British Journal of Pharmacology (1996) 119, 315-320

Inhibition of Ca^{2+}-sensitive K^+ currents in NG 108-15 cells by substance P and related tachykinins

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1 The whole-cell patch-clamp technique was used to investigate the actions of substance P and other agonists at neurokinin (NK) receptors on voltage-gated K^+ and Ca^+ channel currents in undifferentiated mouse neuroblastoma × rat glioma NG 108-15 cells.

2 Both substance P (0.3-30 μM) and the NK_1 receptor selective agonist GR73632 (10 nM-10 μM) caused concentration-dependent inhibition of K^+ currents. GR64349 and senktide (agonists at NK_2 and NK_3 receptors respectively) also inhibited K^+ currents, but only at concentrations which were several orders of magnitude greater than GR73632, suggesting that current inhibition was mediated via NK_1 receptors.

3 Substance P and GR73632 were without effect on residual K^+ currents recorded in the presence of extracellular Co^{2+} (4 mM) to abolish the Ca^{2+}-sensitive component ($I_{K_{Ca}}$) of the K^+ current. Ca^{2+} channel currents, recorded with either Ba^{2+} or Ca^{2+} as charge carrier, were unaffected by NK_1, NK_2 and NK_3 receptor ligands.

4 Iontophoretic application of GR73632 produced a current-dependent reduction of K^+ currents. In the presence of the non-peptide NK_1 antagonists, CP-99,994 and RP67580, and the peptide antagonist, GR82334, the current-response relationship was reversibly shifted to the right. This indicates that the response is mediated by NK_1 receptors.

5 Our results indicate that activation of NK_1 receptors leads to the selective inhibition of $I_{K_{Ca}}$ in undifferentiated NG 108-15 cells.

Keywords: Substance P; GR73632; NG 108-15 cells; whole-cell patch-clamp; $I_{K_{Ca}}$; Ca^{2+} channel currents; NK_1 receptor; neurokinin

Introduction

Mammalian tachykinins are a family of neuropeptides including substance P, neurokinin A (NKA) and neurokinin B (NKB). These interact with specific tachykinin receptors (NK_1, NK_2 and NK_3 respectively), with each agonist displaying around 1 to 2 orders of magnitude potency over the other peptides for its respective receptor. To use tachykinins as pharmacological tools, peptidase-resistant analogues of the major endogenous ligands have been produced which display higher potency and selectivity than the endogenous ligands; for example GR73632 for NK_1 receptors (Hagan *et al.*, 1989); GR64349 for NK_2 receptors (Hagan 1989) and senktide for NK_3 receptors (Wörmser *et al.*, 1986). The discovery of receptor-selective, non-peptide tachykinin receptor antagonists has also accelerated the characterization of tachykinin receptor interactions. The non-peptide antagonists such as CP-99,994 (McLean *et al.*, 1992), CP-96,345 (Snider *et al.*, 1991), RP 67580 (Garret *et al.*, 1991), for NK_1 receptors, and SR48,968 (Advenier *et al.*, 1992) for NK_2 receptors have also indicated the possibility of receptor heterogeneity for the respective receptors they act upon (Beresford *et al.*, 1991; Gitter *et al.*, 1991). The NK_1 and NK_2 receptors were subdivided into A and B forms in guinea-pig/human and rat/mouse/hamster tissues, respectively (Regoli, 1992; Maggi *et al.*, 1993). Peptide antagonists, as compared with the non-peptide antagonists, tend to display an ambiguity of antagonism with respect to tachykinin receptor heterogeneity; this may be linked to their generally lower potency than the non-peptide antagonists (Beresford *et al.*, 1992).

Activation of tachykinin receptors typically results in hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) producing inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) causing intracellular Ca^{2+} release and activation of protein kinase C (PKC: Berridge & Irvine, 1984; Nishizuka, 1984). Receptor-induced activation of adenylate cyclase has also been shown to occur, but generally at higher tachykinin concentrations than PIP_2 hydrolysis (Nakajima *et al.*, 1992).

