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The relationship between the agonist-induced activation and desensitization of the human tachykinin NK_2 receptor expressed in *Xenopus* oocytes

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1 Repeated applications of neurokinin A (NKA) to oocytes injected with 25 ng wild-type hNK_2 receptor cRNA caused complete attenuation of second and subsequent NKA-induced responses while analogous experiments using repeated applications of GR64349 and [Nle^{10}]NKA(4–10) resulted in no such desensitization. This behaviour has been previously attributed to the ability of the different ligands to stabilize different active conformations of the receptor that have differing susceptibilities to receptor kinases (Nemeth & Chollet, 1995).

2 However, for *Xenopus* oocytes injected (into the nucleus) with 10 ng wild-type hNK_2 receptor cDNA, a single 100 nM concentration of any of the three ligands resulted in complete desensitization to further concentrations.

3 On the other hand, none of the ligands caused any desensitization in oocytes injected with 0.25 ng wild-type hNK_2 receptor cRNA, even at concentrations up to 10 μM.

4 The two N-terminally truncated analogues of neurokinin A have a lower efficacy than NKA and it is likely that it is this property which causes the observed differences in desensitization, rather than the formation of alternative active states of the receptor.

5 The peak calcium-dependent chloride current is not a reliable measure of maximal receptor stimulation and efficacy is better measured in this system by studying agonist-induced desensitization.

6 The specific adenylyl cyclase inhibitor SQ22536 can enhance NKA and GR64349-mediated desensitization which suggests that agonist-induced desensitization involves the inhibition of adenylyl cyclase and the subsequent down-regulation of the cyclic AMP-dependent protein kinase, possibly by cross-talk to a second signalling pathway.

Keywords: Tachykinin; neurokinin; NKA; agonist-induced desensitization; *Xenopus* oocyte expression; efficacy

Introduction

The human tachykinin NK_2 receptor (hNK_2 receptor) is a member of the superfamily of G protein-coupled receptors (GPCRs; see Regoli *et al*., 1994). Three mammalian tachykinin receptors have been identified each apparently showing a preference for a corresponding endogenous tachykinin peptide, i.e. the NK_1 receptor for substance P (SP), the NK_2 receptor for neurokinin A (NKA) and the NK_3 receptor for neurokinin B (NKB) (but see Maggi & Schwartz, 1997). The tachykinin NK_1, NK_2 and NK_3 receptors all display an ability to stimulate intracellular signalling pathways involving phospholipase C, leading to phosphoinositide breakdown and elevation of intracellular calcium ($[Ca^{2+}]_i$; Guard & Watson, 1991), and to a lesser degree, modulation of adenylyl cyclase (Nakajima *et al*., 1992). All three receptor types additionally share the potential to exhibit rapid desensitization upon repeated agonist application (Hershey & Krause, 1990). The mechanisms for GPCR desensitization have not been satisfactorily resolved but may involve agonist-induced phosphorylation of serine and threonine residues by intracellular kinases e.g. adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase (PKA), protein kinase C (PKC) and specific receptor kinases such as β-adrenoceptor kinase (β-ARK; Lefkowitz *et al*., 1990). The target of the phosphorylation event may be the receptor and/or downstream G-proteins or effector enzymes and therefore the role of kinase phosphorylation in agonist-induced desensitization may be highly complex and may interfere with the transduction signal at a multitude of levels. However, there is a common functional outcome of these desensitization processes which manifests itself as a reduced coupling of the receptor to the signalling system and a potential sequestration of receptors into the cytoplasm (Harden, 1983; Premont *et al*., 1995).

Tachykinin peptides have a highly conserved C-terminal pentapeptide sequence (Phe-X-Gly-Leu-Met-NH_2, in which the variable residue X is an aromatic or branched aliphatic amino acid) which is essential for their ability to activate the mammalian tachykinin receptors. The amino-terminal sequence of the tachykinin peptides, on the other hand, has been proposed to confer selectivity between the endogenous ligands for their preferred receptor subtypes (Cascieri *et al*., 1992), though all the endogenous ligands can act as full agonists at all the mammalian tachykinin receptors. N-terminally truncated peptide analogues have been synthesized for all of the endogenous mammalian tachykinins. Analogues of SP and NKA, such as GR73632 (δAva-[L-Pro^9, N-$MeLeu^{10}$]-SP(7–11)) and GR64349, ([Lys^3, Gly^8, R-γ-lactam-Leu^9]NKA(3–10)), respectively, can display a greater resistance to cell surface endopeptidase lysis and a higher degree of receptor specificity than their endogenous counterparts (Patacchini *et al*., 1989; Hagan *et al*., 1991). In most systems these analogues resemble the endogenous ligands in their ability to induce full agonist responses and to incur desensitization. However, truncation of endogenous mamma-

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lian tachykinins has been noted to result in a reduced potency at their preferred receptor (Regoli *et al*., 1988).

In the experiments presented here, the signalling behaviour and desensitization capability of the hNK_2 receptor were studied using the *Xenopus laevis* oocyte heterologous expression system. The *Xenopus* expression system has been widely used to study the activity of heterologously expressed GPCRs for agonists that induce PLC activation (e.g. Williams *et al*., 1988). Foreign receptors can be expressed in oocytes from cRNA transcripts injected into the cytoplasm or from cDNA encoding the required protein (downstream of a suitable promoter) injected directly into the nucleus. GPCRs coupling to PLC induce lysis of phosphatidyl inositol 4,5-bisphosphate (PiP_2) producing inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG have been shown to cause an increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$) via release from IP_3-sensitive Ca^{2+} stores and activation of protein kinase C (Berridge & Irvine, 1984), respectively. In *Xenopus* oocytes, such a resulting elevation in $[Ca^{2+}]_i$ levels leads to activation of an endogenous Ca^{2+}-dependent chloride current ($I_{Cl(Ca)}$) which can be readily measured using the two-electrode voltage-clamp technique (Smith *et al*., 1985). Activation of the $I_{Cl(Ca)}$ channel causes a rapid efflux of Cl^- ions down their electrical gradient if the clamped holding potentials are negative to the reversal potential of Cl^- ions in the oocyte and the perfusate. In an unclamped cell, depolarization from the resting potential (around -20 to -60 mV) would occur due to the net inward current equivalent to the induced efflux of Cl^- ions.

Methods

Vector constructs and RNA synthesis

A 1260-base pair *Bam*HI/*Nde*I restricted fragment containing the human NK_2 receptor cDNA (Arkinstall *et al*., 1994) was introduced into the polylinker region of pEF-BOS using *Bst*XI adaptors (Bhogal *et al*., 1994) and was also subcloned, downstream of the T7 polymerase initiation site, into the vector pcDNA3. The pEF-BOS construct was used for cDNA injection into oocytes while the pcDNA3 construct was used to allow *in vitro* transcription of receptor cRNA primed with cap dinucleotide $m^7G(5')ppp(5')G$ using a MEGAscript RNA synthesis kit (Ambion).

