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Characterization of the cloned neurokinin A receptor transfected in murine fibroblasts

Henderson, Alden Keith, Ph.D.
The University of Arizona, 1992
CHARACTERIZATION OF THE CLONED NEUROKININ A RECEPTOR
TRANSFECTED IN MURINE FIBROBLASTS

by

Alden Keith Henderson

A Dissertation Submitted to the Faculty of the
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In Partial Fulfillment of the Requirements
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DOCTOR OF PHILOSOPHY
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THE UNIVERSITY OF ARIZONA
1992
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Alden Keith Henderson entitled Characterization of the Cloned Neurokinin A Receptor Transfected in Murine Fibroblasts and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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Ka lei hā 'ule 'ole, he lūau 'i makua, keiki kane, e ke kumu.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>9</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>11</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>12</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>14</td>
</tr>
<tr>
<td>Physiological Effects of Neurokinins</td>
<td>16</td>
</tr>
<tr>
<td>Discovery of Tachykinin Peptides</td>
<td>17</td>
</tr>
<tr>
<td>Biosynthesis of Neurokinin Peptides</td>
<td>19</td>
</tr>
<tr>
<td>Location of Neurokinin Peptides</td>
<td>20</td>
</tr>
<tr>
<td>Agonist and Antagonist Development</td>
<td>23</td>
</tr>
<tr>
<td>Neurokinin Receptors</td>
<td>26</td>
</tr>
<tr>
<td>Cloning of Neurokinin Receptors</td>
<td>28</td>
</tr>
<tr>
<td>Location of Neurokinin Receptors</td>
<td>35</td>
</tr>
<tr>
<td>Hypothesis</td>
<td>37</td>
</tr>
<tr>
<td>EXPRESSION OF THE BOVINE STOMACH cDNA SKR56S IN B82 FIBROBLASTS</td>
<td>39</td>
</tr>
<tr>
<td>Materials</td>
<td>40</td>
</tr>
<tr>
<td>Methods</td>
<td>41</td>
</tr>
<tr>
<td>Expression of the Bovine Stomach cDNA SKR56S in B82 Fibroblasts</td>
<td>41</td>
</tr>
<tr>
<td>NK-2 Receptor Density in Transfected Cells</td>
<td>43</td>
</tr>
<tr>
<td>Decreased Expression of the NK-2 Receptor</td>
<td>44</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>45</td>
</tr>
<tr>
<td>Results</td>
<td>45</td>
</tr>
<tr>
<td>Incorporation of SKR56S into the Expression Vector</td>
<td>45</td>
</tr>
<tr>
<td>NK-2 Receptor Densities in the Transfected B82 Fibroblasts</td>
<td>49</td>
</tr>
<tr>
<td>Decreased Expression of the NK-2 Receptor</td>
<td>49</td>
</tr>
<tr>
<td>Discussion</td>
<td>51</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (continued)

DEMONSTRATION OF NEUROKININ A BINDING SITES IN SKLKB82#3 CELLS .................................................. 53
Materials .............................................................................. 54
Methods .............................................................................. 54
  Radioligand Binding Assays .............................................. 54
    SKLKB82#3 Cells ......................................................... 54
    Bovine Stomach Membranes ........................................... 55
Data Analysis ....................................................................... 55
Results .............................................................................. 56
Discussion ........................................................................... 59

THE COUPLING OF SECOND MESSENGERS TO THE NK-2 RECEPTORS EXPRESSED IN B82 FIBROBLASTS .......... 60
Materials .............................................................................. 61
  Accumulation of [3H]Inositol Monophosphates ................... 61
  Intracellular Calcium Mobilization ..................................... 61
  3',5' Cyclic Adenosine Monophosphate ............................... 62
  Arachidonic Acid Release .................................................. 62
Methods .............................................................................. 62
  Accumulation of [3H]Inositol Monophosphates ................... 62
  Intracellular Calcium Mobilization ..................................... 64
  3',5' Cyclic Adenosine Monophosphate ............................... 65
  Arachidonic Acid Release .................................................. 66
Data Analysis ....................................................................... 67
  Calcium Mobilization ....................................................... 67
  3',5' Cyclic Adenosine Monophosphate ............................... 68
Results .............................................................................. 68
  Agonist Induced [3H]IP_1 Accumulation .............................. 68
  Intracellular Calcium Mobilization ..................................... 69
  Formation or Inhibition of cAMP ........................................ 70
  Arachidonic Acid Release .................................................. 70
Discussion ........................................................................... 76
TABLE OF CONTENTS (continued)

DEMONSTRATION OF A NOVEL NK-2 RECEPTOR SUBTYPE IN TRANSFECTED B82 FIBROBLASTS ................. 79

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials</td>
<td>80</td>
</tr>
<tr>
<td>Methods</td>
<td>80</td>
</tr>
<tr>
<td>Radioligand Binding Assays</td>
<td>80</td>
</tr>
<tr>
<td>[3H]IP$_1$ Accumulation and pA$_2$ Determination</td>
<td>80</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>81</td>
</tr>
<tr>
<td>Results</td>
<td>81</td>
</tr>
<tr>
<td>Discussion</td>
<td>87</td>
</tr>
</tbody>
</table>

DESENSITIZATION OF NK-2 RECEPTORS EXPRESSED IN B82 FIBROBLASTS .................. 90

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials</td>
<td>91</td>
</tr>
<tr>
<td>Methods</td>
<td>91</td>
</tr>
<tr>
<td>Accumulation of [3H]Inositol Monophosphates</td>
<td>91</td>
</tr>
<tr>
<td>Desensitization Time Course</td>
<td>91</td>
</tr>
<tr>
<td>Recovery from Desensitization</td>
<td>92</td>
</tr>
<tr>
<td>Calcium Mobilization</td>
<td>92</td>
</tr>
<tr>
<td>Phorbol Ester Treatment</td>
<td>92</td>
</tr>
<tr>
<td>Desensitization Selectivity</td>
<td>93</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>93</td>
</tr>
<tr>
<td>Results</td>
<td>93</td>
</tr>
<tr>
<td>Desensitization Time Course</td>
<td>93</td>
</tr>
<tr>
<td>Recovery of [3H]IP$_1$ Accumulation</td>
<td>94</td>
</tr>
<tr>
<td>Calcium Mobilization</td>
<td>94</td>
</tr>
<tr>
<td>Phorbol Ester Treatment</td>
<td>95</td>
</tr>
<tr>
<td>Desensitization Selectivity</td>
<td>95</td>
</tr>
<tr>
<td>Discussion</td>
<td>95</td>
</tr>
</tbody>
</table>

DISCUSSION ........................................ 105

CONCLUSIONS ...................................... 108

LIST OF REFERENCES ................................. 111
### LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Biosynthesis of Neurokinin Peptides</td>
<td>21</td>
</tr>
<tr>
<td>2.</td>
<td>Putative model of the NK-2 receptor</td>
<td>32</td>
</tr>
<tr>
<td>3.</td>
<td>Coupling of PIP₂ hydrolysis and calcium mobilization to the NK-2 receptor</td>
<td>34</td>
</tr>
<tr>
<td>4.</td>
<td>Map of the expression vector, pHβAPr-1-neo-SRK56S, transfected into B82 fibroblasts</td>
<td>42</td>
</tr>
<tr>
<td>5.</td>
<td>Autoradiographic visualization of the hybridization of SKR56S and pGEM-3-SKR56S constructs</td>
<td>46</td>
</tr>
<tr>
<td>6.</td>
<td>Autoradiographic visualization of the hybridization of SKR56S and pHβAPr-1-neo-SKR56S constructs</td>
<td>47</td>
</tr>
<tr>
<td>7.</td>
<td>Restriction enzyme analysis of pGEM-3-SKR56S and pHβAPr-1-neo-SKR56S constructs</td>
<td>48</td>
</tr>
<tr>
<td>8.</td>
<td>Competitive binding of (^{[125]}\text{I})NKA with NKA, substance P, or senktide in intact SKLKB82#3 cells</td>
<td>58</td>
</tr>
<tr>
<td>9.</td>
<td>Agonist induced (^{3}\text{H})IP₁ accumulation in SKLKB82#3 cells</td>
<td>71</td>
</tr>
<tr>
<td>10.</td>
<td>Time course for the change in fluorescence intensity after the addition of NKA to a SKLKB82#3 cell preparation</td>
<td>72</td>
</tr>
<tr>
<td>11.</td>
<td>Concentration-dependent increase in Ca(^{2+}) upon stimulation with NKA in SKLKB82#3 cells</td>
<td>73</td>
</tr>
<tr>
<td>12.</td>
<td>Competitive binding of (^{[125]}\text{I})NKA with MEN 10207 or MEN 10208 in SKLKB82#3 cells</td>
<td>83</td>
</tr>
<tr>
<td>13.</td>
<td>The inhibition of NKA-induced (^{3}\text{H})IP₁ accumulation in SKLKB82#3 cells by different concentrations of MEN 10207</td>
<td>84</td>
</tr>
<tr>
<td>14.</td>
<td>The inhibition of NKA-induced (^{3}\text{H})IP₁ accumulation in SKLKB82#3 cells by different concentrations of MEN 10208</td>
<td>85</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>15.</td>
<td>Arunlakshana-Schild linear regression for the effect of MEN 10207 and MEN 10208 on NKA-induced (^{3}H)IP(_{1}) accumulation in SKLKB82#3 fibroblasts</td>
<td>86</td>
</tr>
<tr>
<td>16.</td>
<td>The effect of chronic NKA stimulation on (^{3}H)IP(_{1}) accumulation in SKLKB82#3 cells</td>
<td>96</td>
</tr>
<tr>
<td>17.</td>
<td>Percent inhibition of (^{3}H)IP(_{1}) accumulation from pretreatment with 1 (\mu)M NKA for various time periods.</td>
<td>98</td>
</tr>
<tr>
<td>18.</td>
<td>The recovery of (^{3}H)IP(_{1}) accumulation in desensitized SKLKB82#3 cells</td>
<td>99</td>
</tr>
<tr>
<td>19.</td>
<td>The effect of chronic NKA stimulation on intracellular calcium mobilization in SKLKB82#3 cells</td>
<td>100</td>
</tr>
<tr>
<td>20.</td>
<td>The effect of phorbol ester treatment on (^{3}H)IP(_{1}) accumulation in SKLKB82#3 cells</td>
<td>101</td>
</tr>
<tr>
<td>21.</td>
<td>The selectivity of NK-2 receptor desensitivity in SKLKB82#3 cells</td>
<td>102</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Amino acid sequence of tachykinins</td>
<td>15</td>
</tr>
<tr>
<td>2. Properties and features of neurokinin receptors</td>
<td>33</td>
</tr>
<tr>
<td>3. NK-2 receptor density in selected tissues and cell lines</td>
<td>50</td>
</tr>
<tr>
<td>4. Comparison of the affinity of neurokinin agonists in intact SKLKB82#3 cells and bovine stomach membranes</td>
<td>57</td>
</tr>
<tr>
<td>5. The effect of neurokinin A on the formation or inhibition of cAMP in SKLKB82#3 cells</td>
<td>74</td>
</tr>
<tr>
<td>6. The effect of neurokinin A on arachidonic acid release in SKLKB82#3 cells</td>
<td>75</td>
</tr>
<tr>
<td>7. Comparison of the affinity and potency of neurokinin agonists and antagonists in SKLKB82#3 cells, bovine stomach membranes, rabbit pulmonary artery, and hamster trachea</td>
<td>82</td>
</tr>
<tr>
<td>8. Half-maximal stimulatory concentrations for NKA-induced [³H]IP₁ accumulation in desensitized SKLKB82#3 cells</td>
<td>97</td>
</tr>
</tbody>
</table>
ABSTRACT

The neurokinins comprise a family of peptides with similar structure and physiological effects. Several limitations arise when investigating their biochemical and pharmacological properties. Because neurokinins contain similar amino acids in their carboxyl terminus, radiolabeled neurokinins and biosynthesized antibodies may lack the specificity to distinguish between the various neurokinin receptors. Tissue preparations used to characterize their pharmacological responses often have several types of neurokinin receptors, different receptor densities, or signal transduction mechanisms. These factors can complicate the interpretation of results from functional assays. Developing a cell line that expresses a high density of one type of neurokinin receptor with similar biochemical and pharmacological characteristics as the endogenous receptor may overcome these limitations.

To facilitate this aim, the neurokinin-2 receptor (NK-2) was expressed by transfecting a bovine stomach cDNA that encoded for the NK-2 receptor into the murine fibroblast B82 L cell line. This de novo protein displayed high affinity and selectivity for neurokinin A (NKA) in competitive radioligand binding assays and was functionally coupled to phosphatidylinositide 4,5-bisphosphate (PIP₂) hydrolysis and intracellular calcium mobilization. The expressed receptor also exhibited a time dependent, homologous desensitization of PIP₂ hydrolysis.
The rank order of affinity in competitive $[^{125}\text{I}]$NKA binding and potency for accumulation of $[^{3}\text{H}]$inositol monophosphates ($[^{3}\text{H}]$IP$_1$) in transfected B82 fibroblasts was NKA > substance P > senktide which classified the expressed protein as an NK-2 receptor. The expressed NK-2 receptors in B82 fibroblasts and the endogenous NK-2 receptors in bovine stomach had similar pharmacological properties. This implies that the in vitro expression system produced a protein whose pharmacological and biochemical characteristics resemble the endogenous receptor. The antagonists MEN 10207 and MEN 10208 blocked NKA-induced PIP$_2$ hydrolysis. In addition, the different pA$_2$ values in bioassays using transfected B82 cells, rabbit pulmonary artery, and hamster trachea of these peptides suggest receptor heterogeneity among the NK-2 receptors in these tissues.

The availability of a cell line expressing a homogeneous population of NK-2 receptors that mimics the pharmacological characteristics of the endogenous receptor allows closer examination of ligand-receptor-effector interactions in a controlled environment. The novel reporting of an NK-2 receptor subtype and desensitization may provide a mechanism for intervention of physiological responses mediated by this receptor subtype.
INTRODUCTION

The survival of an organism depends on its ability to detect, respond, and adapt to environmental changes. Multicellular organisms often utilize cell-to-cell communication to coordinate these biological responses. This form of communication relies on specialized molecules released into the extracellular medium by cells detecting these alterations. These chemical messengers then bind either to membrane or intracellular proteins or to DNA in responsive cells. Receptors are cellular macromolecules with the ability to discriminate among the multitude of molecules they encounter and bind their distinct chemical messenger. The receptor then transduces these external signals intracellularly by specific biochemical pathways which ultimately results in a precise physiological response.

