Properties of receptor-controlled inositol trisphosphate formation in parotid acinar cells

Debra L. AUB and James W. PUTNEY, JR.

Division of Cellular Pharmacology, Medical College of Virginia, Box 524, MCV Station, Richmond, VA 23298-0001, U.S.A.

(Received 5 September 1984/Accepted 7 November 1984)

Activation of muscarinic receptors in rat parotid cells results in breakdown of polyphosphoinositides liberating inositol phosphates, including inositol trisphosphate. Formation of inositol trisphosphate appears independent of agonist-induced Ca^{2+} mobilization, since neither formation nor degradation of inositol trisphosphate are appreciably altered in low-calcium media, and elevation of cytosolic Ca^{2+} with a calcium ionophore does not cause an increase in cellular inositol trisphosphate. Further, activation of substance P receptors and α_1 -adrenoreceptors, but not β -adrenoreceptors, increases inositol trisphosphate formation. The dose–response curve for methacholine activation of inositol trisphosphate formation more closely approximates the curve for receptor occupancy than for Ca^{2+} -activated K^+ release. These results are all consistent with the suggestion that inositol trisphosphate could function as a second messenger linking receptor occupation to cellular Ca^{2+} mobilization.

In the rat parotid gland, muscarinic cholinergic agonists activate a variety of cellular responses by mobilizing Ca^{2+} (Butcher & Putney, 1980). Previous studies have shown that muscarinic and other Ca^{2+} -mobilizing receptors also activate a calcium-independent turnover of phosphoinositides (Oron *et al.*, 1975; Jones & Michell, 1975), which has been suggested to play a role in Ca^{2+} mobilization (Michell, 1975). Recently we have reported (Aub & Putney, 1984) that muscarinic activation of isolated rat parotid acinar cells results in a rapid phosphodiesteratic breakdown of phosphatidylinositol 4,5-bisphosphate. The IP_3 thus generated has been reported to act as a second messenger to release Ca^{2+} from intracellular stores, about $1\text{ }\mu\text{M-IP}_3$ being sufficient for a maximum response (Streb *et al.*, 1983; Burgess *et al.*, 1984; Berridge, 1984). Thus it appears that receptor-activated polyphosphoinositide breakdown may be the link between agonist receptor binding and mobilization of intracellular Ca^{2+} necessary for an appropriate physiological response (Michell, 1975). This is also likely to be the case for the rat parotid gland, where IP_3 is formed rapidly (Downes & Wusteman, 1983) and in sufficient quantity ($20\text{ }\mu\text{M/min}$) (Aub & Putney, 1984) to act

as a second messenger. Here we further examine the generation of IP_3 in the rat parotid gland; specifically, we have examined the role of calcium in the generation of IP_3 , the effects of agents acting on other Ca^{2+} -mobilizing receptors, and the dose–response relationship for methacholine-induced IP_3 formation. In previous studies (Weiss & Putney, 1981), the dose–response curve for phosphatidylinositol labelling was found to be about an order of magnitude to the left (lower concentrations) of the curve for receptor occupancy. In most systems, however, a close relationship between receptor occupancy and phosphoinositide turnover has been noted (Michell, 1975). Thus it was of particular interest to examine the dose–response relationship for IP_3 formation, since this is believed to be a more direct indicator of the biochemical reaction most closely coupled to receptor activation.

Methods

Isolated rat parotid cells were prepared as previously described (Aub & Putney, 1984) from male Sprague–Dawley rats (125–150g) and were routinely more than 80% viable as determined by Trypan Blue exclusion. Cells were suspended in a Tris-buffered Krebs–Ringer medium containing

Abbreviations used: IP_3 , inositol trisphosphate.

1% bovine serum albumin and 5 μ Ci of [3 H]inositol/5 ml. In some experiments designed to measure IP₃ formed by agents less efficacious than methacholine, the [3 H]inositol was increased to 10 μ Ci/ml. After 60 min the [3 H]inositol was removed by washing and cells were resuspended in the standard medium with 1% bovine serum albumin. In some experiments, cells were resuspended in a zero-calcium medium (standard solution without CaCl₂ and with 10⁻⁴ M-EGTA).