Tachykinins are present in a wide variety of neuronal (peripheral and central) and non-excitable tissues. In the central nervous system, tachykinins display a high density in nociceptive primary afferent neurones (Helke *et al.*, 1990), implicating them in the transmission of nociceptive signals into the central nervous system. The action of tachykinins as neurotransmitters is supported by their presence in presynaptic vesicles and the existence of postsynaptic G-protein coupled receptors. Tachykinins have a direct depolarizing action upon neurones (Konishi & Otsuka, 1974) and a modulatory effect upon the action of other neurotransmitters, e.g. excitatory amino acids (Rusin *et al.*, 1993). Direct activation of neurones typically takes place via modulation of ion channels, e.g. suppression of K^+ conductances (Stanfield *et al.*, 1985; Tokimasa *et al.*, 1993) or the activation of non-selective cation conductances (Spigelman & Puil, 1991; Shen & North, 1992). Here, we demonstrate that activation of NK_1 receptors leads to the selective inhibition of Ca^{2+}-sensitive K^+ channels in mouse neuroblastoma × rat glioma hybrid (NG 108-15) cells (Hamprecht, 1977), whilst voltage-gated K^+ currents and Ca^{2+} channel currents are unaffected.

Methods

NG 108-15 cell culture

Mouse neuroblastoma × rat glioma hybrid (NG 108-15) cells were continuously grown in culture in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum,

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HAT (0.1 mM hypoxanthine, 0.4 μM aminopterin and 160 μM thymidine), 100 iu ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine. Cells were maintained in a humidified incubator (37°C, 10% CO_2) and no chemicals were added to promote or induce cell differentiation. When required for study, cells were removed from culture flasks with gentle mechanical agitation and plated onto poly-L-lysine coated coverslips in 35 mm culture dishes where they were allowed to adhere for between 3 and 7 days.

Electrophysiological recording

On each experiment day, fragments of coverslip were transferred to a continually-perfused recording chamber (volume approximately 80 μl, flow rate 1 ml min^{-1}) on the stage of an inverted microscope. The whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) was used to record ionic currents and recordings were limited to cell bodies lacking prominent processes, to avoid possible space-clamp errors. To record K^+ currents, cells were perfused with a solution of (in mM): NaCl 135, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 2.5, HEPES 10, D-glucose 10 (pH 7.4) and patch electrodes were filled with (in mM): KCl 117, EGTA 11, HEPES 11, $CaCl_2$ 1, $MgSO_4$, NaCl 10, Na_2ATP 2 (pH 7.2). To record current flowing through Ca^{2+} channels in the absence of outward K^+ currents, cells were perfused with a solution of composition (in mM): NaCl 110, $BaCl_2$ 10, $MgCl_2$ 0.6, tetraethylammonium Cl 20, CsCl 5, HEPES 5, D-glucose 10 (pH 7.4) and electrodes contained (in mM): CsCl 130, NaCl 10, HEPES 10, $MgCl_2$ 2, EGTA 1.1, $CaCl_2$ 1.1, Na_2ATP 2 (pH 7.2). Electrodes were of 4–10 MΩ resistance, giving series resistances of 10–20 MΩ during experiments, which were not compensated. All recordings were made at room temperature (21–24°C).

