ELSEVIER

FEBS Letters 338 (1994) 75–80

FEBS 13564

FEBS LETTERS

Calcium influx and protein kinase Cα activation mediate arachidonic acid mobilization by the human NK-2 receptor expressed in Chinese Hamster Ovary cells

Steve Arkinstall*, Isabelle Emergy, Dennis Church, Andre Chollet, Eric Kawashima

Department of Biological Chemistry, Biochemistry Section, Glaxo Institute for Molecular Biology, 1228 Plan-les-Ouates, Geneva, Switzerland

Received 8 December 1993

Abstract

We have cloned a cDNA encoding the human ileal neurokinin-2 (NK-2) receptor which mediates powerful neurokinin A-stimulated arachidonic acid (AA) and prostaglandin release when expressed in CHO cells. Two major signal transduction events appear to underlie this response. Firstly, AA liberation is critically dependent upon agonist-stimulated influx of extracellular Ca^{2+} although not release from intracellular stores. Secondly, NK-2 receptor-linked AA mobilization requires concomitant PKC activation and based upon limited subtype immunodetectability as well as NKA-stimulated translocation, PKCα could play a major role. While NKA-stimulated Ca^{2+} mobilization is insensitive to preincubation with pertussis toxin, identical pretreatment inhibits AA release partially and blocks PKCα translocation completely. These observations indicate that in this cell system AA liberation reflects NK-2 receptor-dependent activation of two distinct but converging signal transduction pathways regulated by different G-protein species and involving Ca^{2+} influx and PKCα activation.

Key words: NK-2 receptor (human); Neurokinin A; Arachidonic acid; Eicosanoid; Ca^{2+} influx; PKC

1. Introduction

Arachidonic acid (AA) as well as products of its oxidative metabolism (eicosanoids) are powerful local modulators of diverse physiological responses including smooth muscle contraction, renal absorption, sleep–wake cycles, hyperalgesia, neurotransmission and mitogenesis [1–5]. Eicosanoids have also been implicated in a number of inflammatory disease states [6,7]. Although many neurotransmitters and hormones stimulate enhanced generation of eicosanoids through liberating AA, cellular mechanisms underlying this process are unclear. One important question relates to the pathways leading to activation of crucial phospholipases mobilizing membrane AA as this has been reported to reflect both direct G-protein coupling and indirect processes involving increased cytosolic Ca^{2+}, and/or phosphorylation [1,3,8–11]. It is unclear whether this reflects a redundancy of mechanisms with overlapping activities or whether discrete pathways predominate either within different cellular environments or to mediate the actions of specific receptors.

Neurokinins (tachykinins) are a family of neuropeptides dependent on cellular arachidonic acid mobilization for several important biological actions. They stimulate eicosanoid generation in endothelium [12], macrophages [13], astrocytes [14], rheumatoid synoviocytes [15] and heart [16] while blocking prostaglandin (PG) synthesis inhibits neurokinin-dependent spinal algesia [17], tracheal relaxation [18], control of myenteric cholinergic neurotransmission and colonic ion transport activity [19,20]. As part of an investigation of mechanisms underlying neurokinin-stimulated eicosanoid generation we have cloned a human ileal NK-2 receptor and, following stable expression in CHO cells, identified early signalling events crucial for AA liberation. We demonstrate that at least two distinct but converging NK-2 receptor-linked pathways, one involving Ca^{2+} influx the other PKCα activation, play a major role mobilizing AA.

2. Materials and methods

2.1. Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (180–240 Ci/mmol), prostaglandin (PG) E_2 [^{125}-I]-radioimmunoassay kits as well as ^{125}I-labelled sheep anti-mouse and anti-rabbit antibodies were purchased from New England Nuclear (Regensdorf, Switzerland). Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/F-12) medium, fetal calf serum, monoclonal antibodies directed against PKC α, β, (βI and βII) and γ peptide sequences as well as rabbit polyclonal antibodies against PKC δ and ε were purchased from Gibco (Grand Island, NY, USA). Remaining reagents were from the following companies: reduced streptolysin-O from Wellcome Diagnostics (Dartford, UK), Fura 2-AM and BAPTA-AM from Calbiochem (La Jolla, CA, USA), organic solvents from Fluka (Buchs, Switzerland) and all other chemicals from Sigma (St. Louis, MO, USA).

*Corresponding author. Fax: (41) (22) 794 69 65.

