PLC and identified as Gq (Smrcka *et al.*, 1991).

In contrast to direct G-protein coupling to phosphoinositide hydrolysis, weak NKA-stimulated adenylyl cyclase activation in washed membrane fractions indicates an indirect signaling pathway for cAMP formation in intact cells. We have found that rather than involving

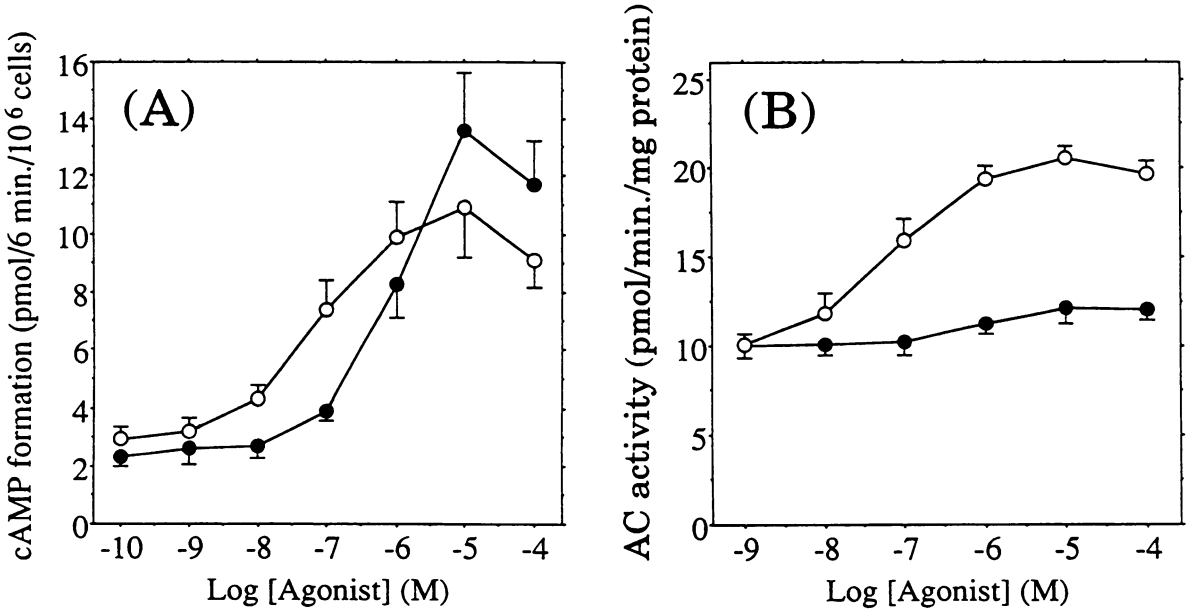


***Figure 6.*** **NKA- and $PGE_2$-stimulated adenylyl cyclase.** (A) Intact CB/4 cells were stimulated for 6 min at 37°C with increasing concentrations of either NKA (●) or $PGE_2$ (○) and cAMP (cAMP) levels (pmol/6 min/$10^6$ cells) measured by [$^3$H]cAMP binding competition assay. Values represent the means ± SEM of eight (NKA) or five ($PGE_2$) experiments each performed in triplicate. (B) Membrane fractions were prepared from CB/4 cells and adenylyl cyclase (AC) activity (pmol/min./mg protein) measured during stimulation with NKA (●) or $PGE_2$ (○) for 10 min at 30°C in the presence of $10^{-7}$ M GTPγS. Values are the means ± SEM of four experiments each performed in triplicate. Membrane preparation, assays, and [$^{32}$P]cAMP extraction were all performed as described in Materials and methods.