Cells were voltage-clamped at −70 mV using a patch-clamp amplifier built in-house and step depolarizations of 100 ms applied at a frequency of 0.2 Hz to elicit outward K^+ currents. To activate voltage-dependent Ca^{2+} channels, cells were voltage clamped at −100 mV and step depolarizations of 200 ms to 0 mV at 0.1 Hz. were applied. Data were filtered at 1 kHz, digitized at 2.5 kHz and stored on computer for subsequent analysis using VCAN software (J. Dempster, Strathclyde University). K^+ currents were sustained throughout and depolarizing step and so were measured by averaging the amplitude over the last 10–15 ms of the depolarization. Ca^{2+} channel currents were measured at their peak and over the last 10–15 ms of each step since they showed time-dependent inactivation. Linear leak subtraction was performed by the appropriate scaling and subtraction of leak currents evoked by small (<30 mV) hyperpolarizing and depolarizing steps. However, since in the case of K^+ currents leak accounted for only approximately 5% of unsubtracted current amplitude, and subtraction procedures rarely removed all capacitance artefacts, unsubtracted traces are used for illustrative purposes. The degree of inhibition caused by agents studied in this paper takes into account the slight degree of run-down of K^+ current amplitudes observed when cells were repeatedly step depolarized, by estimating the predicted current amplitude (expected in the absence of drug) by extrapolation of the pattern of run-down seen during the pre-drug recording period. This ensures that we do not over-estimate the inhibitory actions of any agonists studied.

Multibarrel borosilicate glass micropipettes were manufactured by hand from individual glass capillaries. The iontophoretic pipette was placed in the bathing solution 2–5μm upstream of the voltage clamped cell. Positive iontophoretic currents (50–1000 nA) were applied via chloridized silver wires inserted into the GR73632 (in 0.1 M saline solution) containing barrels. Current compensation was achieved by simultaneous ejection of Cl^- ions from adjacent barrels containing 1 M NaCl. A retaining current of 10–50 nA of opposite polarity to the barrels' respective ejection current was applied for periods when the assembly was placed near the cell under study. Ionotophoretic equipment was all built in-house.

Statistical comparisons were made using Student's paired *t* test, unless otherwise indicated, and results are presented as means ± s.e.mean.

Results

Effects of tachykinins on whole-cell K^+ currents in NG 108-15 cells

Outward K^+ currents recorded in a representative undifferentiated NG 108-15 cell are shown in Figure 1. These currents begin to activate at approximately −30 to −20 mV, and thereafter increase in amplitude linearly with increasing depolarization, as has been previously described (Reeve & Peers, 1992). This outward K^+ current is composed of a Ca^{2+}-sensitive component ($I_{K_{Ca}}$) and a Ca^{2+}-insensitive, voltage-gated K^+ component (I_{K_v}). Using a pipette solution of low free [Ca^{2+}], I_{K_v} can be recorded in isolation in the presence of extracellular Co^{2+} to prevent Ca^{2+} influx via voltage-gated Ca^{2+} channels (Reeve & Peers, 1992). Bath application of substance P (30 μM) for 1–2 min reversibly inhibited K^+ currents in NG 108-15 cells (Figure 1a), and the degree of inhibition was similar at all activating test potentials studied (Figure 1b). From experiments such as this, a concentration-