SSDI 0014-5793(93)E1481-Z

2.2. NK-2 receptor cDNA cloning and generation of cell lines

The coding region of the rat NK-1 receptor gene was subcloned by PCR from a sample of rat brain mRNA using primers designed from the published sequence [21]. A cDNA bank in λgt10 consisting of 4×10^7 independent phage was constructed with mRNA isolated from a piece of human ileum. Approximately 1×10^7 phage were screened at high stringency (hybridization, 20% formamide/5 × SSC/42°C; wash, 65°C/0.1 × SSC) with the rat NK-1 cDNA radiolabeled by random priming (Boehringer-Mannheim). Inserts were isolated from positive phage and sequenced using standard techniques. The cDNA inserts were subcloned under the control of the HTLV-I/LTR promoter in the mammalian expression vector pcDL-SRα296 [22] for recombinant expression studies. CHO:DUKX-B11 cells devoid of endogenous dihydrofolate reductase activity [23] were used as host cell for NK-2 receptor expression. The human NK-2 gene expression plasmid, pCDL/hNK2, was co-transfected in a 9:1 ratio with pNeo/DHFR, a plasmid containing the neomycin resistance gene and the dihydrofolate reductase gene behind the adenovirus major late promoter. Recombinant transfectants were selected after 2 days in 0.6 mg/ml G418 (Geneticin, Gibco) and assayed after 12 days by flow cytometry (FACSCAN, Becton and Dickinson) using the fluorescein-labelled NKA [24]. Cells were grown in DMEM/F12 supplemented with 10% fetal calf serum (Gibco), 2 mM glutamine, penicillin (200 IU/ml) and streptomycin (200 mg/ml) at 37°C and 5% CO_2. Cells were routinely used for assays when 80–90% confluent.

2.3. Prostaglandin E_2 and [^{3}H]AA liberation

Cells were cultured in 6-well plates ($1–2 \times 10^6$/well) and labelled for 18 h by incubation using serum-free DMEM/F-12 containing fatty acid-free bovine serum albumin at 2 mg/ml (FAF-BSA) and [^{3}H]AA (0.5–1.0 μCi/ml). For stimulation, cells were washed three times with 2 ml of warmed PBS containing FAF-BSA and then incubated at 37°C for 20 min under 5% CO_2 in 1 ml of serum-free medium with FAF-BSA and stimulatory agents as indicated. Incubations were terminated by transferring 800 μl to ice-cold tubes and centrifugation at 800 × *g* for 5 min after which aliquots were taken for either direct measurement of [^{3}H]AA release or for assay of PGE_2. When permeabilization was performed cells grown in 6-well plates were incubated for 10 min with DMEM/F-12 containing FAF-BSA and 1.0 U/ml of reduced streptolysin-O. Cells were then washed twice and incubated for 20 min at 37°C in the same medium supplemented with 2 mM EGTA and concentrations of $CaCl_2$ to give free Ca^{2+} levels between 10^{-7} M and 10^{-3} M.

2.4. Electrophoresis and immunoblotting

Cells grown in 9-cm Petri dishes were stimulated with NKA, phorbol ester or A23187 in DMEM/F-12 containing FAF-BSA as indicated. Cells were then washed twice in ice-cold PBS and harvested by scraping and centrifugation at 200 × *g* for 5 min. Cells were resuspended in 0.5 ml of 20 mM Tris, 2 mM EDTA, 10 mM EGTA, 5 mM DTT, 1 mg/ml leupeptin, 50 μg/ml aprotonin, 10 μM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride (pH 7.5) and broken with a 30 s burst of an Ultra-Turrax homogenizer (Bender and Hobein, Zürich, Switzerland). Following centrifugation at 50,000 × *g* for 30 min at 4°C the supernatent was taken as CHO/T cell soluble fraction while the pellet was washed in 25 ml of the same buffer, re-centrifuged and suspended in 0.5 ml of the same buffer containing 1% Triton X-100. Following 30 min on ice the extract was centrifuged again at 50,000 × *g* and the supernatent used as Triton-soluble membrane fraction. Protein extracts were separated by SDS-PAGE using 10% gels, transferred to nitrocelluose membranes and immunoblot analysis performed as decribed [25].

3. Results and discussion

Using a rat NK-1 gene probe, a human ileum cDNA library was screened and one phage with a 1.9 kbp insertion was found to contain the entire coding region for the human NK-2 receptor as published previously [27]. cDNA was transfected into Chinese hamster ovary cells (CHO/DUKX-B11; see section 2) and one G418-resistant cell population (CHO/T) selected for NK-2 receptor