The tachykinin peptides and their receptors comprise a cell-to-cell signaling system. These small, biologically active peptides have a phenylalanine-X-glycine-leucine-methionine carboxyl terminus where X is valine, phenylalanine, tyrosine, or isoleucine. Although named for an ability to contract intestinal tissues rapidly, the physiological actions of tachykinins include inflammation (Lotz et al., 1988), behavioral effects (Massi et al., 1987), and pain regulation (Hokfelt et al., 1975). Tachykinins found in mammalian species are called neurokinins, since they are synthesized, stored, and released by neurons. Members consist of neurokinin A (NKA), substance P (SP), neurokinin B (NKB), neuropeptide K (NPK), and neuropeptide gamma (NPγ) (Table 1). Only NKA, SP, and NKB have defined roles as neurotransmitters.
<table>
<thead>
<tr>
<th>Tachykinin Peptide</th>
<th>Amino Acid Sequence</th>
<th>Tissue Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mammalian</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substance P</td>
<td>RPKPQFFGLM</td>
<td>equine brain</td>
<td>von Euler and Gaddum, 1931</td>
</tr>
<tr>
<td>Neurokinin A</td>
<td>HKTDSFVGLM</td>
<td>bovine spinal cord</td>
<td>Kimura et al., 1983</td>
</tr>
<tr>
<td>Neurokinin B</td>
<td>DMHDFVGLM</td>
<td>porcine spinal cord</td>
<td>Kangawa et al., 1983</td>
</tr>
<tr>
<td>Neuropeptide K</td>
<td>DADSSIEKQVALLKALYGHGQISHKRMHKTSFVGLM</td>
<td>porcine brain</td>
<td>Tatemoto et al., 1985</td>
</tr>
<tr>
<td>Neuropeptide γ</td>
<td>DAGHGQISHKRMHKTSFVGLM</td>
<td>rabbit intestine</td>
<td>Kage et al., 1988</td>
</tr>
<tr>
<td><strong>Nonmammalian</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eledoisin</td>
<td>PSKDAFGLM</td>
<td>octopus salivary gland</td>
<td>Esrpamer and Anastasi, 1962</td>
</tr>
<tr>
<td>Physalaemin</td>
<td>ADPNKFYGLM</td>
<td>amphibian skin</td>
<td>Esrpamer et al., 1964</td>
</tr>
<tr>
<td>Kassinin</td>
<td>DVPKSDQFVGLM</td>
<td>amphibian skin</td>
<td>Anastasi et al., 1977</td>
</tr>
<tr>
<td>** Synthetic**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Senktide</td>
<td>DDFFmPLM</td>
<td></td>
<td>Laufer et al., 1986</td>
</tr>
<tr>
<td>MEN 10207</td>
<td>DYWVWWD</td>
<td></td>
<td>Maggi et al., 1990a</td>
</tr>
<tr>
<td>MEN 10208</td>
<td>TDYWVWWD</td>
<td></td>
<td>Maggi et al., 1990a</td>
</tr>
</tbody>
</table>

Table 1. Amino acid sequence of tachykinins. All tachykinins share a common C-terminus of FXGLM where X is either valine, isoleucine, phenylalanine, or tyrosine. The C-terminus accounts for most of the fundamental properties of tachykinins and must be amidated to be biologically active. The N-terminus displays considerable variability in amino acid composition and accounts for the differences in potency, efficacy, and duration of action. These peptides had multiple names which represented different nomenclature used by various authors: eledoisin was called moschatine; neurokinin A was either neurokinin α, neumodin L, or substance K; and neurokinin B was neurokinin β or neumodin K. The phenylalanine residue (P) in senktide is methylated (m) and tryptophan residues (W) in MEN 10207 and MEN 10208 are D-amino acids.
Physiological Effects of Neurokinins

Neurokinins evoke a common spectrum of biological activities consisting primarily of the contraction or relaxation of peripheral smooth muscles in the gastrointestinal, respiratory, urinary, or vascular systems. Other ancillary roles include sensory transmission in the nervous system, vascular permeability, inflammation, and secretion. While neurokinins produce similar physiological effects, their biological potencies can differ due to the number and type of neurokinin receptors on target cells. The neurokinin peptides are the most potent bronchoconstrictors known. NKA is the most powerful neurokinin bronchoconstrictor in guinea-pigs (Barnes, 1988) and in humans (Fuller et al., 1987a). This peptide also shows strong activity in increasing the micturition reflex and duodenum motility in rats (Maggi et al., 1987). Lesser roles in humans include contraction of the detrusor muscle of the urinary bladder (Dion et al., 1988), increased heart rate (Fuller et al., 1987a), and production of the wheal and flare reaction (Fuller et al., 1987b).

Substance P acts as a potent sialogen and hypotensive agent in rats (Holzer-Petsche et al., 1985). In humans, the main physiological roles for this peptide include increased heart rate, contraction of airway smooth muscle (Lundberg et al., 1983), secretion of airway mucus (Rogers et al., 1989), increased skin temperature (Fuller et al., 1987a) and induction of the wheal and flare reaction (Fuller et al., 1987b). Neurokinin B primarily increases the micturition reflex and plasma extravasation in rats (Coutre and Kerouac, 1987). Lesser roles include increasing salivation in rats (Maggi
et al., 1987) and participating in the wheal and flare reaction in humans (Fuller et al., 1987b).

Discovery of Tachykinin Peptides

Peptides constitute the newest class of molecules considered to be intercellular messengers. Of the more than twenty peptides currently identified as neurotransmitters, substance P has the longest history and is probably the best characterized. The potent activity of tachykinins on intestinal tissue ultimately led to their discovery. While mapping the tissue location of the newly characterized transmitter acetylcholine, von Euler and Gaddum (1931) extracted a white powder from alcoholic extracts of equine brain and intestine. This novel compound lowered blood pressure, stimulated the isolated rabbit duodenum preparation, and differed in chemical and biological characteristics from other known compounds. Unlike acetylcholine, it was not antagonized by atropine. Von Euler named this compound "substance P" for preparation. The small amount of substance P present in natural sources limited biochemical and pharmacological investigations for the next forty years. Consequently, most of the discoveries in the tachykinin field during this period dealt with isolation of novel peptides rather than physiological or biochemical discoveries.

Esrpamer (1949) isolated a compound from the posterior salivary glands of the Mediterranean octopod Eledone moschata which lowered blood pressure, stimulated isolated preparations of intestinal smooth muscle, and caused profuse salivary secretion. It was called eledoisin and showed biological properties similar to substance P. The
structure of eledoisin was elucidated and characterized as a peptide (Esrpamer and Anastasi, 1962). This was soon followed by the isolation of other biologically active peptides. Physalaemin (Esrpamer et al., 1964) and kassinin (Anastasi et al., 1977) were isolated from amphibian skin. They shared similar biological activities and amino acid structure. Currently twelve nonmammalian tachykinins and five mammalian neurokinins have been identified.

The idea that peptides transmit information between cells in the nervous system was a novel idea in the 1970’s. At that time only a few neuropeptides had been discovered. Substance P was the subject of many early attempts to define the role of these peptide transmitters. By 1983 many other tachykinins were isolated but only substance P was found in mammalian nervous system. Other members of this peptide family had been isolated from octopod salivary glands, Hydra neural net, Helix nervous system, and amphibian skin (Esrpamer, 1981). This soon changed.

Tachykinin research increased dramatically with the re-isolation of substance P in bovine hypothalamus (Leeman and Hammerschlag, 1967), the elucidation of its amino acid sequence (Chang et al., 1971), and its consequent commercial availability. In addition, two novel neurokinin peptides were almost simultaneously isolated by several independent groups. Kimura et al. (1983) isolated two neurokinins while searching for novel neuropeptides in porcine spinal cord. Of the four fractions separated by Sephadex G-15 chromatography exhibiting ileum-contracting activity, two fractions contained substance P and its oxidized derivative. The remaining two fractions contained the
novel peptides, "neurokinin α" and "neurokinin β". At the same time, Kangawa et al. (1983) isolated a unique mammalian tachykinin from porcine spinal cord by using bioassays for a tachykinin-like effect on the contractility of guinea-pig ileum. They called this peptide "neuromedin K" due to its remarkable structural homology to kassinin. Shortly afterward, Maggio et al. (1983) reported that extracts of bovine spinal cord contained abundant kassinin-like immunoreactivity. This was attributed to a novel peptide which they named "substance K" to indicate an immunochemical similarity to kassinin. During this same period, Nawa et al. (1983) studied the structure of the substance P gene. They sequenced a bovine striatum mRNA which not only encoded for substance P but also a new tachykinin they named substance K. This peptide had a sequence identical to neurokinin α and to the substance K previously described.

**Biosynthesis of Neurokinin Peptides**

Unlike classical transmitters which are products of enzymes in nerve terminals, neurokinin peptides are synthesized ribosomally as large protein precursors in the neuronal cell body. They are then transported via the axon to the nerve terminus where enzymatic processing converts them to active forms. Then, like the classical transmitters, these peptides are stored in vesicles and released from presynaptic membranes upon stimulation from chemical or electrical signals. The precursor molecules are derived from two distinct but similar genes: preprotachykinin gene A (PPT-A) and preprotachykinin gene B (PPT-B) (Nawa et al., 1983). These genes exhibit a marked structural similarity such that the 5'‑untranslated region, a signal
peptide, the neurokinin amino acid sequence, and the carboxyl-terminal sequence are specified by the corresponding exons between the two genes. This dramatic structural similarity may imply that the two genes evolved from a common ancestor.

Alternate splicing of the primary PPT-A mRNA transcript and different exon usage produces one of three mature mRNAs: alpha-preprotachykinin (α-PPT); beta-preprotachykinin (β-PPT); or gamma-preprotachykinin (γ-PPT) (Figure 1). While α-PPT contains one copy of SP, β-PPT and γ-PPT contain one copy of substance P and NKA (Nawa et al., 1983). More recently, N-terminally extended forms of NKA (neuropeptide K) (Tatemoto et al., 1985) and neuropeptide gamma (Kage et al., 1988) have been identified which appear to be products of β- and γ-PPT, respectively. Thus through posttranslational processing, multiple neurokinin peptides can be produced from the PPT-A protein. Transcription of PPT-B, unlike the PPT-A gene, yields one primary mRNA transcript which eventually produces NKB (Kotani et al., 1986).

Expression of the PPT-A gene is influenced by estradiol, testosterone, and thyroxine (Jonassen and Leeman, 1991). In addition, regulatory elements in the promoter or enhancer region of the PPT-I gene include sites conferring sensitivity to 3',5' cyclic adenosine monophosphate (cAMP), estrogen, phorbol esters, and nerve growth factor (Helke et al., 1990).

Location of Neurokinin Peptides

Once released by nervous or chemical stimulation, neurokinins are susceptible to endogenous peptidases. Thus, target cells are usually across a synaptic cleft from
Figure 1. Biosynthesis of neurokinin peptides. The preprotachykinin A and B genes are translated, differentially spliced and transcribed into the proteins, α-, β-, γ-PPT-A or PPT-B, respectively. These peptides are then enzymatically cleaved and amidated to yield the final biologically active peptide and, upon release, bind a neurokinin receptor.
neurons releasing these neurotransmitters. The peptides in the neurokinin family are generally located in the central and peripheral neuronal structures and the epithelial cells of the gastrointestinal tract. The mammalian central nervous system contains all three major neurokinins with the highest concentrations in the substantia nigra and the dorsal horns of the spinal cord. Substance P and NKA share a similar distribution pattern in the rat central nervous system, although the concentration on substance P is often four-fold higher than NKA. Levels of neurokinin B in the cerebral cortex is generally higher than substance P or NKA (Munekata, 1991). In peripheral tissues, high concentrations of NKA are found in the airways and lungs of the respiratory system (Martling et al., 1987), autonomic nerves of the intestine (Wattchow et al., 1988), and urinary bladder (Sundler et al., 1985). Since respiratory and gastrointestinal tissues are derived from the same embryological tissue, it is not surprising to find NKA in both areas. In regards to specific neurons, substance P is primarily found in small, unmyelinated fibers of sensory nerves in the central nervous system and periphery. Both substance P and NKA are located in peripheral endings of capsaicin-sensitive primary afferent neurons (Regoli, 1985) and neuronal cell bodies of the enteric nervous system (Snider et al., 1991). While the dorsal horn of the spinal cord contains the highest concentration of neurokinin B (Arai and Emson, 1986), this peptide is also found in the hypothalamus, intestinal tract, and gall bladder.

One reason for the differential expression of the neurokinin peptides is that mRNAs coding for the two preprotachykinins are produced from a single gene whose
expression is regulated differentially in a tissue specific-manner (Nawa et al., 1984a). Thus, α-PPT forming substance P and PPT-B producing NKB are preferentially synthesized in the brain, whereas β-PPT and γ-PPT containing substance P and NKA are predominantly produced in sensory or autonomic neurons (Nawa et al., 1984b; Carter and Krause, 1990). The biosynthetic scheme for neurokinins provides for the co-synthesis of substance P and NKA and the development of regional cell populations of substance P and NKB.

**Agonist and Antagonist Development**

Because of their potency to constrict bronchial smooth muscles and to increase bronchial vascular permeability, considerable effort was expended on a search for neurokinin agonists and antagonists. This task was difficult because the endogenous neurokinin peptides were nonselective and biodegraded by peptidases. While a neurotransmitter may act on several types of receptors, the neurokinins introduced another level of complexity. These biologically active peptides not only bind to different neurokinin receptors but also may produce similar effects in complex tissues or whole animals. Consequently, selective receptor ligands were highly desirable. Unlike the muscarinic and nicotinic receptors, there were no natural compounds from plants or animals that stimulated or antagonized the effects of neurokinin peptides or their receptors. The search for biologically active agents was then focused on finding synthetic analogues of neurokinin peptides.

Developing selective neuropeptide agonists and antagonists is difficult because
they are small linear molecules with considerable conformational freedom. One rationale for synthesizing agonists that discriminate between the neurokinin receptors was based on the assumption that naturally occurring transmitters exist in several different and interchangeable conformations and that restricted analogues can be selective towards specific receptor subtypes (Hruby, 1985). While this strategy produced inactive or weakly active substance P molecules (Wormser et al., 1986; Rovero et al., 1989), cyclic dimerization and introduction of a lactam ring in substance P analogues produced several NKA antagonists: L-659,837 (cyclo[Gln, Trp, Phe(R), Gly(ANC), Leu, Met]) and L-659,877 (cyclo(Gln, Trp, Phe, Gly, Leu, Met)) (Williams et al., 1988). These synthetic peptides had limited use because of their poor receptor selectivity, this probably arising from being modifications of substance P.

To overcome lack of activity, low selectivity, and metabolism of cyclic peptides, Wormser et al. (1986) limited conformational mobility by the N-alkylation of a single peptide bond and increased metabolic stability by methylation. Several biologically active compounds were developed: septide ([pGlu⁶, Pro⁹]SP₆₋₁₁), a selective substance P peptide for NK-1 receptors (Wormser et al., 1986), and senktide (succinyl-[Asp⁶, Me-Phe⁸]SP₆₋₁₁), a selective neurokinin B peptide for NK-3 receptors (Laufer et al., 1986).

Another strategy used structure-activity data from other peptides to modify the neurokinin peptide sequence and produce more potent and selective agonists. Structure-activity studies generally determine the minimal chain length that produces biological activity and identify the contribution of each amino acid residue in the expression of
biological activity. For the neurokinin peptides, shorter fragments than seven amino acids results in inactive or low potency molecules (Rovero et al., 1989). Some of the agonists developed for the NK-2 receptor with this strategy were NKA_{4-10} (Regoli et al., 1989) and [β-Ala^3]NKA_{4-10} (Maggi et al., 1990b).

Structure-activity assays also showed that introducing D-amino acids into analogues of substance P produced compounds that blocked certain biological effects of the tachykinins (Folkers et al., 1982). This led to the development of several antagonists. A substance P antagonist peptide, spatide ([D-Arg^1, D-Tyr^7,8, Leu^{11}]-substance P, was developed in the early 1980’s and served as the first generation prototype of neurokinin antagonists (Folkers et al., 1984). While it did produce antagonism, problems such as neurotoxicity, low potency, and selectivity limited its use. Introduction of two D-Trp residues in positions six and eight along with pyroglutamic acid in position four or N-leucine in position ten of the NKA molecule, [Nle^{10}]NKA_{4-10} (Drapeau et al., 1987), gave rise to weak but partially selective NK-2 antagonists (Rovero et al., 1990). While replacing selected amino acids in neurokinin peptides by D-amino acids produced antagonists, these altered molecules have limited use because of neurotoxicity, low potency, and selectivity (Maggi, in press). Like other biologically active peptides, enzymatic degradation often decreases the potency of neurokinins. Acetylation of the N-terminal amide of NKA_{4-10} provided protection from aminopeptidases (Rovero et al., 1989).
Neurokinin Receptors

Historically, receptors are classified and named after their most potent agonist. Nomenclature becomes complicated when compounds with greater potency are subsequently discovered. With the isolation of several novel tachykinin peptides, different agonist rank orders of potency emerged. The existence of multiple tachykinin receptors was suggested to explain the conflicting data (Esrpamer and Falconieri-Esrpamer, 1962; Iversen et al., 1981; Lee et al., 1982; Watson et al., 1983; Buck et al., 1984; Nawa et al., 1984b; Laufer et al., 1985). The hypothesis shared by these authors was based upon the analogy of multiple peptides and multiple receptors in the opioid receptor family and the different rank orders of pharmacological potency or radioligand binding specificities in different tissue preparations for natural tachykinins or their fragments.

