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Cytoskeletal inhibitors impair Ca^{2+} elevations via neuropeptide Y and other G_i-coupled receptors

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Abstract

Neuropeptide Y, α_2-adrenoceptors, thrombin and certain muscarinic acetylcholine receptors can couple to elevations of intracellular free Ca^{2+} concentrations via G_i-proteins. We have studied the effects of inhibitors of microtubules (colchicine, nocodazole, vinblastine) and microfilaments (cytochalasin B, cytochalasin D) on these effects in human erythroleukemia (HEL) cells. Both types of inhibitors reduced neuropeptide Y-, adrenaline- (via α_{2A}-adrenoceptors) and thrombin-stimulated Ca^{2+} elevations while the inactive analog ß-lumicolchicine was without inhibitory effects. Similarly, in SK-N-MC cells vinblastine inhibited neuropeptide Y and carbachol (via muscarinic receptors) stimulated Ca^{2+} elevations. In HEL cells the inhibitory effects of the microfilament inhibitor cytochalasin D and the microtubule inhibitor colchicine were not additive. Colchicine, nocodazole or cytochalasin D did not affect the binding of the agonist neuropeptide Y. On the other hand, neuropeptide Y and thrombin significantly stimulated GTPγS binding in the absence but not in the presence of colchicine, vinblastine or cytochalasin B. This was not due to sequestration of G-protein α-subunits, since nocodazole did not affect the distribution of immunodetectable $G_{i\alpha 1/2}$ or $G_{i\alpha 3}$ between membrane and cytosolic fractions. We conclude that disruption of microfilaments or microtubules impairs Ca^{2+} elevations by neuropeptide Y and other G_i-coupled receptors by inhibiting receptor/G_i-protein interaction; this does not involve impairment of agonist binding to the receptor or redistribution of G_i-protein α-subunits.

Keywords: G_i-protein; Neuropeptide Y receptor; α_{2A}-Adrenoceptor; Ca^{2+}; Microtubule; Microfilament

1. Introduction

Many neurotransmitters and hormones exert their physiological effects via receptors coupling to GTP-binding regulatory proteins, G-proteins, which in turn regulate the function of effector enzymes and/or ion channels. Classically it has been assumed that there is a direct chain of events initiated by agonist binding to a cell surface receptor. This induces G-protein binding to the receptor followed by G-protein dissociation and activation of effector mechanisms. This chain of events has been assumed to be based mainly on collision coupling in the plasma membrane. However, the validity of this theory has been challenged on theoretical grounds, and it has been proposed that a more structured interaction, possibly involving the cytoskeleton, could take place (Peters, 1988). A cytoskeletal involvement in G-protein-mediated signal transduction was originally demonstrated upon interventions such as osmotic swelling (Watson, 1991), shear stress (Reinhart, 1994) or other forms of mechanical stress (Komuro and Yzaki, 1994). Meanwhile it has also been found that the cytoskeleton might be involved in 'classical' receptor/G-protein/effector coupling. For example it has been shown that disruption of microtubules or microfilaments enhances G_s-mediated accumulation of cAMP in S49 lymphoma cells (Leiber et al., 1993; Jasper et al., 1995), and domains in $G_{s\alpha}$ which interact with tubulin have been defined (Popova et al., 1994). However, little information is available on a possible role of the cytoskeleton in signal transduction occurring via G_q- or G_i-type G-proteins.

It is difficult to investigate the role of the cytoskeleton in receptor coupling to G_i using adenylyl cyclase inhibition as the read-out because enhancement of G_s activation can occur concomitantly (see above). On the other hand, various G_i-coupled receptors, e.g. neuropeptide Y (Motulsky and Michel, 1988), α_2-adrenoceptors (Michel et al., 1989), thrombin (Brass et al., 1991) or muscarinic acetylcholine

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receptors (Michel et al., 1992) can also mediate elevations of intracellular free Ca^{2+} concentration, an effect which occurs independently of intracellular cAMP levels (Motulsky and Michel, 1988; Michel et al., 1989). While all of these receptors increase intracellular Ca^{2+} via a form of G_i, they use distinct mechanisms down-stream of G_i to yield Ca^{2+} elevations. For example α_{2A}-adrenoceptors in human erythroleukemia (HEL) cells (Michel et al., 1989) and neuropeptide Y receptors in HEL (Motulsky and Michel, 1988) and SK-N-MC cells (Michel et al., 1992) cause mobilization of Ca^{2+} from intracellular stores without apparent involvement of inositol phosphates. On the other hand thrombin receptors in HEL cells (Brass et al., 1991) or muscarinic acetylcholine receptors in SK-N-MC cells (Michel et al., 1992) mobilize Ca^{2+} from intracellular stores and stimulate influx of extracellular Ca^{2+}, and this apparently involves the intermediate formation of inositol phosphates. While the mechanisms underlying inositol phosphate-independent Ca^{2+} mobilization remain to be defined, this response allows to test the role of the cytoskeleton in receptor coupling to G_i.