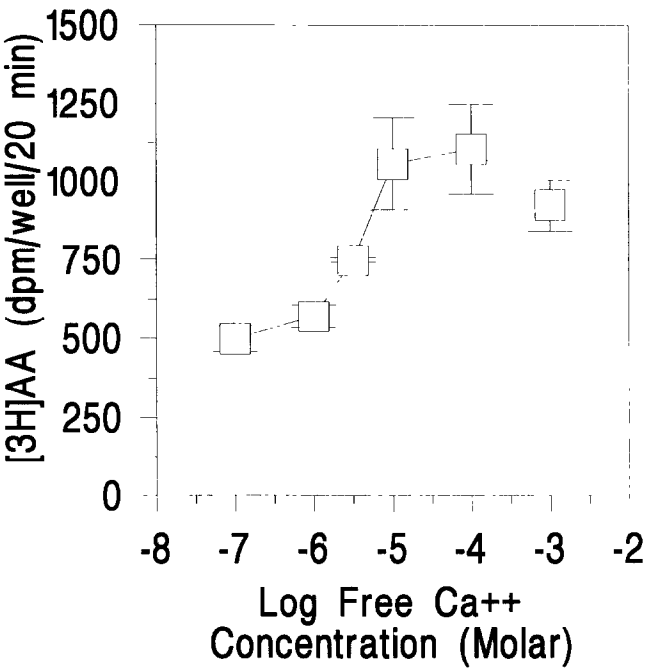

Fig. 1. Ca^{2+} is a crucial determinant of [^{3}H]AA liberation from CHO/T cells. Prelabelled CHO/T cells were grown in 6-well plates and permeabilized with reduced streptolysin-O. [^{3}H]AA mobilization was measured following 20 min incubation at 37°C with free-Ca^{2+} levels between 10^{-7} M and 10^{-3} M achieved by varying $CaCl_2$ concentrations in the presence of 2 mM EGTA. Points are the mean of 5 experiments each performed in triplicate.

expression by flow cytometry using a fluorescent NKA peptide analogue. Binding of [^{125}I]neurokinin A ([^{125}I]NKA) to CHO/T cells (not shown) indicated 28,000 high-affinity sites/cell which was displaced by natural neurokinins with the rank-order NKA>NKB>>SP (NKA $IC_{50} = 6 \pm 1$ nM) consistent with expression of the NK-2 receptor subtype [28,29]. As observed for CHO cells expressing the bovine NK-2 receptor [26], CHO/T responded to NKA stimulation with increased [^{3}H]AA and PGE_2 liberation (not shown) and these cells were employed for all studies described here.

In contrast to our previous report that NK-2 receptor-linked IP_3 generation is GTP-dependent in streptolysin-O permeabilized CHO cells [26], NKA and GTPγS fail to stimulate [^{3}H]AA mobilization under identical conditions at any concentration of free Ca^{2+} between 10^{-7} M and 10^{-3} M (data not shown). This suggests that rather than direct G-protein coupling, NK-2 receptor stimulated AA liberation reflects, at least in part, indirect signalling processes critically dependent upon an intact cell enviroment. Fig. 1 shows that in permeabilized cells physiologically relevant concentrations of free Ca^{2+} trigger increased [^{3}H]AA mobilization by up to 232 ± 32% ($n = 5$) suggesting that increased cytosolic Ca^{2+} could normally be an important signal. Since NK-2 receptors expressed in CHO cells are known to mobilize Ca^{2+} [26], we employed conditions optimized to inhibit selectively either receptor-linked Ca^{2+} influx or its liberation from

