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PERTUSSIS AND CHOLERA TOXINS INHIBIT PROSTAGLANDIN SYNTHESIS IN RAT ASTROCYTE CULTURES AT DISTINCT METABOLIC STEPS

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Abstract—The influence of pertussis and cholera toxin-sensitive G-proteins in the prostaglandin synthetic pathway has been investigated. Prostaglandin D_2 (PGD_2) synthesis was stimulated by the calcium ionophore A23187, the phorbol ester tetradecanoylphorbol acetate (TPA), or by extracellular ATP. Pretreatment of cultures with pertussis toxin (Ptx) resulted in a partial inhibition of PGD_2 synthesis in both stimulated and unstimulated cells. A23187-stimulated PGD_2 synthesis was affected less than ATP- or TPA-stimulated synthesis. Furthermore, Ptx also inhibited A23187-, ATP-, and TPA-stimulated arachidonic acid release. Basal and stimulated PGD_2 synthesis were also inhibited, when cultures were preincubated with cholera toxin (Ctx). Here, ATP-stimulated synthesis was affected the most. Arachidonic acid release, in contrast, was enhanced by cholera toxin, even without addition of stimuli. These data suggest that regulation of prostaglandin synthesis in rat astrocyte cultures involves Ptx- and Ctx-sensitive G-proteins. Ptx substrates affect events at or proximal to phospholipase A_2, whereas Ctx substrates influence events proximal or distal to phospholipase A_2.

A very early step in intracellular signaling pathways entails an activation of receptor-coupled GTP-binding proteins. Activated G-proteins are able to either up- or down-regulate the activities of cellular key enzymes, or regulate ion channels [1–3]. Pertussis and cholera toxins (Ptx and Ctx†) have proved to be an invaluable aid in elucidating the involvement of G-proteins in intracellular signaling cascades by their specific ability to ADP-ribosylate the α-subunits of a variety of G-proteins, resulting in their irreversible inactivation or long-lasting activation, respectively [1, 4]. Pertussis toxin sensitive G-proteins have not only been implicated as inhibitors of adenylate cyclase (G_i) but also as regulators of phospholipase C [5–8] and phospholipase A_2 activities [1, 9, 10]. The present report gives evidence of an involvement of both Ptx- and Ctx-sensitive G-proteins in the regulation of prostaglandin synthesis in astroglial cultures. Pertussis toxin substrate(s), however, appears to control some early event in the biosynthesis of prostaglandins, whereas Ctx substrate(s) may interfere primarily with later events.

MATERIALS AND METHODS

Materials. Ptx and Ctx were purchased from List laboratories (Campbell, CA, U.S.A.); [^{3}H]PGD_2 and [^{3}H]arachidonic acid were from Amersham and Buchler, (Braunschweig, Germany); fatty acid free BSA, ATP, TPA and A23187 were from the Sigma Chemical Co. (Deisenhofen, Germany); LPS (*Salmonella typhii*) and fetal calf serum were from Sebak/Pan Systems (Aidenbach, Germany); and DMEM was from Gibco (Eggenstein, Germany). PGD_2 antisera were made according to a procedure described previously [11]. Cross-reactivity with PGE_2 and other prostaglandins, or with AA was less than 1%.

Cell cultures. Astroglial cultures were prepared from newborn rats as described elsewhere [12] and maintained at precisely 10 ng LPS/mL media [13].

Prostaglandin release and radioimmunoassays. Cultures were preincubated in HEPES-buffered DMEM, pH 7.4 and cells were then stimulated for another 15 min in the same media containing substances as indicated. Reactions were stopped by mixing supernatants into ice-cold phosphate-buffered gelatin solution, pH 7.4. Cells were solubilized in 0.1 M NaOH and protein was determined according to Lowry *et al.* [14]. Supernatant–gelatin (200–500 μL, 0.1%) mixture was used for radioimmunoassays specific for PGD_2, as described previously [11].