Therefore, we have investigated the effects of microtubule (colchicine, vinblastine, nocodazole) and microfilament (cytochalasins B and D) disrupting agents on Ca^{2+} elevations caused by stimulation of α_2-adrenoceptors, neuropeptide Y and thrombin receptors in HEL cells. Studies on agonist-stimulated [^{35}S]GTP[γS] binding, agonist binding and subcellular localization of G_i α-subunits were added to define the molecular mechanism underlying the effects of cytoskeletal inhibitors on Ca^{2+} elevations.

2. Materials and methods

2.1. Cell culture and treatment

HEL cells were originally obtained from Dr Papayannopoulou (Department of Medicine, University of Washington, Seattle, WA, USA) and maintained in the chemically defined CG medium (Camon, Wiesbaden, Germany) in an atmosphere of 95% air-5% CO_2 at 37°C at a density of 400000–600000 cells/ml by daily dilution with fresh medium. SK-N-MC cells were obtained from American Type Culture Collection (Rockville, MD, USA) and grown in minimal essential medium containing fetal calf serum (10%), L-glutamine (4 mM), non-essential amino acids (1%), sodium pyruvate (1 mM), and penicillin/streptomycin (100 I.U. and 100 μg/ml, respectively) as previously described (Feth et al., 1991).

Cells were treated with the microtubule inhibitors colchicine (10 μM), nocodazole (10 μg/ml) and vinblastine (10 μM), the microfilament inhibitors cytochalasin B and D (10 μg/ml), or the inactive colchicine analog β-lumicolchicine (10 μM) for 1 h at room temperature; the inhibitors remained present during the Ca^{2+}, [^{125}I]neuropeptide Y binding and [^{35}S]GTP[γS] binding experiments.

2.2. Ca^{2+} measurements

The free intracellular Ca^{2+} concentration was determined with the fluorescent indicator dye fura-2 in a Hitachi F2000 spectrofluorometer as previously described (Feth et al., 1991, 1992). Briefly, experiments with HEL cells were performed in buffer of the following composition (mM): Hepes 20, NaCl 120, KH_2PO_4 5, magnesium acetate 1, $CaCl_2$ 1 and 1 mg ml^{-1} glucose at pH 7.4 using approximately $6–8 \times 10^5$ cells ml^{-1}; experiments with SK-N-MC cells were performed in phosphate-buffered saline. After 1 h of loading with the dye the cells were washed twice, resuspended in fresh buffer, and used for fluorescence measurements within the next hour. Excitation was alternating at 340 and 380 nm with emission being read at 510 nm. Fluorescence data were converted into Ca^{2+} concentrations using the software supplied by the manufacturer. In the Ca^{2+} experiments all drugs were added to the cell suspension in 100-fold concentrated stock solutions.

2.3. Neuropeptide Y binding

Binding studies with HEL cell neuropeptide Y receptors were performed as described previously (Feth et al., 1992) except that the endogenous neuropeptide Y analog peptide YY was used as the radioligand since [^{125}I]peptide YY yielded less non-specific binding than [^{125}I]neuropeptide Y (data not shown). Briefly, [^{125}I]peptide YY binding to intact HEL cells was performed in a total volume of 500 μl of binding buffer of the following composition (mM): Hepes 10, NaCl 150, KCl 5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and 10 mg ml^{-1} bovine serum albumin. Additionally, the following protease inhibitors were added to the incubation mixture: 1 μg/ml aprotinin, 40 μM amastatin, 10 μg/ml leupeptin, 10 μM pepstatin, 1 mM phenylmethylsulphonylfluoride, 50 μg/ml typsin inhibitor. Binding was carried out for 90 min at 25°C and terminated by centrifugation for 5 min at $3000 \times g$. Non-specific binding was defined by binding in the presence of 100 nM neuropeptide Y.

2.4. G-protein immunoblotting

For the immunoblotting studies membrane and cytosolic fractions were prepared from HEL cells as follows: cells were resuspended in 10 ml HMEN buffer (20 mM Hepes 2 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl, pH 7.4) and subjected to nitrogen cavitation at 40 bar. Thereafter, they were centrifuged for 5 min at 4°C and $50 \times g$. The supernatant was centrifuged again for 20 min at $50000 \times g$, and the resulting pellet was considered as the membrane preparation. The supernatant was incubated for 10 min with ice-cold acetone at −20°C, and the precipitated proteins were then pelleted by a 5 min centrifugation at $10000 \times g$.

After evaporation of the acetone the pellet was considered to contain the cytosolic fraction.

