Effect of dual agonists on phosphoinositide pools in WRK-1 cells

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Both vasopressin and bradykinin activate the phosphoinositide cycle in WRK-1 rat mammary tumour cells. When the two agonists are added simultaneously, partial additivity is observed with respect to disappearance of prelabelled phosphoinositides and accumulation of inositol phosphates; no additivity is observed with respect to resynthesis of phosphatidylinositol as assessed by monitoring [32 P]P_i incorporation. Lack of complete additivity can be explained, at least in part, by heterologous desensitization. In order to determine whether the two agonists were accessing a common or individual hormone-sensitive phosphoinositide pools, cells were incubated with [32 P]P_i in the presence of either vasopressin or bradykinin and subsequently restimulated with the alternative agonist. The lipid pool labelled in the presence of either agonist was sensitive to subsequent treatment by the other ligand, suggesting a common phosphoinositide pool. However, when cells were incubated with [32 P]P_i in the absence of agonists, the time course of labelling of the hormone-sensitive pool was different for bradykinin and vasopressin, with that for bradykinin becoming labelled within a much shorter time. Thus although there is significant overlap between the phosphoinositide pools responding to vasopressin and bradykinin, there is a small fraction of the hormone-sensitive lipid which responds only to bradykinin.

INTRODUCTION

WRK-1 cells possess an active phosphoinositide cycle which is responsive to a variety of agonists, including vasopressin [1,2], bradykinin [2] and 5-hydroxytryptamine [3]. With respect to the vasopressin-responsive cycle, we have previously demonstrated the existence of a vasopressin-sensitive phosphoinositide pool which is distinct from insensitive phosphoinositide within the cell. We wondered whether this hormone-sensitive phosphoinositide pool was specific for vasopressin or accessible to other Ca²⁺-mobilizing agonists as well. Results of the experiments presented here suggest the existence of a common hormone-sensitive phosphoinositide pool, although a small portion, previously considered to be hormone-insensitive, appears to be responsive to bradykinin.

MATERIALS AND METHODS

Tissue culture media, trypsin, Dulbecco's phosphate-buffered saline, antibiotics, antimycotics and glutamine were purchased from Gibco. Plasticware for tissue culture was from Costar. Anion-exchange resin (AG 1-X8, 200–400 mesh, formate form) was from Bio-Rad. [32 P]P_i (carrier-free) was purchased from ICN and [3 H]inositol (15 Ci/mmol) was from American Radiolabelled Chemicals. Aquasol-2 was from DuPont–New England Nuclear. Vasopressin was purchased from Sigma.

Cell culture

WRK-1 cells were established from a dimethylbenz[*a*]anthracene-induced rat mammary tumour as previously described [4]. The variant used in these experiments (1B) arose spontaneously and has been described previously [5]. Monolayer cultures were maintained in Ham's F-12 medium supplemented with Earle's salts and fetal calf serum (10%), rat serum (2%), penicillin (100 units/ml), Fungizone (0.25 µg/ml) and streptomycin (100 µg/ml). For experiments, cells were harvested with

a solution of 0.05% trypsin/0.02% EDTA in 0.9% NaCl and replicately plated into 22 mm wells. Details for each experiment appear in the Figure legends.

Extraction and analysis of phospholipids and inositol phosphates (IPs)

Lipids and their breakdown products were extracted and analysed as previously described [6,7].

RESULTS

Effect of vasopressin and bradykinin on phosphoinositide turnover

Fig. 1 depicts dose-response curves for vasopressin and bradykinin with respect to stimulation of [3 H]IP accumulation and disappearance of prelabelled phosphoinositides. Maximal effects of vasopressin were seen with concentrations of between 10 and 50 nM; maximal bradykinin effects required concentration of 500 nM. In the experiment shown, between 65 and 75% of the total lipid radioactivity was lost when cells were maximally stimulated with either agonist in the presence of LiCl. Addition of a V_{1a} inhibitor {[$(\text{CH}_2)_5$ -β-Mpa¹, Tyr(OMe)², Arg⁸]vasopressin} blocked the effect of vasopressin, but not that of bradykinin (results not shown), indicating the existence of separate receptor systems for the two agonists. In order to determine whether any additivity of effect was apparent when the agonists were used together, a time course experiment was carried out to compare IP accumulation in the presence of lithium and either one or both agonists (Fig. 2). Partial additivity was observed at every time point tested. To determine whether the additivity represented a further breakdown of labelled phosphoinositides, the effect of both agonists on the disappearance of prelabelled lipid was monitored. As seen in Fig. 3, enhanced accumulation of IPs in the presence of both agonists was reflected in the enhanced disappearance of prelabelled

Abbreviations used: PI, phosphatidylinositol; PIP, PIP₂, phosphatidylinositol mono- and bis-phosphate respectively; IP, inositol phosphate.

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Fig. 1. Dose-response curves for vasopressin (a) and bradykinin (b)

Cells were preincubated for 48 h in growth medium with $5 \mu\text{Ci}$ of $[^3\text{H}]\text{inositol/ml}$. The medium was replaced with serum-free minimal essential medium containing 10 mM-LiCl and the concentration of hormone indicated. Following a 60 min incubation, both loss of radioactivity from the lipid fraction ($\circ$) and accumulation of radioactivity in the aqueous IP fraction ($\text{IP}_1 + \text{IP}_2 + \text{IP}_3$) ($\bullet$) were quantified. Methods were as described in the text. Values represent the means of triplicate determinations. Standard deviations around each point averaged $8.6 \pm 5.2\%$, with a range from 0.5 to 20%.

phosphoinositides. The same additivity was observed even when the second agonist was added following a 90 min incubation with the first agonist; re-addition of the first agonist was without further effect (Fig. 4). The extent of the additivity observed varied from experiment to experiment, ranging from 0 to 100%, although both of these extremes were rare. In 16 out of 16 experiments the effect of both agonists was greater than that of either agonist alone with respect to disappearance of prelabelled $[^3\text{H}]\text{phosphoinositides}$; in 21 out of 29 experiments accumulation of IPs was partially additive.