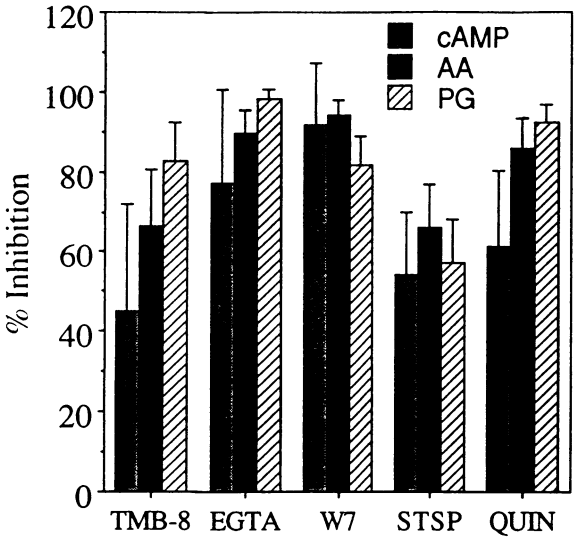


***Figure 7.*** **Parallel inhibition of NKA-stimulated cAMP, [$^3$H]arachidonic acid (AA), and $PGE_2$ (PG) formation.** Intact CB/4 cells were preincubated for 15 min at 37°C with growth medium containing TMB-8 (100 $\mu$M), EGTA (2 mM), W7 (100 $\mu$M), staurosporin (STSP; 1 $\mu$M), or quinacrine (QUIN; 100 $\mu$M) after which time cells were stimulated with NKA for measurement of [$^3$H]arachidonic acid, $PGE_2$ cAMP formation as described in legends to Figures 4 and 5. Data is expressed as percentage inhibition of control responses measured after 15 min preincubation with medium only. Bars represent the means ± SEM of five or six experiments each performed in quadruplicate.

a direct interaction with the G-protein $G_S$, NK-2 receptor-dependent cAMP formation is likely to reflect, at least in part, autocrine stimulation by endogenously generated eicosanoids, particularly $PGE_2$. In fact, NKA stimulates rapid release of arachidonic acid and $PGE_2$, whereas exogenously applied $PGE_2$ activates adenylyl cyclase and raises cAMP levels potently in both intact cells and membranes. Also, a number of agents suppressing prostanoid formation cause parallel inhibition of the cAMP response. An alternative potential mechanism for mediating indirect cAMP formation involves $Ca^{2+}$-dependent stimulation of a calmodulin-sensitive adenylyl cyclase. Although our data do not allow us to exclude this possibility definitively, CB/4 cell membrane activity is insensitive to changes in $Ca^{2+}$ concentration between $10^{-8}$ and $10^{-4}$ M, indicating that such a regulation is unlikely. This is the first report of neurokinin receptor-linked generation of either cAMP or eicosanoids and it raises the question of whether similar responses are of functional importance under physiological conditions. Indeed, if endogenously expressed NK-2 receptors elicit similar effects, NKA-stimulated eicosanoid release could mediate many functional activities regulated by this class of neuropeptide. This is illustrated by neurokinin actions to modulate gastric myenteric cholinergic neurotransmission and control of colonic transport activity that are blocked after inhibition of prostanoid synthesis (Mayer *et al.*, 1990; Rangachari *et al.*, 1990). Moreover, exogenous eicosanoids mimic neurokinin actions to produce mechanical hyperalgesia (Ferreira and Nakamura, 1979; Naka-