Esrpamer and Falconieri-Esrpamer (1962) first suggested multiple neurokinin receptor subtypes. Later Iversen et al. (1981), Lee et al. (1982), and Watson et al. (1983) provided more evidence. They classified tachykinin receptors on the agonist rank order of potency in several in vitro bioassays. Two different pharmacological profiles were observed. The rank order of potency of physalaemin $\geq$ substance P $\geq$ eledoisin $\geq$ kassinin in guinea-pig ileum, rat bladder, and guinea-pig vas deferens indicated that the receptors were of the SP-P type. The rank order of eledoisin = kassinin $>$ physalaemin and substance P in hamster and mouse bladder, rat duodenum, and rat vas deferens indicated SP-E receptors.
By 1984, three neurokinins were identified: substance P, NKA, and NKB, but only two receptors SP-P and SP-E were recognized. Using labeled tachykinins with radioiodinated Bolton-Hunter reagent, Buck et al. (1984) determined the binding characteristics of these peptides in crude membrane suspensions of rat cortex, rat duodenum, mouse bladder, and guinea-pig intestine. In cerebral cortex, labeled eledoisin had high-affinity binding that was inhibited by tachykinins with a rank order of potency indicating an SP-E site. In gastrointestinal smooth muscle and bladder, binding of labeled substance-P was blocked in a pattern which indicated a SP-P site. Whereas in intestinal smooth muscle and bladder, labeled substance K and eledoisin both showed a preference for substance K. These results suggested existence of three distinct types of neurokinin receptors: SP-P, SP-E, and SP-K. Further evidence for three neurokinin receptors were provided by Laufer et al. (1985). Experiments measuring the contraction of guinea-pig ileum from a selective NK-1 agonist, succinly-[Asp²,Me-Phe⁸]-substance P₆₋₁₁, showed that this compound was potent in guinea-pig ileum through the SP-N receptor and inactive in SP-P and SP-E receptor preparations.

With the discovery of these two novel peptides, neurokinin receptor pharmacology became increasingly complex. Researchers that created their own nomenclature added to this confusion. To remedy this situation, the XXX International Congress of Physiology Satellite Symposium on Substance P and Neurokinins held in Montreal on July 23, 1986, decided that the binding sites preferring substance P were to be referred to as NK-1 sites, the sites preferring NKA as NK-2 sites, and the sites
preferring NKB as NK-3 sites (Henry et al., 1987). They also designated neurokinin A as the new name to replace the names neurokinin α, neuromedin L, and substance K; and neurokinin B as the new name for both neurokinin β and neuromedin K.

To complicate matters, McKnight et al. (1987) postulated a fourth neurokinin receptor based on a novel potency order of tachykinins on the contraction of guinea-pig isolated trachea. Ireland et al. (1988) disputed this finding and suggested that the anomalous rank order of potencies was due to substantial peptidase activity in the preparation. In response, McKnight et al. (1988) included several peptidase inhibitors in bioassays using selective neurokinin agonists and antagonists and confirmed his previous findings.

In summary, there is considerable pharmacological evidence for three distinct neurokinin receptors, NK-1, NK-2, and NK-3. These receptors have been identified by several researchers who used the rank order of potency of various tissues as the basis of this classifications. There is considerable controversy for the existence of the NK-4 receptor because several investigators failed to reproduce the findings of McKnight et al. (1987). Pharmacological analysis of neurokinin receptor subtypes is difficult because of the inherent problems with these peptides or tissue preparations. Isolation and cloning of distinct genes for each putative neurokinin receptor subtype could verify the pharmacological evidence for distinct neurokinin receptors.

**Cloning of Neurokinin Receptors**

A fundamental step in cloning a receptor is to obtain sufficient purified receptor
protein to determine its amino acid sequence. This is difficult because receptor proteins usually constitute less than 0.1% of the total cell membrane protein mass and they are tightly embedded, integral parts of the membrane lipid bilayer. Despite these setbacks, the genes for the opsin, nicotinic, beta-adrenergic, and muscarinic receptors have been cloned.

The cloning of the NK-2 receptor began with the expression of the exogenous mRNAs of two different neurokinin receptors in Xenopus oocytes (Harada et al., 1987). Responses to different tachykinins were measured by electrophysiological techniques that detected inward ion current in oocytes injected with mRNAs extracted from rat brain or bovine stomach. On the basis of agonist potency, rat brain mRNA produced the NK-1 receptor and bovine stomach mRNA produced the NK-2 receptor. This strategy of in vitro expression was used to clone the NK-2 receptor (Masu et al., 1987). A cDNA library from bovine stomach poly(A) RNA was constructed with a cloning vector that allowed in vitro transcription of mRNAs. These mRNAs were systematically screened by injection into Xenopus oocytes and tested for an electrophysiological response after NKA application. This was the first time that the structural characteristics of a neuropeptide receptor was defined. The hydropathicity profile of the deduced amino acid sequence revealed seven putative alpha-helical transmembrane domains. With this information, the NK-2 receptor was placed in the guanine-nucleotide-binding regulatory protein (G protein) receptor family along with rhodopsin, adrenergic, and muscarinic receptors. More importantly, this information provided the means to produce specific
tools to investigate the regulation, function, and molecular anatomy of the NK-2 receptor with respect to ligand binding and signal transduction. In addition, the nucleic acid sequence of the NK-2 receptor was used to develop DNA probes which led to the isolation of other receptors.

There was speculation whether the NK-2 cDNA isolated by Masu et al. (1987) actually encoded for the NK-2 receptor (Krause et al., 1989). Criticism was based upon the classification of the receptor by the rank order of potency of three endogenous and somewhat nonselective tachykinins. More definitive characterization would determine whether this clone represents the NK-2 receptor or another neurokinin subtype. Unfortunately, selective ligands were not available at that time.

Soon afterward, the NK-1 receptor was cloned from rat brain cDNA (Yokota, et al., 1989; Hershey and Krause, 1990) and was followed by the cloning of an NK-2 receptor from the rat stomach (Sasai and Nakanishi, 1989). In addition, the genomic sequence of the human NK-2 receptor was determined by generating a cDNA fragment of the human NK-2 receptor. Using RNA from human tracheal tissue and oligonucleotide primers derived from bovine NK-2 receptor cDNA sequences from reverse transcriptase-polymerase chain reactions, an NK-2 receptor gene was isolated (Gerard et al., 1990). This gene had remarkable homology to the bovine stomach NK-2 gene and contained five exons and four introns. This was unusual because, of the more than thirty G protein coupled receptors, only bovine rhodopsin (Nathans and Hogness, 1983), D₂ dopamine receptor (O'Malley et al., 1990), and substance P (Hershey and
Krause, 1990) contained introns. A human NK-2 receptor was cloned from a jejunal cDNA by Kris et al. (1991). The human jejunal and bovine stomach NK-2 receptors have a 90% amino acid homology. Finally the NK-3 receptor was isolated from a rat brain cDNA library by cross-hybridization with brain cDNA libraries (Shigemoto et al., 1990).

The neurokinin receptors belong to the G protein-coupled receptor family. Members of this group have considerable sequence similarity with one another and all share a topological structure consisting of seven hydrophobic domains that span the lipid bilayer (Figure 2). Each domain contains twenty to twenty five uncharged amino acids in an alpha helix structure. These receptors possess potential N-glycosylation sites at the extracellular amino terminus and many serine and threonine residues as possible phosphorylation sites at their intracellular carboxyl terminus (Table 2).

Among the neurokinin receptors, there is a high degree of similarity in amino acid residues found in transmembrane domains (54-66%) and intracellular loops (Sasai and Nakanishi, 1989). There are three conserved cysteine residues; two in the first and second extracellular loops and may form disulfide bonds, and the other conserved cysteine residue in the intracellular tail may be palmitolyated to anchor the receptor to the plasma membrane. Histidine residues in the fifth and sixth transmembrane domains are characteristic of the neurokinin receptors. The receptors are G protein linked and coupled to phospholipase C which generates the second messengers inositol 1,4,5-triphosphate and diacylglycerol (Figure 3).
Figure 2 Putative model of the NK-2 receptor. The hydropathy profile of the NK-2 receptor amino acid sequence shows seven transmembrane domains containing twenty one to twenty four amino acids in an alpha helix. The extracellular amino terminus contains two glycosylation sites and the carboxyl intracellular tail has seventeen phosphorylation sites.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NK-1</th>
<th>NK-2</th>
<th>NK-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model tissue</td>
<td>dog carotid artery</td>
<td>rabbit pulmonary artery</td>
<td>rat portal vein</td>
</tr>
<tr>
<td>Potency of agonists</td>
<td>SP &gt; NKA &gt; NKB</td>
<td>NKA &gt; NKB &gt; SP</td>
<td>NKB &gt; NKA &gt; SP</td>
</tr>
<tr>
<td>Selective agonist</td>
<td>[Sar⁹, Met(O₂)¹¹]SP</td>
<td>[Nle¹⁰]NKA₄₁₀</td>
<td>succinyl-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[Asp⁶,MePhe⁸]SP₆₁₁</td>
</tr>
<tr>
<td>Selective antagonist</td>
<td>[D-Pro⁴,D-Trp⁷⁹¹⁰, Phe¹¹]SP₄₁₁</td>
<td>[Tyr⁵, D-Trp⁶⁸⁹, Arg¹⁰]⁻¹⁰</td>
<td>[D-Pro²,D-Trp⁶⁸,Nle¹⁰]NKB</td>
</tr>
<tr>
<td>Second messengers</td>
<td>IP₃/Ca²⁺</td>
<td>IP₃/Ca²⁺</td>
<td>IP₃/Ca²⁺</td>
</tr>
<tr>
<td>Amino acids</td>
<td>407 (rat brain)</td>
<td>384 (bovine stomach)</td>
<td>452 (rat brain)</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>46,364</td>
<td>43,851</td>
<td>51,104</td>
</tr>
<tr>
<td>N-glycosylation sites</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Phosphorylation sites</td>
<td>31</td>
<td>17</td>
<td>30</td>
</tr>
<tr>
<td>Relative desensitization</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Location in the nervous system</td>
<td>hypothalamus, olfactory bulb,</td>
<td>deeper cortical layers of</td>
<td>cerebral cortex, olfactory</td>
</tr>
<tr>
<td></td>
<td>spinal cord, striatum</td>
<td>neonatal guinea-pig</td>
<td>bulb, hypothalamus, hippocampus</td>
</tr>
<tr>
<td>Location in the peripheral tissues</td>
<td>urinary bladder, salivary glands,</td>
<td>urinary bladder, large</td>
<td>ileum, vascular smooth</td>
</tr>
<tr>
<td></td>
<td>small and large intestines</td>
<td>intestines, stomach, adrenal</td>
<td>muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gland, lungs</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Properties and features of neurokinin receptors.
Figure 3. Coupling of PIP$_2$ hydrolysis and calcium mobilization to the NK-2 receptor. NKA binds to a site in the pore of the NK-2 receptor (NK-2R). This activates the NK-2 receptor and cleaves $G_{p_{\alpha}G_{\gamma}}$ into $G_{p_{\alpha}}$ and $G_{\gamma}$ subunits. This stimulates phospholipase C (PLC) to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ then binds to the IP$_3$ receptor on calcium storing vesicles to release intracellular calcium. DAG activates protein kinase C which then phosphorylates cell proteins. A stimulatory effect is indicated by the plus sign.
Guanine-binding proteins (G proteins) regulate many effector pathways including the adenylyl cyclase or phospholipase C enzymes and the K\(^+\) and Ca\(^{2+}\) ion channels. The first conclusive evidence for neurokinin receptors coupling to G proteins came from the observation that G proteins inhibited the binding of tachykinin agonists to their receptors (Cascieri and Liang, 1983). Further experimental evidence of the coupling of PIP\(_2\) hydrolysis to the NK-1 receptor was observed in rat parotid gland (Hanley et al., 1980), to NK-2 receptors in rat ileum longitudinal smooth muscle (Watson and Iversen, 1984), and to NK-3 receptors in guinea-pig myenteric plexus (Guard et al., 1990).

Although several other receptors are coupled to adenylyl cyclase and arachidonic acid release, there is no conclusive evidence that the neurokinin receptors are coupled to these second messengers. However, a single report exists of neurokinin-induced inhibition of adenylyl cyclase in rat submandibular gland (Laniyonu et al., 1988).

**Location of Neurokinin Receptors**

The distribution of receptors is an important factor for understanding peptide function, since the sensitivity of target cells to an intracellular messenger is related to receptor distribution and concentration. Unfortunately, analysis of the tissue distribution of neurokinin receptors has been hindered by the lack of highly specific and selective antibodies, antagonists, and radioligands. Therefore, past studies lacked the rigorous specificity needed to distinguish between various endogenous neurokinin receptors. In addition, endogenous neurokinin peptides cross-reacted with other neurokinin receptors. Also tissue preparations used in ligand-binding and pharmacological studies often
contained several neurokinin receptors. Immunological probes used to label neurokinin receptors were generally nonspecific because of the common tachykinin C-terminus. The cloning of these receptors allowed the construction of specific cDNA probes. Use of these probes has recently provided definitive localization of neurokinin receptors. In general, peripheral tissues contain all three receptor types, while the brain contains only NK-1 and NK-3 receptors (Okubo and Nakanishi, 1990). There is controversy on whether NK-2 receptors are present in the brain (reviewed by Guard and Watson, 1991).

The NK-1 receptor mRNA is widely distributed in the central nervous system with the highest concentrations in the hypothalamus, olfactory bulb, spinal cord, and striatum. In the peripheral tissues, NK-1 receptor mRNA is found in the urinary bladder, salivary glands, and small and large intestines. The NK-2 receptor mRNA is predominant in peripheral tissues such as the urinary bladder, stomach, large intestine, and adrenal gland (Tsuchida et al. 1990). Presence of NK-2 receptors in the central nervous system remains controversial and has only been reported in the deeper cortical layers of the neonatal guinea-pig brain (Quirion and Dam, 1985). The NK-3 receptor mRNA is found in the cerebral cortex, olfactory bulb, hypothalamus, and hippocampus and in low density in peripheral tissues such as the ileum and smooth muscle of the portal vein.

As for specific cells, the NK-1 receptor is primarily found on postganglionic cholinergic neurons and smooth muscle cells, the NK-2 receptor on smooth muscle cells, and the NK-3 receptor on postganglionic cholinergic neurons (Bartho and Holzen, 1985;
Laufer et al., 1985; Kilbinger et al., 1986; Regoli et al., 1987a).

In several situations, there is no apparent correlation between the distribution of neurokinin peptides and their receptors. Substance P is found in high levels in the substantia nigra, yet NK-1 receptors are absent (Buck et al., 1986b). Neurokinin A is readily detected in the brain but the presence of NK-2 receptors in this tissue remains to be confirmed. The endogenous ligand-receptor mismatch may arise from cross-talk between neurokinin peptides and neurokinin receptors.

Hypothesis

Currently there is no cell line that expresses a high density of NK-2 receptors. Such a cell line would enable ready access to a homogenous population of cells expressing a high density of a single type of neurokinin receptor. In addition the environment of the cell can be precisely controlled to simulate physiological or other conditions. Construction of this cell line would reduce some of the limitations associated with neurokinin peptides and their receptor preparations. These include low receptor density and the presence of different tachykinin receptor subtypes.

Stable expression of functional receptor genes has been accomplished by several investigators (Lefkowitz et al., 1984; Chung et al., 1988; Lai et al. 1988). Biochemical and pharmacological properties of these de novo proteins reflected their respective endogenous receptor.

The hypothesis set forth by this dissertation is that the bovine stomach cDNA SKR56S encodes for a functional NK-2 receptor. The specific aims include stable
expression of the bovine stomach NK-2 receptor in B82 fibroblasts, comparison of pharmacological properties of the *de novo* receptor to the endogenous NK-2 receptors, demonstration of biochemical coupling to second messengers, classification of receptor type with agonists and antagonists, and demonstration of receptor regulation via the reduction of a biochemically coupled response.
EXPRESSION OF THE BOVINE STOMACH cDNA SKR56S IN B82 FIBROBLASTS

There are several important limitations associated with the use of endogenous tissue preparations to characterize neurokinin receptors and their ligands. These include the presence of different neurokinin receptors in endogenous tissues (Burcher and Buck, 1986; Regoli et al., 1987b), the lack of specific agonists and antagonists (Quirion and Dam, 1988; Buck and Shatzer, 1988), and the ability of agonists to induce similar biological responses (Guard and Watson, 1991). Currently, no cell line expressing a high density of functional NK-2 receptors has been identified although several tissues have been characterized as having only one population of neurokinin receptors. For example, the dog carotid artery has been identified as containing NK-2 receptors and has been useful for studying the relaxation of blood vessels (Regoli et al., 1987a). Other uses are limited because of the low NK-2 receptor density and the absence of PIP₂ hydrolysis after NKA stimulation (Moskowitz et al., 1987).

The purpose of this study was to establish a cell population expressing functional NK-2 receptors that will enable examination of ligand specificity, second messenger coupling, and receptor regulation. To circumvent low receptor density and the presence of multiple neurokinin receptor subtypes, a gene encoding for the NK-2 receptor was transfected into an established B82 cell line to produce a homogeneous population of cells which express a high density of the de novo receptor. Stable expression of these
biologically active proteins by host cells initially devoid of these receptors overcame some of the limitations associated with tissue preparations. This cell line may enable closer and more specific examination of ligand-receptor-effector interactions.