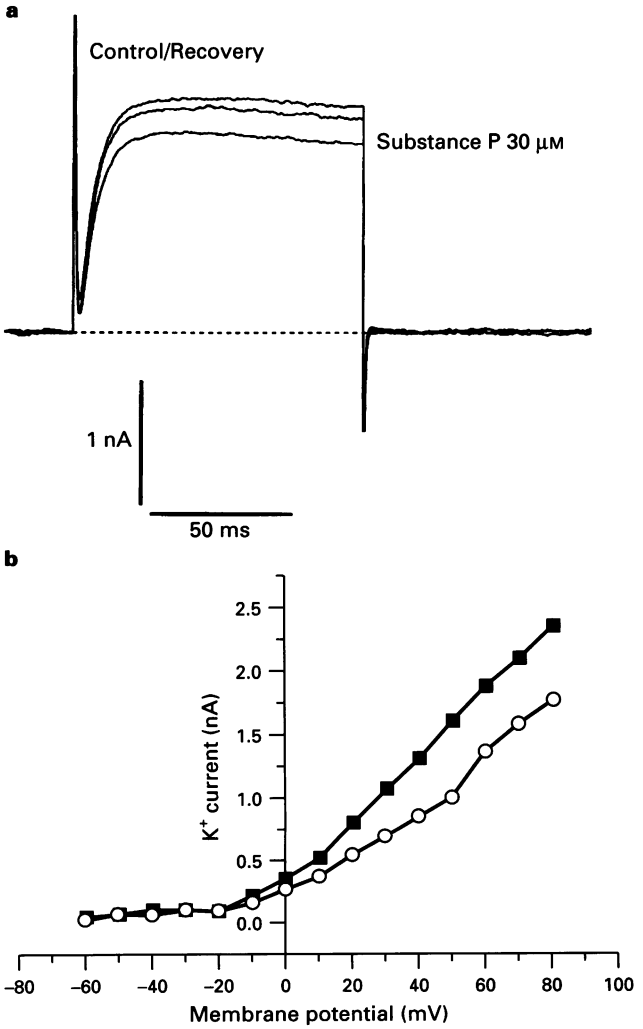

Figure 1 (a) Superimposed currents, obtained by step depolarization to +60 mV from the holding potential of −70 mV, displaying the outward K^+ current before, during and after bath application of 30 μM substance P. (b) Current-voltage relationships obtained from the same NG 108-15 cell under control conditions (■) and in the presence of bath applied substance P for 1–2 min (○).

response curve was constructed for the inhibitory actions of substance P (Figure 2, open circles), using currents activated by step depolarizations to +60 mV. At high concentrations substance P activates all three major tachykinin receptor types (see Introduction). To investigate which subtype of receptor mediated the inhibitory actions of substance P in NG 108-15 cells, we examined the relative potencies of GR73632 (delta Ava[L-Pro^9,N-MeLeu^{10}]SP(7–11)), GR64349 ([Lys^3,Gly^8-R-gamma-lactam-Leu^9]NKA(3–10)) and senktide, selective agonists at NK_1, NK_2 and NK_3 receptors respectively, to inhibit K^+ currents when applied in the bath. Figure 2 plots concentration-response relationships for these compounds, constructed exactly as described for substance P, and indicates that the NK_1 agonist, GR73632 was approximately 2 orders of magnitude more potent than the NK_3 agonist, senktide and the NK_2 agonist, GR64349, giving a potency order of GR73632 > substance P > > senktide ≥ GR64349. This finding strongly suggests that the inhibitory actions of substance P were mediated by NK_1 receptors.

Effect of tachykinins on IK_v and Ca^{2+} channel currents

As described earlier, K^+ currents in NG 108-15 cells can be divided into Ca^{2+}-sensitive and Ca^{2+}-insensitive components. To investigate whether NK_1 receptor-mediated inhibition was selective for either component, we tested the ability of GR73632 to inhibit residual currents recorded in the presence of 4 M Co^{2+}, which maximally inhibits IK_{Ca} in these cells (Reeve & Peers, 1992). Figure 3a compares the actions of 10 μM GR73632 to inhibit total K^+ current in one cell with its effects on IK_v recorded in another cell in the presence of Co^{2+}. As can be seen, residual currents recorded in the presence of Co^{2+} were unaffected by 10 μM GR73632, whereas with no Co^{2+} present total K^+ current was reversibly reduced in amplitude (Figure 3a): no response to GR73632 was observed in the presence of 4 mM Co^{2+} in 6 out of 6 cells tested. Such a finding suggests that activation of NK_1 receptors leads to a selective inhibition of IK_{Ca} in NG 108-15 cells. However, this raised the possibility that receptor activation might block IK_{Ca} indirectly, by inhibiting the Ca^{2+} current itself in these cells. To investigate this possibility, we recorded Ca^{2+} currents directly, using either 10 mM Ba^{2+} or 10 mM Ca^{2+} as charge carrier and solutions designed to inhibit outward K^+ currents (see Methods). Under these conditions, step depolarizations evoked inward currents