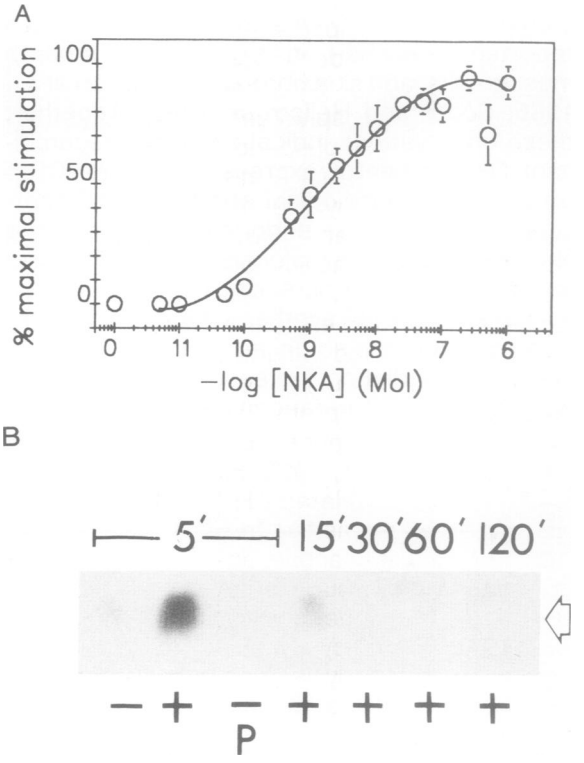


***Figure 8.*** **NKA-stimulated de novo DNA synthesis and c-fos expression.** (A) Dose-dependence of NKA-stimulated DNA synthesis in hyper-confluent CB/4 cultures that had been synchronized by serum-depletion as described (Ashkenazi *et al.*, 1989a). [$^3$H]thymidine incorporation was measured 22 h after stimulation of cells with varying concentrations of NKA as described in Materials and methods. Data are presented as percentage of maximal stimulation that was set to 100%. Data points are the means ± SEM of six individual experiments each performed in triplicate. Basal thymidine incorporation varied from $3 \times 10^4$ to $10^5$ cpm/$10^6$ cells in different experiments. (B) Confluent starved CB/4 cells were cultured in the absence (−) or presence (+) of 1 $\mu$M NKA for 5 to 120 min as indicated. After cell lysis and RNA-extraction, Northern blots were carried out using a c-*fos* probe as described in Materials and methods. Approximately 10 $\mu$g of total RNA were loaded per lane. The arrow points to the 2.2 kb c-*fos* transcript. P symbolizes the use of PMA (100 ng/ml) in the cultures. (The presence of PMA for even 16 h did not influence c-*fos* expression; data not shown).

mura-Craig and Smith, 1989), to inhibit acetylcholine release from gastric myenteric plexus (Mayer *et al.*, 1990), to resist gastric mucosal injury after noxious challenge (Whittle and Lopez-Belmonte, 1991), and to enhance airway mucus secretion (Rogers *et al.*, 1989; Jacquot *et al.*, 1990). Finally, adenylyl cyclase activation could underly neurokinin-stimulated striatal dopamine release (Reid *et al.*, 1990) because this response is mimicked by treatments raising cAMP levels (Santiago and Westerink, 1990).

Despite many reports on receptor-linked eicosanoid formation, the biochemical pathways underlying this response are unclear. Because the level of arachidonic acid is rate limiting in the generation of eicosanoids (Shimizu and Wolfe, 1990), phospholipase activation to hydrolyse membrane phospholipids is a key event. The release of arachidonic acid can be increased directly by the hydrolytic actions of phospholipase $A_2$ and also indirectly by several phospholipase C and D subtypes (Axelrod *et al.*, 1988; Exton, 1990). Several potential pathways for receptor-linked eicosanoid generation have been identified and include activation of phospholipases through 1) direct G-protein coupling to cell surface receptors, 2) increased cytosolic $Ca^{2+}$ levels, and 3) phosphorylation by activated PKC (Axelrod *et al.*, 1988; Exton, 1990). In CB/4 cells, NKA-stimulated [$^3$H]inositol phosphate generation is insensitive to EGTA, TMB-8, W7, indomethacin, quinacrine, and increased slightly by staurosporin, suggesting that arachidonic mobilization is independent of the metabolic breakdown of diacylglycerol formed by phos phoinositide hydrolysis (S.J. Arkinstall and P.P. Godfrey, unpublished observations). Nevertheless, observations presented in this paper indicate that $Ca^{2+}$ and potentially PKC activation could play a key role in activating phospholipase(s) for eicosanoid formation. The nature of these enzymes, the phospholipid substrates, as well as the potential involvement of direct phospholipase $A_2$ activation by G-protein-linked signaling pathways is currently unknown.

The genes encoding several members of the seven transmembrane domain receptor family have now been cloned and expressed in a variety of host cells. Many of these studies have demonstrated that activation of a single recombinant receptor subtype can regulate the activity of several signal transduction pathways simultaneously. A picture emerging from these investigations is that many of these receptors, which in the same cell regulate both adenylyl cyclase activity and phosphoinositide hydrolysis, can be subdivided into two distinct groups. The first group includes 1) $5HT_{1a}$ expressed in COS-7 and HeLa cells (Fargin *et al.*, 1989), 2) $D_2$ dopaminergic and $\alpha 2$ adrenergic in fibroblasts (Cotecchia *et al.*, 1990; Vallar *et al.*, 1990), and 3) muscarinic M2 and M4 in CHO cells (Peralta *et al.*, 1988; Ashkenazi *et al.*, 1989b) all of which mediate both activation of phosphoinositide hydrolysis and inhibition of adenylyl cyclase activity through pathways involving pertussis toxin-sensitive G-proteins such as $G_o$ and $G_i$. The second group is represented by muscarinic M1 and M3 expressed in CHO and A9L cells (Conklin *et al.*, 1988; Peralta *et al.*, 1988; Ashkenazi *et al.*, 1989b; Felder *et al.*, 1989) and also $\alpha_1$-adrenergic receptors in COS-7 cells (Cotecchia *et al.*, 1990) that mediate a powerful phosphoinositide hydrolysis and a less potent adenylyl cyclase activation involving pathways insensitive to pretreatment with pertussis toxin. The second class of receptor could also mediate increased arachidonic acid mobilization and an important mitogenic action as described for recombinant M1 and M3 muscarinic receptors expressed in A9L and CHO cells, respectively (Conklin *et al.*, 1988; Ashkenazi *et al.*, 1989a; Felder *et al.*, 1989).