Arachidonic acid release. Cells were incubated overnight in [^{3}H]AA (0.25 μCi/mL; sp. act. 207 Ci/mmol) and 10 ng/mL unlabeled AA (maximum ^{3}H-incorporation was achieved at this concentration, which probably ensured an even distribution in all cellular compartments). After removal of the media, they were incubated sequentially in DPBS (5 min), DPBS/1% BSA (fatty acid-free) (5 min) and stimulated with ATP, A23187, or TPA in PBS/BSA (30 min). Supernatants were transferred into scintillation vials and radioactivity determined. AA release was calculated as the percentage of total

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† Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate buffered saline; IP_3, inositol trisphosphate; PGD_2, prostaglandin D_2; LPS, lipopolysaccharide; PIP_2, phosphoinositoldiphosphate; PLA_2, phospholipase A_2; Ptx, pertussis toxin; Ctx, cholera toxin; TPA, tetradecanoyl phorbol acetate.

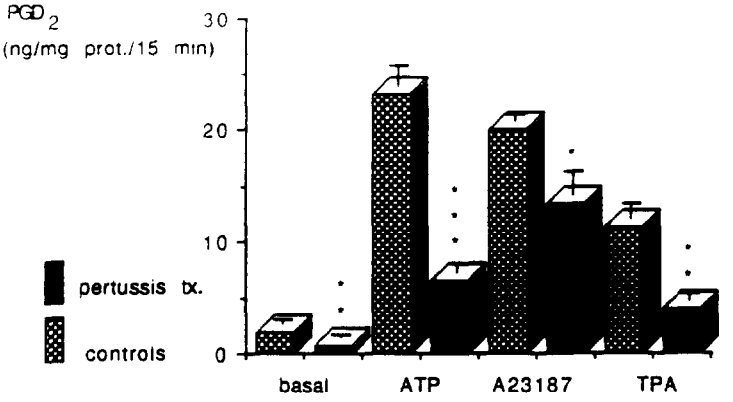

Fig. 1. Pertussis toxin-mediated inhibition of basal and stimulated prostaglandin D_2 synthesis in astroglial cultures. A23187, TPA [10^{-6} M] each, ATP [10^{-3} M]. Cultures were incubated with Ptx, 50 ng/mL, overnight. In previous experiments these conditions turned out to be optimal. Experiments were each performed three times, in triplicate (N = 9). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to controls.

radioactivity incorporated into cells. As verified in previous experiments by HPLC and TLC, radiolabel in those supernatants was exclusively associated with AA. Preferential binding of AA to albumin has been reported by others [15, 16].

Statistics. Data represent arithmetic means ± SEM. Significant differences between values were evaluated by the Student's *t*-test.

RESULTS

Pertussis toxin inhibits PGD_2 synthesis and arachidonic acid release

Two to three-week-old astroglial cultures were incubated with Ptx (50 ng/mL) for 16–20 hr and subsequently stimulated with A23187 (10^{-6} M), ATP (10^{-3} M) or TPA (10^{-6} M) for 15 min. In previous experiments, concentrations of Ptx [17] and stimuli were found to be in the saturation range [18, 19]. Compared to untreated controls, Ptx inhibited both basal (non-stimulated) and stimulated PGD_2 synthesis (Fig. 1). Inhibition of A23187-stimulated synthesis was weaker than inhibition of ATP- or TPA-stimulated synthesis. Since Ptx also reduced basal levels, no significant net inhibition was observed with A23187.

Figure 2 shows the effect of Ptx on arachidonic acid release in astrocyte cultures. Inhibition occurred in a similar way to that of PGD_2 synthesis, ATP- and TPA-stimulated release being affected the most. Surprisingly, basal release was unchanged. Taken together, these results suggest an involvement of Ptx-sensitive G-proteins at or proximal to phospholipase A_2.

Cholera toxin inhibits PGD_2 synthesis but not arachidonic acid release

Cholera toxin-preincubation (250 ng/mL, overnight) of astroglial cultures had a strong inhibitory effect on non-stimulated and ATP-stimulated PGD_2 synthesis (Fig. 3). It also inhibited A23187- and TPA-stimulated synthesis but to a much lesser degree. Compared to the marked inhibition of basal synthesis by Ctx, these reductions were not significant.

Arachidonic acid release was in no case inhibited by Ctx. Control values were markedly increased, whereas stimulated values were moderately increased (Fig. 4). Apparently, Ctx did not inhibit PGD_2 synthesis at the level of arachidonic acid release, as observed above with Ptx.