Inositol phosphates were extracted and then separated on Dowex anion exchange resins as described by Berridge *et al.* (1983) with the modifications described by Aub & Putney (1984). Aliquots of the cell suspension were homogenized in HClO₄. The HClO₄-insoluble pellets were assayed for DNA (Burton, 1968), and the supernatants were neutralized, applied to anion-exchange columns, and the water-soluble [3 H]inositol phosphates eluted as described previously (Aub & Putney, 1984; Berridge *et al.*, 1983). For each preparation, the specific radioactivity of phosphatidylinositol was estimated and, by assuming similar specific radioactivity for the inositol phosphates (Aub & Putney, 1984), could be used to convert c.p.m. to nmol of inositol phosphate formed.

Results and discussion

If IP₃ is a second messenger for Ca²⁺ mobilization, then the generation of IP₃ should not be blocked by the absence of extracellular Ca²⁺, or formed as a result of increases in cytosolic free Ca²⁺. Fig. 1 summarizes experiments in which we examined the stimulation of the formation of inositol phosphates by 10⁻⁴ M-methacholine in the presence of extracellular calcium (1.8 mM), and in its absence (no added calcium plus 10⁻⁴ M-EGTA). LiCl (10 mM) was present to prevent degradation of inositol 1-phosphate (Berridge *et al.*, 1982). At 40 min, 10⁻⁴ M-atropine was added, causing inositol bisphosphate and trisphosphate levels to decay. Removal of extracellular calcium slightly inhibited, but did not completely block, the generation of the three inositol phosphates measured. The observed decreases may result from a calcium requirements for optimal activity of one or more of the enzymes involved in inositol lipid metabolism. That IP₃ was still formed and broken down in the absence of extracellular calcium indicates that its metabolism is not dependent on Ca²⁺ mobilization. In the absence of extracellular calcium, methacholine can cause a transient release of internal Ca²⁺ (Butcher & Putney, 1980; Aub *et al.*, 1982); however, this release can support calcium-dependent responses for 1–2 min only, yet IP₃ production remains elevated for 40 min (Fig.

Fig. 1. Formation of [3 H]inositol phosphates by parotid acinar cells in response to 10⁻⁴ M-methacholine

Basal values have been subtracted. Parotid cells were incubated in media containing a physiological concentration of calcium (1.8 mM) (---), or with no added calcium and 10⁻⁴ M-EGTA (—). Atropine (10⁻⁴ M) was added at $t = 40$ min. $\square$, Inositol phosphate; $\circ$, inositol bisphosphate; $\triangle$, inositol trisphosphate. Values are means $\pm$ S.E.M. for four independent experiments.

1). Furthermore, the calcium ionophore, A23187, at a concentration (2×10^{-6} M) sufficient to elevate intracellular free Ca²⁺ levels did not significantly increase IP₃ levels above that of control (A23187, 1.46 ± 0.10 nmol of IP₃/mg of DNA; control, 1.12 ± 0.25 nmol of IP₃/mg of DNA), indicating that IP₃ formation is not a consequence of an elevation in free cytosolic Ca²⁺ concentration.

The dose-response curve (Fig. 2) for IP₃ generation is also consistent with the second

Fig. 2. $[^3\text{H}]\text{IP}_3$ formation in parotid acinar cells as a function of methacholine concentration

The increase in $[^3\text{H}]\text{IP}_3$ after 30 min of exposure of parotid cells to 10^{-7}M – 10^{-3}M methacholine was determined and expressed as a percentage of the average value for 10^{-3}M . ●—●, IP_3 formation (means $\pm$ S.E.M. for five independent experiments); ---, ···, theoretical curves constructed from published (Putney *et al.*, 1980) values for EC_{50} for K^+ release (---) and K_d for receptor binding (····).