Our data on NK-2 receptor-dependent arachidonic acid mobilization, cAMP, and $IP_3$ formation together with increased de novo DNA synthesis and interaction with a pertussis toxin insensitive G-protein(s) indicates that this receptor subtype falls into the second of these classes when expressed in CHO cells. Within this classification there appear to be important differences in the mechanisms for adenylyl cyclase activation. Although neither NK-2, M1, or $\alpha_1$ receptors interact directly with the stimulatory G-protein $G_S$, eicosanoid formation appears important only for the neurokinin response. In A9L cells M1 receptor-stimulated adenylyl cyclase reflects a calmodulin-dependent activation by $Ca^{2+}$ (Felder *et al.*, 1989), whereas in fibroblasts $\alpha_1$-adrenergic receptor effects appear to be mediated, at least in part, by PKC (Cotecchia *et al.*, 1990). It is unclear whether these differences originate at the level of the receptor subtype or host cell system employed.

Mechanisms underlying NKA-stimulated transcription of the immediate-early genes c-*fos* and c-*jun* are unclear, although both $Ca^{2+}$/calmodulin- and cAMP-dependent protein kinases could play a role, potentially through activation of transcription factors cAMP-responsive element binding protein and serum responsive factor (Sheng and Greenberg, 1990).

Although short-term treatment with PMA had no effect on c-*fos* expression, it did increase c-*jun* transcription in CB/4 cells. This suggests that NK-2 receptor-mediated activation of c-*fos* may reflect $Ca^{2+}$/cAMP-dependent processes, whereas PKC is likely to trigger NK-2 receptor-induced c-*jun* transcription in CB/4. Both proto-oncogenes encode DNA-binding proteins that form bimolecular complexes to initiate and modulate gene expression, thereby playing a key role in cell differentiation, development, and proliferation (Schonthal, 1990). Although the physiological relevance of the c-*fos* and c-*jun* responses to CB/4 cells remains unclear, these proto-oncogenes are likely to play a crucial role in the signal transduction mechanisms of NK-2 receptors. Addition of NKA stimulated their expression both in proliferating (data not shown) and growth-arrested CB/4 cells. Thus, one of the consequences of their actions might be an increase in de novo DNA synthesis in growth-arrested CB/4 cells, an effect that was also observed after stimulation with NKA. Although the CB/4 clone is derived from a highly mitogenic parental cell line (CHO:K1) and does not represent an ideal cell system for analysing proliferative mechanisms under normal growth conditions, our results suggest strongly a mitogenic role for NK-2 receptors and support previous findings on primary cells (Nilsson *et al.*, 1985). Moreover, since during brain development NK-2 receptors are expressed transiently at high levels (Dam *et al.*, 1988), NK-2 receptor-dependent stimulation of c-*fos* and c-*jun* expression might be required for stage-specific cell proliferation in the developing CNS.

In summary, data presented in this paper demonstrate that recombinant bovine NK-2 receptors expressed in CHO cells retain their pharmacological characteristics, and moreover, modulate either directly or indirectly multiple signal transduction pathways. These studies also demonstrate that the NK-2 receptor can regulate both immediate and long-term cell function through signal transduction processes organized at three levels: 1) direct coupling to phosphoinositide hydrolysis and $Ca^{2+}$ mobilization through pertussis toxin-insensitive G-protein(s), 2) secondary autocrine stimulation after arachidonic acid release and subsequent eicosanoid generation, and 3) control of gene expression and cell division. The multiple cellular responses to recombinant NK-2 receptor activation could be applicable to NK-2 receptors expressed endogenously in various tissues and may prove to be important for the wide-ranging biological actions of neurokinins. Currently, analysis of primary tissues and cells is underway to validate which of these activities is responsible for neurokinin actions under physiological conditions because the pattern of signal transduction may vary depending on cellular content of G-proteins, effector systems, and other regulatory components controlling receptor activity. These variations will allow for a multitude of functional responses after stimulation of a single neurokinin receptor subtype.

