Pertussis toxin selectively abolishes hormone induced lowering of cytosolic calcium in GH_3 cells

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Received 10 July 1985

Pertussis toxin, PT, abolishes inhibitory regulation of adenylate cyclase by cell surface receptors. Inhibitors of adenylate cyclase in GH_3 cells, namely somatostatin and the muscarinic cholinergic agonist carbachol, lower the cytosolic free Ca^{2+} concentration. $[Ca^{2+}]_i$ and cause hyperpolarization. These responses are selectively abolished by PT. It is concluded that the effects of somatostatin and carbachol to lower $[Ca^{2+}]_i$ and to hyperpolarize are secondary to their inhibitory action on adenylate cyclase. In contrast, PT does not impair the TRH induced rise in $[Ca^{2+}]_i$ in GH_3 cells demonstrating that the coupling of TRH receptors to Ca^{2+} mobilization is not mediated by a PT substrate.

Pertussis toxin *Cytosolic Ca^{2+}* *Somatostatin* *Muscarinic cholinergic agonist* *TRH* *Pituitary*

1. INTRODUCTION

Regulation of adenylate cyclase activity by stimulatory or inhibitory receptors is mediated by the GTP-regulatory proteins N_s and N_i [1,2]. N_s and N_i are substrates for the ADP ribosylation reaction catalysed by the A subunits of cholera toxin and PT, respectively, a toxin isolated from the culture medium of *Bordetella pertussis* [3]. PT selectively impairs inhibitory regulation of adenylate cyclase [4] by the ADP ribosylation of a 41 kDa protein, the α subunit of N_i [5,6].

We have shown previously that inhibitors of adenylate cyclase such as somatostatin and carbachol, a muscarinic cholinergic agonist, lower the $[Ca^{2+}]_i$ and cause hyperpolarization of GH_3 cells [7,8], a cell line derived from rat pituitary tumors [9]. In this study we use PT to probe for the involvement of N_i [4] in mediation of the effects of somatostatin and carbachol on $[Ca^{2+}]_i$ and membrane potential.

Recent reports have shown that PT abolishes the action of the chemotactic peptide fMLP in activating neutrophils, inhibiting the hydrolysis of PIP_2 [10,11], the generation of IP_3 [12,13] and the transient rise in $[Ca^{2+}]_i$ [13,14]. It was concluded that a PT substrate is involved in receptor stimulation of phospholipase C and possibly the subsequent mobilization of Ca^{2+}.

Likewise, it has been reported that GTP analogues can enhance diacylglycerol formation in leaky platelets [15] and stimulate phospholipase C in cell free systems [11,16,17]. Taken together these findings suggest that a GTP binding protein similar or identical to N_i is involved in the coupling of receptors to enhanced PIP_2 breakdown. We and others have shown that TRH, similar to fMLP in neutrophils, acts in GH_3 cells to enhance PIP_2

Abbreviations: N_s, N_i, the guanyl nucleotide regulatory units which mediate hormonal stimulation (N_s) or inhibition (N_i) of adenylate cyclase; PT, pertussis toxin; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; fMLP, *N*-formyl-Met-Leu-Phe; IP_3, *myo*-inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bis-phosphate; TRH, thyrotropin releasing hormone; bis-oxonol, bis(1,3-diethylthiobarbiturate)trimethineoxonol; VIP, vasoactive intestinal polypeptide

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00145793/85/$3.30

hydrolysis by phospholipase C, generating IP_3 [18–21] which in turn mobilizes Ca^{2+} from intracellular stores [22,23]. A further aim of this study was therefore to investigate whether PT would affect the Ca^{2+} mobilizing action of TRH in GH_3 cells.

2. MATERIALS AND METHODS

GH_3 cells obtained from the American type culture collection were maintained in monolayer culture as described [10]. Prior to the experiments they were incubated for 16 h with PT, 30 ng/ml, added to the culture medium. The cells were then detached from their substrate, left in a suspension culture for 1–3 h and loaded with quin2 as described [24]. PT, 30 ng/ml, was present during the suspension culture period. $[Ca^{2+}]_i$ was determined from quin2 fluorescence [25] and the average membrane potential was monitored with the fluorescent probe bis-oxonol [26] as described [7,24]. Statistical analysis of the data in table 1 was performed by analysis of variance followed by an a posteriori test (DUNCAN test). Single experiments shown represent at least 3 repetitions performed on different batches of cells. PT was obtained from List Biological Laboratories, Campbell California. For a preliminary experiment PT was kindly provided by Dr R.G. Larkins. Bis-oxonol was a gift from Dr R. Tsien, quin2 acetoxy methyl ester was from Sigma or Amersham, carbachol (carbamoylcholine chloride) and