**Materials**

The bovine stomach cDNA, pSKR56S, was a gift from Dr. Shigetada Nakanishi, Institute of Immunology, Kyoto University Faculty of Medicine, Kyoto, Japan. The expression vector, pHβAPr-1-neo (Gunning *et al.*, 1987) and host cell, B82 murine fibroblasts of the L cell line, were gifts from Dr. Josephine Lai. The restriction enzyme HindIII and SalI were purchased from Promega, Madison, WI; XbaI from GIBCO-BRL, Grand Island, NY; BamHI from Boehringer Mannheim, West Germany; pGEM-3 vector from Promega, Madison, WI; Dulbecco’s Modified Eagle Medium (DMEM), Ham’s F-12 Medium, Iscove’s Modified Dulbecco’s Medium (IMDM), fetal calf serum, and geneticin® (Antibiotic G418) from GIBCO-BRL, Grand Island, NY; newborn calf serum from Irvine Scientific, Santa Anna, CA; the tissue culture supplies (cell scrapers, roller bottles, flasks, and plates) from Costar, Cambridge, MA; (2-[¹²⁵I]Iiodohistidyl)-NKA (specific activity 2000 Ci/mmol) from Amersham Corporation, Arlington Heights, IL, and GF/B filters from Whatman, Maidstone, England. Neurokinin A was purchased from Peninsula Laboratories, Belmont, CA. Stock solutions were diluted in 0.01 M acetic acid with 1% mercaptoethanol, aliquoted, and frozen at -70°C until used. All other compounds were purchased from Sigma Chemical Co., St. Louis, MO.
Methods

Expression of the Bovine Stomach cDNA SKR56S in B82 Fibroblasts

The bovine stomach cDNA, SKR56S, was excised with the restriction enzymes XbaI and BamHI and ligated into the corresponding restriction sites in the pGEM-3 vector. The open reading frame was then cleaved with HindIII and BamHI and inserted into the eukaryotic plasmid expression vector, pHβAPr-1-neo (Figure 4). B82 fibroblasts were transfected with 20 μg of this recombinant DNA by the calcium phosphate precipitation method (Maniatis et al., 1982). At each subcloning step, DNA hybridization assays and restriction enzyme analysis confirmed the incorporation and orientation of the SKR56S open reading frame into the recipient vector.

The pHβAPr-1-neo vector was used because it contains a constitutive eukaryotic promoter capable of directing high-level expression, several unique restriction enzyme sites, and the ability to confer a rapid screening marker, neomycin resistance, through induction of aminoglycoside phosphotransferase. The B82 cell line was chosen for several reasons. First, it lacks neurokinin receptors, since [3H]IP1 accumulation remained at basal levels despite stimulation with substance P, NKA, or senktide. B82 cells have favorable culturing characteristics such as a short doubling time (16 hours), viability under suboptimal conditions, ease of harvesting, and minimal growth requirements. In addition, previous reports demonstrated the functional coupling of transfected receptors to preexisting G proteins that mediate phospholipase C (Lai et al., 1988) or adenylyl cyclase in the B82 fibroblasts (Chung et al., 1988; Lai et al., 1991).
Figure 4. Map of the expression vector, pHβAPr-1-neo-SKR56S, transfected into B82 fibroblasts. The bovine stomach cDNA SKR56S was inserted between the HindIII and BamHI restriction sites in the polylinker region which lies downstream from the human β-actin promoter. This plasmid consists of a 1.2 kB bovine Substance K receptor (SKR) open reading frame (o.r.f.), a 5' untranslated region of the β-actin gene (5' UT), an intervening sequence (IVS 1), a simian virus 40 polyadenylation region (SV40), an ampicillin resistance gene (Amp'), an origin of replication (Ori), and a neomycin resistance gene (Neo').
Transfected cells were cultured at 37°C in a humidified atmosphere of 95% air, 5% CO₂ in 45% (v/v) Dulbecco's Modified Eagle Medium, 45% (v/v) Ham's F-12 Medium, and supplemented with 5% (v/v) fetal calf serum, 5% (v/v) newborn calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. The addition of 500 μg/ml geneticin to this media selected for cells resistant to neomycin due to the expression of neomycin resistance by the incorporation of the pHβAPr-1-neo-SKR56S construct. Resistant cells formed clonal colonies which eventually produced individual cell lines. Viable clones were maintained under these conditions for three to six passages before propagation in media in the absence of geneticin. Cells were then maintained in DMEM/Ham's F-12 medium at 37°C in a humidified atmosphere of 95% air, 5% CO₂.

**NK-2 Receptor Density in Transfected Cells**

Viable clones were grown in DMEM/Ham's F-12 media in roller bottles until confluent (approximately 10⁸ cells per bottle). Cells were removed with cell scrapers, washed twice in 10 ml of IMDM, and centrifuged at 1500 × g for 10 minutes. The supernate was discarded and the pellet weighed and frozen at -70°C until used.

Membrane pellets were homogenized with a Polytron (setting #6 for 15 to 30 seconds) in 15 volumes of cold (4°C) 50 mM Tris HCl buffer (pH 7.4, 4°C) containing 120 mM NaCl and 5 mM KCl. The homogenate was centrifuged at 48,000 × g for 10 minutes at 4°C, and the pellet resuspended, homogenized (as above) in 15 volumes of 50 mM Tris HCl buffer (pH 7.4, 4°C) containing 10 mM EDTA and 300 mM KCl, and allowed to set on ice for 30 minutes. After centrifuging at 48,000 × g for 10 minutes,
the pellet was washed twice by resuspension and centrifugation in 50 mM Tris HCl buffer (pH 7.4, 4°C). The final pellet was diluted to a concentration of 20 mg/ml in incubation buffer (50 mM Tris HCl, pH 7.4, room temperature, containing 0.1% bovine serum albumin, 2 mM MnCl₂, 40 μg/ml bacitracin, 4 μg/ml leupeptin, 4 μg/ml chymostatin, and 1 μM thiorphan) and incubated at room temperature for 15 minutes.

The radioligand [¹²⁵I]NKA was diluted into several concentrations (0.05 to 1.3 nM) in incubation buffer and added to appropriate tubes. The assay was started by the addition of 250 μl of well-mixed tissue suspension and incubation at room temperature for 120 minutes. The assay was terminated by rapid addition of 3.5 ml of 50 mM Tris HCl buffer (pH 7.4, 4°C) and filtered under vacuum through GF/B filters which had been soaked overnight in 0.5% bovine serum albumin. The filters were rapidly washed with two 3.5 ml portions of the same buffer and transferred to 12 mm × 75 mm polystyrene culture tubes for direct counting in a gamma spectrometer (Packard Auto-Gamma 5650, Packard Instrument Co., Downers Grove, IL). Nonspecific binding was determined in the presence of 1 μM unlabeled NKA. Under these conditions, specific binding ranged from 90 to 95% of total binding and less than 10% of total radioactivity.

**Decreased Expression of the NK-2 Receptor**

SKLKB82/3 cells with 30 and 106 passages were plated at 100,000 cells per well and incubated in DMEM/Ham's F-12 medium in presence or absence of 500 μg/ml G418. After 24 hours, cell counts and viability were determined by the trypan blue exclusion method.
Data Analysis

Except where noted, all data represents the arithmetic mean ± standard error of mean (SEM) of at least three independent experiments with duplicate determinations. Receptor concentrations ($B_{max}$) and dissociation constants ($K_D$) were determined by nonlinear least-squares regression.

Results

Incorporation of SKR56S into the Expression Vector

The ligation of the SKR56S into the pHβAPr-1-neo vector was successful. DNA hybridization autoradiography of $^{35}$P-labeled SKR56S probes and pGEM-3-SKR56SXbaI/BamHI (Figure 5) or pHβAPr-1-neo-SKR56SXbaI/BamHI fragments (Figure 6) indicated that these plasmids contained the SKR56S open reading frame. The dark areas, marked 5 and 6 in figure 5, and 1, 2, 4, 5, and 6 in figure 6, indicate hybridization of these fragments and are similar in density to the positive control. The negative control provided the background level and was comparable to areas blotted with samples 3 and 7 in figure 6.

The restriction enzyme analysis of pHβAPr-1-neo-SKR56S verified the size and the 5'→3' orientation of the SKR56S gene to the β-actin promoter (Figure 7). The excised fragments are approximately 1.6 kilo bases which corresponds to the size of the SKR56S gene. If the orientation of this gene was reversed, the molecular weight of these fragments would be considerably less.
Figure 5. Autoradiographic visualization of the hybridization of SKR56S and pGEM-3-SKR56S constructs. pSKR56S was cut with XbaI and BamHI restriction enzymes and ligated into the corresponding restriction enzyme sites in pGEM-3. This construct was amplified in competent *E. coli* and processed as plasmid preparations. A [\(^{35}\)P]SKR56S probe was prepared by cleaving the original SKR56S cDNA with XbaI and BamHI enzymes and \(^{35}\)P-end labeling. DNA from the plasma preparations was cut with XbaI and BamHI, blotted on filter paper, and hybridized with the [\(^{35}\)P]SKR56S XbaI/BamHI probe. Autoradiography film was exposed for one hour at -70°C. Dark areas indicate hybridization of the [\(^{35}\)P]SKR56S XbaI/BamHI probe and the pGEM-3-SKR56S XbaI/BamHI fragment. The positive control (+) represents the hybridization of labeled and unlabeled SKR56S XbaI/BamHI fragments while the negative control (-) represents absence of [\(^{35}\)P]SKR56S XbaI/BamHI and pGEM-3 XbaI/BamHI hybridization.
Figure 6. Autoradiographic visualization of the hybridization of SKR56S and pHβAPr-1-neo-SKR56S constructs. The pGEM-3-SKR56S construct was cut with HindIII and BamHI restriction enzymes and ligated into the corresponding restriction enzyme sites in the pHβAPr-1-neo vector. This construct was amplified in competent *E. coli* and processed as plasmid preparations. A [*³²P*]SKR56S probe was prepared by cleaving the original SKR56S cDNA with XbaI and BamHI enzymes and *³²P*-end labeling. DNA from the plasma preparations containing the pHβAPr-1-neo-SKR56S construct was cut with XbaI and BamHI, blotted on filter paper, and hybridized with the [*³²P*]SKR56S XbaI/BamHI probe. Autoradiography film was exposed for one hour at -70°C. Dark areas indicate hybridization of the [*³²P*]SKR56S XbaI/BamHI probe and the pHβAPr-1-neo-SKR56S XbaI/BamHI fragment. The positive control (+) represents the hybridization of labeled and unlabeled SKR56S XbaI/BamHI fragments while the negative control (-) represents absence of [*³²P*]SKR56S XbaI/BamHI and pHβAPr-1-neo XbaI/BamHI hybridization.
Figure 7. Restriction enzyme analysis of pGEM-3-SKR56S and pHβAPr-1-neo-SKR56S constructs. Lanes 1 and 12 contain a molecular weight ladder with weight in kilobases indicated on the right. Lanes 2 to 6 contain DNA from pGEM-3-SKR56S plasmid preparations cut HindIII/BamHI restriction enzymes. Lanes 7 to 11 contain DNA from pHβAPr-1-neo-SKR56S plasmid preparations cut with SalI/BamHI restriction enzymes.
NK-2 Receptor Densities in the Transfected B82 Fibroblasts

Transfection of B82 fibroblasts with the pHβAPr-1-neo-SKR56S construct produced twelve neomycin-resistant clones. The NK-2 receptor density of each clone was measured by [125I]NKA saturation binding assays and ranged between 4 to 147 fmol/mg protein. These findings, provided by Stephen Buck, allowed us to select the clone, SKLKB82#3, which expressed the highest density of NK-2 receptors, 147 fmol/mg protein. This cell line was chosen for further characterization. The control cell lines, B82 (parent cells), LK4V (B82 cells transfected with pHβAPr-1-neo), and LK3-3 (B82 cells transfected with pHβAPr-1-neo-m) did not show significant NK-2 receptor density or specific [125I]NKA binding (Table 3).

The binding of [125I]NKA to SKLKB82#3 membranes was saturable. Nonspecific binding increased linearly with increasing concentrations of ligand (data not shown). Rosenthal analysis of the data points from [125I]NKA binding to SKLKB82#3 membranes produced a linear plot ($r = 0.99$) with a Hill coefficient of 1.00. A $K_d$ and $B_{max}$ value of $0.59 \pm 0.10$ and $147 \pm 14$ fmol/mg protein, respectively, was calculated (Van Giersbergen et al., 1991).

Decreased Expression of the NK-2 Receptor

After a 24 hour incubation in the presence of 500 μg/ml geneticin, the cell density of SKLKB82#3 cells with 106 passages was 161,000 cells per well (62% viable) while the cell count in absence of neomycin was 235,000 cells per well (96% viable). SKLKB82#3 cells with 30 passages incubated in the presence and absence of neomycin
<table>
<thead>
<tr>
<th>Cell Line/Tissue</th>
<th>NK-2 Receptor Density (fmol/mg protein)</th>
<th>Specific Binding (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster Urinary Bladder</td>
<td>304</td>
<td>96</td>
</tr>
<tr>
<td>Bovine Stomach</td>
<td>3</td>
<td>58</td>
</tr>
<tr>
<td>B82 Fibroblasts</td>
<td>69</td>
<td>13</td>
</tr>
<tr>
<td>LK4V Cells</td>
<td>8</td>
<td>37</td>
</tr>
<tr>
<td>LK3-3 Cells</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SKLKB82#3 Cells</td>
<td>147</td>
<td>90</td>
</tr>
</tbody>
</table>

Table 3. NK-2 Receptor density in selected tissues and cell lines. B82 fibroblasts are the parent cell line, LK4V cells are B82 fibroblasts transfected with pHβAPr-1-neo, the LK3-3 cells are B82 fibroblasts transfected with pHβAPr-1-neo-m1 which expresses m1 muscarinic receptors, and SKLKB82#3 cells are B82 fibroblasts transfected with pHβAPr-1-neo-SKR56S which express NK-2 receptors. Receptor density was measured by [125I]NKA saturation binding in SKLKB82#3 cell membranes.
had a cell density of 303,000 cells per well (100% viable) and 289,000 cells/well (100% viable), respectively. The lower counts and viability in the older cell line probably reflects two populations of cells, one lacking and one having neomycin resistance.

**Discussion**

A cell line stably expressing a high density of NK-2 receptors was constructed by transfecting a bovine stomach cDNA encoding for the NK-2 receptor into the B82 fibroblast cell line. DNA hybridization of XbaI/BamHI endonuclease fragments of the SKR56S gene and the construct pHβAPr-1-neo-SKR56S show that the expression vector contained the open reading frame of the SKR56S. Restriction enzyme analysis indicated the 5'3' orientation of the gene in respect to the β-actin promoter in the pHβAPr-1-neo expression vector and also the size of this fragment.

The highest NK-2 receptor density obtained from this transfection was 147 fmol/mg protein. This level is lower than hamster urinary bladder which has a high density of NK-2 receptors (304 fmol/mg protein), but 50-fold higher than bovine stomach, the original tissue of the SKR56S cDNA clone (3 fmol/mg protein) (Van Giersbergen et al., 1991).

The expression of the NK-2 receptor in SKLKB82#3 cells remained stable after 60 passages. However, after 106 passages, cells became susceptible to neomycin and had lower [³H]inositol monophosphate accumulation response. Examination for expression of neomycin resistance, indicative of the presence of the introduced
expression vector, showed only 68% with this trait. Consequently, the absence of environmental pressure (500 µg/ml neomycin) to retain the pHβAPr-1-neo-SKR56S construct may have resulted in the reduction of neomycin resistance after 106 passages. The older cell line probably contains two populations, one with neomycin resistance and the other lacking this trait.

Tissues respond to neurokinins in a complex manner because they often contain several neurokinin receptor subtypes. These responses become less distinct when the receptors mediate similar biological responses and have affinity for other neurokinins. A cell line expressing high density of functional NK-2 receptors offers the advantage of a well-characterized receptor in a controlled environment. The cell line, SKLKB82#3, provides a system for the examination of NK-2 receptor expression, agonist affinities, and second messenger coupling.
A receptor selectively recognizes and binds only those hormones, transmitters, or drugs with a complementary molecular structure. Thus, it discriminates between the agonists, antagonists, and other molecules to which it is exposed. Endogenous neurokinin peptides exhibit low selectivity toward other neurokinin receptors because a common carboxyl terminus enables cross-talk with other neurokinin receptor subtypes. Consequently, classification of these receptors should be established by the rank order of several agonists. Although [¹²⁵I]NKA saturation assays measured a high affinity NKA binding site in cells, additional pharmacological assays can establish whether the de novo protein expressed by the SKLKB82#3 cells represents an authentic NK-2 binding site or an NK-2 receptor subtype.

