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BRADYKININ STIMULATION OF INOSITOL PHOSPHATE AND CALCIUM RESPONSES IS INSENSITIVE TO PERTUSSIS TOXIN IN NG115-401L NEURONAL CELLS

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SUMMARY: Stimulation of NG115-401L neuronal cells with bradykinin produces a dose-dependent increase in inositol phosphate production which is not blocked, rather slightly increased, after treatment with pertussis toxin. Nevertheless, pertussis toxin stimulates ADP-ribosylation of a 41K membrane protein, and blocks opioid receptor-mediated inhibition of stimulated cAMP production in these cells. These results suggest that bradykinin responses in the NG115-401L cells are pertussis-insensitive, unlike bradykinin responses resported in other neuronal cell lines.

INTRODUCTION: The bacterial toxin, pertussis toxin, is now recognised to act on several membrane substrates by ADP-ribosylation (1). These substrates include Gi, (2), and other GTP-binding proteins, termed Go(3) Gp(4) and Gc(5). The effect on Gi is inactivation of its activity in coupling receptors to the negative regulation of adenyl cyclase (2). Thus, it is of considerable interest that in several cell populations, receptors coupled to inositol phosphate production and intracellular calcium discharge can be blocked by pertussis toxin (5). However, not all inositol lipid receptors are sensitive to pertussis toxin (5). One interpretation is that a given signalling pathway may use more than one G-protein, as has been suggested for mitogen stimulation of CCL39 fibroblasts (6). In this regard, it is unclear whether neuronal activation through an identified receptor, using a G-protein coupled second messenger pathway, always works through the same G-protein. Accordingly, we have examined the pertussis-toxin sensitivity of a receptor-coupled inositol lipid response characterised in detail; the activation of NG115-401L cells by the neural peptide bradykinin (7). For purposes of comparison,two closely-related hybrid cell lines, NG108-15 and F11, express bradykinin receptors which are pharmacologically identical to those in the 401L cells (8,9), and, in both instances, pertussis toxin treatment blocks bradykinin-induced generation of inositol phosphates. On the contrary, similar pertussis toxin treatment, fully effective in ADP-ribosylation and blocking a Gi-mediated opioid receptor response, is shown here to be ineffective in blocking bradykinin actions.

MATERIAL AND METHODS: NG115-401L cells were grown as monolayers in antibiotic-free medium, as described earlier (7). Pertussis toxin (gift of Dr J. Munoz, NIH) was added to a final concentration of 300 ng/ml for the final 16 hours in vitro.

For the measurement of inositol phosphate production, cells were grown in 24-well plates and maintained for the final 16 hours in culture in DMEM containing 10 μM myo-inositol (Sigma), 2 μCi/ml [^{3}H]myo-inositol (New

Abbreviations used: DMEM; Dulbecco's Modified Eagle's Medium; DAGO, [D-Ala^2-N-MePhe^4,Gly^{5}ol]-enkephalin;DSLET, [D-Ser^2]-leu-enkephalin-thr; EDTA, ethylenediaminetetracetic acid; FCS, foetal calf serum; Ins 1-P, inositol 1-monophosphate; Ins 1,4,5-P3, inositol 1,4,5-trisphosphate; Ins P, Ins P2 and Ins P3, inositol mono, bis and trisphosphates; NAD, nicotinamide adenine dinucleotide; PGE1, prostaglandin E1; TES, N-tris [hydroxymethyl] methyl-2-aminoethanesulfonic acid.

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England Nuclear, 14 Ci/mmol) and 5%dialysed FCS. Labelled cells were washed in 0.25 ml balanced salt solution (7), and preincubated in the same medium supplemented with 10 mM LiCi for 15 min (10). After peptide addition (10µl), incubations were continued (30 min, 37°C) and quenched by the addition of 0.25 ml ice-cold 10% perchloric acid. After 5 minutes on ice, extracts were neutralised as described (11), and applied to Dowex AG 1 x 8 (Bio-Rad, formate form) mini-columns (0.5ml). Elution conditions were as described earlier (7) and pooled fractions containing Ins P, Ins P2 and Ins P3 were counted by liquid scintillation.

For the measurement of cyclic AMP production, cells were grown in 6-well plates. The growth medium was aspirated, the monolayers washed by a balanced salt solution (2 x 4 mls), and the cells preincubated for 60 min at 37°C. Drugs were added for 10 min., and the incubations quenched by ice-cold 5% percholoric acid (0.5 ml). Extracts were centrifuged and neutralised for determination of cAMP content by a competitive protein binding assay (12).

For examination of ADP-ribosylation, homogenates were prepared by harvesting cells with a teflon-coated spatula into TES-KOH buffer (10 mM, pH 7.4) containing 10mM $MgSO_4$, 1 mM EDTA, 1 mM benzamidine-HCl, 1mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.01% bacitracin. Cells were centrifuged at 1500 x g (10 min., 40°C). The cell pellet was resuspended in ice-cold buffer and homogenised with a Potter-Elvehjem homogeniser (protein concentration 1 mg/ml). The homogenate was incubated (30 min., 4°C) and centrifuged at 40,000 x g (30 min., 8°C). The final pellet was resuspended in ice-cold TES-KOH buffer (protein concentration 1 mg/ml). ADP-ribosylation was performed essentially as described (13) with slight modifications indicated in the legend to Figure 2.