Xenopus *oocyte preparation for injection*

Wild-type (Blades) and lab-bred (Nottingham University) female frogs were anaesthetized by immersion in 7.7 mM 3-aminobenzoic acid ethyl ester (methanesulphonate) at room temperature for 20–40 min and killed by decapitation. Ovarian follicles were removed and then placed in 2 mg ml^{-1} collagenase 1A in Ca^{2+}-free frog Ringers solution (in mM: NaCl 82, KCl 2, $MgCl_2$ 1, pH 7.2 with NaOH) and mechanically agitated for 1.5–2 h. After collagenase treatment, any extraneous follicular cell layers were removed manually with forceps. Only state V and VI oocytes were selected for experimentation. Oocytes were then washed five times in Ca^{2+}-free frog Ringers solution and either injected 2 to 4 h after defolliculation or stored overnight at 19.6°C in a modified Barth's solution (in mM: NaCl 88, KCl 1, $NaHCO_3$ 2.4, $MgSO_4.7H_2O$ 0.82, $Ca(NO_3)_2.4H_2O$ 0.33, $CaCl_2.6H_2O$ 0.41 and Tris-HCl 7.5; pH 7.6 supplemented with 10 μg ml^{-1} benzylpenicillin and streptomycin sulphate) for injection the next day.

Injection of hNK_2 cRNA and cDNA

Oocytes were microinjected using a manually driven Drummond 10 μl piston injector. Injection micropipettes for cRNA injection were treated with 0.1% diethyl pyrocarbonate dH_2O and then autoclaved. RNA (50 nl, 0.25–25 ng) was injected into the oocyte vegetal pole but cDNA (20 nl, 10 ng) was injected into the oocyte nucleus which was clearly visible in the animal pole after the oocytes were subjected to centrifugation for 5 min at 600 *g* in a Centaur 2 MSE centrifuge swing-out rotor.

Electrophysiological recording

Standard two-electrode voltage-clamp recordings were made from oocytes at 24 and 72 h post injection of cRNA and cDNA, respectively. Oocytes were voltage-clamped at -80 mV (to minimize any extraneous potassium currents) with two 3 M KCl microelectrodes (1–2 MΩ) using an Axoclamp 2 current- and voltage-clamp amplifier (Axon Instruments, Axon Industries, Burlingame, CA, U.S.A.). Amplifier outputs (sampled at 1 kHz and filtered at 500 Hz) were directly recorded onto computer hard disk via a CED-1401 digital interface and CED current and voltage-clamp software on a 486 PC. Agonists and other reagents were dissolved in the perfusate, ND96 (comprising in mM: NaCl 96, KCl 2, $MgCl_2$ 1, HEPES 5 and $CaCl_2$ 1.8; pH 7.4 with NaOH), and applied to the oocyte via a continuous flow system (2 ml min^{-1} at 22°C). The solution exchange time for the oocyte perfusion chamber was approximately 7 to 8 s. Agonist-induced responses were quantified by measurement of the peak endogenous Ca^{2+}-dependent Cl^- current ($I_{Cl(Ca)}$). For studies of agonist-induced desensitization, agonists were applied at 5–10 min intervals for a minimum of 10 s. When the enzyme inhibitors staurosporine, SQ22536 and Ro-31-8220 were used, they were allowed to equilibrate in the oocyte recording chamber for 5 min before agonist application and then maintained in the perfusate between agonist applications.

In some experimental procedures oocytes were permeabilized to extracellular calcium by incubation with Ca^{2+}-free ND96 perfusate supplemented with the calcium ionophore A23187. Insertion of the ionophore in the oocyte cell membrane was achieved by perfusing the oocyte with a modified ND96 perfusate in which the $CaCl_2$ was omitted, the $MgCl_2$ was increased to 5 mM and 1 mM EGTA was added. The concentration of A23187 employed was 2 μM and was allowed to equilibrate with the cell for five minutes in the perfusion chamber. After this five minute incubation period A23187 was removed from the Ca^{2+}-free ND96 media. Calcium-activated chloride currents were activated in such cells upon the restoration of extracellular calcium to the cell perfusate.

Assessment of the reversal potential (E_{rev}) of the agonist-induced chloride conductances was made using a ramped voltage pulse protocol. In such experiments the cell potential was ramped from -80 to $+50$ mV over 500 ms at a frequency of 0.5 Hz before and after agonist application. Introduction of the Ca^{2+}-chelator EGTA to the interior of the oocyte was achieved by direct injection into the cell of a sterilised 2 mM aqueous EGTA solution (pH 7.4). Estimating the mean oocyte volume to be 1 μl, injection of 50 nl of this solution into the cell 30 min before electrophysiological recording would result in a final intracellular concentration of 100 μM. The chloride channel blocking agent niflumic acid (2-(α,α,α-trifluoro-m-toluidino)nicotinic acid) was introduced to the oocytes by its addition to the ND96 perfusate. Application of the niflumic

acid vehicle, 0.1% DMSO-ND96, to the oocyte did not have any significant effect upon the electrophysiological properties of the oocyte.

Materials

NKA, staurosporine, Ro-31-8220 (3-[1-[3-(amidinothio)propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)-maleimide-methane sulfonate) and SQ22536 ([9-(tetrahydro-2′-furyl)adenine]) were obtained from Calbiochem. [Nle^{10}]NKA(4–10) was obtained from Peninsula Laboratories and GR64349 was generously donated by GlaxoWellcome. [^{3}H]-SR48968 (N-methyl-N-(4-(4-acetamido-4-phenylpiperidinyl)-(2S)-(3,4-dichlorophenyl)butyl)benzamide; 22.6 Ci $mmol^{-1}$) was obtained from Dupont-NEN. Collagenase 1A and basic chemical reagents were obtained from Sigma. SR48968[*] was kindly supplied by Sanofi Recherche.

Results

Functional expression of the hNK_2 receptor in oocytes

Oocytes injected with 25 ng of cRNA for the WT hNK_2 receptor exhibited large $I_{Cl(Ca)}$ current responses after application of neurokinin agonists (0.02–10 μM, for 10 s) to the perfusate; a typical response generated is depicted in Figure 1a. The agonist-induced response was not observed when the oocytes were first incubated for 5 min with 10 nM of the NK_2 receptor antagonist SR48968, further indicating the expression of functional hNK_2 receptor ($81 \pm 3.8\%$; $n=4$). Tachykinin agonist-induced responses were also greatly attenuated by the pre-injection of EGTA and by the addition of 500 μM niflumic acid to the extracellular perfusate. Treatment of oocytes injected with 25 ng WT hNK_2 receptor cRNA with EGTA or niflumic acid inhibited control current responses to 100 nM NKA by $89 \pm 5\%$ ($n=4$) and by $87 \pm 6\%$ ($n=6$), respectively.

Using an oocyte membrane isolation procedure and membrane binding assay similar to that of Nemeth & Chollet (1995) an estimate of the K_d and B_{max} for the non-peptide NK_2 receptor antagonist [^{3}H]-SR48968 was determined from oocytes injected with 25 ng WT hNK_2 receptor cRNA. Analysis of data gained from competitive, homologous binding assays enabled the K_d to be estimated at approximately 6.5 nM and B_{max} at approximately 3.5×10^9 receptors per oocyte (data not shown).

Maximal current responses were observed with a minimum concentration of 80 nM for the three ligands used in the majority of these experiments. However, a high degree of variation in the peak current magnitude was observed between different oocytes injected with the same amount cRNA and stimulated with the same concentration of each agonist (Figure 1b).