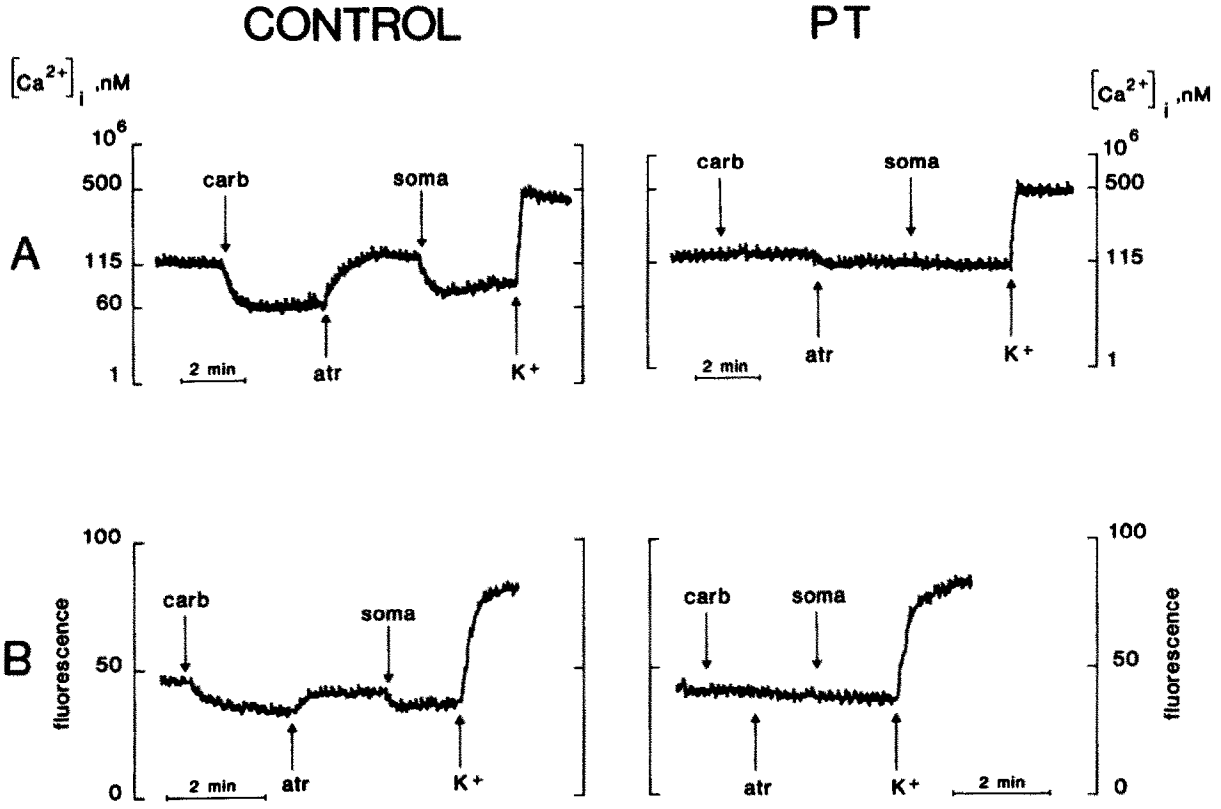

Fig.1. PT abolishes the effects of carbachol (carb) and of somatostatin (soma) on $[Ca^{2+}]_i$ and on the average membrane potential of GH_3 cells. (A) Quin2 fluorescence traces calibrated for $[Ca^{2+}]_i$ in cells (2×10^6 per ml) exposed to PT (30 ng/ml) or control cells from the same batch; arrows denote the time of addition of carbachol (carb), atropine (atr), somatostatin (soma) and KCl (K^+) to the final concentrations of 10 μM, 1 μM, 0.1 μM and 54 mM, respectively. (B) Fluorescence of bis-oxonol, 50 nM (expressed in arbitrary units), monitoring the average membrane potential of GH_3 cells (5×10^5 per ml), exposed to PT or control cells from the same batch, under conditions identical to those in the experiment shown in panel A.

atropine (atropine sulphate) were from Fluka, somatostatin from Clin. Midi Corp. or CRB, and other materials from sources described in [7,24].