G-protein α-subunits were determined by quantitative Western blotting as previously described (Michel-Reher et al., 1993). Briefly, membrane preparations (≈ 100 μg protein/sample) were diluted 4 : 1 with sample buffer (4% sodium dodecyl sulphate, 20% glycerol, 10% 2-mercaptoethanol, 125 mM TrisHCl, pH 8.0), boiled for 5 min, and separated on sodium dodecyl sulphate/polyacrylamide gels with 10% acrylamide in the running gel. The separated proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham, Braunschweig, Germany) with an electric field of 55 V overnight. Following the transfer the blots were washed for 90 min in TBS (20 mM Tris, 100 mM NaCl, pH 7.5) at room temperature in the presence of 2% non-fat dry milk and twice for 10 min each in TTBS (TBS supplemented with 500 μl/l Tween-20), and then incubated overnight at 4°C in 15 ml of TTBS containing 1% non-fat dry milk and a 1 : 500 dilution of the antisera. After removal of the antisera the blots were washed twice for 10 min each with TTBS and then incubated for 1 h at room temperature in 100 ml of TTBS which had been supplemented with 1% non-fat dry milk and 70 μl [^{125}I]protein A solution. Finally, the [^{125}I]protein A was washed out 4 times for 10 min each with TTBS and the blots were used for autoradiography at −80°C. Using the autoradiograms the molecular weights of the specific bands were identified and the corresponding sections were cut from the blots and counted in a scintillation counter.

2.5. GTP[γS] binding assay

GTP[γS] binding in permeabilized cells was determined as previously described under conditions which have been optimized for the detection of agonist-induced enhancement (Wieland et al., 1995). Briefly, cells were harvested by centrifugation (10 min at 2000 × g) and resuspended in 50 ml of an ice-cold buffer containing 150 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4. Cells were again pelleted (10 min at 2000 × g) and resuspended in a buffer containing 50 mM triethanolamine-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA and 150 mM NaCl. 10^6 cells/tube were added to the thermoequilibrated reaction mixture (total volume 200 μl), containing 50 mM triethanolamine-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl, 10 μM digitonin and 1 μM GDP, and permeabilized for 15 min at 30°C. Thereafter, the binding reaction was started by the addition of [^{35}S]GTP[γS] (1–1000 nM, 0.1 μCi/tube) and conducted, unless otherwise indicated, for 10 min at 30°C. Separation of protein-bound and -free radioactivity was performed by rapid filtration through nitrocellulose filters. The filters were washed 5 times with 2.5 ml each of an ice-cold washing buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$) and counted in a liquid scintillation spectrometer. Non-specific binding was defined as the binding not competed for by 10 μM unlabelled GTP[γS].

2.6. Chemicals

Fura-2 AM was obtained from Molecular Probes (Eugene, OR, USA), human neuropeptide Y from Saxon Biochemicals (Hannover, Germany), (−)-adrenaline bitartrate, carbachol HCl, thrombin (from bovine plasma), colchicine, ß-lumicolchicine, nocodazole, vinblastine sulphate, cytochalasin B, and cytochalasin D from Sigma (Munich, Germany). The antisera AS/7 and EC/2 were purchased from New England Nuclear (Dreieich, Germany). [^{125}I]Peptide YY (specific activity 2200 Ci/mmol) was obtained from Biotrend (Cologne, Germany), [^{125}I]protein A (specific activity 2–10 μCi/μg, 30–150 μCi/ml) and [^{35}S]GTP[γS] (specific activity 1340 Ci/mmol) from New England Nuclear.

Fura-2-AM was dissolved at 1 mM in dimethylsulphoxide and used at 1 μM to load the cells. Adrenaline, carbachol and neuropeptide Y were dissolved (at 10 mM, 10 mM and 1 mM, respectively) and further diluted in 10 mM HCl. Adrenaline and carbachol were dissolved fresh daily, whereas neuropeptide Y solutions were stored at 4°C for up to three months; no decline in neuropeptide Y activity could be observed during this period as assessed by its EC_{50} to mobilize Ca^{2+} in HEL cells. Colchicine was dissolved fresh daily at 1 mM in H_2O. Nocodazole was dissolved at 10 mg/ml in dimethylsulphoxide, diluted 1 : 10 in ethanol; stock solutions in dimethylsulphoxide were stored at −20°C for up to 4 weeks. Vinblastine was dissolved at 10 mM in 50% ethanol, and stock solutions were stored at 4° for up to 4 weeks. Cytochalasin B and D were dissolved at 10 mg/ml in ethanol, and stock solutions were stored at 4°C for up to 4 weeks.

2.7. Data analysis

Data are means ± S.E.M. of n experiments. Statistical significance of differences between groups was evaluated by paired two-tailed t-tests if two groups were compared. If multiple groups were compared a repeated measures one-way analysis of variance was performed; if this indicated a significant variance between groups a Dunnett's multiple comparison test was performed. A $P < 0.05$ was considered significant. All statistical calculations were performed with the Instat program (GraphPAD Software, San Diego, CA, USA).