DISCUSSION

Cultured astrocytes possess three Ptx substrates with M_r 41,000, 40,000 and 39,500, as reported earlier [17]. The M_r 41,000 protein is believed to be the α-subunit of G_i, whereas the functions of the others are unknown. Quantitatively, the M_r 40,000 protein predominates in astroglial cultures. The present data clearly show an inhibition of arachidonic acid and PGD_2 release by Ptx under basal and stimulatory conditions. Since Ptx inactivates the G-proteins by ADP-ribosylation, one can conclude that one or more G-protein(s) is essential for normal arachidonic acid release and biosynthesis of prostaglandins in astrocyte cultures.

Sources of arachidonic acid are phosphatidyl inositol, phosphatidyl choline and phosphatidyl ethanolamine [20]. Beside the extensively studied pathway of PIP_2 degradation by phospholipase C yielding the two second messenger molecules IP_3 and DAG, another signal transduction mechanism has been described recently, mediated by a phospholipase D that uses phosphatidyl choline as substrate [21]. In this signaling system no IP_3 is formed and consequently no increase of intracellular calcium is observed [22]. DAG, however, containing AA esterified to its 2′-position, is one product of this cascade. DAG has also been reported to be released upon stimulation of phorbol esters via phospholipase D activation [23, 24]. Finally, phosphatidyl choline can be used directly by PLA_2 to liberate AA [25]. This depicts at least three ways of

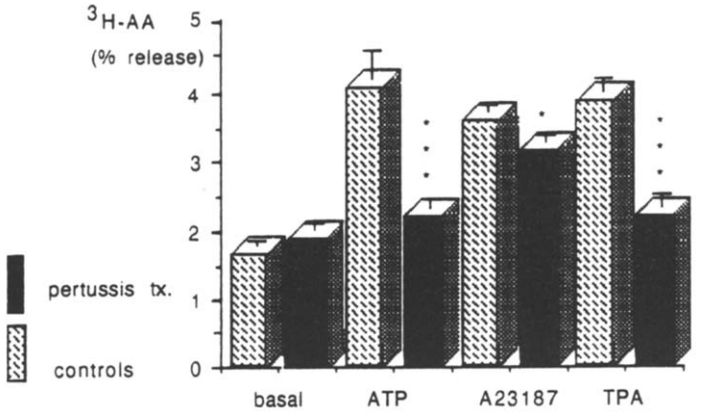

Fig. 2. Pertussis toxin-mediated inhibition of stimulated arachidonic acid release. Concentrations of stimuli and Ptx as in Fig. 1. N = 9 for each condition. * $P < 0.05$; *** $P < 0.001$.

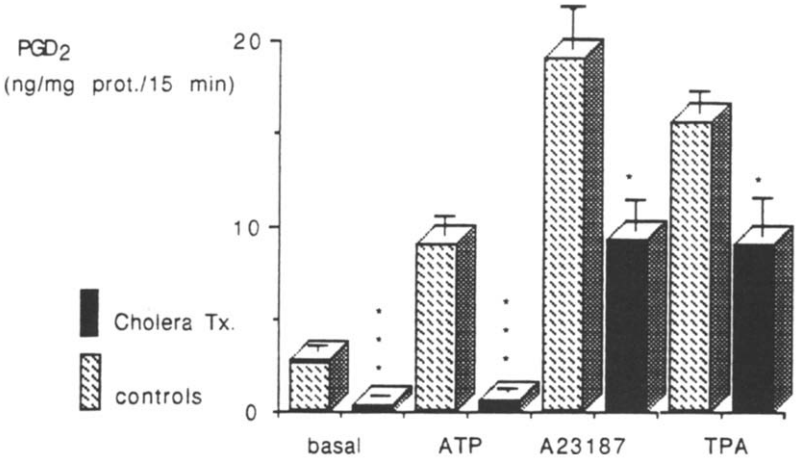

Fig. 3. Cholera toxin-mediated inhibition of basal and stimulated prostaglandin D_2 synthesis in astroglial cultures. Concentrations of stimuli as in Fig. 1. Cultures were incubated with Ctx, 250 ng/mL overnight. Experiments were each performed three times, in triplicate (N = 9). * $P < 0.05$; *** $P < 0.001$ compared to controls.