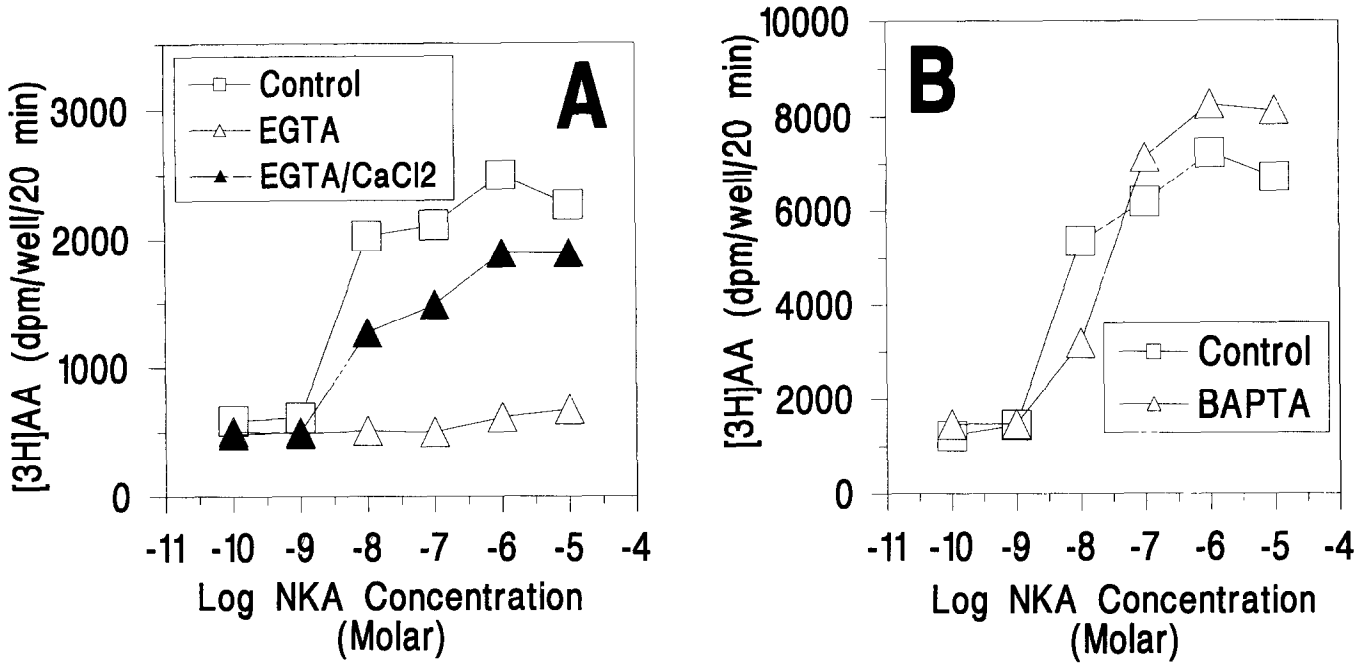

Fig. 2. Ca^{2+} is a crucial determinant of [^{3}H]AA liberation from CHO/T cells. Prelabelled CHO/T cells were grown in 6-well plates and [^{3}H]AA liberation measured following stimulation with increasing concentrations of NKA for 20 min at 37°C in the presence of 1 mM free Ca^{2+}, following Ca^{2+} chelation with 2 mM EGTA, or after addition of 2 mM EGTA/3 mM $CaCl_2$ (A). In similar experiments [^{3}H]AA release was tested with 1 μM extracellular Ca^{2+} after loading cells with the intracellular chelator BAPTA-AM (50 μM for 5 min) (B). Points are the mean of 3 (A) or 5 (B) experiments each performed in triplicate.

intracellular stores (not shown) to test which of these Ca^{2+} components is quantitatively more important for [^{3}H]AA liberation. While blocking NKA-stimulated Ca^{2+} influx using extracellular EGTA (2 mM, added <2 min before stimulation) abolished NK-2 receptor-linked [^{3}H]AA mobilization, supplementing medium with 3 mM $CaCl_2$ restored cellular responsiveness (Fig. 2A) suggesting that extracellular Ca^{2+} plays a crucial role. Surprisingly, loading cells with the chelator BAPTA-AM (50 μM; pretreatment for 5 min) to abolish rapid NKA-stimulated Ca^{2+} mobilization did not effect NKA-stimulated [^{3}H]AA release (Fig. 2B). These results suggest that NKA-stimulated Ca^{2+} influx and not release from intracellular stores is a major trigger mediating [^{3}H]AA liberation.

Stimulation of NK-2 receptors expressed in CHO cells is reported to increase phospholipase (PL) C-dependent phosphoinositide hydrolysis [26,29,30]. One likely consequence of this hydrolytic reaction is activation of PKC [31] which is an additional potentially important early step in processes leading to AA liberation. To test this we employed two treatments optimized to inhibit endogenous PKC activities in intact CHO/T cells (data not shown). Following either treatment with staurosporine (10^{-6} M for 15 min) or long-term exposure to phorbol ester (10^{-6} M PMA for 18 h), NKA-stimulated [^{3}H]AA mobilization was suppressed by ~50% (Fig. 3A). A general inhibitory effect on NK-2 receptor-linked signalling and phospholipase activation is unlikely as NKA stimulated [^{3}H]inositol phosphate generation is increased by up to 5-fold following identical pretreatment (data not shown). These observations support a central role for PKC in an NK-2 receptor-linked pathway liberating AA.

Molecular cloning indicates that mammalian PKC exists as a large family consisting of at least 10 subspecies with closely related structures. These have been subdivided into Ca^{2+}-sensitive (PKCα, βI, βII and γ) and Ca^{2+}-independent (PKCδ, ε, ζ, η, θ and λ) isoforms with different activation requirements, kinetic properties, substrate specificities, tissue distribution and subcellular localization [31]. To test which of these subtypes are expressed in CHO/T cells as well as which are regulated by the NK-2 receptor and could be involved in AA mobilization we have employed specific antibodies for immunoblot analysis. While isoforms β (either βI and βII), γ and ε were not detectable by this approach, bands of expected molecular weight (~80 kDa) were observed using antibodies selective for PKCα (Fig. 4A) and PKCδ (not shown). PKC activation is characterized by translocation from cytosolic to particulate fractions and such a relocalization of PKCα was observed following stimulation with either NKA or phorbol ester (Fig. 4A). Although PKCδ was found localized in both particulate and soluble fractions in resting cells, low expression levels prevented clear assessment of whether this distribution is sensitive to NKA stimulation. Increased membrane association was however observed in the presence of phorbol ester (not shown). Together these observations suggest that PKC plays a role mediating NKA-stimulated AA liberation and that PKC subtypes δ and

particularly α are potential mediators of this response. Such a conclusion is not restricted to NK-2 receptors in CHO cells as PKCα has also been proposed as the major isoform mediating bradykinin-stimulated AA release in MDCK cells [32] while heterologous expression of this PKC subtype facilitates considerably PDGF-stimulated AA efflux in NIH 3T3 fibroblasts [33].