The agonist-induced response was unaffected by 24 h incubation with 2 μg ml^{-1} pertussis toxin (PTX); 100 nM NKA induced currents in control and PTX-treated cells were 1062 ± 212 nA ($n=15$) and 1155 ± 337 nA ($n=8$), respectively. Oocytes not injected with cRNA or cDNA (or injected with a volume of RNase-free dH_2O water corresponding to the volumes of cDNA and cRNA injected) displayed no induced chloride current upon application of any of the tachykinin agonists at any concentration.

The agonist-evoked current reversed in cRNA injected oocytes at -23 ± 2.4 mV ($n=3$) which is similar to the reversal potential obtained in oocytes for Cl^- ions (Guillemare *et al.*, 1994). Evoked currents comprised a rapid initial current reaching peak within 10 s followed by a slow current reaching peak in up to 120 s after agonist application (Figure 1a). The initial current is assumed to be due to $I_{Cl(Ca)}$ activated by $[Ca^{2+}]_i$ mobilization while the slow component is mediated by

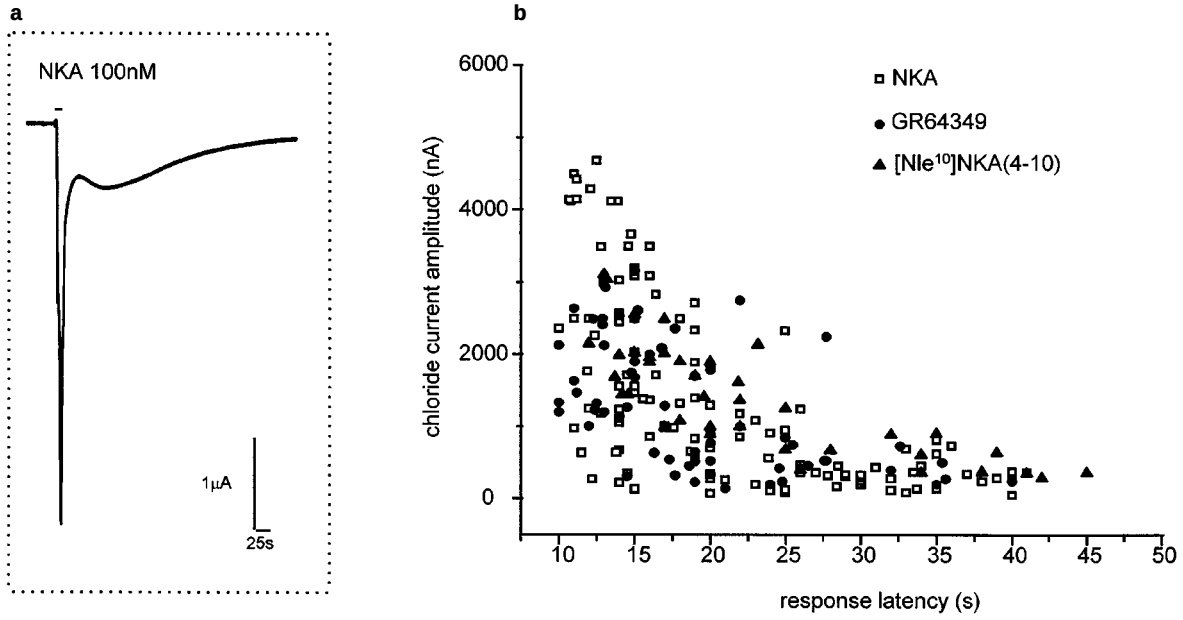

Figure 1 Chloride current responses induced by tachykinin agonists in oocytes expressing the human NK_2 receptor. (a) A typical chloride current response initiated in a cell injected 24 h previously with 25 ng WT hNK_2 receptor cRNA by the application of the tachykinin agonist NKA (horizontal bar). The N-terminally truncated NKA analogues generated similar currents when applied at the same concentrations as NKA. A relationship common to all the agonists was evident between the response amplitude and latency. (b) Demonstrates that for a single agonist concentration (100 nM) of each agonist, a considerable variation in the induced current amplitude is seen between individual oocytes. The data plotted represent individual responses to agonist challenges in separate cells. The latency of the response was measured as the time between agonist application and the point at which the current response was maximal. All the three ligand types display a similar relationship between the response amplitude and its latency.

the $I_{Cl(Ca)}$ but activated by Ca^{2+} entry from the perfusate via the Ca^{2+}-release activated channel (I_{crac}; Berridge, 1995). During the tachykinin agonist-induced current responses the membrane potential of the cell was continuously monitored to ensure that an accurate control of the holding potential was maintained during the alteration in membrane conductance induced by the opening of the $I_{Cl(Ca)}$ channels. Even with the activation of maximal current responses ($\sim 5\ \mu A$) the measured clamped potential of the membrane did not significantly deviate from the holding potential of -80 mV.

Desensitization of agonist-induced responses

Oocytes were challenged with tachykinin agonists applied via the perfusate and second agonist challenges were made at either 5 or 10 min after the primary challenge. The degree of agonist-induced desensitization was expressed as a ratio of the second current peak amplitude (R2) to that of the first (R1) and will be referred to as the response ratio (R2/R1). Using a ratio can avoid problems resulting from the high degree of variation seen in peak $I_{Cl(Ca)}$ induced by the same concentration of agonist in different oocytes (Figure 1b).

The degree of agonist-induced desensitization was highly dependent upon the amount of cRNA injected into the oocytes, as well as upon both the agonist type and the agonist concentration. Figure 2 shows that oocytes injected with 0.25 ng cRNA (a) show no significant desensitization to either 100 nM or 10 μM applications of either GR64349 or NKA. In oocytes injected with 2.5 ng cRNA (b), the 10 μM application of NKA was able to induce desensitization. In oocytes injected with 25 ng cRNA (c), desensitization was induced by a lower concentration of NKA (100 nM) and also by 10 μM GR64349, although not by 100 nM GR64349. When 10 ng cDNA was injected (d), both ligands induced desensitization at both concentrations.

The absence of desensitization at certain cRNA levels facilitated the construction of log-concentration response curves by utilizing multiple responses from a single oocyte thus avoiding the variation in the peak current observed across different oocytes (Figure 1b). Figure 3a shows a series of applications at 10 min intervals of varying concentrations of GR64349 to a single oocyte which shows no significant accumulating desensitization. The log-concentration response curve (Figure 3b) was constructed using the data from multiple oocytes which were combined only after the current amplitudes within each series had been normalized to the maximal current within each individual oocyte (obtained 15–20 min after the completion of the series by applying 1 μM GR64349).

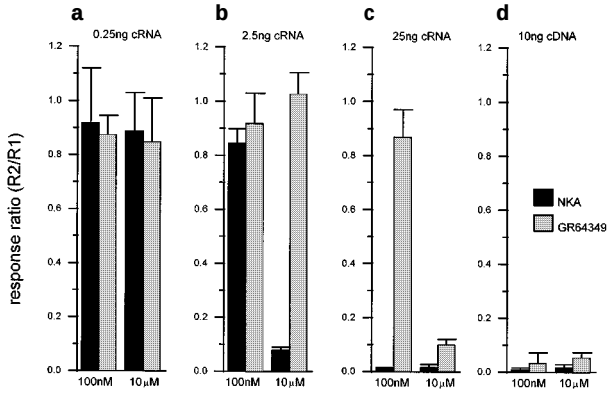

Figure 2 Modulation of the degree of agonist-induced desensitization generated by high and low concentrations of GR64349 and NKA across various receptor expression paradigms. (a–d) Represent the mean ± s.e.mean R2/R1 response ratio ($n \geqslant 4$ for each column) obtained from two successive agonist applications (separated by 10 min) at the concentration indicated below the columns. The quantity and type of nucleic acid injected are indicated above the respective panels, (a) to (d).