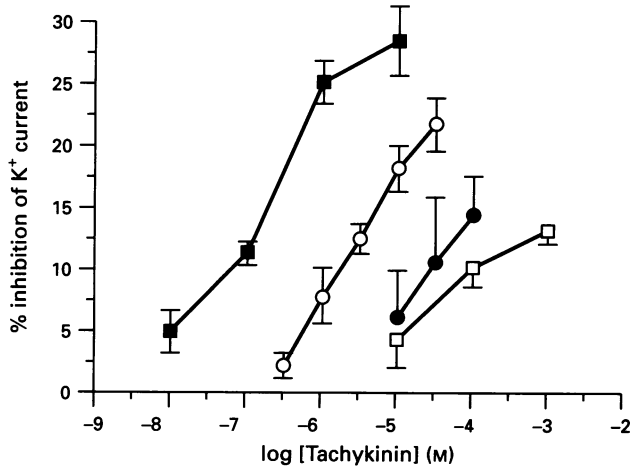

Figure 2 Log concentration-response relationships for tachykinin inhibition of depolarization activated outward whole-cell K^+ currents. Step depolarizations were applied from the holding potential of −70 mV to +60 mV at 0.1 Hz to elicit currents. Bath application of the tachykinin agonists caused partially reversible, concentration-dependent inhibition of the outward K^+ current. The NK_1 agonists GR73632 (■) displays approximately 2 orders of magnitude greater potency than the NK_2 and NK_3 agonists, GR64349 (□) and senktide (●). The lower relative potency of substance P (○) to GR73632 may be attributable to metabolism of the ligand by membrane surface peptidases (see Introduction). Percentage inhibitions of control current were calculated from leak-subtracted currents in the presence and absence of tachykinin agonist. Each point represents the mean ± s.e.mean for $4 \leqslant n < 6$.