3. Results

Basal free intracellular Ca^{2+} concentrations in HEL cells typically ranged between 50 and 100 nM and did not undergo major changes upon preincubation with or addi-

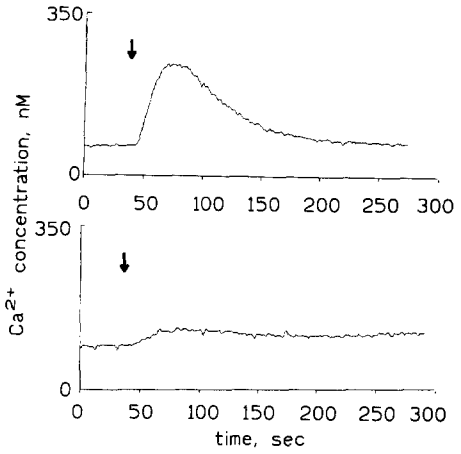

Fig. 1. Neuropeptide Y-induced Ca^{2+} elevation in control (upper panel) and cytochalasin B (10 μg/ml)-treated HEL cells (lower panel). Neuropeptide Y (100 nM) was added at the time point indicated by the arrow. Data are from a representative experiment. The mean ± S.E.M. of 11 such experiments is shown in Fig. 2.

tion of any of the cytosekeletal inhibitors (data not shown). As reported previously (Michel et al., 1989; Feth et al., 1992), neuropeptide Y (100 nM), adrenaline (1 μM) and thrombin (0.1 U/ml) typically increased intracellular Ca^{2+} by approximately 150, 50 and 300 nM, respectively (data not shown). Treatment with the microtubule inhibitors colchicine (10 μM), nocodazole (10 μg/ml) and vinblastine (10 μM) or the microfilament inhibitors cytochalasin B and D (10 μg/ml) reduced Ca^{2+} responses to all three agents although the reduction did not reach statistical significance in all cases. Raw data from an individual experiment with neuropeptide Y and cytochalasin B is shown in Fig. 1, Fig. and Fig. , and a summary of all data is depicted in Fig. 2. In contrast, no inhibition of adrenaline-, neuropeptide Y- or thrombin-induced Ca^{2+} elevations was detectable with 10 μM of the inactive colchicine analog, ß-lumicolchicine (Fig. 2). The effect of combined exposure to colchicine and cytochalasin D on neuropeptide Y-, adrenaline- or thrombin-evoked Ca^{2+} responses was not significantly different from that produced by either agent alone (Fig. 3), indicating a lack of additivity.

In SK-N-MC cells basal free intracellular Ca^{2+} was typically 60–70 nM, and exposure to cytochalasin D (10 μg/ml) did not cause major alterations (data not shown). Neuropeptide Y (100 nM) and carbachol (1 mM) elevated intracellular Ca^{2+} by approximately 25 and 150 nM, respectively (data not shown). Exposure to cytochalasin D significantly reduced Ca^{2+} responses to both agonists by ≈ 20% (Fig. 4).

Since all of the above Ca^{2+} responses occur via a pertussis toxin-senstive G-protein, i.e. a form of G_i (see Discussion), we tested whether cytoskeletal inhibitors interfere with Ca^{2+} elevations at the level of G-protein activation. In agreement with our previous data (Wieland et al., 1995) neuropeptide Y and thrombin significantly enhanced GTP[γS] binding in permeabilized HEL cells with the more efficacious Ca^{2+} elevating agent, thrombin, yielding larger enhancements (Fig. 5). No significant enhancement of GTP[γS] binding by neuropeptide Y or