Due to the lack of potent and selective ligands, neurokinin receptors have been classified by the rank order potency of agonists. However, degradative enzymes, access to the receptors, different signal transduction mechanisms, spare receptors, and partial agonists can complicate the interpretation of results from functional assays. Radioligand binding assays can reduce these limitations. These experiments are designed to demonstrate that the de novo protein expressed in SKLKB82#3 cells selectively binds NKA and to compare the pharmacological characteristics of this binding site to endogenous NK-2 receptors in bovine stomach.
Materials

The agonists, neurokinin A and substance P, were purchased from Peninsula Laboratories, Belmont, CA; senktide (a selective NK-3 agonist) from Bachem, Philadelphia, PA; and (2-[125I]iodohistidyl)-NKA (specific activity 2000 Ci/mmol) from Amersham Corporation, Arlington Heights, IL. Stock solutions of the peptides were made in 0.01 M acetic acid containing 1% 2-mercaptoethanol, aliquoted, and frozen at -70°C until used.

Triton X-100 was purchased from ICN Radiochemicals, Inc., Irvine, CA; Dulbecco’s Modified Eagle Medium (DMEM), Ham’s F-12 medium, and Iscove’s Modified Dulbecco’s Medium (IMDM) from GIBCO-BRL, Grand Island, NY; tissue cultures supplies from Costar, Cambridge, MA; and all other compounds from Sigma Chemical Co., St. Louis, MO.

Methods

Radioligand Binding Assays

SKLKB82#3 Cells. Competitive [125I]NKA/ligand binding experiments were modified from a procedure described in Womack et al., (1985). Twenty four hours prior to each experiment, SKLKB82#3 cells were detached by a 30 to 60 second exposure to 0.02% ethylenediaminetetraacetic acid (EDTA)/0.25% trypsin, diluted in DMEM/Ham’s F-12 media, plated at 100,000 cells per well in a 24 well tissue culture plate and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO₂. After this
treatment, intact SKLKB82#3 cells were placed in 0.5 ml of IMDM with 50 pM $^{125}$I-NKA and one of several concentrations of a competing drug (NKA, substance P, or senktide). The incubation media also contained 1 mg/ml bovine serum albumin to reduce nonspecific binding and 40 µg/ml bacitracin, 4 µg/ml leupeptin and 4 µg/ml chymostatin to inhibit endopeptidase activity. SKLKB82#3 cells were incubated at 26±1°C for 30 minutes in a humidified atmosphere of 95% air, 5% CO$_2$. The assay was terminated by aspirating the incubation media and washing the cells twice for five minutes with 0.5 ml of cold (4°C) IMDM. The cells were then detached and solubilized by a 30 minute exposure to 0.5 ml of 1% Triton X-100 and each well washed with 0.5 ml of 1% Triton X-100. Solutions from each well were pooled in borosilicate test tubes and the radiation counted by a gamma counter (Packard Auto-Gamma 5650, Packard Instrument Co., Downers Grove, IL).

**Bovine Stomach Membranes.** Bovine stomach was obtained from a local slaughter house. The smooth muscle layer was separated from the mucosa with a scalpel and stored at -80°C until used. Tissue was homogenized and processed as described on pages 41-43 in the "Expression of the bovine stomach cDNA SKR56S in B82 fibroblasts methods section. Each sample contained 100 pM $^{125}$I-NKA and between 50 and 100 µg of protein which was measured by a protein dye binding method (Bradford 1976).

**Data Analysis**

Specific binding was calculated by subtracting nonspecific counts from total
counts. Nonspecific levels were measured in presence of 1 μM NKA and ranged from 90-95% of total binding. The percent of specific bound [12S I]NKA for each agonist concentration was calculated as the percent of specific counts of 10 pM NKA. Data points represent the average ± SEM of at least three determinations performed in duplicate. A nonlinear least-squares regression program (GraphPAD, 1990) was used to draw each curve and calculate their IC50 value and the Hill slope.

Results

Table 4 lists the IC50 value of the compounds assayed with SKLKB82#3 cells or bovine stomach membranes. In both preparations the IC50 values displayed a rank order of affinity of NKA > substance P > senktide (Figure 8), with nanomolar affinity for NKA, and significantly lower affinity for substance P and senktide. The SKLKB82#3 cells had a 100-fold greater selectivity between NKA and substance P in contrast to the 50-fold difference present in the bovine stomach preparation.

In addition to neurokinin agonists, other drugs were assayed for their ability to compete with [125I]NKA binding in SKLKB82#3 cells. Atropine, a nonselective muscarinic antagonist; naloxone, a nonselective opioid antagonist; propranolol, a beta adrenergic antagonist; and spiperone, a serotonin antagonist did not inhibit the binding of [125I]NKA to SKLKB82#3 cells (data not shown).
Table 4. Comparison of the affinity of neurokinin agonists in intact SKLKB82#3 cells and bovine stomach membranes. SKLKB82#3 cells were incubated in IMDM with 50 pM [125I]NKA and various concentrations of peptides for 30 minutes at 26°C. Bovine stomach membranes were incubated in Tris buffer with 100 pM [125I]NKA and various concentrations of peptides for 120 minutes at room temperature.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>SKLKB82#3 Cells IC$_{50}$±SEM (nM)</th>
<th>Bovine Stomach Membranes IC$_{50}$±SEM (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurokinin A</td>
<td>10±2.6</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>Substance P</td>
<td>1,350±220</td>
<td>53±25</td>
</tr>
<tr>
<td>Senktide</td>
<td>82,200±13,700</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>
Figure 8. Competitive binding of $[^{125}I]$NKA with NKA, substance P, or senktide in intact SKLKB82#3 cells. $[^{125}I]$NKA concentration was 50 pM. Data points lacking error bars contain the error bar within the symbol.
Discussion

The transfected B82 fibroblasts expressed a *de novo* protein with high affinity and selectivity for NKA. This binding site had a rank order of affinity of NKA > substance P > senktide which corresponds to an NK-2 binding site. Atropine, naloxone, propranolol, and spiperone failed to inhibit [125I]NKA binding to SKLKB82#3 cells. The expressed receptor not only recognized and bound NKA and other neurokinin agonists in a pattern characteristic of an NK-2 binding site, but also discriminated other receptor antagonists.

The expressed NK-2 receptor in the SKLKB82#3 cells and the endogenous NK-2 receptor in bovine stomach a have similar pharmacological profiles. This implies that transcription and translation of the transfected NK-2 receptor gene by the host cell did not sufficiently alter the pharmacological properties of this protein. Different experimental conditions in the radioligand binding assays for intact SKLKB82#3 cells and bovine stomach membrane preparations probably accounted for the different IC50 values of each peptide.
THE COUPLING OF SECOND MESSENGERS

TO THE NK-2 RECEPTORS EXPRESSED IN B82 FIBROBLASTS

A wide variety of membrane receptors for neurotransmitters and peptides are coupled to guanine-nucleotide-binding regulatory proteins (G proteins). They achieve their physiological effects by modulating the flow of ions through membrane channels or by the enzymatic production of second messengers such as cyclic 3',5'-adenosine monophosphate, inositol 1,4,5-triphosphate, diacylglycerol, or arachidonic acid. Furthermore, inositol 1,4,5-triphosphate binds to specific receptors on calcium storing organelles to raise intracellular calcium (Ca²⁺) levels.

The neurokinin receptors belong to the family of receptors coupled to G proteins. The NK-2 receptor has been shown to be coupled to phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis in hamster urinary bladder (Bristow et al., 1987) and calcium mobilization in small lung cancer cells (Takuwa, et al., 1990). While numerous neurotransmitter, hormone, and peptide receptors have been shown to be coupled to adenylyl cyclase modulation or arachidonic acid release (Axelrod et al., 1988), the NK-2 receptor has not been reported to couple to these second messengers. The purpose of these experiments is to demonstrate that the high affinity NKA binding site in SKLKB82#3 cells is functionally coupled to PIP₂ hydrolysis and calcium mobilization. Coupling to other second messenger systems will also be examined.
Materials

Accumulation of $[^3\text{H}]$Inositol Monophosphates

Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagle medium, fetal calf serum, and Ham's F-12 medium were purchased from GIBCO-BRL, Grand Island, NY; myo-$[^2,3\text{H}](\text{N})$inositol ($[^3\text{H}]$MI) (20 Ci/mmol specific activity) from NEN, Boston, MA; neurokinin A and substance P from Peninsula Laboratories, Belmont, CA; and senktide from Bachem, Philadelphia, PA. The Poly-Prep$^8$ chromatography column and the AG1-X8 100-200 mesh anion exchange resin in formate form were obtained from Bio-Rad Laboratories, Richmond, CA; sodium tetraborate and sodium formate from J. T. Baker Chemical Co., Phillipsburg, NJ; ammonium formate from Eastman Kodak Co., Rochester, NY; and Aquamix from ICN, Irvine, CA. All other chemicals were obtained from Sigma, St. Louis, MO.

Intracellular Calcium Mobilization

The 75 cm$^2$ culture flasks were purchased from Costar, Cambridge, MA; Dulbecco's Modified Eagle Medium (DMEM) and fetal calf serum from GIBCO-BRL, Grand Island, NY; Fluo-3, AM (acetoxymethyl ester) from Molecular Probes, Eugene, OR; dimethyl sulfoxide from ATCC, Rockville, MD; and Pluronic acid from BASF Wyandotte Corp., Wyandotte, MI. Dr. Paulo Rovero, Chemistry Department, A. Menarini Pharmaceuticals, Firenze, Italy, synthesized the NK-2 antagonist, $[^{\text{Tyr}^5, \text{D-Trp}^{6,8,9}, \text{Arg}^{10}]$-NKA$\text{A}_{4,10}$ (MEN 10207).
3',5' Cyclic Adenosine Monophosphate

Cyclic AMP-dependent protein kinase and 3-isobutyl-1-methyl xanthine (IBMX) were purchased from Sigma Chemical Co., St. Louis, MO; [2,8-$^3$H]-adenosine 3',5'-cyclic phosphate ($[^3$H]cAMP) (31.3 Ci/mmol specific activity) from NEN, Boston, MA; Norit Ultra C activated charcoal from American Norit Company Inc., Jacksonville, FL; and forskolin from Calbiochem, La Jolla, CA.

Arachidonic Acid Release

Fatty acid-free bovine serum albumin (FAF BSA) was purchased from Boehringer Mannheim Corp., Indianapolis, IN; [5,6,8,9,11,12,14,15-$^3$H(N)]Arachidonic acid ($[^3$H]AA) (specific activity 100 Ci/mmol) from NEN, Boston, MA; and A23187 from Calbiochem Corp., La Jolla, CA.

Methods

Accumulation of $[^3$H]Inositol Monophosphates

The quantification of $[^3$H]IP$_1$ accumulation was modified from a procedure described by Berridge et al., (1982). SKLKB82#3 cells were grown in 75 cm$^2$ Costar culture flasks to confluence (approximately 20-25 million cells per flask). To detach the cells, the media was aspirated, the cells washed with phosphate buffered saline, and treated with 0.02% EDTA/0.25% trypsin for 30 to 60 seconds. Trypsin was neutralized with 5 ml of IMDM containing 10% (v/v) fetal calf serum. This suspension was counted, diluted with DMEM/Ham's F-12 media to a density of 125,000 cells per ml,
and aliquoted into a 24 well tissue culture plate with 1.6 cm diameter culture wells. After an incubation at 37°C in a humidified atmosphere of 95% air, 5% CO₂ (all incubations were under these conditions) for 24 hours, the media was removed and 0.5 ml of IMDM with 0.2 μM [³H]MI was added to each well. After a second incubation of 20 to 24 hours, the media was removed and the cells washed with 1.0 ml of IMDM and incubated for 10 minutes in 0.45 ml of assay buffer (IMDM with 10 mM LiCl, 40 μg/ml bacitracin, 4 μg/ml leupeptin, and 4 μg/ml chymostatin). An eighty percent decrease in [³H]IP₁ accumulation was observed in the absence of these peptidase inhibitors (data not shown). The agonists, NKA, substance P, and senktide, were diluted with assay buffer and 50 μl of various concentrations were added to the 0.45 ml of assay buffer present in each well. The plates were incubated for 60 minutes (optimum assay conditions) and the reaction was terminated by aspirating the media and adding 0.5 ml methanol to each well. The cells were scraped from each well and transferred to chloroform resistant tubes. A second aliquot of 0.5 ml methanol was added to each well to collect the remaining cells and pooled with the initial methanol cell suspension. This solution was mixed with 1.0 ml chloroform and 0.5 ml double distilled water. After centrifugation at 1520 × g for 10 minutes, a 0.9 ml aliquot of the aqueous phase was added to 2.0 ml of distilled water and vortexed. This mixture was passed through a chloroform resistant Poly-Prep® chromatography column which contained 2.0 ml of a 10% slurry of AG1-X8 100-200 mesh anion exchange resin in formate form. The columns were washed three times with 5.0 ml of distilled water and two times with
5.0 ml of 5 mM sodium tetraborate/60 mM sodium formate. [3H]IP$_1$ was eluted with 2.0 ml of 0.2 M ammonium formate/0.1 M formic acid. The eluate was mixed with 9.0 ml of Aquamix and the radioactivity counted for two minutes in a liquid scintillation counter (Searle Model 6880, Searle Analytic Inc., Des Plains, II., 43% efficiency).

**Intracellular Calcium Mobilization**

The SKLKB82#3 cells were grown in 75 cm$^2$ Costar culture flasks to confluency (approximately 20-25 million cells per flask) before being detached by a 30 to 60 second exposure to 0.02% EDTA/0.25% trypsin and immediately neutralized with 5 ml of DMEM with 10% (v/v) fetal calf serum. The cells were harvested and washed three times with 3 ml of a 10 mM HEPES buffer (pH 7.35) containing 110 mM NaCl, 5.3 mM KCl, 1 mM MgCl$_2$, 80 mM sucrose, and 25 mM glucose (buffer A). The washed cells were then resuspended in buffer A containing 1.8 mM CaCl$_2$ (buffer B) to a concentration of 12 million cells per ml. The viability of the SKLKB82#3 cells was determined by trypan blue exclusion and ranged from 72 to 95%. The cells were loaded with the cell permeant calcium indicator, Fluo-3, AM (acetoxymethylester) essentially as described previously (Smith, 1990). Briefly, 2.5 ml of suspended cells were incubated in the presence of 25 µl of a 1.0 mM stock solution of Fluo-3, AM dissolved in dimethyl sulfoxide containing 25% (w/v) Pluronic acid at 35°C for 30 to 120 minutes with continuous shaking.

Fluorescence determinations were made using a T-format Series 300 spectrofluorometer (H&L Instruments, Burlingame, CA) with excitation and emission
wavelengths set at 506 nm and 526 nm, respectively. Fluorescence intensity, F, was monitored continuously with an on-line IBM-compatible personal computer. Immediately prior to each determination, a 100 μl aliquot of the cell suspension containing 1.0 to 1.2 million cells was washed two times with 3 ml of buffer A and resuspended in 3 ml of buffer B. All washings and resuspensions were performed in buffer kept at 35°C. The cell suspension was then transferred to a quartz cuvette containing a perforated square teflon bar designed for rapid and continuous sample mixing. After this cell suspension reached 35°C, one of several concentrations of NKA was added and the subsequent changes in F recorded.

To demonstrate the antagonistic effect of MEN 10207, SKLKB82#3 cells were preincubated with this selective NK-2 antagonist 3 to 4 minutes prior to the addition of NKA. In experiments designed to determine the source of the receptor-mediated rise in Ca^{2+}, SKLKB82#3 cells were resuspended in buffer without calcium (buffer A) for 3 to 5 minutes prior to stimulation by NKA.