3. RESULTS

$[Ca^{2+}]_i$ is lowered in GH_3 cells in response to carbachol [8] and somatostatin [7]. The $[Ca^{2+}]_i$ response to these inhibitors of adenylate cyclase can be demonstrated in a single experiment, as is shown in fig.1A. After the addition of carbachol, $[Ca^{2+}]_i$ is lowered from a basal level averaging 116 nM (table 1) to about 70 nM. Atropine, an inhibitor of the muscarinic acetylcholine receptor, reverses the action of carbachol such that $[Ca^{2+}]_i$ returns within 2 min to the original basal levels. The subsequent addition of somatostatin causes a rapid and marked lowering of $[Ca^{2+}]_i$ and upon depolarization with K^+ $[Ca^{2+}]_i$ rises immediately to a level of about 500 nM. Performing an identical experiment on the same batch of cells after exposure to 30 ng/ml PT for 16 h, the inhibitory responses to both carbachol and somatostatin are no longer observed, whereas there is a similar rise in $[Ca^{2+}]_i$ following depolarization with K^+. The PT effect to abolish inhibitory regulation of $[Ca^{2+}]_i$ is maximal at the dose of 30 ng/ml. A partial loss of somatostatin and carbachol effects can be observed already at 3 ng/ml PT, whereas experiments performed with 300 ng/ml PT give similar results to those observed with 30 ng/ml (not shown).

Fig.1B shows that upon PT treatment the effects of somatostatin and carbachol on the average membrane potential are lost concomitantly with the effects on $[Ca^{2+}]_i$. In an experiment performed identically to that described in fig.1A on both control and PT treated cells, the monitoring of fluorescence by the membrane potential probe bis-oxonol reveals that the hyperpolarization by carbachol, its reversal by atropine and the hyperpolarization by somatostatin are completely abolished by the toxin, however the enhancement of fluorescence by K^+ reflects that its depolarizing effect on GH_3 cells is unaltered by PT.

The selectivity of the PT effect can be appreciated from the data presented in fig.2 and table 1. VIP, a stimulator of adenylate cyclase, causes a transient rise of $[Ca^{2+}]_i$ in both control and PT treated cells. In the same experiment PT causes a complete loss of the action of somatostatin and carbachol to lower $[Ca^{2+}]_i$. The peak $[Ca^{2+}]_i$ values reached in GH_3 cells after stimulation by VIP are not significantly different in toxin treated vs control cells (table 1). It should be noted that the kinetics of the $[Ca^{2+}]_i$ response to a maximal dose of VIP remains unaltered by PT.

Similar to the $[Ca^{2+}]_i$ response to VIP, the rise in $[Ca^{2+}]_i$ due to TRH remains unaltered by PT. As can be seen in fig.2B, control cells respond to somatostatin and TRH by lowering and raising $[Ca^{2+}]_i$, respectively. In PT treated cells the somatostatin effect is completely abolished whereas following TRH there is a rapid and tran-

Table 1

$[Ca^{2+}]_i$ in GH_3 cells: the effect of PT on hormonal responses

	Control nM ± SE (*n*)	PT nM ± SE (*n*)	Difference PT vs control
Basal	116 ± 3 (45)	111 ± 3 (17)	n.s.
Carbachol, 100 μM	68 ± 3 (15)	120 ± 5 (5)	$p < 0.01$
Somatostatin, 0.1 μM	78 ± 5 (9)	100 ± 4 (7)	$p < 0.05$
TRH, 0.1 μM	276 ± 37 (13)	253 ± 37 (7)	n.s.
VIP, 0.1 μM	213 ± 25 (5)	164 ± 12 (4)	n.s.

n.s., not significant; GH_3 cells were exposed to PT, 30 ng/ml, for 16 h. Steady state $[Ca^{2+}]_i$ in the presence or absence of carbachol or somatostatin and peak values for the transient elevation in $[Ca^{2+}]_i$ following stimulation by TRH or VIP were determined from quin2 fluorescence as described [8,9,26]. Results are expressed in nM as the mean ± SE (*n* = number of experiments)