Maximal receptor stimulation

Oocytes injected with 2.5 ng cRNA were used to compare the maximal chloride current induced by the truncated agonists relative to that induced by NKA. The protocol used involved the application of an increasing concentration of one of the truncated ligands to a single oocyte at 10 min intervals. Once the maximal current had been obtained, this series of amplitudes was normalized relative to the peak current obtained from a single application of 20 μM NKA applied to the same oocyte 15–20 min after the completion of the series. Figure 4a shows the results from one such experiment. The normalized results from multiple oocytes can then be combined and plotted as a log-concentration response curve as shown in Figure 4b. The maximal currents induced by GR64349 and [Nle^{10}]NKA(4–10) were $39 \pm 7\%$ ($n = 5$) and

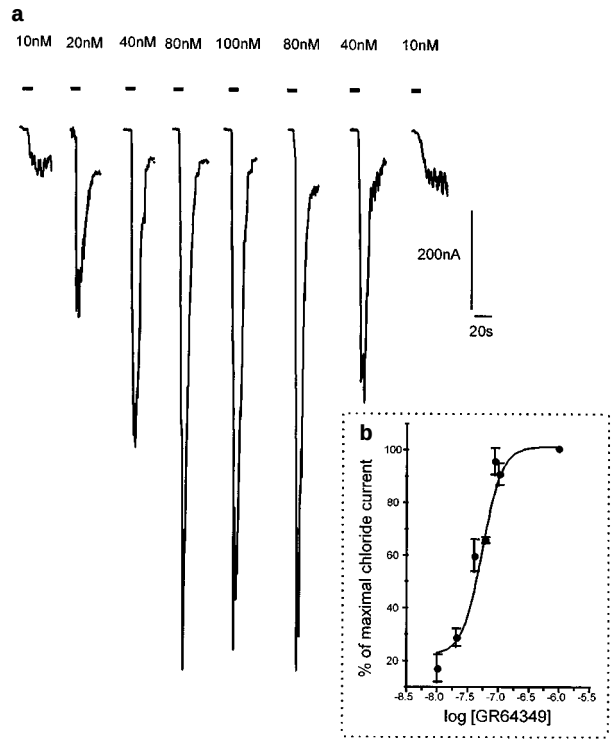

Figure 3 Construction of GR64349 log concentration-response relationships. (a) Chloride current responses from a single oocyte, injected with 25 ng WT hNK_2 receptor cRNA, induced by ascending and then descending GR64349 agonist concentrations. Each agonist application, all to the same cell, were separated by 10 min intervals. Little agonist-induced desensitization is observed in this paradigm of agonist concentration and receptor expression which facilitated the estimation of the full response series at least twice in each oocyte. (b) The resultant averaged log concentration-response relationship produced from a series of multiple applications of GR64349 (10 nM to 1 μM) to single cells. A GR64349 response series created in a single oocyte was first normalized to the cells GR64349-induced maximal response (R_{max}), thus the percentage response for each specific agonist concentration could be averaged between multiple oocytes. The GR64349-R_{max} was estimated using a single applied concentration of 1 μM concentration applied at the end of the series. Each data point represents the mean and vertical lines s.e.mean of $\geqslant 3$ separate cells. The estimated GR64349 EC_{50} for the activation of the endogenous $I_{Cl(Ca)}$ was ~ 45 nM.

$35 \pm 3\%$ ($n=5$) of the maximal NKA response, respectively, within single cells. Hence both the truncated NKA analogues demonstrated a significantly ($P<0.05$) lower ability to stimulate the chloride current response in such cells. Although the same trend was observed in oocytes injected with 25 ng cRNA, the difference in the maximal peak sizes were not significant at this level.

In oocytes injected with 25 ng WT hNK_2 receptor cRNA, the application of 100 nM and 10 μM GR64349 to the same oocyte yielded similar peak chloride currents. A protocol of four successive GR64349 or [Nle^{10}]NKA(4–10) applications at 10 min intervals to individual oocytes was utilized to study the relationship between measured peak chloride current and desensitization threshold. In such protocols (Figure 5a and b), two consecutive 100 nM applications were followed by two successive 10 μM applications. The two 100 nM GR64349 applications induced minimal desensitization (response ratio of 0.89 ± 0.04, $n=4$) whereas, despite a similar level of chloride current activation, the successive 10 μM applications induced a marked degree of desensitization (response ratio of 0.11 ± 0.04, $n=4$ Figure 5a). A similar result was obtained with [Nle^{10}]NKA(4–10); the 100 nM agonist applications yielded a response ratio of 0.97 ± 0.10 ($n=4$) while the two successive 10 μM applications produced a response ratio of 0.22 ± 0.06 ($n=4$: Figure 5b). In control experiments where four consecutive 100 nM agonist applications were applied there was observed to be no significant degree of desensitization induced by these applications (data not shown). Figure 5c shows a comparison of the concentration-dependance of chloride current activation and desensitization for GR64349 using oocytes injected with 25 ng cRNA. The EC_{50} value for desensitization was approximately 14 fold greater than that for chloride current activation.