Although these observations implicate PKC as a mediator of AA liberation in CHO cells, we were surprised to find that stimulation with PMA under conditions mediating PKCα and δ translocation (Fig 4A) failed to enhance [^{3}H]AA release (Fig. 5). In the presence of the calcium-ionophore A23187, however (which alone stimulated [^{3}H]AA liberation by ~14-fold), phorbol ester augments [^{3}H]AA release up to ~35-fold above basal levels with half maximal effect observed at 3 nM PMA (Fig. 5). NKA mimicks this A23187 action to augment PMA-stimulated [^{3}H]AA output although effective phorbol ester concentrations are ~10-fold higher (Fig. 5). This synergistic stimulation of [^{3}H]AA output reflects concomitant Ca^{2+} mobilization and PKC activation. Hence, 2 mM EGTA abolished all stimulatory actions in the presence of A23187 while supplementing with excess $CaCl_2$ (3 mM) restored full responsiveness (data not shown). In addition, all PMA actions were abolished following 18 h incubation with 10^{-6} M phorbol ester to down regulate PKC (Fig. 5). Interestingly, PKC down-regulation also inhibited A23187-stimulated [^{3}H]AA release by 63 ± 6.0% (n = 6) (Fig. 5) suggesting that as well as PMA, a large proportion of ionophore action reflects PKC activation. This is consistent with A23187-stimulated translocation of PKCα from cytosolic to membrane fractions (Fig. 4A). These observations demonstrate that although PKC activation is an important component in mediating NKA-stimulated [^{3}H]AA liberation, this effect is crucially dependent upon concomitant Ca^{2+} influx.

A further interesting observation in our cell system was that pretreatment with pertussis toxin under conditions which maximally ADP-ribosylate G-proteins endogenous to CHO/T cells [26] blocks totally NKA-stimulated PKCα translocation (Fig. 4B). This is consistent with an important role for pertussis toxin sensitive PKCα activation in mediating NK-2 receptor-dependent [^{3}H]AA liberation as this was also inhibited by 73 ± 5.0% (n = 4) following identical pretreatment (Fig. 3B). Importantly, inhibition of AA release following combined pretreatment with pertussis toxin and PMA to down regulate PKC is no greater than with either agent acting alone (Fig. 3B). It is of note that as reported previously for NK-2 receptor-linked IP_3 generation [26], both Ca^{2+} influx and mobilization from intracellular stores are insensitive to CHO/T cell pretreatment with pertussis toxin (data not shown). This indicates that similar to muscar-