**Cyclic 3',5' Adenosine Monophosphate**

The cells were rinsed with 1.0 ml of IMDM and covered with 0.4 ml of 5 mM IBMX in IMDM one minute before the addition of 100 μl of one or a combination of forskolin, NKA, or MEN 10207. After 3 minutes, cold Tris/EDTA buffer (50 mM Trizma HCl/Trizma base containing 4 mM Na<sub>2</sub>EDTA, pH 7.5 at 25°C) were added to each well to stop the reaction and placed on ice. The cells were dislodged from the wells with a Costar cell scraper, transferred to microfuge tubes, and heated in a boiling
water bath for 5 to 15 minutes. After centrifugation at 500 \times g for 2 minutes, a 50 \mu l aliquot of the supernatant was analyzed for cAMP content using a competitive protein binding assay according to a modified method of Gilman (1970). In brief, cAMP-dependent protein kinase was prepared in a 50 mM Tris HCl/4 mM EDTA/0.1% bovine serum albumin buffer (60 \mu g protein kinase/ml, pH 7.5 at 25°C). A 50 \mu l aliquot of either the supernatant from each sample or a cAMP standard solution (0.125 to 64 pM cAMP) was incubated with 50 \mu l (0.9 pmol/50 \mu l) of \[^{3}\text{H}]\text{cAMP} and a 100 \mu l aliquot of the protein kinase preparation on ice for 2 hours. Separation of bound from free \[^{3}\text{H}]\text{cAMP} was achieved by adding 100 \mu l of ice cold activated charcoal in 50 mM Tris HCl/4 mM EDTA/2% bovine serum albumin to each tube. The samples were gently mixed and immediately centrifuged at 5600 \times g for 45 seconds. A 200 \mu l aliquot of each supernatant was transferred to a scintillation vial, mixed with 4.0 ml of Aquamix, and the radioactivity was counted (Searle Model 6893, Searle Analytic Inc., Des Plains, IL., 45% efficiency).

**Arachidonic Acid Release**

Arachidonic acid release was measured according to a procedure described by Kanterman et al. (1990). SKLKB82#3 cells were plated at a density of 100,000 cells per well in a 24 well tissue culture plate. After an incubation at 37°C in a humidified atmosphere of 95% air, 5% CO₂ (all incubations were under this condition) for 24 hours, the media was removed and replaced with IMDM containing 0.2 \mu Ci \[^{3}\text{H}]\text{arachidonic acid (0.2 \mu Ci/well). After an 18 to 24 hour incubation, the
[³H]arachidonic acid media was aspirated, the cells washed three times with 1.0 ml of IMDM/0.2% FAF BSA, and 450 μl of incubation media (IMDM in absence or presence of 10 μM A23187) was added to each well. To initiate the assay, 50 μl of various concentrations of drugs were added. After a 60 minute incubation, the media was transferred into borosilicate tubes and centrifuged for one minute at 12,000 x g. Four hundred microliters of supernatant was carefully removed, placed in scintillation vials with 9.0 ml Aquamix, and counted for 10 minutes in a liquid scintillation counter (Beckman Liquid Scintillation Counter LS 6000SE, Fullerton, CA, 45% efficiency).

**Data Analysis**

For all experiments, the data points represent the average ± SEM of at least three experiments performed in duplicate. Basal level was defined as the amount of radioactivity present in SKLKB82#3 cells with no NKA treatment. Stimulated levels were calculated as percent above basal level with basal level equal to one hundred percent. A nonlinear least-squares regression program (GraphPAD, 1990) was used to construct and calculate the EC₅₀ value and the Hill slope of each curve.

**Calcium Mobilization.** The concentration of free intracellular calcium (Ca²⁺) was calculated as described previously using a Kᵦ of 450 nM for Fluo-3, AM (Tsien et al., 1982). Separate calibrations were performed on each sample. F_max was determined in the presence of 0.1% sodium dodecyl sulfate and a saturating concentration of calcium. F_min was determined in the presence of excess ethylenebis(oxyethylenenitrilo)tetraacetic acid. Stimulated Ca²⁺ was defined as the difference between resting Ca²⁺
and that calculated from the peak fluorescence intensity observed after the addition of agonist. Leakage of Fluo-3, AM over the period of measurement (30 to 40 seconds) was approximately 5% of the fluorescence values. Similar resting and stimulated Ca\(^{2+}\) values were obtained from aliquots of cells incubated with Fluo-3, AM over the period of 30 to 120 minutes. Total fluorescence was corrected for both autofluorescence and dye leakage prior to calculation of Ca\(^{2+}\) values. The concentration response curve was fitted to the data points by a nonlinear least-squares computer program from GraphPad (GraphPAD Software, 1990). The level of significance was assessed with the student's t-test. A probability value greater that 95% was considered significant.

3',5' Cyclic Adenosine Monophosphate. A standard curve was constructed for each assay and disintegrations per minute converted to cAMP levels as pmol cAMP/50 μl by interpolation of the standard curve.

**Results**

**Agonist Induced \(^{3}H\)IP\(_1\) Accumulation**

NKA, substance P, and senktide induced a concentration-dependent accumulation of \(^{3}H\)IP\(_1\) in SKLKB82#3 cells with a rank order of potency of NKA > substance P > senktide (Figure 9). These agonists had no significant stimulatory or inhibitory effect on \(^{3}H\)IP\(_1\) accumulation in the parent cells (B82 fibroblasts), parent cells transfected with pHβAPr-1-neo (LK4V cells), or parent cells transfected with pHβAPr-1-neo-m1 (LK3-3 cells) (p > 0.05).
The NKA dose response curve was drawn by a four parameter fit (minimum, maximum, EC$_{50}$, and Hill slope) of the data points with a maximal [$^3$H]IP, accumulation of 7.2 times basal level, a Hill slope of 1.3, and an EC$_{50}$ value of 10 nM. The SP and senktide curves were drawn by a three parameter fit with a maximum fixed at the NKA maximum. SP had an EC$_{50}$ value of 4.9 µM and a Hill slope of 0.6. Senktide did not reach an EC$_{50}$ level under experimental conditions but at 100 µM it increased [$^3$H]IP, accumulation 1.8 times basal level.

**Intracellular Calcium Mobilization**

The change in fluorescence intensity of Fluo-3, AM after the addition of NKA to an SKLKB82U3 cell preparation increased sharply then gradually returned to basal level (Figure 10). Fluorescence rapidly increased to a maximum value within 5 to 10 seconds after the addition of NKA and gradually declined to near resting levels over a period of 90 seconds. There were no additional peaks during this period.

This cell exhibited a concentration-dependent increase in Ca$^{2+}$, upon stimulation with NKA over the range of 1 nM to 10 µM (Figure 11). Nonlinear regression analysis of the curve yielded an EC$_{50}$ value of 24 nM. In the presence of 1 µM NKA, Ca$^{2+}$ rose to a maximum value of $58\pm3$ nM above resting levels ($82\pm3$ nM). When stimulated with 1 µM NKA, the parent cell line, B82, had a Ca$^{2+}$ level of $86\pm8$ nM which did not differ from resting levels.

In similar experiments, the effect of MEN 10207, a selective NK-2 antagonist, on receptor-stimulated Ca$^{2+}$ was determined. The rise in Ca$^{2+}$, $38\pm5$ nM, induced by
0.1 μM NKA was abolished by 1 μM MEN 10207. When increasing NKA to 1 μM, the Ca\(^{2+}\) response in the presence of 1 μM MEN 10207 was 34% of that observed in the absence of MEN 10207, indicating that the inhibition by the antagonist is reversible. Therefore, these results suggest that the stimulation of Ca\(^{2+}\) by NKA is mediated by NK-2 receptors.

In other experiments, the effect of extracellular calcium on the magnitude of the receptor mediated rise in Ca\(^{2+}\) was evaluated. In SKLKB82#3 cells resuspended for 3 to 5 minutes in the absence of calcium (buffer A), the Ca\(^{2+}\) response to 0.1 μM NKA rose 33±2 nM as compared to 38±5 nM in the presence of 1.8 mM Ca\(^{2+}\) in the medium. These results indicate that most of the receptor-mediated rise in Ca\(^{2+}\) is generated from internal Ca\(^{2+}\) stores.

**Formation or Inhibition of cAMP**

At 1 μM, a concentration that produced maximum \(^{3}H\)IP\(_{1}\) accumulation in SKLKB82#3 cells, NKA failed to increase cAMP formation or decrease forskolin stimulated cAMP levels in SKLKB82#3 cells (Table 5) (p>0.05). Both the sham-transfected LK4V cells and the parent B82 fibroblasts did not display a change in cAMP level.

**Arachidonic Acid Release**

At 1 μM, a concentration that produced maximum \(^{3}H\)IP\(_{1}\) accumulation in SKLKB82#3 cells, and in the presence or absence of A23187, NKA had no effect on arachidonic acid release in SKLKB82#3 cells (Table 6). A positive control, LK3-3 cells
Figure 9. Agonist induced $[^3]$H]IP$_3$ accumulation in SKLKB82#3 cells. Data points are arithmetic means ± SEM of three separate experiments done in duplicate. Data points without error bars contain the bar within the symbol. NKA had an $E_{max}$ seven times basal level. The EC$_{50}$ value for NKA was 10 nM and substance P was 4.9 μM. Senktide did not reach an EC$_{50}$ value. At 100 μM, senktide stimulated $[^3]$H]IP$_3$ accumulation 1.8 times basal level.
Figure 10. Time course for the change in fluorescence intensity after the addition of NKA to a SKLKB82#3 cell preparation. The curve has been corrected for drift from dye leakage from the cells. No evidence of additional fluorescence peaks was observed within 90 seconds after the addition of 1 μM NKA. The peak corresponds to an increase of 50 nM Ca^{2+}. The arrow indicates when NKA was added to the preparation. The curve represents data from a typical experiment.
Figure 11. Concentration-dependent increase in $\text{Ca}^{2+}$ upon stimulation with NKA in SKLKB82#3 cells. NKA induced a maximum $\text{Ca}^{2+}$ concentration of $58 \pm 3$ nM. Basal $\text{Ca}^{2+}$ levels were $82 \pm 3$ nM. Nonlinear regression analysis of the curve yielded an $\text{EC}_{50}$ value of 24 nM. When stimulated with 1 $\mu$M NKA, the parent cell line, B82, had a $\text{Ca}^{2+}$ level of $86 \pm 8$ nM which did not differ from resting levels.
Table 5. The effect of neurokinin A on the formation or inhibition of cAMP in SKLKB82#3 cells. Cells were treated with 5 mM IBMX and then for 3 minutes with various drugs. cAMP was measured by a competitive protein binding assay.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>cAMP (fmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.89 ± 0.42</td>
</tr>
<tr>
<td>1 μM NKA</td>
<td>5.77 ± 1.25</td>
</tr>
<tr>
<td>10 mM Forskolin</td>
<td>36.9 ± 8.1</td>
</tr>
<tr>
<td>10 mM Forskolin + 1 μM NKA</td>
<td>36.5 ± 7.4</td>
</tr>
</tbody>
</table>
Table 6. The effect of neurokinin A on arachidonic acid release in SKLKB82/3 cells. Cells were loaded with 0.2 Ci/well [3H]arachidonic acid for 24 hours and then stimulated with drugs for one hour.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Arachidonic Acid Released (percent of basal level)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(no A23187)</td>
</tr>
<tr>
<td></td>
<td>(100 µM A23187)</td>
</tr>
<tr>
<td>1 µM NKA</td>
<td>103 ± 0.5</td>
</tr>
<tr>
<td>1 µM MEN 10207</td>
<td>92 ± 5.0</td>
</tr>
<tr>
<td>1 µM NKA +</td>
<td>99 ± 9.0</td>
</tr>
<tr>
<td>1 µM MEN 10207</td>
<td>100 ± 8.5</td>
</tr>
</tbody>
</table>
expressing the muscarinic m$_1$ receptors stimulated with 100 $\mu$M carbachol and 100 $\mu$M A23187, had a 4.5-fold increase in arachidonic acid release.

**Discussion**

A cell line with stable expression of a high density of NK-2 receptors was constructed by transfecting a bovine stomach cDNA encoding for this receptor into B82 fibroblasts. The transfected cells increased [$^3$H]IP$_1$ accumulation upon stimulation with NKA in a dose dependent manner and displayed a relative order of agonist potency of NKA > substance P > senktide. The EC$_{50}$ values in the transfected cells were 10 nM for NKA and 4.9 $\mu$M for SP. These results agree with a previous observation of neurokinin stimulated [$^3$H]IP$_1$ accumulation in hamster urinary bladder where NKA induced a dose response in PIP$_2$ hydrolysis, had an EC$_{50}$ value of 13 nM, and was more potent than substance P (EC$_{50}$ = 5.1 $\mu$M) (Bristow et al., 1987). In addition, the rank order of agonist potency, NKA > substance P > senktide, in the SKLKB82#3 cells is similar to that reported for hamster urinary bladder, a tissue with a high density of NK-2 receptors (Buck and Shatzer, 1988).

The present investigation also demonstrated that the activation of NK-2 receptors by NKA produced a rapid and transient increase in Ca$^{2+}$$_i$ which is inhibited by MEN 10207, a selective NK-2 antagonist. Although NKA has been reported to increase Ca$^{2+}$$_i$ in small cell lung cancer cells, the specific receptor type mediating this response was not characterized (Takuwa et al., 1990). Omission of calcium from the external medium
did not change the calcium response to NKA in the SKLKB82#3 cell line. These results suggest that intracellular sources of Ca\(^{2+}\), such as inositol 1,4,5-triphosphate-sensitive Ca\(^{2+}\) pools within the endoplasmic reticulum, account for the rise in Ca\(^{2+}\) observed in the presence of NKA. Similar observations have also been made in small cell lung cancer cells (Takuwa et al., 1990).

Upon stimulation with NKA, the SKLKB82#3 cells accumulated inositol monophosphates in a concentration-dependent manner. The EC\(\text{so}_{50}\) value of this response (10 nM) compared favorably with the EC\(\text{so}_{50}\) value of calcium mobilization (24 nM). The similarity of these values supports the hypothesis that the release of membrane-bound intracellular calcium is sensitive to the amount of inositol triphosphate produced upon receptor activation. In the SKLKB82#3 cells, the IC\(\text{so}_{50}\) value for \[^{125}\text{I}]\text{NKA}/\text{NKA}\) competitive binding and the EC\(\text{so}_{50}\) value for \[^{3}\text{H}]\text{IP}_1\) accumulation are similar (10 nM) and suggest the absence of spare receptors since 50% occupation resulted in approximately 50% response.

In SKLKB82#3 cells, cAMP levels are not modulated by the NK-2 receptor. The parent cell line, B82 fibroblasts, does contain the G proteins that couple to adenylyl cyclase because transfection of the muscarinic-2 receptor gene results in functional coupling of adenylyl cyclase (Lai et al., 1991). One possibility for the absence of an adenylyl cyclase response is that the NK-2 receptor lacks the optimal sequence (Arg-Arg-X-Ser) for cAMP-dependent protein kinase that occurs on the third cytoplasmic loop of the β-adrenergic receptor (Lefkowitz et al., 1984). Nakajima et al. (1992) reported
that the NK-1, NK-2, and NK-3 receptors were coupled to adenylyl cyclase in Chinese ovary cells transfected with cDNAs for these receptors. The transfected cells demonstrated an agonist induced concentration-dependent increase of cAMP. They were able to demonstrate coupling of cAMP to the neurokinin receptors because the transfected cells expressed 100-fold more NK-2 receptors than the SKLKB82#3 cells and they optimized the experimental conditions. While most cells produce arachidonic acid, the release of this compound is not mediated by activation of the NK-2 receptor in SKLKB82#3 cells.

The primary function of cell surface receptors is to discriminate the appropriate ligands from among multiple extracellular stimuli and then to activate an effector system that produces an intracellular signal. Receptors not only transmit but also amplify and integrate extracellular signals, thereby controlling cellular processes. These mechanisms allow cells to communicate and coordinate their development and actions. To accomplish this, upon agonist binding, the NK-2 receptor activates phospholipase C which then cleaves PIP$_2$ to produce the intracellular second messenger, inositol 1,4,5-triphosphate, which mobilizes intracellular calcium and probably induces smooth muscle contraction or secretion. Adenylyl cyclase modulation and arachidonic acid release are not mediated by the NK-2 receptor in the SKLKB82#3 cells.
DEMONSTRATION OF A NOVEL NK-2 RECEPTOR SUBTYPE
IN TRANSFECTED B82 FIBROBLASTS

In the absence of selective antagonists, neurokinin receptors were classified by
their agonist rank order of potency. These experiments provided evidence for three
distinct neurokinin receptors: NK-1 receptors with high affinity for substance P; NK-2
receptors with high affinity for NKA; and NK-3 receptors with high affinity for NKB
(Buck et al., 1984). A fourth tachykinin receptor type, NK-4, was hypothesized when
the rank potency of tachykinin agonists in contracting guinea-pig tracheal smooth muscle
did not concur with the pharmacological profiles of previously identified tachykinin
receptors (McKnight et al., 1987).