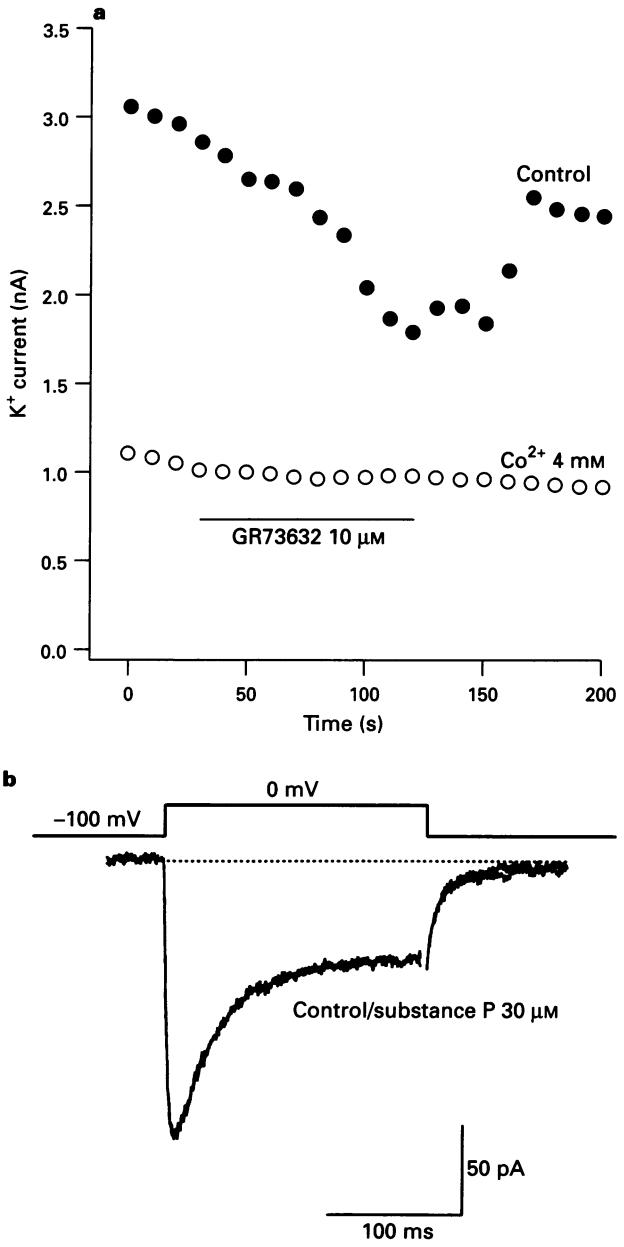

Figure 3 (a) Outward K^+ current time series plots for depolarizing pulses from the holding potential of −70 mV to +60 mV at 0.1 Hz. Current values indicated are for leak-subtracted K^+ currents. Contemporaneous bath application of 10 μM GR73632 causes suppression of the outward K^+ current in control conditions (●), but with 4 mM Co^{2+} present (○), there is no suppression of the outward current. The difference in magnitude between the K^+ current before GR73632 application is due to the loss of the Ca^{2+}-sensitive component from the whole-cell outward K^+ current. The two time series, in the presence and absence of Co^{2+}, were obtained from two separate cells. (b) Voltage-dependent whole-cell Ca^{2+} channel currents recorded using 10 mM Ba^{2+} as the charge carrier. Currents were obtained by 200 ms step depolarizations from a holding potential of −100 mV to 0 mV applied at 0.1 Hz. Activated currents shown are leak-subtracted with a leak current averaged from 10 mV depolarizing steps from the holding potential. Bath application of substance P produced no significant effect upon evoked whole-cell Ba^{2+} currents at any test depolarizing potential that elicited Ba^{2+} currents ($n = 6$).

which consisted of transient and sustained components (Figure 3b), as expected since these cells possess both low- (T-type) and high (L-type) voltage activated Ca^{2+} channels (Peers *et al.*, 1990; high threshold N-type channels are also present following differentiation (see e.g. Kasai & Neher, 1992)). Bath application of 30 μM substance P, which caused marked inhibition of K^+ currents (Figure 1), was without a significant effect on the inward Ca^{2+} channel current in 6 out of 6 cells examined using Ba^{2+} as charge carrier (e.g. Figure 3b) or in 5 out of 5 cells examined using Ca^{2+} as charge carrier (not shown). This suggests that the inhibition of $I_{K_{Ca}}$ was not secondary to an inhibition of Ca^{2+} channel currents in these cells.

Antagonism of the GR73632-mediated response

Current-compensated positive current ejection of GR73632 (50–100 nA: containing 0.1 mM GR73632 in 0.1 M saline solution) produced an ejection current-dependent attenuation of the outward whole-cell K^+ current. Maximal current inhibition attainable was approximately 25% of the whole-cell K^+ current; thus iontophoretic ejection could produce a local cell concentration similar to that achieved by bath application, as