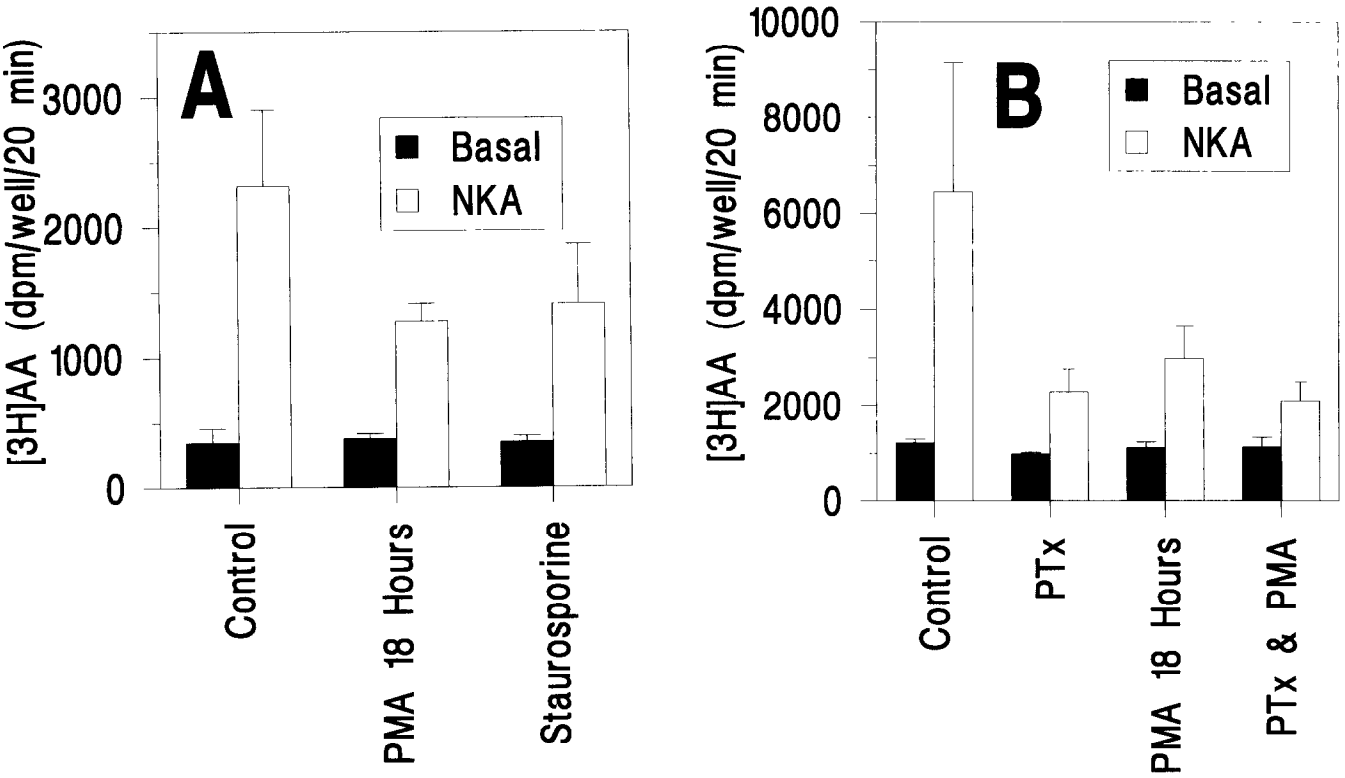

Fig. 3. Inhibition of NKA-stimulated [^{3}H]AA generation following pretreatment with phorbol ester, staurosporine or pertussis toxin. CHO/T cells grown in 6-well plates were prelabelled and NKA-stimulated [^{3}H]AA liberation measured for 20 min after pretreatment with either 1 μM PMA for 18 h, 1 μM staurosporine for 15 min (A) or with pertussis toxin (PTx; 500 ng/ml for 18 h) with and without PMA (1 μM PMA for 18 h) (B). Bars represent the mean ± S.D. of 4 experiments each performed in triplicate.