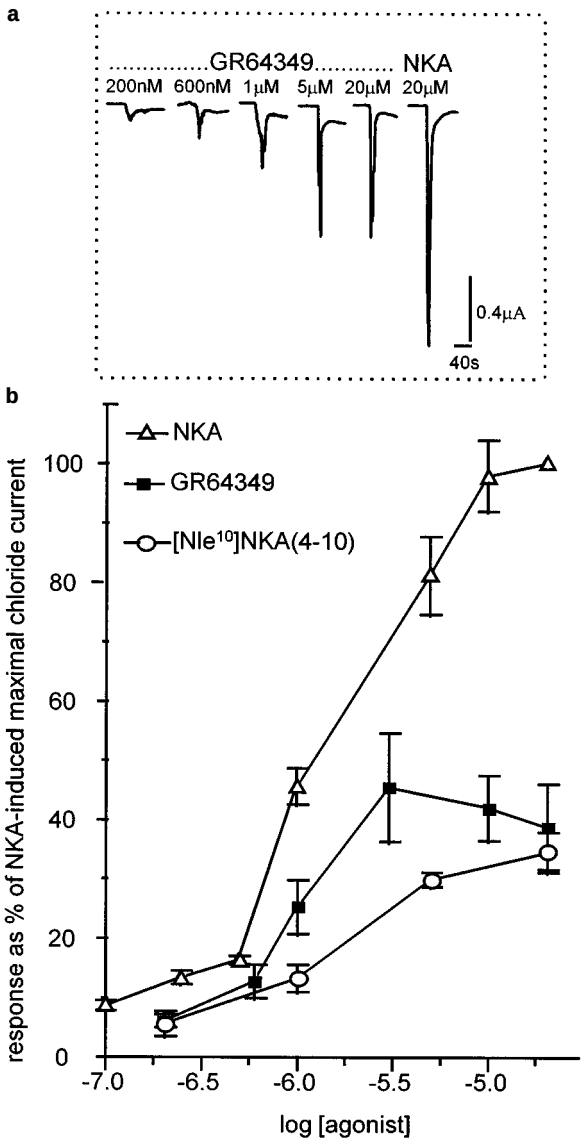

Figure 4 Partial agonist activity of the N-terminally truncated NKA analogues. A typical concentration application protocol employed to create the response series is displayed in (a). Successively increasing concentrations of the truncated ligands were applied at 10 min intervals to the same cell until a maximal response (R_{max}) was achieved. These current responses were then normalized to the NKA-R_{max} within that cell. The NKA-R_{max} was assessed using a 20 μM concentration applied after the concentration-response series. The NKA concentration-response series were generated and normalized to NKA in a similar manner. (b) Concentration-response series for NKA and the N-terminally truncated NKA analogues gained from oocytes injected with 2.5 ng WT hNK_2 receptor cRNA. The relationship shown for each ligand represents the mean of at least four separate normalized series generated from different cells. Hence when normalized to the NKA-R_{max} within the same cell both of the N-terminally truncated ligands appeared to stimulate the chloride current to a significantly lesser degree than NKA.

Modulation of agonist-induced receptor desensitization: involvement of endogenous kinase enzymes

In a recent study, the non-desensitizing behaviour of GR64349 was used to demonstrate the facilitation of agonist-induced desensitization by an intracellular protein kinase inhibitor staurosporine (Nemeth & Chollet, 1995). We have reproduced this effect using a non-desensitizing paradigm (application of 80 nM GR64349 to 25 ng hNK_2 receptor cRNA injected oocytes, applied at 5 min intervals yielding a response ratio of 0.80 ± 0.3, $n=3$) and have observed that desensitization was facilitated by a 5 min pre-incubation with 1 μM staurosporine (response ratio of 0.11 ± 0.07, $n=3$; Figure 6a). However, we have also observed a similar facilitation of agonist-induced desensitization with NKA by 1 μM staurosporine (Figure 6a) when we used a paradigm that did not produce a marked degree of agonist-induced desensitization. Injection of 2.5 ng cRNA for the WT hNK_2 receptor with applications of 100 nM NKA at 5 min intervals yielded a response ratio of 0.6 ± 0.03 ($n=3$). With a 5 min pre-incubation with 1 μM staurosporine, the response ratio decreased to 0.08 ± 0.04 ($n=3$).

In contrast pre-incubation with the specific PKC inhibitor Ro-31-8220 did not facilitate agonist-induced receptor desensitization in the same protocols as for staurosporine (Nemeth & Chollet, 1995; Figure 6b). Repetition of these experiments with 5 min pre-incubation with the adenylyl cyclase inhibitor SQ22536 (10 μM) facilitated desensitization for both NKA and GR64349 in a similar fashion to staurosporine (Figure 6c): response ratios for NKA and GR64349 in the absence of SQ22536 were 0.61 ± 0.01 ($n=5$) and 1.04 ± 0.04 ($n=5$), respectively, but in the presence of 10 μM SQ22536 these were reduced to 0.1 ± 0.07 ($n=7$) and 0.12 ± 0.09 ($n=4$), respectively.

With identical enzyme inhibitor application protocols, the direct effect of staurospine and SQ22536 upon the chloride channel itself was assessed. Hence, chloride currents were activated, in the absence of cell surface stimulation, by the re-application of extracellular Ca^{2+} to A23187-permeabilized cells that had been maintained in Ca^{2+}-free ND96. Restoration of extracellular calcium generated a biphasic chloride current at the holding potential of -80 mV. Upon removal of