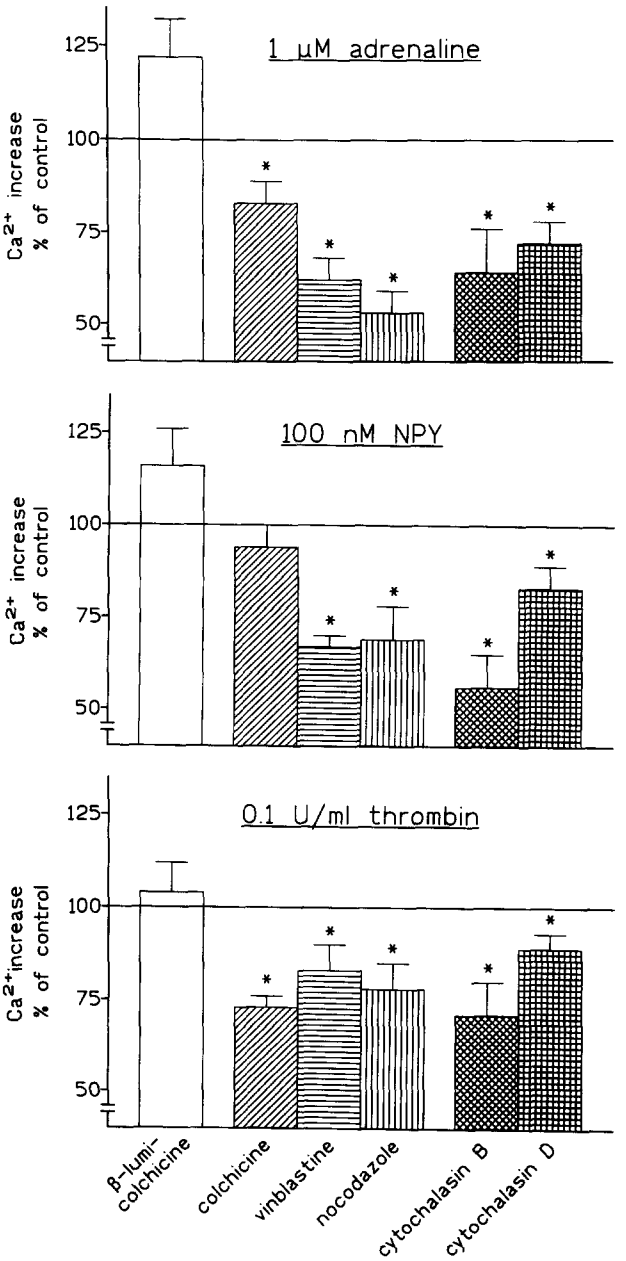

Fig. 2. Effects of cytoskeletal inhibitors on adrenaline, neuropeptide Y and thrombin induced Ca^{2+} increases in HEL cells. Adrenaline (1 μM, upper panel), neuropeptide Y (100 nM, middle panel), and thrombin (0.1 U/ml, lower panel) induced peak Ca^{2+} elevations were quantified in the absence ('control') and presence of 10 μM ß-lumicolchicine, 10 μM colchicine, 10 μM vinblastine, 10 μg/ml nocodazole, 10 μg/ml cytochalasin B or 10 μg/ml cytochalasin D. Data are means ± S.E.M. of 5–12 experiments and expressed as % of control peak Ca^{2+} elevation. Control level responses are indicated by the horizontal line. * $P < 0.05$ vs. control in a one-way analysis of variance for repeated measures.

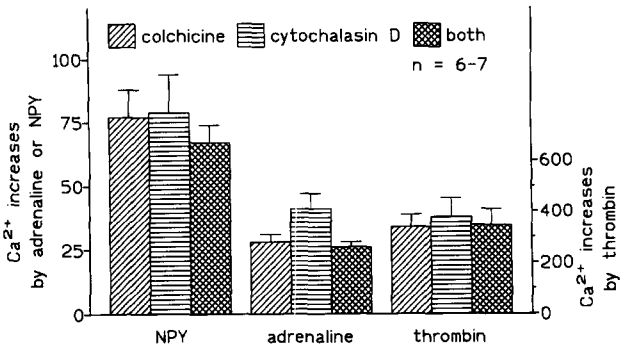

Fig. 3. Effects of colchicine (10 μM) and cytochalasin D (10 μg/ml) alone and in combination on neuropeptide Y (100 nM)-, adrenaline (1 μM)- and thrombin (0.1 U/ml)-induced Ca^{2+} increases in HEL cells. Data are means ± S.E.M. of 6–7 paired experiments. Differences between experimental groups for one receptor agonist were not significantly different. Note different scale of y-axis for thrombin.

thrombin was observed in the presence of the cytoskeletal inhibitors. Moreover, vinblastine significantly inhibited the neuropeptide Y effect ($P < 0.05$) and colchicine and vinblastine significantly inhibited the thrombin effect ($P < 0.01$), while the inhibitory effects of the other drugs did not reach statistical significance with the given number of experiments (Fig. 5). Neuropeptide Y- and thrombin-stimulated GTP[γS] binding to isolated membranes was not affected by colchicine (data not shown).

Exposure to colchicine did not alter the amount of specific [^{125}I]peptide YY binding to intact HEL cells (Fig. 6). The potency of neuropeptide Y to compete for [^{125}I]peptide YY binding was also not significantly altered ($-\log IC_{50}$ 8.41 ± 0.16 vs. 8.62 ± 0.09 in control and treated cells, respectively; $n = 4$; Fig. 6). Similarly, nocodazole or cytochalasin D failed to affect specific [^{125}I]peptide YY binding or neuropeptide Y competition for [^{125}I]peptide YY binding (Fig. 6).

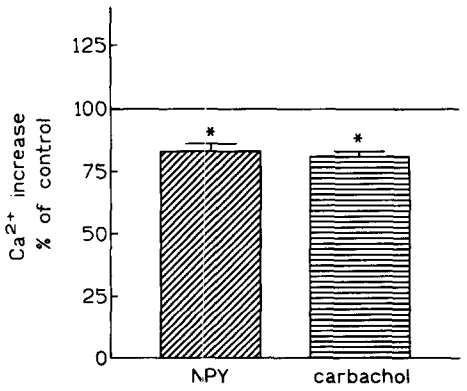

Fig. 4. Effects of cytochalasin D (10 μg/ml)- on neuropeptide Y (100 nM)- and carbachol (1 mM)-induced Ca^{2+} increases in SK-N-MC cells. Data are means ± S.E.M. of 6–7 experiments and expressed as % of control peak Ca^{2+} elevation. Control level responses are indicated by the horizontal line. * $P < 0.05$ vs. control in a paired two-tailed t-test.