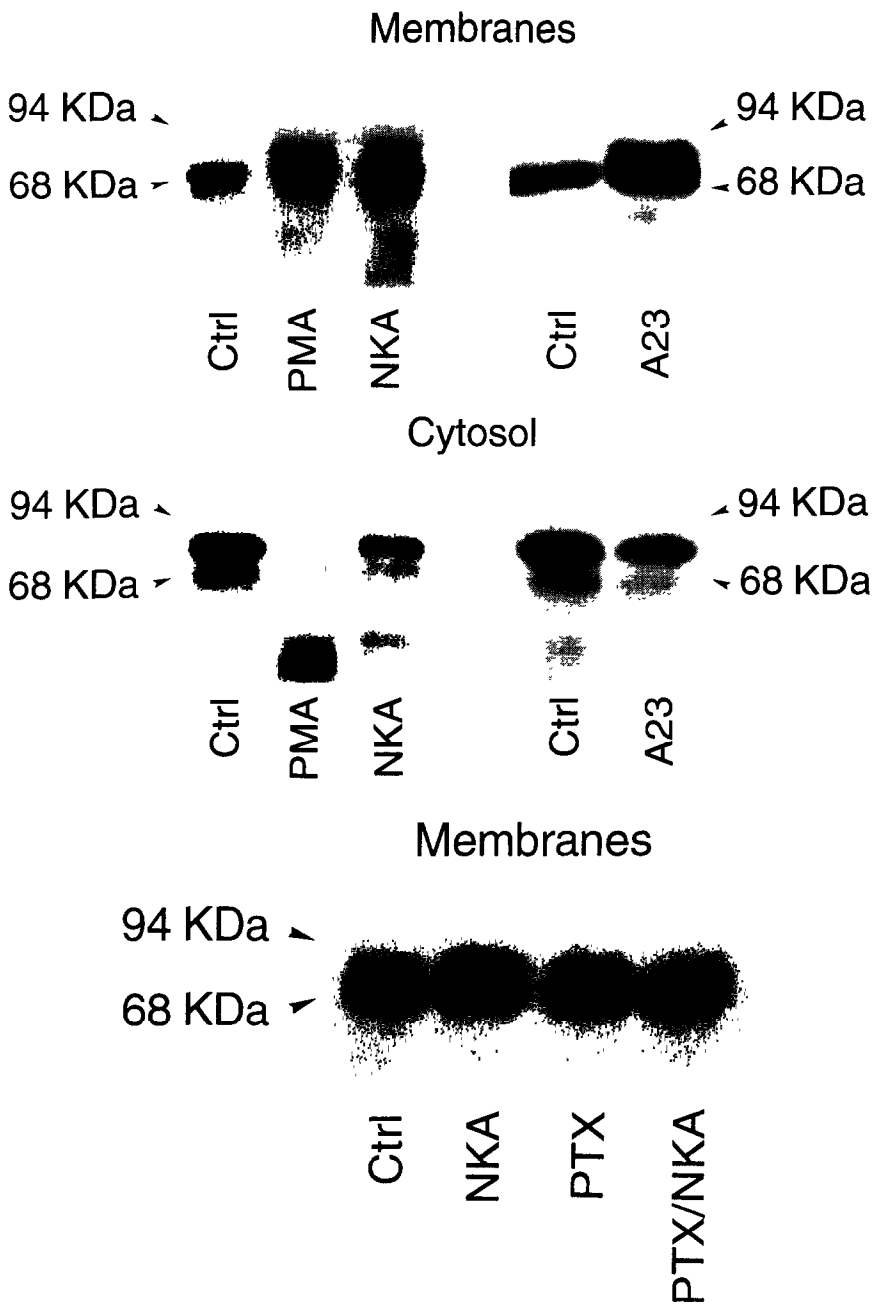

Fig. 4. Immunodetection of PKCα and δ in CHO/T cell extracts. CHO/T cells grown in 9 cm Petri dishes were stimulated with NKA (1 μM), PMA (0.1 μM) or A23187 (A23; 10 μM) for 20 min at 37°C and cell soluble and membrane fractions analysed by SDS-PAGE and immunoblotting with PKCα-selective antibodies as described in section 2. Where indicated cells were pretreated with pertussis toxin (PTX) at 500 ng/ml for 18 h.

inic and metabotropic glutamate receptors expressed in CHO cells [34,35], human NK-2 receptors interact with multiple G-proteins to activate independent second messenger responses (Ca^{2+} influx and PKCα activation) which converge at a level crucial in processes mediating AA liberation. While G-proteins of the G_q class are expressed in CHO cells [36] and are likely candidates mediating pertussis toxin insensitive NK-2 receptor-linked phosphoinositide hydrolysis and Ca^{2+} mobilization [26], $G_{\alpha i2}$ is important for AA release in this cell type [37] and could underly pertussis toxin sensitive events such as PKC activation.

Acknowledgements: We thank C. Gillieron, A. Chollet, C. Losberger and K. Ceszowski for excellent technical assistance. We are also grateful to Dr. J. Knowles for support during these studies. This paper is dedicated to the memory of Philip Godfrey.

References

[1] Axelrod, J., Burch, R.M. and Jelsema, C.L. (1988) Trends Neurosci. 11, 117–123.
[2] Smith, W.L. (1989) Biochem. J. 259, 315–324.
[3] Shimizu, T. and Wolfe, L.S. (1990) J. Neurochem. 55, 1–15.
[4] Hayaishi, O. (1991) FASEB. J. 5, 2575–2581.

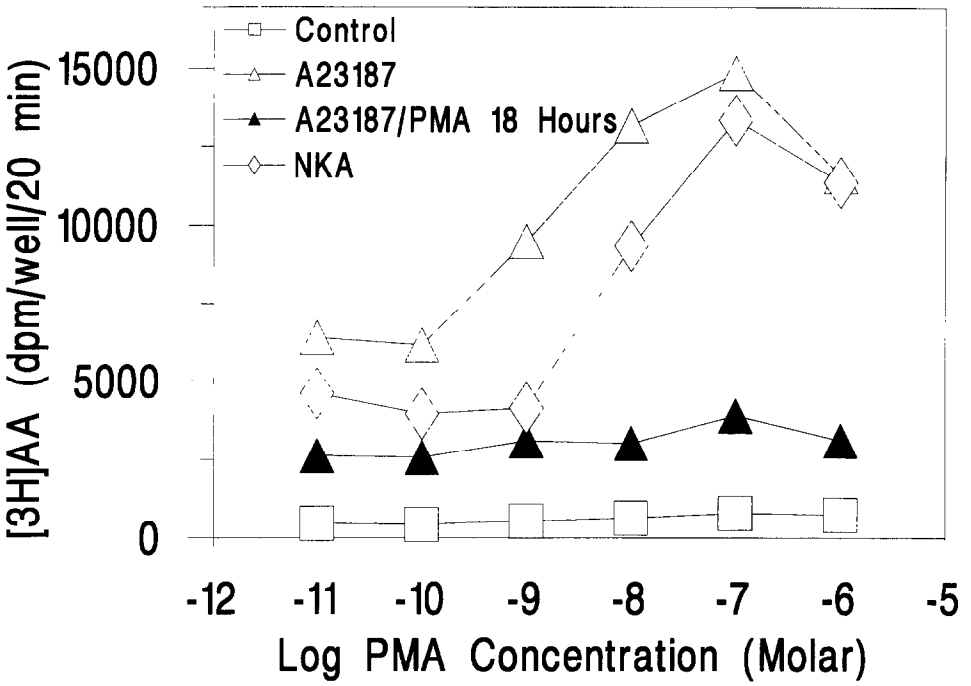

Fig. 5. Phorbol ester stimulates [^{3}H]AA liberation only in the presence of A23187 or NKA. Prelabelled CHO/T cells grown in 6-well plates were incubated for 20 min at 37°C with increasing concentrations of PMA either alone or together with 1 μM NKA or 10 μM A23187 and [^{3}H]AA liberation measured as described in section 2. Comparison was made with CHO/T pretreated with 1 μM PMA for 18 h. Data points represent the mean of 5 (PMA alone, PMA with A23187), 3 (PMA with NKA) or 4 (following 18 h with PMA) experiments each performed in triplicate.