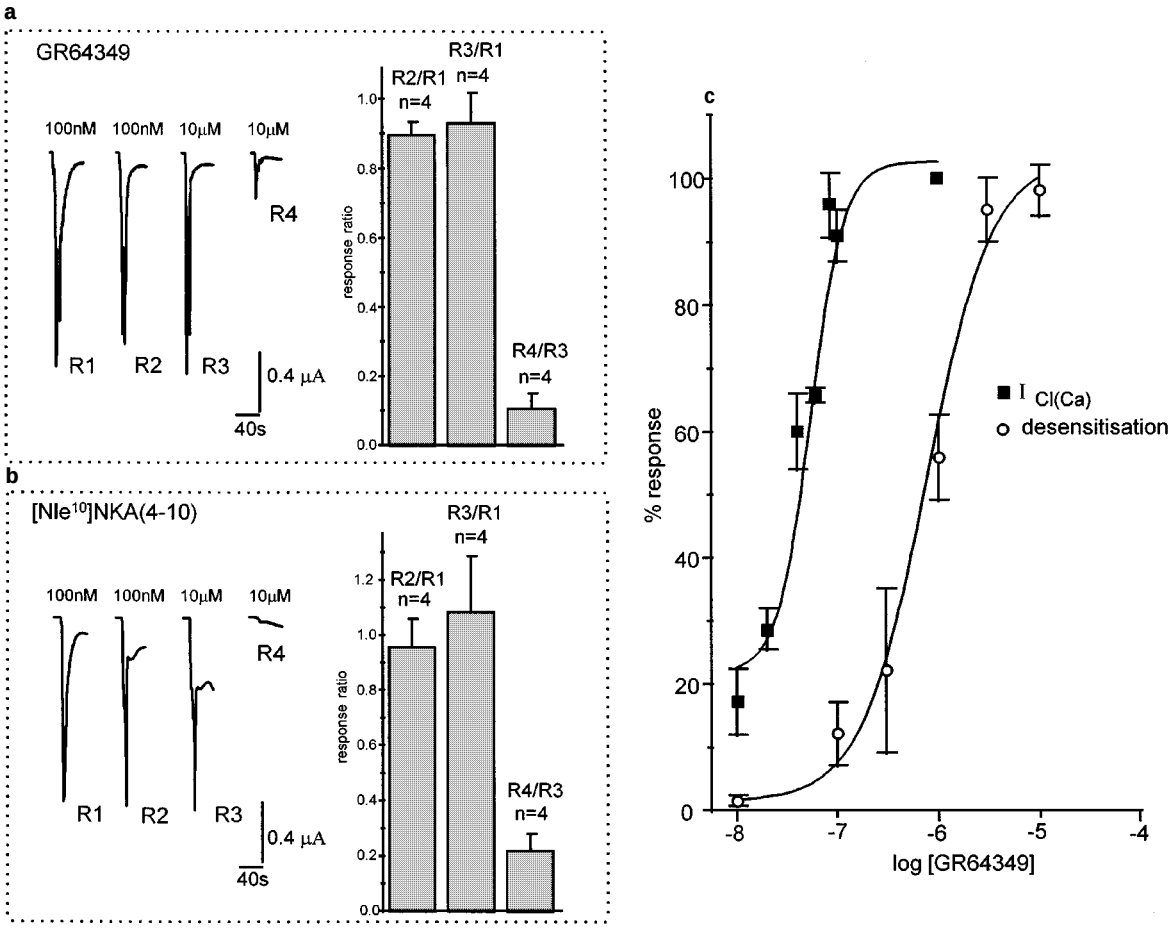

Figure 5 Separation of agonist concentrations required to activate the chloride conductance and desensitization. (a) and (b) The ability of agonist concentration increases, for both the N-terminally truncated NKA analogues, to augment desensitization without significantly increasing the amplitude of the current response itself. All the responses measured for both ligands in these experiments were from oocytes injected with 25 ng WT hNK_2 receptor cRNA. In both (a) and (b), the four current records R1 to R4 represent four consecutive applications of the agonist to a single respective cell. The salient response ratios generated from such an application series: R2/R1; R3/R1 and R4/R3, were averaged over several such protocols applied to multiple cells. (c) Concentration-'response' relationships for chloride current activation and for the generation of response desensitization for specific agonist concentrations. The creation of the chloride current-response curve has already been described. The agonist-induced desensitization 'response' was measured as the complement of the R2/R1 response ratio induced by a pair of consecutive agonist applications of a specific concentration to an individual cell. Hence a respose ratio of 0.6 is represented by a desensitization 'response' of 40%. It is clear that a concentration separation exists between the ability of the agonist ligand to stimulate the chloride current and induce desensitization.

the Ca^{2+} the membrane conductance quickly returned to baseline levels. This Ca^{2+}-activated conductance could be repeatedly generated from a single oocyte without a significant waning of the peak current amplitude. Pre-incubation of oocytes with 1 μM staurosporine or 10 μM SQ22536 did not significantly alter the ability of Ca^{2+}-restoration to activate the chloride current (1215 $\pm$ 113 nA compared to 1198 $\pm$ 171 nA in presence and absence of SQ22536, respectively; 1139 $\pm$ 190 nA compared to 1012 $\pm$ 250 nA in presence and absence of staurosporine, respectively).

Discussion

Efficacy and receptor density

GPCRs are believed to interconvert between multiple states which can be simplified to an inactive R form, an activated R* form and a G-protein-coupled R*G form (Samama *et al.*, 1993). The extent to which a GPCR signal transduction cascade is activated is dependent upon the number of activated receptor-G-protein complexes (R*G), i.e. it is the size of the appropriate R*G population that is responsible for the extent of the pathway activation. Since the proportion of the receptor population which is in the R*G state is dependent upon the concentration of agonist, an increase in agonist concentration produces an increase in the degree of pathway activation. A partial agonist (an agonist possessing a lower efficacy compared to a full agonist) cannot promote the formation of sufficient R*G for a maximal pathway activation. However, it is well known that this effect can be overcome by increasing the total receptor population (Leff, 1995). Indeed, one of the dangers of using heterologous expression systems is that the increased number of expressed receptors can often exaggerate the efficacy of an agonist so that a partial agonist appears to act as a full agonist.

We have attempted to manipulate receptor density by injecting different amounts of cRNA into the oocytes, since other studies have shown that there is a relatively linear relationship between the amount of injected cRNA and the amount of expressed protein product and hence of the biological response mediated, e.g. electrophysiological (Ne-

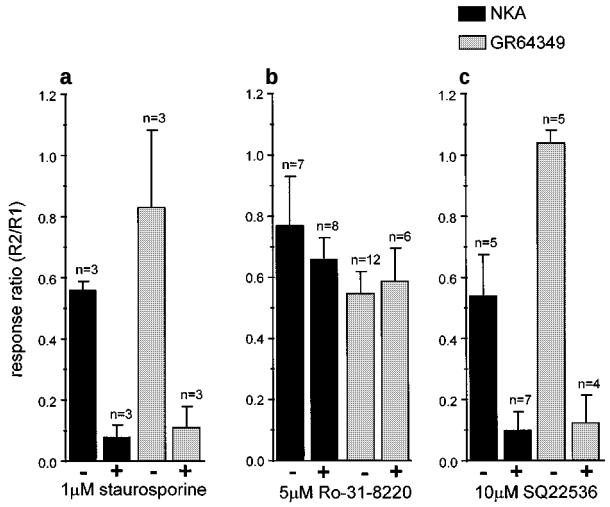

Figure 6 Inhibitors of intracellular enzymes modulate the agonist-induced desensitization generated by NKA or GR64349 receptor activation in non-desensitizing paradigms. The histograms in (a) to (c) depict the response ratios generated by application of two consecutive NKA (100 nM) or GR64349 (80 nM) concentrations, separated by 5 min to oocytes injected with respectively, 2.5 and 25 ng wild-type NK_2 receptor cRNA. In both these receptor-ligand paradigms a minimal degree of agonist-induced desensitization was demonstrated in control conditions. Pre-incubation of the oocytes with either 1 μM staurosporine (a) or 10 μM SQ22536 (c), before agonist application caused a reduction in the response ratio (R2/R1) generated by the subsequent agonist challenges. Thus incubation with these enzyme inhibitors induced an increase in the degree of agonist-induced desensitization generated by the same concentration of NKA or GR64349 used in their respective non-desensitizing paradigms. However, a similar pre-incubation with 5 μM Ro-31-8220 did not potentiate the degree of agonist-induced desensitization (b).

meth & Chollet, 1995) or biochemical (McCaman *et al*., 1988). Injection of cDNA generally yields greater levels of protein expression, compared with injection of cRNA, due to continued production of transcript from the vector injected into the oocyte nucleus (Soreq & Seidmean, 1992). Although we have not measured the level of receptor expression directly, the trend in our data (Figure 2) suggests that the injection of 10 ng cDNA results in greater receptor expression compared to 25 ng cRNA. However, a consequence of using cDNA injection is that there is also an inherent lack of control of the amount of expressed protein and therefore it was for this reason that cRNA was used in most of the experiments in this study.

Figure 2 shows how the alteration of receptor number affects the ability of the agonists to induce receptor desensitization. These data suggest that GR64349, at each particular concentration, requires a greater receptor density to induce desensitization relative to the same concentration of NKA. If receptor desensitization is a measure of the extent of receptor-mediated pathway activation, then this indicates that GR64349 has a lower efficacy than NKA. A more classical measure of pathway activation is the measure of the magnitude of the peak agonist-induced chloride current. Using a relatively low receptor density (2.5 ng injected cRNA), it is clear that both of the truncated ligands are less efficacious than NKA since they can only produce approximately half the maximal chloride current activation of the physiological ligand (Figure 4). At a higher receptor density (25 ng injected cRNA) the difference between the maximal induced currents is not significant, although the different efficacies can be clearly detected by the varying abilities of the ligands to induce desensitization. The latter scenario has previously been interpreted as the result of the different abilities of the various ligands to stabilize active conformations of the receptor that