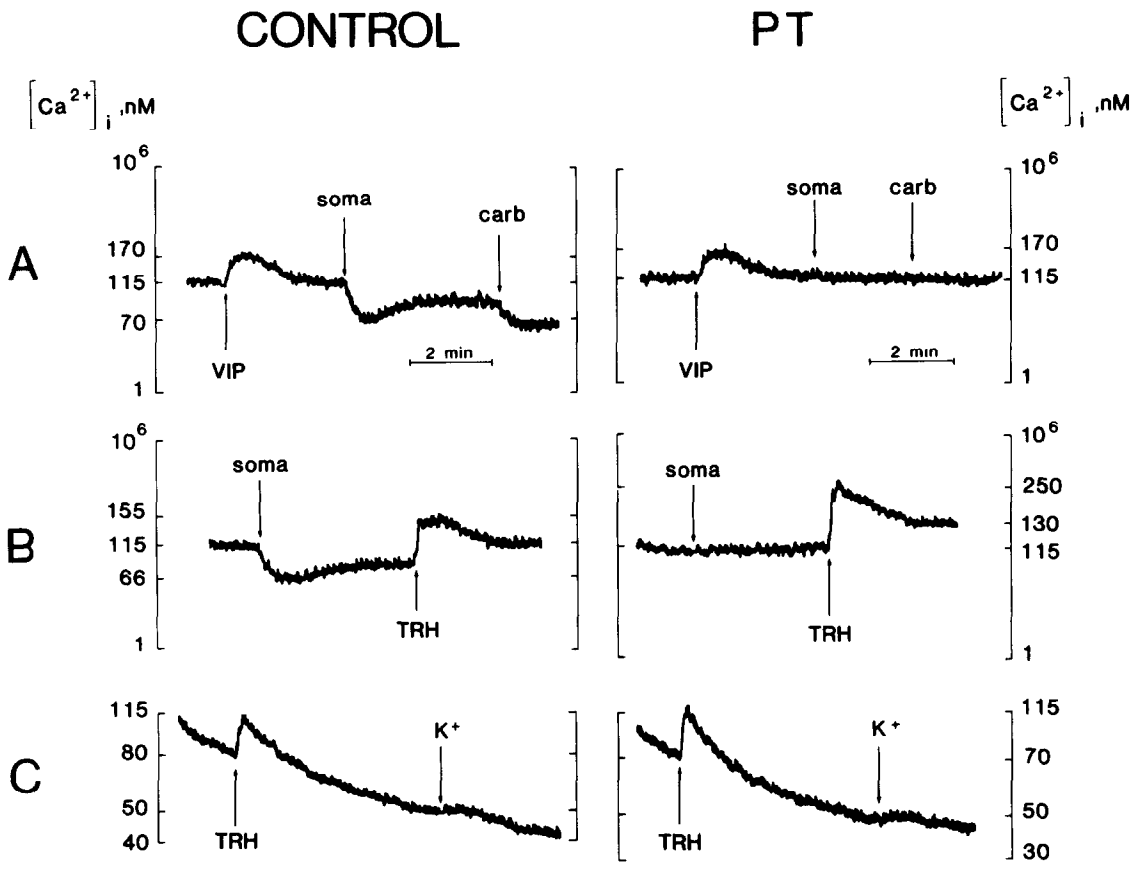

Fig.2. Regulation of $[Ca^{2+}]_i$ in GH cells by VIP, somatostatin (soma), carbachol (carb) and TRH: selective effects of PT. Quin2 fluorescence traces calibrated for $[Ca^{2+}]_i$ in cells (2×10^6 per ml) exposed to PT (30 ng/ml) or control cells from the same batch; arrows denote the time of addition of 0.1 M VIP (final concentration), 0.1 M somatostatin (soma), 0.1 mM carbachol (carb), 0.1 M TRH, and 54 nM KCl (K^+). Traces in panels A and B were monitored at a free extracellular $[Ca^{2+}]$ of 1 mM; traces in panel C were observed in Ca^{2+}-free medium containing 1 mM EGTA, i.e. at a free extracellular $[Ca^{2+}]$ lower than 100 nM.

sient rise in $[Ca^{2+}]_i$, and the PT exposed cells maintain a steady state $[Ca^{2+}]_i$ above baseline as has been described for control cells [24]. The peak value reached after TRH stimulation averages 253 nM in PT treated cells, not unlike the average value of 276 nM observed in control cells (table 1). Submaximal concentrations of TRH elicit a similar rise in $[Ca^{2+}]_i$ in PT treated vs contol cells (not shown) and hence PT does not appear to alter TRH sensitivity. Fig.2C shows that TRH acts equally well in GH_3 cells exposed to PT as in control cells to raise $[Ca^{2+}]_i$ transiently at very low extracellular free $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from an intracellular pool [24].

Table 1 summarizes the effects of PT on the regulation of $[Ca^{2+}]_i$ in GH_3 cells. There is a significant change due to PT in the average steady state $[Ca^{2+}]_i$ observed in the presence of either somatostatin or carbachol. In contrast, neither the average basal $[Ca^{2+}]_i$ nor the peak values of $[Ca^{2+}]_i$ after stimulation by either VIP or TRH are significantly altered.

4. DISCUSSION

There are 2 major aspects of the data presented above: PT treatment of GH_3 cells completely abolishes the effects of somatostatin and carbachol

to lower $[Ca^{2+}]_i$ and to cause hyperpolarization; PT exposed GH_3 cells respond however normally to several stimulators of PRL secretion, in particular to TRH which acts via accelerated PIP_2 hydrolysis to mobilize Ca^{2+}.