Unless otherwise noted, all materials were reagent grade from standard commercial sources. Peptides were from Cambridge Research Biochemicals.

RESULTS: Addition of 100 nM bradykinin to [^{3}H]inositol-labelled NG115-401L cells increased inositol phosphate production roughly twofold over basal (Fig. 1A). Overnight incubation with pertussis toxin did not inhibit

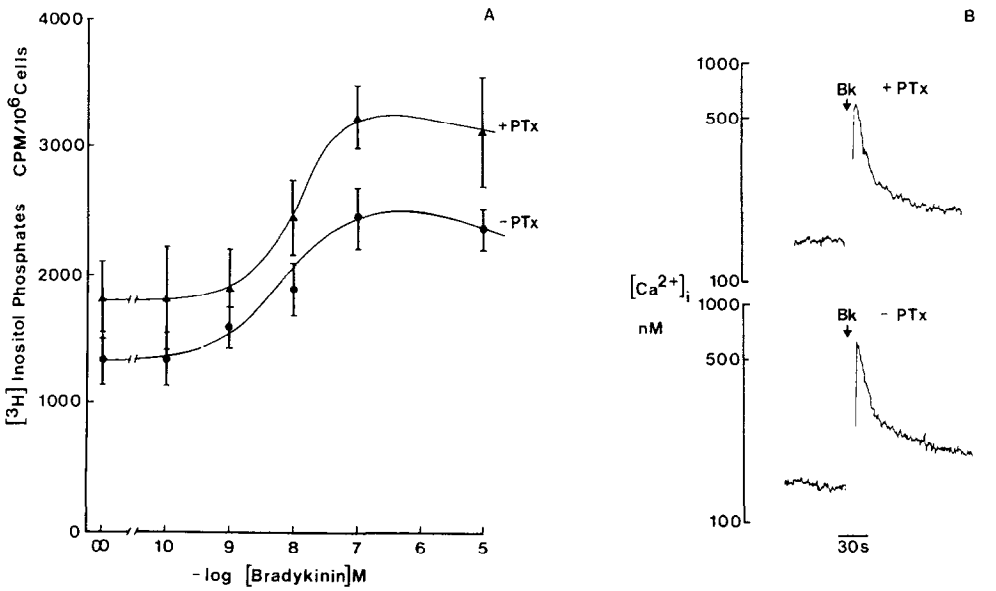

Figure 1. Influence of pertussis toxin treatment on bradykinin stimulation of inositol phosphate production and increase in intracellular free calcium.

A. Cells were labelled with [^{3}H]inositol with (▲) or without (●) 300 ng/ml pertussis toxin, and then prepared as described in Materials and Methods. Cells were incubated with bradykinin for 30 min in the presence of 10 mM LiCl. Data are presented as means ± SEM of inositol phosphate formation/106 Cells. Data are from triplicate determinations from 3 separate experiments.

B. The fluorescence intensities (500 nm emission) from Fura-2-loaded cells grown on coverslips were recorded in a Perkin-Elmer fluorimeter using a thermostatted cuvette holder (37°C), as described in detail elsewhere (7).

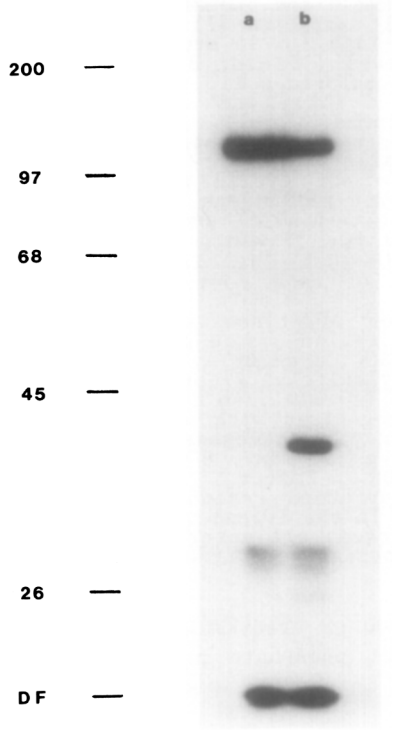

Figure 2. Pertussis toxin-stimulated ADP-ribosylation of NG115-401L cell membranes.