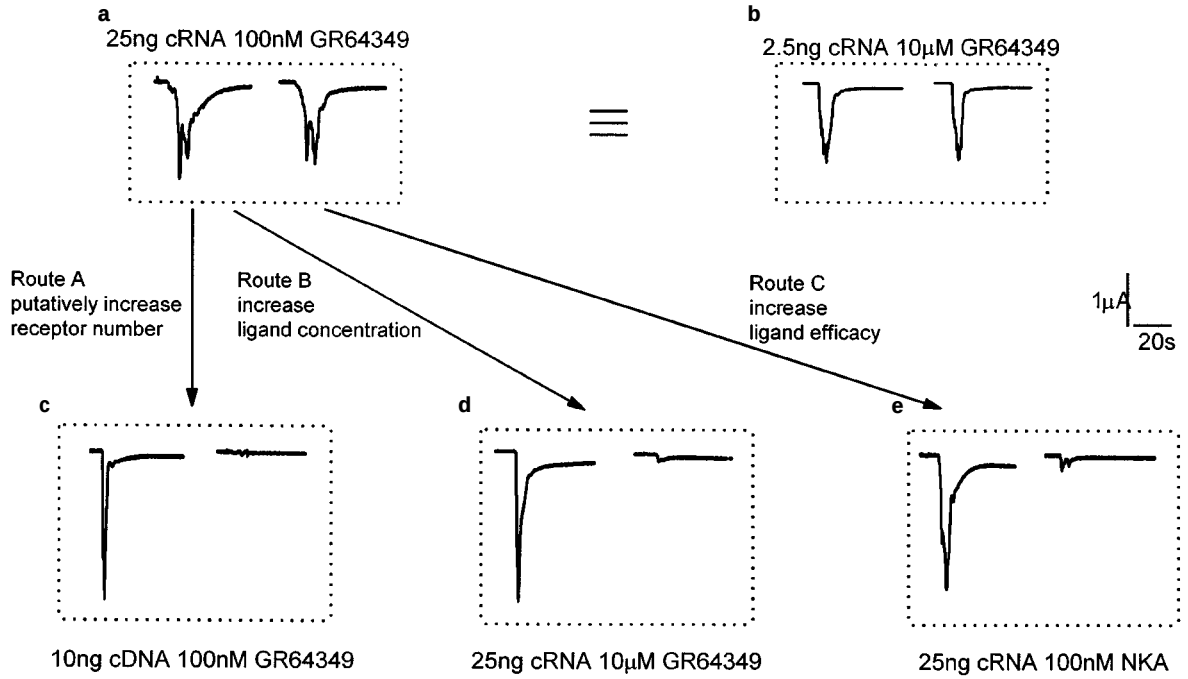

Figure 7 Modulation of GR64349 induced desensitization in *Xenopus* oocytes by changes in: ligand efficacy; ligand concentration and receptor number. The non-desensitizing paradigms for GR64349 current activation are shown in (a) and (b) and paradigms in which significant desensitization occurs, (c, d and e). Desensitization of the GR64349 response can be facilitated by the process in routes A to C. Route A involves a putative increase in the expressed WT hNK_2 receptor resulting from the injection of cDNA rather than cRNA which facilitates desensitization resulting from the same concentration of GR64349, reducing the response ratio from 0.86 ± 0.1 ($n=5$) to 0.03 ± 0.001 ($n=10$). Route B involves the increase of GR64349 concentration to allow desensitization, producing a reduction in response ratio from 0.86 ± 0.1 ($n=5$) to 0.09 ± 0.02 ($n=9$). Route C involves the use of a ligand of higher potency, i.e. NKA, and produces a reduction of response ratio from 0.86 ± 0.1 ($n=5$) to 0.013 ± 0.005 ($n=9$). All current responses shown are from individual cells challenged twice with the specific agonist separated by a 10 min washing interval.

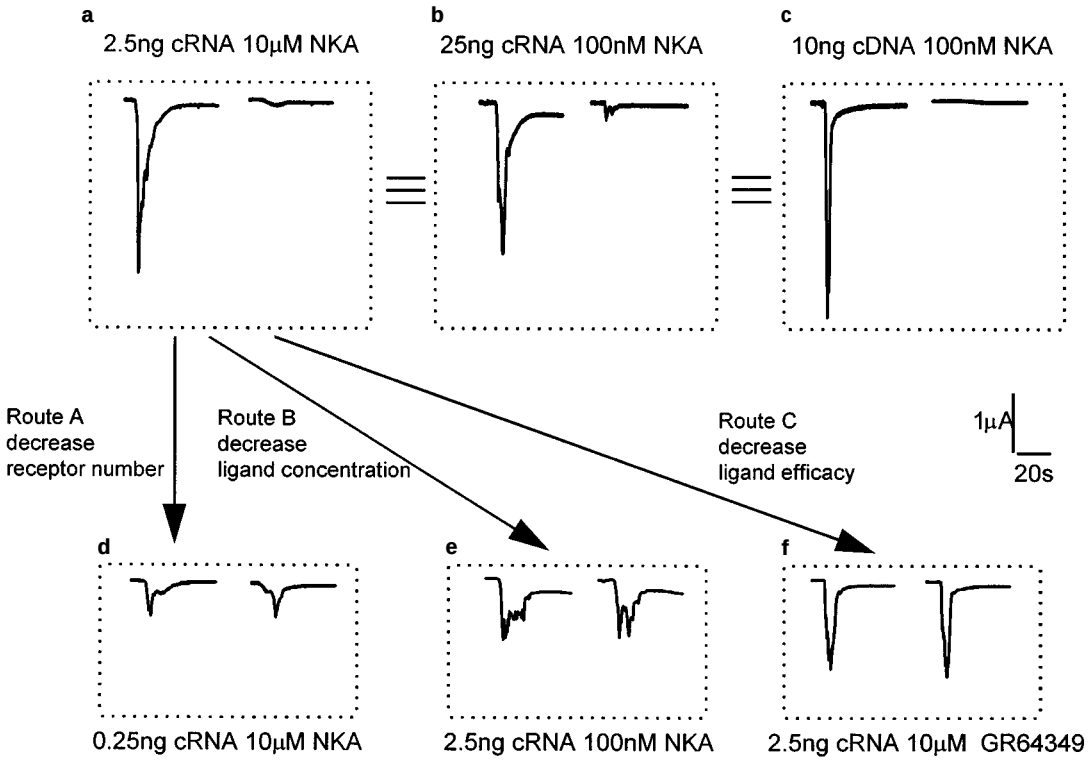

Figure 8 Modulation of NKA induced chloride current responses desensitization in *Xenopus* oocytes by changes in: ligand efficacy; ligand concentration and receptor number. Desensitizing paradigms for NKA are depicted in (a), (b) and (c). Desensitization can be abolished by following any of the routes A to C (equivalent to routes in Figure 7). Therefore the response ratio R2/R1 for NKA induced responses can be raised to non-desensitizing levels by: route A, reduction of the amount of injected cRNA (R2/R1 increase from 0.08 ± 0.02 ($n=5$) to 0.9 ± 0.1 ($n=8$)); route B, reduction of concentration of NKA applied to an oocyte injected with a similar amount of WT hNK_2 receptor cRNA (R2/R1 increase from 0.08 ± 0.02 ($n=5$) to 0.84 ± 0.05 ($n=6$)); route C, reduction of ligand efficacy by using GR64349 at a similar concentration to NKA (R2/R1 increase from 0.08 ± 0.02 ($n=5$) to 1.2 ± 0.3 ($n=10$)).

have differing susceptibilities to receptor kinases (Nemeth & Chollet, 1995). However, our data clearly show that both ligands produce qualitatively similar responses and that the data can be fully interpreted using the efficacy argument.