There is now increasing evidence that GTP binding proteins are involved in the receptor mediated acceleration of phosphoinositide turnover and Ca^{2+} mobilization [10–17]. From the observation that PT blocks the stimulation of neutrophils by fMLP [10–14], mediated by enhanced PIP_2 breakdown, it appears that the N protein linking receptors to the acceleration of phosphoinositide turnover is a PT substrate similar or identical to N_i. The data presented above show that such a conclusion should at present not be generalized: TRH action to mobilize Ca^{2+}, mediated like the effects of fMLP on neutrophils by enhanced PIP_2 hydrolysis, is unaffected by PT. Similarly, it has been reported that muscarinic receptor mediated phosphoinositide hydrolysis and Ca^{2+} mobilization are not inhibited by PT [27]. A straightforward hypothesis to account for the differential sensitivity to PT treatment would propose that – in analogy to the adenylate cyclase system which contains N_s and N_i – similar but not identical N proteins couple the various receptors to the phospholipase C reaction, only part of them being PT substrates. Irrespective of the reasons for the lack of a PT effect on the response to TRH, it should be emphasized that PT cannot be generally used as a probe to investigate receptor coupling to enhanced PIP_2 hydrolysis.

There is evidence that PT abolishes the inhibition of adenylate cyclase by somatostatin and dopamine in pituitary cells [28,29] and of muscarinic agonists in GH_3 cells [30] in which it causes ADP ribosylation of a 41 kDa protein, presumably N_i [31]. In GH_3 cells alterations in adenylate cyclase activity are paralleled by changes in $[Ca^{2+}]_i$ and the average membrane potential. Stimulation of adenylate cyclase by VIP or forskolin raise $[Ca^{2+}]_i$ [32], the inhibitors carbachol and somatostatin lower $[Ca^{2+}]_i$ and cause hyperpolarization. Since PT completely abolishes these effects of somatostatin and carbachol we conclude that their primary action is exerted on adenylate cyclase, and that the lowered $[Ca^{2+}]_i$ and the hyperpolarization are a consequence of reduced cAMP production. In regard to the mechanism of such regulation, it appears that changes in steady state $[Ca^{2+}]_i$ must result from altered Ca^{2+} fluxes. Since in GH_3 cells steady state $[Ca^{2+}]_i$ is largely dependent on Ca^{2+} influx via the voltage-dependent Ca^{2+}-channel [24], reduced steady state $[Ca^{2+}]_i$ observed in the presence of somatostatin or carbachol most probably reflects reduced Ca^{2+} influx. Phosphorylation by cAMP-dependent protein kinase enhances the conductivity of the voltage-dependent Ca^{2+}-channel, whereas it decreases voltage-dependent K^+ currents (review [33,34]). Inhibitors of adenylate cyclase thus could lead to a reduction of the proportion of phosphorylated Ca^{2+}- and K^+-channels, decreased Ca^{2+} conductance leading to the lowering of $[Ca^{2+}]_i$ and concomitantly increased K^+ currents resulting in hyperpolarization.

It has been reported that PT can impair inhibitory regulation of pituitary hormone secretion by somatostatin [28] and dopamine [29] in rat anterior pituitary cells in primary culture and by muscarinic cholinergic agonists in GH_3 cells [30,31]. Inhibition of adenylate cyclase by these ligands, mediated by the PT substrate N_i, leads to reduced stimulation of cAMP levels due to stimulators of secretion, an action which was lost also upon PT treatment [28–30]. These findings suggest that reduced pituitary hormone secretion in the presence of somatostatin, dopamine and muscarinic cholinergic agonists results from reduced cAMP levels. The reports that dopamine [35], somatostatin [7] and carbachol [8], the latter acting through muscarinic acetylcholine receptors, lower $[Ca^{2+}]_i$ in pituitary cells points to an alternative possibility of how these ligands could act to inhibit secretion: all 3 inhibitors reduce basal $[Ca^{2+}]_i$ and appear to attenuate the transient rise in $[Ca^{2+}]_i$ provoked by stimulators of secretion [8,35]. The concomitant loss of the inhibition of secretion, of the lowering of $[Ca^{2+}]_i$, and of the inhibition of adenylate cyclase following PT treatment is consistent with a role for the lowering of $[Ca^{2+}]_i$ – as a consequence of reduced cAMP production – in the mediation of inhibitory regulation of pituitary hormone secretion.

ACKNOWLEDGEMENTS

The authors are indebted to Miss Isabelle Puiz for excellent technical assistance. Financial sup-

port was from the Swiss National Science Foundation, grant no.3.840-0.83.

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