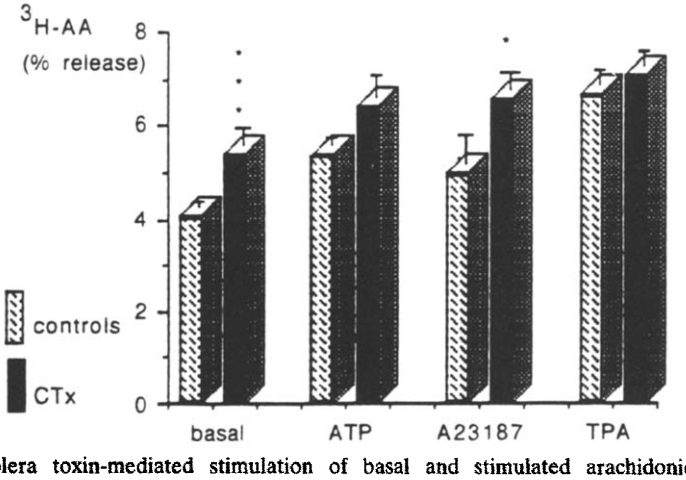

Fig. 4. Cholera toxin-mediated stimulation of basal and stimulated arachidonic acid release. Concentrations of stimuli as in Fig. 1. Cultures were incubated with Ctx, 250 ng/mL overnight. Experiments were each performed three times, in triplicate (N = 9). * $P < 0.05$; *** $P < 0.001$.

obtaining arachidonic acid for further metabolism. Apparently, it depends on the type of stimulus which pathway is activated. It has been reported, that vasopressin [26] and P_2-purinergic agonists [26] stimulate both PIP_2 and phosphatidyl choline hydrolysis, whereas IL-1 [27] and IL-3 [28] do not initiate phosphatidyl inositol breakdown.

Each of these pathways generating arachidonic acid may be under the control of G-proteins. There are reports of Ptx-sensitive G-proteins regulating PLA_2 but not PLC [29, 30] or PLC activities [31], or influencing P_2-purinergic-induced phosphatidyl choline hydrolysis [32]. The data reported here suggest that there are two Ptx-sensitive G-proteins involved in PGD_2 synthesis, since ATP- and TPA-stimulated arachidonic acid release and PGD_2 synthesis were inhibited much more than A23187-stimulated synthesis. From the above, one could conclude that the direct stimulation of PLA_2 by A23187 involves one Ptx substrate, whereas an additional Ptx substrate is inserted between the P_2-purinoceptor or protein kinase C and PLA_2, the inhibition of which by Ptx results in additional inhibition of events prior to PLA_2. ATP-stimulated P_2-purinoceptor, in particular, appears to trigger two intracellular signaling pathways, the PLC and PLD pathways. As reported elsewhere, the stimulation of this receptor involves Ptx-sensitive and Ptx-insensitive mechanisms [32]. The latter probably apply to the PLC pathway. TPA- or DAG-activated PKC, which has been reported to phosphorylate the PLA_2 inhibitory protein lipocortin [33], may be compromised by ADP-ribosylation of this Ptx-sensitive G-protein.

Cholera toxin-mediated inhibition of PGD_2 synthesis clearly affects biosynthetic steps distal to AA release, since the toxin did not inhibit this release. On the contrary, a substantial stimulation of AA-release was observed upon pretreatment of cultures with Ctx. It was less pronounced when ATP or TPA were used as stimulators but showed clearly when no stimulus was used. The stimulation may involve a direct effect on PLA_2, on the reacylating enzymes or on both. Since AA is reacylated very efficiently to maintain low levels of this putative second messenger, interference with this metabolic cycle may result in enhanced quantities of free AA. Arachidonic acid may well be released under steady state conditions without addition of stimulatory substances because cultures are maintained with LPS throughout culture time to suppress proliferation of microglial cells [12]. LPS is a (weak) stimulus for prostaglandin synthesis in our and other culture systems. Another mechanism explaining the effect of Ctx treatment may involve the cAMP system. Cholera toxin is able to increase markedly intracellular cAMP in astroglial cultures. Furthermore, we have shown recently that increasing intracellular cAMP by virtue of β-adrenoceptor stimulation results in an inhibition of both basal and stimulated PGD_2 synthesis [34]. Similar findings have been documented elsewhere [35]. Therefore, events related to elevations of this second messenger molecule may be responsible for inhibition of PGD_2 synthesis distal to AA release.

In conclusion, PGD_2 synthesis appears to be facilitated by more than one PTx-sensitive G-protein. The results with Ctx suggest an involvement of Ctx substrates in the stimulation of AA-release and inhibition of AA-conversion into prostaglandins.

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