Membranes were incubated without (lane A) or with preactivated (10 µg toxin incubated for 1 h 25°C with 50 mM DTT in 25 µl water) pertussis toxin (lane B) in the presence of [^{32}P]NAD^{+}. Membranes (0.1 mg) were incubated with the preactivated toxin in potassium phosphate buffer (100 mM, pH 7.0, 0.1 ml) containing 10 mM thymidine, 1 mM ATP, 0.5 µM [alpha-^{32}P]NAD+ (New England Nuclear) at 30°C for 1 hour. Radiolabelled membranes were immediately precipitated by chloroform/methanol and the dried pellet solubilised in sample buffer (0.1 ml) and analysed by 10% SDS-PAGE. Molecular weight protein standards were: lysozyme (14300); B-lactoglobulin (18400); alpha-chymotrypsinogen (25700); ovalbumin (45000); bovine serum albumin (68000); phosphorylase B (97400) and myosin heavy-chain (200000). The dried gel was autoradiographed with Fuji RX-7 film at -70°C for 1-2 days.

stimulated inositol phosphate production. Indeed it caused increases in both the basal and stimulated inositol phosphate levels (Fig. 1A). Independent confirmation that the initial bradykinin receptor-coupled event was unaffected by pertussis toxin was obtained by measurement of intracellular [Ca^{2+}] using the indicator fura-2 (Fig. 1B)

The insensitivity of the bradykinin responses to pertussis toxin might indicate the cells lacked the appropriate pertussigen receptor or that the modification reaction was incomplete. Accordingly, we examined the ability of pertussis toxin to block a Gi-mediated response. In NG115-401L cells, treatment with PGE1 stimulates cAMP production which can be inhibited by the delta-opioid receptor agonist DSLET in a naloxone-sensitive manner (Table 1). The mu-opioid receptor agonist DAGO had no effect on PGE1-stimulated cAMP accumulation. These results demonstrate that the NG115-401L cells have a delta-opioid receptor coupled to inhibitory regulation of cAMP levels; a response known to be mediated by Gi (2). Incubation with pertussis toxin (300 ng/ml) abolishes the DSLET inhibition without effect on the PGE1 stimulation (Table 1). Thus, pertussis toxin can act in these cells on a known pertussis-sensitive pathway.

Table 1. Effect of PGE1, DSLET, naloxone and pertussis toxin treatment on production of cAMP in NG115-401 cells

	pmol cAMP/10^6 cells	
	control	Pertussis pretreatment
No addition	3.4 ± 0.4*	4.2 ± 0.5*
30 μM PGE1	20.5 ± 1.3π	34.0 ± 1.9≠π
30 μM PGE1 + 5 nM DSLET	11.0 ± 2.2**	33.9 ± 1.8≠
30 μM PGE1 + 5 nM DSLET + 5μM naloxone	22.2 ± 1.9±	N.D.

Cells were incubated with pertussis toxin (300 ng/ml) for 16 hours as appropriate. After a 10 minute incubation with drugs, cAMP was extracted and determined as described in Materials and Methods. Data are means ± SEM of triplicate determinations, of three experiments. Significance was determined by Students t-test.

N.D. Not determined

* not different, P>0.1
≠ not different, P>0.5
π different P<0.01
** different from PGE1-stimulated, P<0.01
± not different from PGE1-stimulated, P>0.1

ADP-ribosylation of NG115-401L cell membranes in the presence of [^{32}P]NAD revealed pertussis toxin-stimulated labelling of a single band of 41,000 Da molecular weight, likely to correspond to the alpha-subunit of Gi. ADP-ribosylation was complete in that pre-incubation of cells with pertussis toxin (300 ng/ml), prior to membrane preparation, quantitatively eradicated stimulated radioactive labelling. Analysis of pertussis toxin-stimulated labelling of rat brain membranes in parallel lanes indicated a well-resolved doublet of 41,000 and 39,000 Da, (data not shown), likely to correspond to the alpha-subunits of Gi and Go respectively (14). Thus, these cells may be deficient in Go, a candidate mediator of receptor coupling to inositol lipid breakdown in those circumstances where pertussis toxin sensitivity is observed (15). The apparent lack of Go lends added weight to the evidence that bradykinin responses do not require pertussis-sensitive G-protein.

DISCUSSION: The comparison of clonal cell lines expressing an identical functional receptor can identify whether or not the associated signalling components are highly conserved. In two neuronal hybrid cell lines, NG108-15 and F11, the bradykinin receptor has been reported to be sensitive to the action of pertussis toxin, unlike the results herein. In all three instances, pertussis toxin has been used in the same concentration range for the same period of time. Moreover, control experiments established that pertussis toxin could abolish the Gi-mediated inhibitory responses to opioid peptides without reducing the Gs-mediated stimulatory responses to PGE1; as has been seen in a variety of other cells and tissues. Consequently, neither Gi nor Go is involved in the bradykinin pathway in these cells, unlike the claim in NG108-15 cells (8). This suggests that the bradykinin receptor can be coupled to inositol lipid pathways through distinct G-proteins, distinguished by their sensitivity to pertussis toxin. Indeed, in other homogeneous cell populations, it has been suggested that both pertussis-sensitive and pertussis-insensitive responses to different receptors working through the same pathway can co-exist (16,17).

Intriguingly, pertussis toxin treatment causes a small but reproducible elevation in basal and bradykinin-stimulated responses. This has been reported earlier in platelets (18) in response to thrombin. Whether these

increases reflect an interaction mediated by Gi or by another pertussis toxin substrate in these cells will be an important issue for further study.

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