While agonist rank order of potency can differentiate receptors into subtypes,
anomalous potency ratios may lead to misclassification when factors such as variation
of receptor density, efficiency of signal transduction pathways, selectivity of agonists,
or tissue uptake of drugs are not considered. Selective antagonists often reduce these
limitations. Recently, Maggi et al. (1990a) developed NK-2 receptor antagonists by
replacing selected amino acids of truncated NKA peptides. In bioassays using these
altered molecules to inhibit NKA-induced smooth muscle contraction in rabbit
pulmonary artery or hamster trachea, the pA₂ values of three NK-2 receptor antagonists
suggested heterogeneity among the NK-2 receptors in these tissues.

The present study uses three neurokinin agonists and the two recently developed
NK-2 antagonists, MEN 10207 and MEN 10208, to characterize the bovine stomach NK-2 receptor transfected into and stably expressed by B82 fibroblasts.

Materials

The source of the materials used in this section can be found on page 61 of the materials section of "The coupling of second messengers to the NK-2 receptors expressed in B82 fibroblasts." Dr. Paulo Rovero, Chemistry Department, Menarini Pharmaceuticals, Firenze, Italy, synthesized the NK-2 antagonists, [Tyr⁴, D-Trp⁶,⁸,⁹, Arg¹⁰]-NKA₄₋₁₀ (MEN 10207) and [Tyr⁵, D-Trp⁶,⁸,⁹, Arg¹⁰]-NKA₃₋₁₀ (MEN 10208), by conventional solid-phase methods.

Methods

Radioligand Binding Assays

The procedures used for radioligand binding assays in bovine stomach membranes and SKLKB82#3 cells can be found on pages 54 to 55 in the methods section of "Demonstration of neurokinin A binding sites in SKLKB82#3 cells."

[^H]IP₁ Accumulation and pA₂ Determination

Phosphatidylinositol hydrolysis was quantified by measuring[^H]IP₁ accumulation as described on pages 62 to 64 of the methods section of "The coupling of second messengers to the NK-2 receptors expressed in B82 fibroblasts." pA₂ values were determined by measuring potency ratios of three different antagonist concentrations in
three independent experiments done in duplicate and determining dose ratios in presence
and absence of antagonists.

Data Analysis

A nonlinear regression program (GraphPAD, 1990) was used to construct and
calculate the IC$_{50}$ value and Hill slope of each curve. The Pharmacologic Calculation
System program (Microcomputer Specialists, Philadelphia, PA, 1986) was used to
calculate the pA$_2$ values of the NK-2 antagonists.

Results

Table 7 lists the IC$_{50}$ values of compounds assayed in several preparations. The
IC$_{50}$ values of the agonists displayed a rank order of affinity of NKA > substance
P > senktide in the SKLKB82#3 cells (Figure 12), bovine stomach membranes, rabbit
pulmonary artery, and hamster trachea. In all preparations, the IC$_{50}$ value for NKA is
in the nanomolar range and at least 50-fold higher affinity than substance P. Based upon
agonist rank potency and NKA affinity, all preparations contain NK-2 receptors.

In contrast to the agonists, the antagonists had a different rank order of affinity. MEN
10207 was more potent than MEN 10208 in SKLKB82#3 cells and bovine
membranes in comparison to high potency in rabbit pulmonary artery and low potency
in hamster trachea. The IC$_{50}$ value of the antagonists had a 30-fold difference in affinity
in SKLKB82#3 cells and bovine stomach membranes. This difference was not present
in rabbit pulmonary artery or hamster trachea and may indicate receptor heterogeneity.
### TABLE 7. Comparison of the affinity or potency of neurokinin agonists and antagonists in SKLKB82#3 cells, bovine stomach membranes, rabbit pulmonary artery and hamster trachea. The IC$_{50}$ and pA$_2$ values are the mean of at least three independent experiments done in duplicate. *Maggi et al., 1990a, bPotency = 10$^{pA_2}$, cPotency = 10$^{pA_2}$, RPA = Rabbit Pulmonary Artery, HT = Hamster Trachea, and ND = Not Determined.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>SKLKB82#3 Cells</th>
<th>Bovine Stomach Membranes</th>
<th>RPA*</th>
<th>HT*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ ± SEM</td>
<td>pA$_2$ ± SE</td>
<td>IC$_{50}$ ± SEM</td>
<td>Potency</td>
</tr>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurokinin A</td>
<td>10 ± 2.6</td>
<td>1.2 ± 0.4</td>
<td>4.9$^b$</td>
<td>2.4$^b$</td>
</tr>
<tr>
<td>Substance P</td>
<td>1350 ± 220</td>
<td>53 ± 25</td>
<td>741$^b$</td>
<td>6700$^b$</td>
</tr>
<tr>
<td>Senktide</td>
<td>82,200 ± 13,700</td>
<td>&gt;100,000</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEN 10207</td>
<td>21 ± 6.4</td>
<td>8.2 ± 0.1</td>
<td>54 ± 14</td>
<td>22$^c$</td>
</tr>
<tr>
<td>MEN 10208</td>
<td>691 ± 113</td>
<td>7.2 ± 0.4</td>
<td>1560 ± 473</td>
<td>33$^c$</td>
</tr>
</tbody>
</table>
Figure 12. Competitive binding of [\(^{125}\text{I}\)]NKA with MEN 10207 or MEN 10208 in SKLKB82#3 cells. Each data point represents the mean ± SEM of at least three independent experiments each performed in duplicate. Data points without an error bar contain the error bar within the data symbol. The IC\(_{50}\) value and Hill slope is 21 nM and 0.98 for MEN 10207 and 691 nM and 0.95 for MEN 10208.
Figure 13. The inhibition of NKA-induced $[^3]$H)$IP_1$ accumulation in SKLKB82#3 cells in presence of 10 nM ($\bullet$), 100 nM ($\triangle$), and 1 $\mu$M ($\blacksquare$) MEN 10207. Cells were loaded with 0.2 $\mu$M $[^3]$H$myoinositol for 24 hours and stimulated with NKA for one hour. MEN 10207 alone had no effect on $[^3]$H)$IP_1$ accumulation.
Figure 14. The inhibition of NKA-induced $[^3H]IP_1$ accumulation in SKLKB82#3 cells in presence of 100 nM (●), 1 μM (▲), and 10 μM (■) MEN 10208. Cells were loaded with 0.2 μM $[^3H]$myoinositol for 24 hours and stimulated with NKA for one hour. MEN 10208 alone slightly reduced basal $[^3H]IP_1$ accumulation.
Figure 15. Arunlakshana-Schild linear regression for the effect of MEN 10207 and MEN 10208 on NKA-induced $[^3H]$IP$_1$ accumulation in SKLKB82#3 fibroblasts. Dose ratios were calculated using the EC$_{50}$ values from dose response curves of figures 13 and 14 for each respective antagonist concentration. The MEN 10207 regression line had a slope of -1.11 and an $r=1.0$ while the MEN 10208 regression line had a slope of -1.23 and an $r=0.966$. 
Both peptides acted as competitive antagonists in inhibiting NKA induced $[^3\text{H}]\text{IP}_1$ accumulation in SKLKB82#3 cells by producing a rightward shift of the NKA dose response curve with no decrease in maximum levels (Figures 13 and 14). The difference in affinity was confirmed for the $pA_2$ values which showed that MEN 10207 had greater affinity than MEN 10208 (Figure 15).

In figure 15, the maximum $[^3\text{H}]\text{IP}_1$ accumulation response appears to decrease as the concentration of MEN 10208 increased, thereby indicating noncompetitive inhibition. Close examination of these curves revealed that the maximum $[^3\text{H}]\text{IP}_1$ accumulation response of NKA in absence of MEN 10208 and basal levels of this response in presence of MEN 10208 also decreased. Consequently, when calculating maximum $[^3\text{H}]\text{IP}_1$ accumulation as percent basal for each MEN 10208 concentration, maximum $[^3\text{H}]\text{IP}_1$ accumulation for each concentration of MEN 10208 was not different from the NKA maximum.

Table 7 also lists the IC$_{50}$ value of the NK-2 agonists and antagonists in bovine stomach, the source of the cDNA transfected into B82 fibroblasts. While the IC$_{50}$ values in stomach membranes are twice the value in SKLKB82#3 cells, both preparations share a thirty-fold difference in affinity between MEN 10207 and MEN 10208.

**Discussion**

This study suggests that the NK-2 receptors in SKLKB82#3 cells and bovine stomach membranes differ pharmacologically from the NK-2 receptors in rabbit
pulmonary artery and hamster trachea. The agonist rank order in these preparations identify an NK-2 receptor but the antagonist rank order implies receptor heterogeneity. The antagonist, MEN 10207, has a thirty-fold greater affinity than MEN 10208 for the NK-2 receptors in SKLKB82#3 cells and bovine stomach membrane preparations. In contrast, Maggi et al. (1990a) reports that both MEN 10207 and MEN 10208 have high affinity for NK-2 receptors in rabbit pulmonary artery and low affinity for NK-2 receptors in hamster trachea. The different pharmacological profiles suggest NK-2 receptor heterogeneity or due to a species difference since NK-2 receptors are being compared in bovine, rabbit and hamster tissues.

This study also confirms that the transfected cDNA expresses a protein with an agonist affinity order of NKA > substance P > senktide from competitive radioligand binding assays. This de novo protein is an NK-2 receptor since it is functionally coupled to intracellular calcium mobilization (Henderson et al., 1991) and polyphosphoinositide hydrolysis (Henderson et al., 1990).

The literature contains several reports of NK-2 receptor heterogeneity. Buck et al. (1990) showed distinct NK-2 receptor subtypes when a neurokinin analogue, MDL 28,564 (Asp-Ser-Phe-Val-Gly-Leu(\(\text{CH}_2\text{NH}\))-Leu-NH₂), acted as a full agonist in guinea-pig trachea and rabbit pulmonary artery, as a partial antagonist in hamster urinary bladder, and as an antagonist in rat vas deferens. Maggi et al. (1990a) demonstrated NK-2 receptor heterogeneity in rabbit pulmonary artery and hamster trachea by showing that MEN 10207 and MEN 10208 blocked NKA-induced smooth
muscle contraction with high affinity in rabbit pulmonary artery ($K_n = 22$ nM and 33 nM, respectively) and with low affinity in hamster trachea ($K_n = 1580$ nM and 1170 nM, respectively). Maggi et al. (1991) also identified NK-2A and NK-2B receptors in guinea-pig trachea by demonstrating different affinities for several NK-2 receptor antagonists.

The similar pharmacological profile for SKLKB82 murine fibroblasts and bovine stomach membranes imply that transcription and translation of the transfected NK-2 receptor gene by the host cell did not sufficiently alter the pharmacological properties of this protein. This finding suggests that the observed difference in affinity between MEN 10207 and MEN 10208 is not an artifact introduced from expression of the transfected gene.

In conclusion, the selective antagonists, MEN 10207 and MEN 10208 demonstrate NK-2 receptor heterogeneity among SKLKB82 murine fibroblasts, rabbit pulmonary artery, and hamster trachea. Consequently, these distinct NK-2 receptor subtypes may each represent potential sites for modifying biological responses by antagonists.
Neuropeptides act as intercellular messengers by traveling from one cell to another and then binding to specific cell surface proteins on target cells. This produces second messengers which culminate in a physiological response. Upon exposure to continuous stimulation, most biological systems become desensitized or down-regulated. This enables cells to respond to both changes in the chronic concentration of a signaling ligand as well as acute changes in the absolute concentration.

The neurokinin receptors, NK-1 and NK-2, dramatically differ in their ability to undergo desensitization. Moskowitz et al. (1987) measured neurokinin-induced relaxation of dog carotid artery and reported that substance P and not NKA desensitized the native receptor. In addition, Harada et al. (1987) distinguished the NK-1 receptors expressed by rat brain mRNA from the NK-2 receptors expressed by bovine stomach mRNA by a difference in their desensitization behavior. In these experiments, a second application of substance P failed to change membrane potential in oocytes injected with rat brain mRNA, whereas repeated applications of NKA produced a small decrease in membrane potential in oocytes injected with bovine stomach mRNAs.

The intensity of a biological response may decrease with time despite the presence of an activating agent. Consequently, desensitization to drugs plays a major factor in the efficacy and duration of action of many pharmacological agents. Cells may...
adjust their response to a drug concentration by either decreasing receptor density by sequestration or degradation. It may also inactivate receptors by phosphorylation or uncoupling of the receptor-G protein complex. These experiments were designed to demonstrate NK-2 receptor desensitization in a cell line expressing functional NK-2 receptors. Accumulation of inositol monophosphates is the functional response measured to assess desensitization. The characteristics of the desensitization response will also be examined.

Materials

The source of the materials used in this section are found on page 61 of the materials section in "The coupling of second messengers to the NK-2 receptors expressed in B82 fibroblasts." All other compounds were purchased from Sigma Chemical Co., St. Louis, MO.

Methods

Accumulation of [3H]Inositol Monophosphates

PIP$_2$ hydrolysis was stimulated with 1 μM NKA for 60 minutes and measured by [3H]IP$_1$ accumulation as described on pages 62 to 64 in the methods section of "The coupling of second messengers to the NK-2 receptors expressed in B82 fibroblasts."

Desensitization Time Course

SKLKB82#3 cells were incubated for various time periods in 500 μl of IMDM/40
μg/ml bacitracin, 4 μg/ml leupeptin, and 4 μg/ml chymostatin (incubation media) supplemented with 1 μM NKA and 0.2 μM [3H]myoinositol. The cells were then washed twice with 1.0 ml of IMDM and immediately followed by addition of 450 μl incubation media containing 10 mM LiCl (assay media). After a 10 minute incubation, the assay media was replaced with 450 μl of fresh assay media and PIP₂ hydrolysis stimulated by the addition of 50 μl of various concentrations of NKA to each well.

**Recovery from Desensitization**

SKLKB82#3 cells were desensitized by a 24 hour exposure to 1 μM NKA and 0.2 μM [3H]myoinositol in incubation media. After this treatment, the cells were washed twice with 0.5 ml IMDM. Recovery was accomplished by incubation of treated SKLKB82#3 cells in 1.0 ml of IMDM for various time periods. IMDM contained a limited amount of nutrients to minimize cell growth during the recovery period.

**Calcium Mobilization**

SKLKB82#3 cells were grown in 75 cm² flasks in incubation media supplemented with 1 μM NKA for 18 to 24 hours. Cells were assayed for calcium mobilization by a method previously described on page 62 in "The coupling of second messengers to the NK-2 receptor expressed in B82 fibroblasts."

**Phorbol Ester Treatment**

After a 20 to 24 hour incubation in 0.2 μM [3H]myoinositol/IMDM, SKLKB82#3 cells were placed in incubation media with various concentrations of phorbol 12-myristate 13-acetate (PMA) for 60 minutes. The cells were then washed twice with 1.0
ml of IMDM and placed in assay buffer for 10 minutes.

**Desensitization Selectivity**

SKLKB82#3 cells were incubated for 3 or 24 hours in incubation media containing 0.2 μM [3H]myoinositol. Maximum inhibiting concentrations of MEN 10207 or MEN 10208, or stimulating concentrations of NKA, substance P, or senktide were also added to the incubation media. The cells were washed twice with 1.0 ml IMDM and then incubated in assay buffer for 10 minutes.

**Data Analysis**

Except where indicated, all data represents the average of at least three independent experiments performed in duplicate. A nonlinear regression program fitted the dose response curves with three variable parameters (minimum, maximum, and EC₅₀) with a Hill slope fixed at unity (GraphPad, 1990). The student’s t-test was used to determine the significant difference between experimental results. A 95% confidence level was considered significant. Individual data points expressed as the percent of response were calculated by determining the percentage of the [3H]IP₁ accumulation response from naive SKLKB82#3 cells stimulated with 1 μM NKA.

**Results**

**Desensitization Time Course**

Naive SKLKB82#3 cells had a [3H]IP₁ accumulation four-fold over the basal level, while cells treated for various periods with 1 μM NKA showed a time dependent
decrease in maximum $[^3]$HJIP$_1$ accumulation (Figure 16). The EC$_{50}$ values for 1, 3, and 6 hour treatments were similar to the EC$_{50}$ value of naive cells (Table 8). Since the 12 and 24 hour treatments did not exhibit a dose response, EC$_{50}$ values for these time periods were not calculated. An hour of pre-exposure to 1 $\mu$M NKA decreased $[^3]$HJIP$_1$ accumulation 30%; by 3 to 6 hours the inhibition approached 50%; and by 12 to 24 hours the inhibition reached 70% (Figure 17).