messenger hypothesis. The amount of IP_3 formed was measured at 30 min for five different methacholine concentrations. As Fig. 2 illustrates, the concentration dependence of IP_3 generation is more closely associated with the curve for receptor occupancy [determined by methacholine displacement of $[^3\text{H}]\text{quinuclidinyl benzilate}$ (Putney & VanDeWalle, 1980)], as compared with the curve for Ca^{2+} -mediated K^+ release (Putney & VanDeWalle, 1980) or phosphatidylinositol labelling [results not shown (Weiss & Putney, 1981)]. These results suggest that for maximal K^+ secretion, only a fraction of the muscarinic receptors needs to be occupied, and that with less than maximal IP_3 levels one obtains the full physiological response. Thus there is not only a receptor reserve, but also a similar reserve of IP_3 -forming capacity, indicating that receptor occupancy and IP_3 formation are closely coupled and precede the Ca^{2+} mobilization necessary for K^+ secretion.

Table 1 shows the results of experiments in which the receptor specificity of the IP_3 response in rat parotid cells was examined. Only agonists that act on receptors associated with calcium mobilization evoked significant IP_3 production. As previously reported by Berridge *et al.* (1983), the α -adrenergic agonist phenylephrine significantly elevated IP_3 ; this is likely to be due to action on the α_1 receptor subtype since prazosin (10^{-6}M) but not yohimbine (10^{-6}M) blocked the increase in IP_3 due to phenylephrine. It is the α_1 receptor subtype, not the α_2 , that is associated with Ca^{2+} mobilization and phosphoinositide turnover

Table 1. Effect of various agonists and antagonists on formation of $[^3\text{H}]\text{IP}_3$ in rat parotid acinar cells

Values are means for three independent experiments $\pm$ S.E.M. * Statistically significant at $P < 0.01$.

Condition	IP_3 formed (nmol/mg of DNA)
Control	1.45 ± 0.21
Phenylephrine (10^{-5}M)	$3.61 \pm 0.35^*$
+ prazosin (10^{-6}M)	1.59 ± 0.22
+ yohimbine (10^{-6}M)	$3.49 \pm 0.29^*$
Substance P (10^{-6}M)	$5.48 \pm 0.03^*$
Isoproterenol (10^{-6}M)	1.16 ± 0.28
Methacholine (10^{-4}M)	$29.13 \pm 2.42^*$

(Jones & Michell, 1978; Fain & Garcia-Sainz, 1980; Uchida *et al.*, 1981). Substance P, which is also believed to act through elevation of cytosolic free Ca^{2+} concentration (Berridge *et al.*, 1983), significantly increased IP_3 levels (Table 1). The β -adrenergic agonist, isoproterenol, however, which stimulates protein secretion through an elevation in cellular cyclic AMP, was ineffective at increasing IP_3 levels. Notably, the increases in IP_3 elicited by maximal concentrations of substance P or phenylephrine were only a fraction of that caused by methacholine. This is consistent with previous findings suggesting that there are no spare receptors for substance P and phenylephrine, as there are for methacholine (Putney *et al.*, 1980). Thus the smaller IP_3 response for agonists acting without a receptor reserve is consistent with the suggested close coupling of receptor occupancy to IP_3 formation, and may also be consistent with a proportionality between the quantity of IP_3 formed and rate of Ca^{2+} mobilization.

In conclusion, these results suggest that IP_3 formation in the parotid acinar cell is closely coupled to receptor occupation, but quantitatively related to Ca^{2+} mobilization. IP_3 formation is not, however, a consequence of Ca^{2+} mobilization. These results are consistent with the hypothesis suggesting that IP_3 functions as a second messenger signalling the release of Ca^{2+} from internal stores (Berridge, 1983; Streib *et al.*, 1983; Burgess *et al.*, 1984).

This study was supported by a grant from the NIH, no. DE-05764.

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