However, we need to explain why, at higher receptor density, desensitization should provide a more sensitive measure of efficacy than the chloride current activation. Figure 5 shows that the activation and desensitization components appear to be initiated within different agonist concentration ranges. It is clear that even when maximal activation of the chloride current has occurred in a non-desensitizing paradigm (e.g. with 100 nM GR64349, Figure 5a; or with 100 nM [Nle^{10}]NKA(4–10), Figure 5b) there is still further capacity for receptor activation since 10 μM of either ligand causes desensitization. This strongly suggests that further agonist binding and receptor activation occurs beyond the maximal stimulation of the chloride channel. This is more apparant in Figure 5c which shows that the EC_{50} for desensitization is shifted rightwards on the log concentration-response curve, compared to that for activation, by greater that one order of magnitude. One interpretation of these data is that the desensitization is mediated through a separate pathway to that of the chloride current activation. Since this second pathway is activated at higher ligand concentrations than the first pathway, it can discriminate agonists of different efficacies at higher receptor densities.

Therefore we can manipulate the size of the R*G population in a number of ways in order to convert a non-desensitizing paradigm to a desensitizing paradigm or *vice versa*. For example, the non-desensitizing paradigm using 100 nM GR64349 as a ligand with oocytes injected with 25 ng WT hNK_2R cRNA can be converted to a desensitizing paradigm by substituting GR64349 with the more potent ligand NKA (Figure 7, Route C). We would suggest that this is the result of the formation of a greater number of R*G complexes and hence a greater activation of the transduction pathway. We can increase the number of R*G complexes by other routes, as shown by the fact that we can also convert the non-desensitizing paradigm to the desensitizing paradigm by either increasing ligand concentration (Figure 7, Route B), or by putatively increasing receptor number (Figure 7, Route A). Figure 8 shows the converse situation in which the desensitizing paradigm (using 10 μM NKA and 2.5 ng WT hNK_2 receptor cRNA) can be converted to the non-desensitizing paradigm by either reducing agonist efficacy (Figure 8, Route C), reducing ligand concentration (Figure 8, Route B) or reducing receptor number (Figure 8, Route A).

Desensitization mechanisms

Homologous agonist-induced receptor desensitization has been studied for other GPCRs in the *Xenopus* oocyte heterologous expression system (Kobilka *et al*., 1987; Sasakawa *et al*., 1994; Lipinsky *et al*., 1995). However, the classical model for GPCR agonist-induced desensitization is based upon work carried out with the β_2-adrenoceptor coupled to G_s and stimulation of adenylyl cyclase (Kobilka *et al*., 1987; Lohse *et al*., 1990; Garcia-Higuera & Mayor, 1994). Generally it has been seen that the desensitization process correlates with increased receptor phosphorylation and often a sequestration of the receptor (Harden, 1983). The targets for phosphorylation are often cited as being the Ser and Thr residues often

present on the C-termini of GPCRs, although some contradictory reports have been made for the need of an intact C-terminus for agonist-induced desensitization in the hNK_2 receptor (Josiah *et al*., 1994; Alblas *et al*., 1995). The tachykinin receptors are generally regarded as preferring the $G_{q/11}$ mediated PLC activation signal transduction cascade, unlike the β_2-adrenoceptor which preferentially stimulates adenylyl cyclase activity via G_s. However, a similar desensitization mechanism may apply to these two different members of the rhodopsin-like GPCR family irrespective of the G-protein coupling, as *in vitro* phoshorylation of the NK_1 receptor by recombinant β-ARK has been observed (Kwatra *et al*., 1993).

Nemeth and Chollet (1995) have shown that the general protein kinase inhibitor staurosporine facilitates GR64349 mediated desensitization of hNK_2 receptors expressed in oocytes, since a non-desensitizing paradigm can be converted to a desensitizing paradigm by the presence of the kinase inhibitor. In our study, pre-incubation with staurosporine also increased the degree of agonist-induced desensitization seen with NKA, providing further evidence that the signalling mechanisms of the two agonists are qualitatively similar (Figure 6a). Furthermore, the application of the membrane permeable and specific adenylyl cyclase inhibitor SQ22536 caused a similar facilitation of agonist-induced desensitization for both NKA and GR64349 (Figure 6c). This suggests the involvement of PKA and adenylyl cyclase in the desensitization process of the hNK_2 receptor in *Xenopus* oocytes. However, the specific PKC inhibitor Ro-31-8220 showed no effect upon the activation of desensitization of the receptor (Figure 6b).

Cross-talk induced desensitization

Hence, our current model for agonist-induced desensitization is that at lower levels of receptor activation, basal PKA levels protect the system from desensitization. At higher levels of receptor activation PKA activity is reduced, via the inhibition of adenylyl cyclase, which allows desensitization to proceed. The cross-talk to the adenylyl cyclase system may not occur via the various $G_{i/o}$ proteins since PTX has no effect, although PTX-resistant agonist-mediated inhibition of oocyte adenylyl cyclase has been demonstrated for progesterone and acetylcholine (Sadler *et al*., 1984). The lack of any qualitative or quantitative effects of PTX incubation could be due to an insufficient incubation time (Blitzer *et al*., 1993) or insensitivity of the oocyte $G_{i/o}$ type proteins to PTX. However, since the chloride channel activation pathway and the desensitization pathway are activated by different agonist concentration ranges, it suggests that the desensitization pathway is activated by cross-talk through an alternative pathway. If the same G-protein was involved in both processes, then we would expect an increased agonist concentration to increase the chloride current as well as the desensitization which is clearly not the case (Figure 5). An increase in receptor number is believed to increase the extent of promiscuous G-protein coupling (e.g. Kenakin, 1995) which, as our observations show, would manifest itself as increased desensitization in our system. Thus the heterologously expressed receptors may be able to couple to multiple G-proteins depending on the number of R*G complexes generated by a specific agonist concentration, the density of receptor expression or the amount and availability of specific G-proteins to the GPCR.

Conclusions

In conclusion our results indicate that the two N-terminally truncated specific NK_2 agonists GR64349 and [Nle^{10}]NKA(4–10) are less efficacious than NKA upon WT hNK_2 receptor heterologously expressed in *Xenopus* oocytes and that it is this property which is responsible for the differences in their abilities to cause agonist-induced desensitization. We have also utilized the oocyte expression system to demonstrate that the use of peak $I_{Cl(Ca)}$ measurements may not be a reliable indicator of maximal receptor stimulation, and that measuring the threshold for agonist-induced desensitization may be a better indicator of the efficacy of specific ligands. However, the size of the submaximal currents, relative to the size of the maximal current within the same cell, is reproducible. Hence, we have shown that the calculation of concentration-response curves in non-desensitizing paradigms is possible by normalizing multiple currents from the same cell. Finally, the facilitation of agonist-induced desensitization in non-desensitizing paradigms by both staurosporine and the specific adenylyl cyclase inhibitor SQ22536 indicates the involvement of adenylyl cyclase and PKA in the desensitization process of PLC linked tachykinin receptors expressed in *Xenopus* oocytes. This pathway, and therefore desensitization, may be activated by pathway cross-talk in situations of high receptor density or high agonist concentrations. The mechanism by which PKA can protect a system from agonist-induced desensitization is of great interest since this kinase is usually associated with the promotion of desensitization.

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