**Recovery of $[^3]$HJIP$_1$ Accumulation**

Desensitized SKLKB82#3 cells showed a time dependent recovery of $[^3]$HJIP$_1$ accumulation (Figure 18). No recovery, as assessed by $[^3]$HJIP$_1$ accumulation was apparent by 3 hour. When the time increased to 12 hours, a 32% recovery had occurred; and by 24 hours a 92% recovery was observed. The half time for recovery is approximately 18 hours. Residual NKA from desensitization treatment was sufficiently removed, since basal, naive cells, and 24-hour desensitized cells had similar $[^3]$HJIP$_1$ accumulation levels.

**Calcium Mobilization**

Desensitized SKLKB82#3 cells had lower calcium mobilization than naive cells (Figure 19). Stimulation by 1 $\mu$M NKA, the $E_{\text{max}}$ concentration for Ca$^{2+}$ mobilization, produced an increase of 19.0 $\pm$ 8.6 nM Ca$^{2+}$ in desensitized cells and a rise of 49.9 $\pm$ 9.4 nM Ca$^{2+}$ in naive cells ($p>0.05$). Stimulation by 30 nM NKA, the EC$_{50}$ concentration for Ca$^{2+}$ mobilization, increased Ca$^{2+}$ 10.7 $\pm$ 5.0 nM in desensitized cells as compared to a rise of 19.0 $\pm$ 4.7 nM Ca$^{2+}$ in naive cells ($p<0.05$). Basal
Ca\(^{2+}\) levels were similar in naive cells (163 ± 29 nM) and naive sham-transfected cells (138 ± 10 nM) thereby indicating that expression vector did not affect the Ca\(^{2+}\) response.

**Phorbol Ester Treatment**

SKLKB82#3 cells preincubated in phorbol ester displayed a threshold effect in NKA-induced \(^{3}\)HJIP\(_1\) accumulation (Figure 20). At concentrations greater than 10 nM, PMA decreased \(^{3}\)HJIP\(_1\) accumulation to basal level, while concentrations less than 10 nM had no effect.

**Desensitization Selectivity**

A three hour pretreatment with substance P, senktide, MEN 10207, and MEN 10208 did not reduce \(^{3}\)HJIP\(_1\) accumulation. After a 24 hour treatment, the neurokinin agonists all decreased \(^{3}\)HJIP\(_1\) accumulation (Figure 21). The rank order of inhibition was NKA > senktide ≡ substance P. Treatment with the NK-2 antagonists, MEN 10207 and MEN 10208, showed no significant difference from untreated cells.

**Discussion**

The NK-2 receptor undergoes a time-dependent homologous desensitization while selective NK-2 antagonists did not induce desensitization or hypersensitization. The neurokinin agonists, substance P, and senktide, produced a smaller degree of desensitization than NKA. Desensitization induced by the neurokinin agonists was not due to heterologous
Figure 16. The effect of chronic NKA stimulation on $[^{3}H]IP_{1}$ accumulation in SKLKB82#3 cells. Cells were treated with 1 μM NKA for various time periods, and PIP$_{2}$ hydrolysis was stimulated with various concentrations of NKA. Basal level represents $[^{3}H]IP_{1}$ accumulation in absence of treatment with NKA. This is a representative experiment.
Table 8. Half-maximal stimulatory concentrations for NKA-induced $[^3H]IP_1$ accumulation in desensitized SKLKB82#3 cells. Cells were desensitized for various periods in 1 $\mu$M NKA and $[^3H]IP_1$ accumulation stimulated with various concentrations of NKA. $[^3H]IP_1$ was measured by anion exchange chromatography. $EC_{50}$ values were calculated by nonlinear regression using a three parameter fit (Hill slope set at unity) of the data points.

<table>
<thead>
<tr>
<th>Desensitization Time (hrs)</th>
<th>EC$_{50}$ (nM)</th>
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<tr>
<td>0</td>
<td>1.7</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
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<td>3</td>
<td>1.2</td>
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Figure 17. Percent inhibition of $[^3]$H]IP$_i$ accumulation from pretreatment with 1 $\mu$M NKA for various time periods. Percent inhibition was calculated by extrapolation of individual data points using basal level as 100% inhibition and 100 nM NKA as 0% inhibition. Data points are from figure 16 when SKLKB82#3 cells were treated with 100 nM NKA for various time periods and a second treatment of 1 $\mu$M NKA. This is a representative experiment.
Figure 18. The recovery of $[^{3}H]IP_{1}$ accumulation in desensitized SKLKB82#3 cells. Desensitization was accomplished by exposure to 1 μM NKA for various time periods. A 100% recovery reflects the level of $[^{3}H]IP_{1}$ accumulation produced by 1 μM NKA in untreated SKLKB82#3 cells.
Figure 19. The effect of chronic NKA stimulation on Ca\textsuperscript{2+}\textsubscript{i} mobilization in SKLKB82#3 cells. Desensitized cells had an 18-20 hour exposure to 1 \mu M NKA, while naive cells had no prior exposure to NKA. Ca\textsuperscript{2+}\textsubscript{i} was measured by the change in fluorescence of fluo-3, AM. Ca\textsuperscript{2+}\textsubscript{i} levels in naive and treated sham transfected cells were not different from basal SKLKB82#3 cells. Ca\textsuperscript{2+}\textsubscript{i} mobilization was stimulated by the E\textsubscript{max} (1 \mu M) or EC\textsubscript{90} (30 nM) concentration for NKA-induced Ca\textsuperscript{2+}\textsubscript{i} mobilization. The bars represent the average ± SEM of at least three independent experiments done in duplicate. The asterisk indicates a p<0.05.
Figure 20. The effect of phorbol ester treatment on $[^3H]IP_1$ accumulation in SKLKB82#3 cells. Cells were incubated for 60 minutes in various concentrations of PMA and stimulated with 1 μM NKA to initiate PIP$_2$ hydrolysis. The curve was drawn by a three parameter fit of the data points with the Hill slope equal to unity. The EC$_{50}$ value was 710 pM. A 100% response corresponds to the maximum $[^3H]IP_1$ accumulation due to 1 μM NKA and a 0% response corresponds to basal level.
Fig 21. The selectivity of NK-2 receptor desensitivity in SKLKB82#3 cells. Cells were incubated for 3 or 24 hours in maximum stimulating concentrations of NKA (1 μM), substance P (10 μM), or senktide (100 μM) or maximum inhibiting concentrations of MEN 10207 (1 μM) or MEN 10208 (10 μM). PIP₂ hydrolysis was stimulated with 1 μM NKA. A 100% response reflects the level of [³H]IP₃ accumulation produced by cells with no pretreatment with NKA.
desensitization because SKLKB82#3 cells lack NK-1 and NK-3 receptors. Substance P and senktide probably desensitized the NK-2 receptor because a neurokinin peptide may cross-react with other neurokinin receptor subtypes (Guard and Watson, 1991). Prolonged exposure to these neuropeptides may increase the probability of cross-talk between the neurokinin peptides and the NK-2 receptor in as much as reducing the exposure from 24 to 3 hours did not produce desensitization.

Since the EC_{50} values in desensitized cells were similar to naive cells, the affinity of NKA to the NK-2 receptor did not change with prior treatment with NKA. Desensitization was reversible with an 90% recovery in 24 hours. Along with decreased [^{3}H]IP_{1} accumulation, NK-2 receptor desensitization reduced NKA-induced Ca^{2+} mobilization. This probably reflects coupling of these two biochemical pathways. The literature contains conflicting reports of NK-2 desensitization. Moskowitz et al. (1987) reported substance P and not NKA induced desensitization of canine carotid artery relaxation response. Fujiwara et al. (1985) corroborated this finding by reporting the desensitization of rabbit iris sphincter muscle by substance P and not NKA. Harada et al. (1987) distinguished the tachykinin receptors induced by rat brain mRNA (NK-1) or bovine stomach mRNA (NK-2) by a difference in their desensitization behavior. A second application of SP in oocytes injected with rat brain mRNA failed to change membrane potential. In contrast, a repeat application of NKA produced a small reduction in membrane potential in oocytes injected with bovine stomach mRNAs. All these experiments utilized a short NKA pretreatment followed by a maximum-response
dose of NKA. While a three hour exposure to NKA, the NK-2 receptor expressed by the SKLKB82#3 cells still retains most of its PIP$_2$ hydrolytic activity. Perhaps longer exposure to NKA in these tissue or oocyte preparations would induce a measurable desensitization response.

Desensitization is a complex phenomenon and may involve more than one mechanism. The NK-2 desensitization curve showed two components, a rapid segment probably representing receptor inactivation by the uncoupling of G proteins or receptor phosphorylation and a slower part that may reflect sequestration. Since the time course for desensitizations occurs over hours, sequestration rather than phosphorylation probably is the major mechanism of desensitization for the NK-2 receptors. Recovery of the receptor took several hours and may reflect recycling of the sequestered receptor.

In many G protein-coupled receptors, the uncoupling, sequestration, and translocation of receptors from the plasma membrane involve receptor phosphorylation or phosphorylation of the receptor-coupled regulatory proteins. PMA, which phosphorylates proteins, reduced $[^3H]IP_1$ accumulation in SKLKB82#3 cells. Different numbers of phosphorylation sites may account for the differences in desensitization among neurokinin receptors. The NK-2 receptor contains 17 potential phosphorylation sites, considerably less than the 31 present in the NK-2 receptor or the 30 in the NK-3 receptor. This is consistent with the greater resistance of the NK-2 receptor to desensitization.
DISCUSSION

The primary outcome of these experiments was the construction of a cell line that stably expresses a high density of functional NK-2 receptors. Recent advances in molecular biology brought in a new era of research on cell surface receptors. While several of these biologically active proteins have been cloned and stably transfected into immortal cell lines for characterization, the NK-2 receptor was the first peptide receptor to be stably expressed. One advantage of this technique is that the pharmacology and properties of each receptor type can be compared under identical conditions and in isolation from other receptors. This is an important consideration in the neurokinin field because it can alleviate inherit problems of pharmacological assays utilizing tissue preparations.

The NK-2 receptor, first postulated by Buck et al. (1984), was based upon different rank orders of potency or affinity for natural tachykinins or their fragments on endogenous tachykinin receptors in tissue preparations. This concept was further supported by data obtained with synthetic receptor agonists and antagonists and the cloning and stable expression of tachykinin receptors in immortal cell lines. While three neurokinin receptors have been identified, this division is based upon agonist rank order of potency and may require modification with the future discovery of selective, peptidase resistant antagonists. Recently developed high affinity agonists and antagonists may be instrumental in the identification of new subtypes. The availability of a homogenous population of cells expressing one subtype on NK-2 receptors also led to the discovery
of a novel NK-2 subtype and homologous desensitization of the NK-2 receptor.

Neurokinin A has important physiological effects in the respiratory system. This peptide constricts large airways, enhances vascular permeability, and stimulates mucus secretion. The novel findings of this dissertation, identification of an NK-2 receptor subtype and desensitization of the NK-2 receptor, may have important implications regarding the targeting of this receptor for drug development. Induction of homologous desensitization or receptor antagonism may be a mechanism to reduce bronchoconstriction due to this potent physiological activity. Drugs that alleviate bronchoconstriction may be more effective if they can block NK-1 and NK-2 receptors instead of just NK-2 receptors, since both receptors mediate this physiological response.

Another important aspect regarding the SKLKB82#3 cells and respiration is that the human tracheal NK-2 receptor has 93% homology to the NK-2 receptor in bovine stomach (Gerard et al., 1990). Consequently, the human and bovine NK-2 receptors share considerable amino acid sequences and perhaps also have similar pharmacology. Currently, most receptors have been cloned from rodent cDNAs. In the future, receptors from human genome will be cloned and stably expressed and studies will be more closely related to man. With genomic clones, regulation of the receptor can be investigated.

Tissues respond to neurokinins in a complex manner because they may contain different densities of several neurokinin receptor types. These responses become less clear when the receptors mediate similar biological responses and have affinity for other
neurokinins. A cell line expressing a high density of functional NK-2 receptors offers the advantage of a well characterized receptor in a controlled environment. The cell line, SKLKB82#3, provides a system for the examination of NK₂ receptor expression, agonist affinities, and second messenger coupling. The availability of a cell line expressing a high density of homogenous NK-2 receptors provides a readily available source of NK-2 receptors to assay new drugs and investigate the pharmacological properties of NK-2 agonists and antagonists.
CONCLUSIONS

In the past four years, considerable advances have been made in neurokinin research. Previously, neurokinin agonists and antagonists lacked sensitivity and selectivity and were highly susceptible to endopeptidases. Currently, several potent and specific agonists and agonists are available; some are nonpeptides. The cloning of the neurokinin receptor genes provided specific molecular biological tools that rigorously distinguish between these receptors. Isolation of these genes and induction of stable expression in immortal cells created cell lines such as the SKLKB82#3 cells.

The host cell, B82 fibroblasts, initially lacked neurokinin receptors. After transfection with SKR56S, a bovine stomach cDNA encoding for the NK-2 receptor, the B82 cells expressed a fifty-fold higher density of NK-2 receptors than the endogenous tissue. This de novo receptor had high affinity and selectivity for NKA and, like the endogenous NK-2 receptors, were functionally coupled to PIP$_2$ hydrolysis and intracellular calcium mobilization. In addition, the NK-2 receptor expressed by SKLKB82#3 cells and the endogenous NK-2 receptor in bovine stomach had pharmacologically similar properties regarding agonist rank order of potency and selectivity. Consequently, the SKLKB82#3 cells contain authentic NK-2 receptors.

The advantages of this in vitro expression system are the production of a high density of receptors, a reduction in endopeptidase susceptibility, and a system that contains only one type of neurokinin receptor. These characteristics diminish several
of the unfavorable properties associated with tissue preparations used in pharmacological studies of neurokinin receptors.

The *de novo* NK-2 receptor, in addition to having similar biochemical and pharmacological properties as endogenous NK-2 receptors, exhibited several novel characteristics. Receptor heterogeneity was demonstrated when selective neurokinin antagonists had different affinities for SKLKB82#3 cells and other tissues. The transfected cells may express a novel NK-2 subtype. Since the protein sequence of human lung and bovine stomach NK-2 receptor have considerable homology, they may also have comparable pharmacological properties. Anatomical and physiological evidence indicates NKA has a role in certain respiratory diseases. The SKLKB82#3 cells can serve as a model for the human lung NK-2 receptor and be utilized to screen drugs that have a therapeutic capacity for treating asthma or bronchial hyperactivity.

Another novel property was that the SKLKB82#3 cells displayed a time-dependent homologous desensitization in PIP₂ hydrolysis. This biological response is characteristic of NK-1 receptors but has not been conclusively demonstrated in the NK-2 receptor. Desensitization to drugs plays a major factor in the efficacy and duration of many chemotherapeutic agents. Further investigation into the desensitization of the NK-2 receptor may identify the mechanisms of this response.

The significance of this research is the construction of a cell line that stably expresses a high density of authentic NK-2 receptors. Currently there is no other cell line with a high density of NK-2 receptors. Although several model tissues for the NK-
2 receptor are available, most have limited use for pharmacological research. Since the NK-2 receptor in SKLKB82#3 cells and human lung have similar amino acid sequence, the pharmacological characteristics of these receptors may be alike. This information can be utilized in developing a drug that alleviates NKA-induced bronchoconstriction.

Future directions for this project include further investigation of receptor heterogeneity, examination of the molecular anatomy of this receptor, and determining the mechanism of the desensitization response. One strategy to resolve the possibility of species differences is to express several NK-2 receptor genes from different species in the same host cell type followed by comparison of the pharmacological properties of these clones. In addition, comparison of the amino acid sequence of the NK-2 receptor gene in rabbit pulmonary artery, hamster trachea, and bovine stomach can determine whether each represents a distinct NK-2 receptor subtype. Site directed mutagenesis can demonstrate the contribution of selected amino acid substitutions or deletions in the biological activity of NK-2 receptors. Since all three neurokinin receptor have been cloned, chimeric analysis can also determine the functional significance of longer sections of the proteins. Comparing the difference in agonist and antagonist binding after modification of histidine and tyrosine residues by alkylating agents can infer the importance of these amino acids in ligand binding to the NK-2 receptor. Finally, desensitization mechanisms can be identified by measuring the change in receptor density and receptor mRNA levels before and after desensitization, and determining the ability of the receptor to become phosphorylated via in vitro phosphorylation.
LIST OF REFERENCES


GraphPAD Ver 3.14 (1990) GraphPAD Software, San Diego, CA.