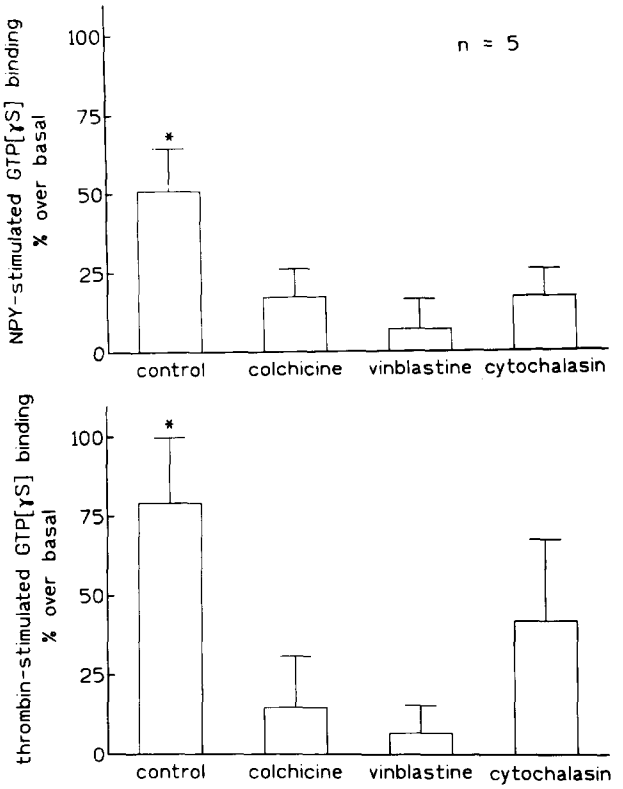

Fig. 5. Effects of cytoskeletal inhibitors on neuropeptide Y- and thrombin-stimulated [^{35}S]GTP[γS] binding to permeabilized HEL cells. Enhancement of [^{35}S]GTP[γS] binding by the agonists neuropeptide Y (1 μM, upper panel) and thrombin (0.1 U/ml, lower panel) was determined in the absence ('control') and presence of 10 μM colchicine, 10 μM vinblastine or 10 μg/ml cytochalasin B. Data are means ± S.E.M. of 5 experiments. Basal [^{35}S]GTP[γS] binding was 36.7 ± 1.7 fmol/10^6 cells. * $P < 0.05$ vs. basal in a paired two-tailed t-test. When a one-way ANOVA for repeated measures followed by a Dunnett's multiple comparison test was used to analyze inhibition of the neuropeptide Y and thrombin effects by the inhibitors, vinblastine significantly inhibited the neuropeptide Y effect ($P < 0.05$) and colchicine and vinblastine significantly inhibited the thrombin effect ($P < 0.01$) while the inhibitory effects of the other drugs did not reach statistical significance with the given number of experiments.

The AS/7 antiserum which recognizes α-subunits of G_{i1} and G_{i2} identified a band with an apparent molecular mass of ≈ 41 kDa in membrane and cytosolic fractions of HEL cells (data not shown). The bands in the cytosolic fraction of HEL cells were much fainter than those in the membrane fraction (721 ± 128 vs. 47 837 ± 7 142 cpm [^{125}I]protein A bound per mg of protein loaded per lane; $n = 7$). Nocodazole treatment of intact HEL cells did not significantly change the amount of immunodetectable $G_{i1/2}$ in HEL cells membrane or cytosol fractions (Fig. 7). Two additional bands with apparent molecular weights of ≈ 32 kDa and ≈ 47 kDa were also seen with the AS/7 antiserum in the cytosolic fraction of HEL cells, but nocodazole also failed to affect the amount of [^{125}I]protein A binding to either of these additional bands (data not shown).

The EC/2 antiserum which recognizes α-subunits of

G_{i3} identified a band with an apparent molecular mass of ≈ 41 kDa in membrane and cytosolic fractions of HEL cells (data not shown). The amount of [^{125}I]protein A binding to that band was again much greater in the membrane than in the cytosolic fraction (76 505 ± 1 509 vs. 4281 ± 802 cpm [^{125}I]protein A bound per mg of protein loaded per lane; $n = 6–7$). Exposure of intact HEL cells to nocodazole did not significantly change the amount of immunodetectable G_{i3} in HEL cell membrane or cytosolic fractions (Fig. 7).