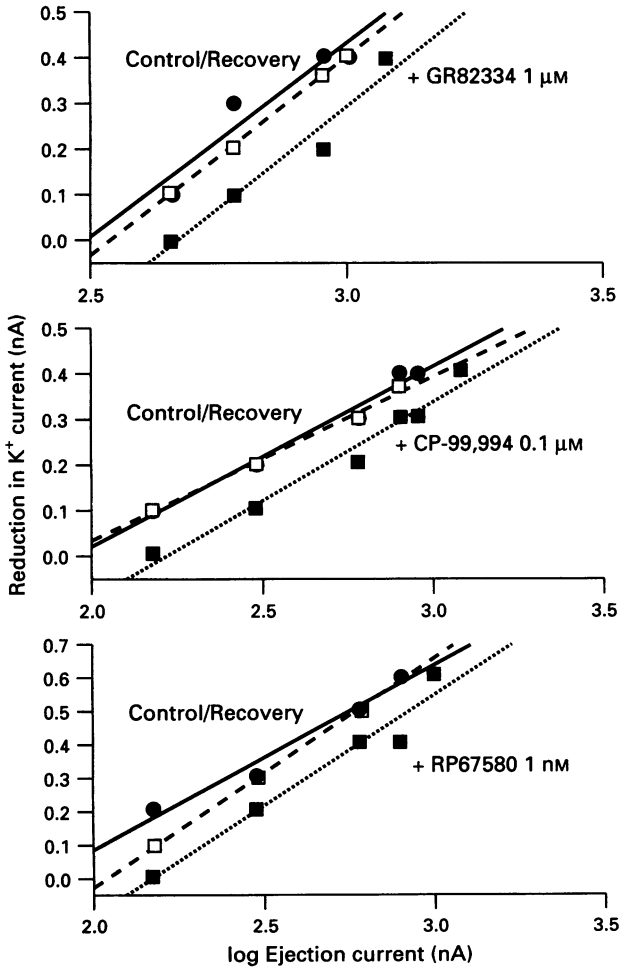

Figure 4 Ejection current-response relationships for the reduction of outward K^+ currents in NG 108-15 cells produced by current-compensated microiontophoresis of GR73632 (●), onto single cells in each case. The bath application of the non-peptide NK_1 antagonists CP-99,994 and RP67580 produce a rightward shift of the response series to increasing ionotophoretic currents of GR73632 (■); a similar effect was observed with the peptide NK_1 antagonist, GR82334. All response shifts seen with bath application of the antagonists were reversible upon repetition of an ejection current-response series after wash out of antagonist (□). Concentrations of antagonists depicted were chosen to illustrate the differences in potency between them in antagonizing the GR73632 response.

the responses are approximately equal. Ejection current-response series of four to five current values were estimated on single cells. Bath application of CP-99,994, RP67580 and GR82334 produced a reversible rightward shift of the repeated GR73632 response series. Representative response shifts for appropriate concentrations of the antagonists studied are shown in Figure 4. Antagonists were used over the following concentration-ranges: RP67580 0.1–2 nM; CP-99,994 0.5–1 μM: GR82334 0.1–10 μM. All concentrations used were effective in producing a shift of the ejection current-response relationship except 0.1 μM GR82334. Using dose-ratios obtained from response levels between 30–60% of the maximal on the particular cell, an average pK_B was estimated using the Gaddum equation:

$$pK_B = \log(\text{dose ratio} - 1) - \log(\text{antagonist concentration})$$

A value for a pA_2 is theoretically comparable to pK_B for competitive antagonists. The two indices are both measures of antagonist potency: pA_2 is the quoted value from an Arunlakshana-Schild plot where the gradient is not significantly different from +1 (Arunlakshana & Schild, 1959), but it is now more usual to constrain the slope of the plot to +1 in calculating the pA_2 value, which then equates to the pK_B value. Using the above range of antagonist concentrations the estimated pK_B values for the three antagonists are as follows: GR82334, 6.21 ± 0.17 ($n=5$); CP-99,994, 7.14 ± 0.14 ($n=6$); RP67580, 8.81 ± 0.07 ($n=7$). The display of 1–2 orders of magnitude greater potency of RP67580 over GR82334 and CP-99,994 indicates that the NK_1 receptor present shows greater similarity to NK_1 receptors in rat/mouse cells as opposed to guinea-pig/human cells (Beresford *et al.*, 1992; Barr & Watson, 1993; Beaujouan *et al.*, 1993).

Discussion

The present study demonstrates that activation of NK_1 receptors leads to a selective inhibition of $I_{K_{Ca}}$ in undifferentiated NG 108-15 cells, whereas I_{K_v} and Ca^{2+} channel currents themselves were unaffected. Our conclusion that the effects of substance P are mediated by NK_1 receptors is based on the results obtained using both receptor-selective agonists and antagonists. Thus, GR73632 (an NK_1 agonist) was by far the most potent compound in inhibiting $I_{K_{Ca}}$ in NG 108-15 cells, whilst GR64349 and senktide (agonists at NK_2 and NK_3 receptors respectively) were orders of magnitude less potent. This is in agreement with other agonist potency orders for typical NK_1 receptor activation (Regoli *et al.*, 1985). It is noteworthy that GR73632 was approximately 50 fold more potent than substance P itself, which is perhaps surprising given that these agents have been shown to be approximately equipotent at NK_1 receptors in other preparations. The discrepancy can be accounted for by the fact that substance P is particularly susceptible to surface membrane peptidases such as neutral endopeptidase (Ireland *et al.*, 1988), whereas GR73632 is not (Hagan *et al.*, 1989). Indeed if the concentration-response curve is shifted to the left by an amount which represents a 50 fold increase in potency (based on the reduction in potency of substance P to cause contraction of the guinea-pig ileum preparation; Hagan *et al.*, 1989), then the concentration-response curves for substance P and GR73632 appear extremely similar. Such a conclusion must be taken with caution since the presence and activity of peptidases in NG 108-15 cells is not well documented, although such membrane surface enzymes have been demonstrated to be present in other neuroblastoma cells (Medeiros *et al.*, 1991). The implications of the NK_1 receptor mediating the response in NG 108-15 cells was reinforced by the parallel rightward shift of the ejection current-response relationship for GR73632 by the NK_1 receptor antagonists CP-99,994, RP67580 and GR82334 (Figure 4). The difference in potency between RP67580 and CP-99,994/GR82334 is in accordance with spe-