## Materials and methods

### Cell culture

Parental and transfected CHO:K1 cells (American Type Culture Collection, Rockville, MD) were grown routinely as monolayers in Dulbecco's Modified Eagle's medium/Ham's F12 (vol/vol) medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (GIBCO), 2 mM glutamine, penicillin, and streptomycin (60 μg/ml each) in a humidified $CO_2$-atmosphere at 37°C. Except where indicated all binding experiments and neurokinin stimulation of intact cells were performed in assay medium (serum-free 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]).

### Expression vector, transfection of cells, and selection of recombinants

Partial digestion of bovine neurokinin-2 receptor cDNA generously donated by S. Nakanishi (Masu *et al.*, 1987) with *Pst* I yielded a 1.6 kb restriction fragment containing the entire coding sequence. This fragment was subcloned into the mammalian expression vector pBGL312 (Cate *et al.*, 1986), which drives expression of the inserted gene by the SV40 early promoter. For constitutive expression, the resulting plasmid pBGL312/SKR was cotransfected with plasmid pSVtkneo (Nicolas and Berg, 1983), conferring neomycin resistance to the cells. Transfectants were selected in 600 μg/ml G418 (Geniticin, GIBCO) and analyzed for their ability to specifically bind [$^{125}$I] labeled neurokinin A (Amersham, Arlington Heights, IL). NK-2 receptor-bearing subclones were then propagated.

### Radioligand binding to intact cells

Cells were grown in 24-well plates, rinsed, and incubated with gentle agitation for 60 min at 20°C in 0.5 ml of assay medium containing [$^{125}$I]NKA (30–40 pM), $MnCl_2$ (3 mM), and competing neurokinins as indicated. After a rapid wash with 2 ml of ice-cold medium, cells were solubilized with 2 M NaOH and bound [$^{125}$I]NKA determined by gamma counting. Nonspecific binding was determined in the presence of 1 μM unlabeled NKA (Bachem, Philadelphia, PA).

### Assessment of $IP_3$ generation

Cells were grown in 6-well plates ($1–2 \times 10^6$/well) and stimulated with different concentrations of NKA in assay medium containing experimental agents as indicated. Reactions were stopped at various times with 0.2 vol of ice-cold 20% (wt/vol) perchloric acid. Precipitates were pelleted by centrifugation and the supernatants neutralized using 10 M KOH.

After sedimentation of $KClO_4$, D-*myo*-$IP_3$ levels were determined using an [$^3$H]-$IP_3$ competition binding assay (Amersham).

For permeabilization, confluent cells were incubated at room temperature for 5–15 min with 10 U/ml of streptolysin-O (Sigma, St. Louis, MO) in serum-free medium. After washing with 50-fold excess of serum-free medium, NKA and guanine nucleotide analogues (Sigma) were added as indicated. Reactions were terminated after 1 min as described above.

### *Pertussis toxin treatment*

For treating intact cells, the cells were exposed to pertussis toxin (Sigma) for 6 h at a concentration of 1000 ng/ml in growth medium. For assessing the completeness of G-protein ADP-ribosylation under these conditions, membrane fractions were prepared by washing and resuspending cells in 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4, containing 1 mM EDTA followed by centrifugation at 35 000 $\times g$ for 30 min at 4°C. The cell pellet was then homogenized in 10 mM Tris, pH 7.4, and centrifuged twice as above with intermediate washing and final resuspension in the same buffer. Membranes were also prepared from control CB/4 cells not pretreated with pertussis toxin. Membranes from control and pretreated CB/4 cells were then incubated at 37°C for 60 min with pertussis toxin (40 $\mu$g/ml; preactivated by treating with 50 mM dithiothreitol [DTT] at 37°C for 30 min) in 50 mM Tris, pH 8.0, containing (final concentrations) 2 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, 1 mM ATP, 10 mM thymidine, 500 $\mu$M GTP, 3 $\mu$M NAD, and 10–20 $\mu$Ci [$^{32}$P]NAD (New England Nuclear, Boston, MA). Membranes were then applied to a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel, which was dried and subjected to autoradiography.

### *Intracellular [$Ca^{2+}$] measurements*

Intracellular $Ca^{2+}$ concentrations were determined by using the fluorescent $Ca^{2+}$ chelating agent *fura-2*. Aliquots of *fura-2*-loaded cells (~2 × $10^6$) were stimulated with neuropeptides as indicated and fluorescence emission was recorded using an MS-5 spectrophotometer (Perkin Elmer, Norwalk, CT). Loading of cells, fluorescence monitoring, calibration procedures, and other experimental details were as previously described (Capponi *et al.*, 1984).