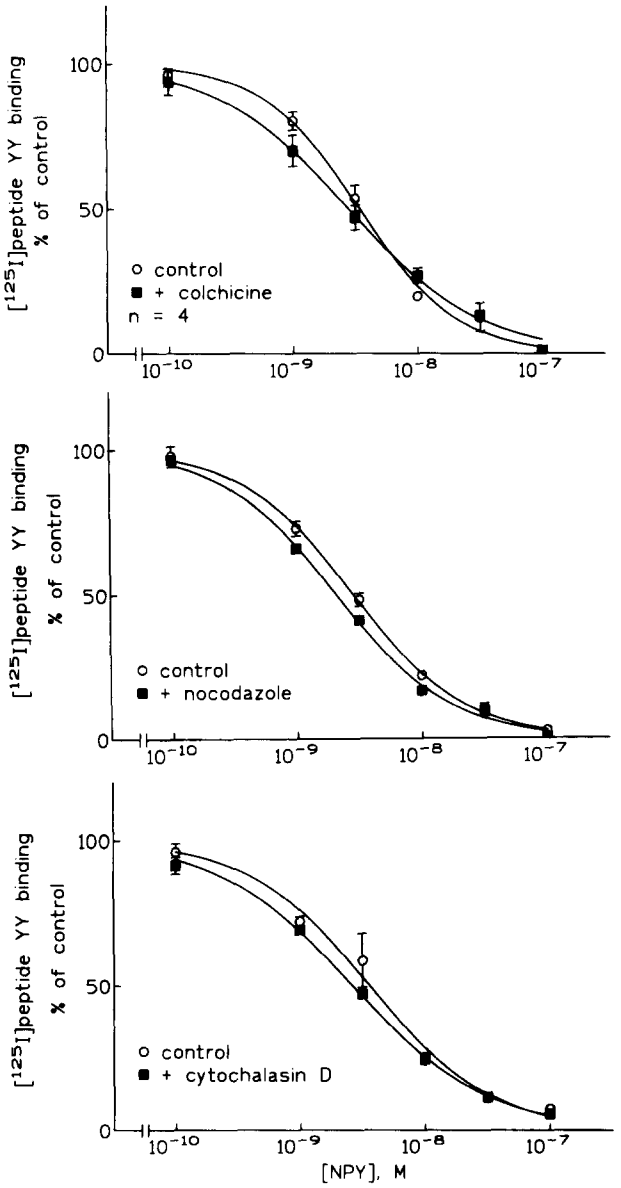

Fig. 6. Effects of colchicine, nocodazole and cytochalasin D on neuropeptide Y competition for [^{125}I]peptide YY binding to intact HEL cells. Data are means ± S.E.M. of 4 paired experiments for colchicine effects (10 μM, upper panel) and expressed as percentage of [^{125}I]peptide YY binding in the absence of neuropeptide Y ('control'). Control binding was 6024 ± 269 and 5939 ± 206 cpm/tube in control and colchicine-treated cells, respectively. Data are means ± S.E.M. of a triplicate determination in a representative paired experiment for nocodazole (10 μg/ml, middle panel) and cytochalasin D effects (10 μg/ml, lower panel).

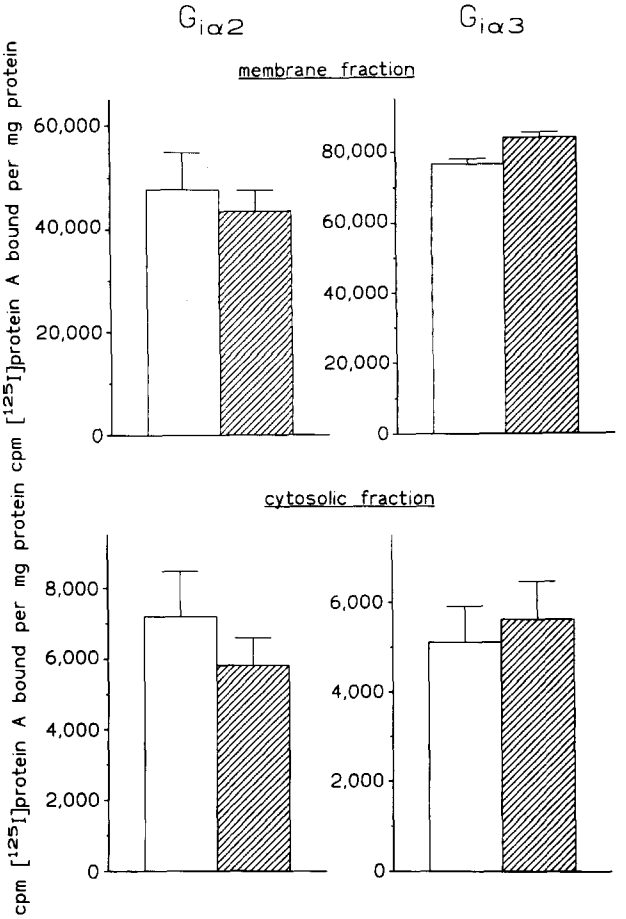

Fig. 7. Effects of nocodazole (10 μg/ml) treatment of intact HEL cells on immunodetectable G_i α-subunits in membrane and cytosolic cell fractions. Immunodetectable $G_{i\alpha 1/2}$ (left panels) and $G_{i\alpha 3}$ (right panels) are shown for membrane (upper panels) and cytosolic fractions (lower panels) in control (open bars) and nocodazole-treated HEL cells (hatched bars). Data are presented as cpm [^{125}I]protein A bound to the specific 41 kDa bands per mg of protein loaded on the respective lane of the gel. Data are means ± S.E.M. of 6–7 paired experiments.

4. Discussion

In the present study the microtubule-disrupting agents, colchicine, vinblastine and nocodazole, and the microfilament-disrupting agents, cytochalasin B and D, inhibited Ca^{2+} responses to stimulation of α_{2A}-adrenoceptors, neuropeptide Y, thrombin and muscarinic acetylcholine receptors. Our experiments were designed to define the molecular mechanism underlying this inhibition in more detail. These studies were performed in HEL cells because Ca^{2+} signals were larger and thus easier to quantify in that cell line than in SK-N-MC cells.