[5] Huang, N.-N., Wang, D.-J. and Heppel, L.A. (1993) J. Biol. Chem. 268, 10789–10795.
[6] Wallace, J.L. (1990) Trends Pharmacol. Sci. 11, 51 53.
[7] Barnes, P.J. (1991) Trends Biochem. Sci. 16, 365–369.
[8] Ho, A.K. and Klein, D.C. (1987) J. Biol. Chem. 262, 11764–11770.
[9] Billah, M.M. and Anthes, J.C. (1990) Biochem. J. 269, 281–291.
[10] Lin, L.-L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A. and Davis, R.J. (1993) Cell 72, 269–278.
[11] Nemenoff, R.A., Winitz, S., Qian, N.-X., Putten, V.V., Johnson, G.L. and Heasley, L.E. (1993) J. Biol. Chem. 268, 1960–1964.
[12] Marceau, F., Tremblay, B., Couture, R. and Regoli, D. (1989) Can. J. Physiol. Pharmacol. 67, 159–162.
[13] Hartung, H.-P., Wolters, K. and Toyka, K.V. (1986) J. Immunol. 136, 3856–3863.
[14] Marriott, D.R., Wilkin, G.P. and Wood, J.N. (1991) J Neurochem. 56, 259–265.
[15] Lotz, M., Carson, D.A. and Vaughan, J.H. (1987) Science 235, 893–895.
[16] Assem, E.S.K., Ghanem, N.S., Abdullah, N.A., Repke, H., Foreman, J.C. and Hayes, N.A. (1989) Immunopharmacology 17, 119–128.
[17] Malmberg, A.B. and Yaksh, T.L. (1992) Science 257, 1276–1279.
[18] Devillier, P., Acker, G.M., Advenier, C., Marsac, J., Regoli, D. and Frossard, N. (1992) J. Pharmacol. Exp. Ther. 263, 767–772.
[19] Rangachari, P.K., Prior, T. and McWade, D. (1990) J. Pharmacol. Exp. Ther. 254, 1076–1083.
[20] Mayer, E.A., Koelbel, C.B.M., Snape, W.J., Vandeventer, G. and Leduc, L. (1990) Am. J. Physiol. 258, G122-G128.
[21] Yokota, Y., Sasai, Y., Tanaka, K., Fujiwara, T., Tsuchida, K., Shigemoto, R., Kakizuka, A., Ohkubo, H. & Nakanishi, S. (1989) J. Biol Chem. 264, 17649–17652.
[22] Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M. and Arai, N. (1988) Mol. Cell Biol. 8, 466–472.
[23] Nicolas, J.F. & Berg, P. (1983) in: Cold Spring Harbor Conference on Cell Proliferation, No. 10 (Cold Spring Harbor, New York, Cold Spring Harbor Laboratories), pp. 469–485.
[24] Ceszkowski, K. and Chollet, A. (1992) Biomed. Chem. Lett. 2, 609–612.
[25] Church, D.J., Braconi, S., Vallotton, M.B. and Lang, U. (1993) Biochem. J. 290, 477–482.
[26] Eistetter, H.R., Church, D.J., Mills, A., Godfrey, P.P., Capponi, A.M., Brewster, R., Schulz, M.-F., Kawashima, E. and Arkinstall, S.J. (1991) Cell Regulation 2, 767–779.
[27] Gerard, N.P., Eddy, R.L., Shows, T.B. and Gerard, C. (1990) J. Biol. Chem. 265, 20455–20462.
[28] Helke, C.J., Krause, J.E., Mantyh, P.W., Couture, R. and Bannon, M.J. (1990) FASEB J. 4, 1606–1615.
[29] Guard, S. and Watson, S.P. (1991) Neurochem. Int. 18, 149–165.
[30] Grandordy, B.M., Frossard, N., Rhoden, K.J. and Barnes, P.J. (1989) Mol. Pharmacol. 33, 515–519.
[31] Nishizuka, Y. (1992) Science 258, 607–614.
[32] Godson, C., Bell, K.S. and Insel, P.A. (1993) J. Biol. Chem. 268, 11946–11950.
[33] Finkenzeller, G., Totzke, F., Fitzke, E., Marme, D. and Dieter, P. (1993) FEBS Lett. 321, 11–14.
[34] Ashkenazi, A., Peralta, E.G., Winslow, J.W., Ramachandran, J. and Capon, D.J. (1989) Cell 56, 487–493.
[35] Aramori, I. and Nakanishi, S. (1992) Neuron 8, 757–765.
[36] Mullaney, I., Mitchell, F.M., McCallum, J.F., Buckley, N.J. and Milligan, G. (1993) FEBS Lett. 324, 241–245.
[37] Gupta, S.K., Diez, E., Heasley, L.E., Osawa, S. and Johnson, G. (1990) Science 249, 662–666.