cies and tissue variation in the activity of NK_1 receptor antagonists reported by other groups (Gitter *et al.*, 1991; Barr & Watson, 1993; Beaujouan *et al.*, 1993).

Substance P and GR73632 at the highest concentrations studied both inhibited K^+ currents in NG 108-15 cells by up to ca. 30% (Figures 1 and 2). This effect was shown to be selective for $I_{K_{Ca}}$ since application of GR73632 in the presence of 4 mM Co^{2+} (to block Ca^{2+} channels and therefore, indirectly, $I_{K_{Ca}}$) was without detectable effect. This degree of inhibition of $I_{K_{Ca}}$ (seen in the absence of Co^{2+}) can therefore be considered to be almost complete, since a previous study has shown $I_{K_{Ca}}$ to account for only approximately 30% of the total K^+ current in undifferentiated NG 108-15 cells at high test potentials (Reeve & Peers, 1992), based on the maximal inhibition caused by bath-applied Co^{2+}. We have not characterized the channels underlying $I_{K_{Ca}}$, but they are believed to be primarily of the low-conductance variety which strongly influence long after-hyperpolarizations in neurones, since they are potently inhibited by apamin and (+)-tubocurarine (Brown & Higashida, 1988). The block of $I_{K_{Ca}}$ was not secondary to inhibition of Ca^{2+} channel currents in these cells, since these were unaffected by substance P (Figure 3b) regardless of whether Ca^{2+} or Ba^{2+} was used as charge carrier. The lack of action of tachykinins upon Ca^{2+} currents is perhaps surprising since there are multiple accounts of tachykinin inhibition and activation of voltage-dependent Ca^{2+} channels in neuronal cells (Murase *et al.*, 1989; Bley & Tsien, 1990). One possible explanation for this is that our cells were undifferentiated, and possess only T- and L-type Ca^{2+} channels (Peers *et al.*, 1990), and neurotransmitters most commonly target N-type channels in neurones (Anwyl, 1991).

All three tachykinin receptor subtypes are capable of inducing PIP_2 hydrolysis, which leads to rises in intracellular Ca^{2+} and could therefore increase $I_{K_{Ca}}$ as has been shown for other transmitters acting in these cells (Brown & Higashida, 1988). However, it has been reported that application of tachykinin agonists to NG 108-15 cells does not raise $[Ca^{2+}]_i$ (Reiser & Hamprecht 1985). Instead, our results indicate a novel mechanism of $I_{K_{Ca}}$ modulation by tachykinin ligands in the NG 108-15 cell line. NG 108-15 cells have long been used as model neurones for the study of receptor-ion channel coupling, and the present study demonstrates that these cells will provide a novel and useful model system for investigating the regulation of electrophysiological properties by NK_1 receptors.

We would like to thank G.G. Wilson and A. Dawson for their assistance with cell culture and Dr P.J. Birch of Glaxo Group Research (Ware, Hertfordshire) for the donation of tachykinin research compounds.

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(Received November 22, 1995
Revised May 24, 1996
Accepted July 2, 1996)