### *Whole cell cAMP determination*

Cells (2.5–5 × $10^5$) were incubated at 37°C for 6 min in 200 $\mu$l of assay medium containing 0.5 mM IBMX, together with NKA or $PGE_2$ as indicated. Incubations were terminated with 50 $\mu$l of ice-cold 25% (wt/vol) trichloroacetic acid followed by immediate freezing in dry-ice/methanol. Samples were then neutralized by three extractions in 5 vol of water-saturated ether, freeze-dried and assayed for cAMP using a [$^3$H]cAMP competition binding kit (Amersham) according to the manufacturer's instructions.

### *Adenylyl cyclase activity in isolated washed membranes*

Membrane fractions were prepared by homogenization in ice-cold 5 mM Tris, pH 7.5, containing 2 mM $MgCl_2$ and 0.1 mM DTT followed by two centrifugations at 35 000 × $g$ for 30 min at 4°C with intermediate washing and final resuspension in 10 mM Tris, pH 7.5, containing 0.1 mM DTT. Final protein concentration was 4–6 mg/ml. Adenylyl cyclase assays were performed by incubation at 30°C for 10 min in a final volume of 100 $\mu$l containing membrane fraction (100–150 $\mu$g of protein), 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM KCl, 0.25 mM [$\alpha$-$^{32}$P]ATP (200–400 c.p.m./pmole), 2.5 mM cAMP, 0.5 mM IBMX, 0.5 mM DTT, 1 mg/ml BSA, 20 mM phosphocreatine, 10 U/assay creatine phosphokinase, $10^{-7}$M GTP$\gamma$S, and NKA or $PGE_2$ as indicated. When required, free $Ca^{2+}$ levels were controlled using $CaCl_2$ in the presence of 1mM EGTA. Reactions were terminated with 200 $\mu$l of 1% SDS containing 20 mM ATP and 20 mM cAMP followed by heating at 95°C for 5 min. After diluting samples with 700 $\mu$l of water [$^{32}$P]cAMP was isolated by chromatography using plastic minicolumns containing 2 ml of Dowex 50 (H+) with further purification performed by $ZnSO_4$/$Ba(OH)_2$ precipitation with 0.5 ml each of 0.2 M solutions.

### *[$^3$H]Arachidonic acid mobilization and $PGE_2$ formation*

Cells were cultured in 6-well plates (1–2 × $10^6$ cells/well) and labeled by incubation for 2 h with serum-free medium containing [$^3$H]arachidonic acid (1$\mu$Ci/ml) and supplemented with fatty-acid free BSA (FAF-BSA; 2 mg/ml). Cells were then washed three times and stimulated for 20 min at 37°C with neurokinins in 1 ml of medium containing FAF-BSA. Eight hundred microliters were then removed to ice-cold tubes, centrifuged at 500 × $g$ for 5 min, and aliquots either counted directly for [$^3$H]arachidonic acid release or taken for assay of $PGE_2$ using an $^{125}$I-RIA kit (New England Nuclear).

### *DNA synthesis*

Cells were grown in 24-well tissue culture dishes. Confluent cultures were synchronized for 16–22 h in serum-free medium before incubation in assay medium with neuropeptides and experimental agents for 22–24 h in the presence of 1–3 $\mu$Ci/ml [$^3$H]thymidine (Amersham). TCA-precipitable material was quantified by liquid scintillation counting.

### *c-fos and c-jun expression*

Cells were stimulated with NKA or various compounds as indicated. Total cytoplasmic RNA was extracted at different time points as described (Miller *et al.*, 1982). Before transfer onto nylon membranes (Genescreen, New England Nuclear), 10–20 $\mu$g RNA aliquots were electrophoresed in 1% agarose gels using denaturing conditions (Maniatis *et al.*, 1982). Hybridizations were carried out at 37°C in 5× SSC, 20% formamide, 5× Denhardt's, 5 mM $PO_4$ (pH 6.5), 1 $\mu$g/ml $Na_2H_2P_2O_7$, and 0.1% SDS for 16 h. Antisense-oligonucleotides, 5'-labelled, (according to the nucleotide sequences encoding amino acids 1–15 and 316–327 of human c-*fos* and c-*jun*, respectively) were used as hybridization probes. Final washes were at 43°C in 2× SSC.

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