Firstly, our data demonstrate that inhibition of G_i-mediated Ca^{2+} signalling was detectable in multiple cell types,

i.e. HEL and SK-N-MC cells. On the other hand, we could not detect inhibition of G_i-mediated Ca^{2+} elevations by cytoskeletal inhibitors in HL-60 cells differentiated towards a neutrophil-like state (data not shown). Such cell type specificity is not surprising since the cytoskeleton plays distinct roles in various cell types. Secondly, we found that the inhibitory effects of the cytoskeletal inhibitors were specific since they were not mimicked by the inactive colchicine analog, ß-lumicolchicine. Thirdly, inhibition of peak Ca^{2+} elevations was only 10–50% although the cytoskeletal drug concentrations used were rather high. This partial inhibition indicates that the cytoskeleton plays a modulatory role rather than being a mandatory requirement for Ca^{2+} signalling. Fourthly, our data suggest that this modulatory role may involve both types of cytoskeletal structures, i.e. microtubules and microfilaments, since the combination of colchicine and cytochalasin D was not more effective than either agent alone. Enhancements of cAMP formation in S49 lymphoma cells by cytoskeletal inhibitors can also involve microtubules and microfilaments, but this effect has been reported to be additive for colchicine and cytochalasin B (Jasper et al., 1995). Fifthly, the inhibitory effect of the microtubule- and microfilament-discrupting drugs was found for agonists which use distinct pathways down-stream of G_i to yield Ca^{2+} elevations (see Introduction). Thus, it is likely that the cytoskeleton affects signalling at the levels of receptors, G-proteins or their interaction. These possibilities were therefore studied in more detail.

We have previously demonstrated that agonist-stimulated GTP[γS] binding is a direct indicator of receptor/G-protein interaction in intact cells (Wieland et al., 1995). Using this approach we have now investigated the effects of cytoskeletal inhibitors on neuropeptide Y and thrombin receptor signalling. The effects on α_2-adrenoceptor signalling could not be evaluated by this assay for technical reasons, possibly because HEL cells express much fewer α_2-adrenoceptors than neuropeptide Y receptors (Feth et al., 1992). Our data demonstrated that microtubule (colchicine and vinblastine) and microfilament inhibitors (cytochalasin B) diminish agonist-stimulated GTP[γS] binding. A lack of G-protein activation by the neuropeptide Y or thrombin receptors could result from impairment of agonist binding to the respective receptors or from sequestration of G-protein α-subunits away from the cell membrane to a compartment where they are no longer accessible for receptors.

We have tested the effects of cytoskeletal inhibitors on the cellular ability to bind receptor agonists with high affinity using the neuropeptide Y receptor as an example. Previously we have demonstrated that this type of assay can detect alterations of receptor/G-protein interaction, e.g. those caused by increasing intracellular GTP or by pertussis toxin-catalysed ADP-ribosylation (Feth et al., 1991). Cytoskeletal inhibitors, however, did not interfere with the binding of the radiolabelled agonist, [^{125}I]peptide YY, or with competition of the unlabelled agonist, neuropeptide Y. Thus, the affinity and capacity of HEL cell neuropeptide Y receptors to bind agonist were apparently not affected by the cytoskeletal inhibitors.

Another possibility tested in our study is whether cytoskeletal inhibitors might cause sequestration of G-protein α-subunits to a cellular compartment where they can no longer productively interact with receptors. Among the various pertussis toxin substrates, HEL cells contain mostly α-subunits of the G_{i2} type with less G_{i3} type and only very little if any G_{i1} (Williams et al., 1990) while α-subunits for G_o isoforms are missing (Michel et al., 1989). In the present study most immunodetectable G_{i2} and G_{i3} in control cells were found in the membrane fraction with only very little immunoreactivity detectable in the cytosolic fraction, the latter possibly reflecting a contamination of the crude fraction with plasma membranes. This finding is in good agreement with other studies describing that α-subunits of G_i in contrast to those of G_s are restricted to the plasma membrane. This compartmentalization was not noticeably affected by the nocodazole treatment.

Since neither agonist binding nor G-protein α-subunit compartmentalization appears to be altered by cytoskeletal inhibitors, their inhibitory effect on Ca^{2+} elevations may occur at the level of receptor/G-protein interaction. Therefore, we propose that microtubule and microfilament elements of the cytoskeleton might play a modulatory role in the coupling of receptors to G_i-like proteins. Disruption of either of these elements can impair coupling to Ca^{2+} elevations and possibly to inhibition of adenylyl cyclase. In contrast the effects of cytoskeletal inhibitors on the G_s/adenylyl cyclase pathway appear to occur mainly at the level of G-protein/adenylyl cyclase interaction (Jasper et al., 1995). In a broader sense it appears that disruption of the cytoskeleton can interfere with signal transduction at different levels to enhance G_s- and concomitantly impair G_i-mediated pathways. Since cAMP plays an inhibitory role in the control of leukocyte effector functions (Kammer, 1988), it is possible that these effects contribute to the well-known clinically used anti-gout actions of cytoskeletal drugs such as colchicine.

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