Desensitizing effect of heterologous ligands

Failure to observe full additivity in the presence of both agonists could result from heterologous desensitization. One

type of such desensitization could involve a change in the receptor affinity, such that additional ligand would be required to produce a comparable effect. Fig. 5 illustrates the dose-response curve for bradykinin with respect to IP accumulation in the presence of a maximally stimulatory concentration of vasopressin. As is the case for bradykinin alone, maximal stimulation was observed with 500 nM -bradykinin. Kirk *et al.* [2] have previously demonstrated that the potency of vasopressin remains unchanged in the presence of bradykinin. Thus failure to observe full additivity with the two agonists cannot be explained on the basis of a change in receptor affinity for one or the other agonist.

Fig. 2. Time course of accumulation of IPS ($\text{IP}_1 + \text{IP}_2 + \text{IP}_3$) in the absence ($\circ$) or presence of vasopressin ($\bullet$), bradykinin ($\square$), or both together ($\blacksquare$)

Cells were treated as described for Fig. 1. Values represent the means of triplicate determinations. The effect of the two agonists was significantly different ($P < 0.05$) from that of either agonist alone at 30, 90, 120 and 150 min. At the 60 min time point, the effects of vasopressin plus bradykinin were different from those of bradykinin alone, but not from those of vasopressin alone. All treated samples were significantly different from controls. $[\text{Vasopressin}] = 0.1 \mu\text{M}$; $[\text{bradykinin}] = 1 \mu\text{M}$.

Fig. 3. Effect of hormones on disappearance of prelabelled phosphoinositides

Cells were prelabelled with $[^3\text{H}]\text{inositol}$ and treated with hormones as described for Fig. 1. Values shown represent the means of triplicate determinations $\pm$ S.D. Hormone concentrations were the same as in Fig. 2.

Fig. 4. Effect of hormone readdition following maximal stimulation with vasopressin (VP; ▲), bradykinin (BK; △) or both (□)

Cells were preincubated with [³H]inositol and treated with hormone as described for Fig. 1. Following hormonal treatment, cells were further treated with the hormone indicated for an additional 90 min, without washout of the original incubation medium. Results are the means of triplicate determinations. In each case, the result in the presence of two hormones is significantly different from that obtained in the presence of a single hormone (*P* < 0.005); identical results were seen in several other experiments.

A second type of desensitization involves a reduction in the number of active receptors. Such a reduction, unlike a change in affinity, might attenuate the maximal response attainable. Cantau *et al.* [8] have previously demonstrated that following an 18 h incubation with vasopressin, the response of WRK-1 cells to bradykinin was attenuated by 17 %. To test whether heterologous desensitization might explain the failure to see full additivity in

Fig. 5. Dose-response curve for bradykinin in the presence of vasopressin (0.1 μM)

Incubation with 0 and 5 μM-bradykinin in the absence of vasopressin is indicated by △. Results are the means of triplicate determinations ± s.d. Methods were as described for Fig. 1.

Table 1. Desensitization of hormonal response

Cells were grown in the presence of [³H]inositol (5 μCi/ml) for 48 h. Experiments were carried out in serum-free minimal essential medium. Preincubations were done in the absence of LiCl for the times indicated. Incubations were done in the presence of 10 mM-LiCl for 45 min. IPs were quantified as described in the text. Results are the means of triplicate determinations ± s.d. Desensitization of the bradykinin (BK) response following preincubation with vasopressin (VP) for 15 min was significant, *P* < 0.05; desensitization of the BK response following preincubation with VP for 90 min was significant, *P* < 0.025; and desensitization of the VP response following preincubation with BK for 90 min was significant, *P* < 0.0125.

Preincubation time (min)	Preincubation addition	Incubation addition	Inositol phosphates (c.p.m./dish)
15	None	None	149 ± 47
		VP	2292 ± 410
		BK	3150 ± 219
		VP	175 ± 9
		BK	1758 ± 99
		BK	2546 ± 329
	VP	None	486 ± 157
		VP	2443 ± 230
		BK	2719 ± 109
90	None	None	112 ± 9
		VP	2319 ± 186
		BK	3174 ± 188
		VP	150 ± 19
		BK	1326 ± 159
		BK	2515 ± 345
	VP	None	260 ± 54
		VP	1849 ± 120
		BK	2697 ± 225

the presence of both vasopressin and bradykinin at the times employed in the studies shown here (30–90 min), [³H]inositol-prelabelled cells were incubated without LiCl for 15 or 90 min in the presence of either vasopressin or bradykinin, followed by a second incubation with hormone in the presence of LiCl (Table 1). A 15 min preincubation with vasopressin resulted in a 19 % decrease in the effect of bradykinin; however, a 15 min preincubation with bradykinin did not attenuate the vasopressin response. A 90 min preincubation decreased these respective responses by 21 and 20 %. Thus heterologous desensitization could explain failure to see full additivity, especially at earlier times when the quantity of lipid is not limiting. At later times there is not enough phospholipid to permit full additivity.

Effect of agonists on [³²P]_i labelling of phosphatidylinositol (PI)

Cells were incubated with radioactive phosphate in the presence of vasopressin, bradykinin or both, and the labelled PI was quantified (Table 2). Both agonists stimulated a large increase in the incorporation of radioactivity into PI; however, no additivity was seen. Unlike the results observed for IP accumulation and disappearance of phosphoinositides, maximal stimulation was seen with either agonist alone. These results rule out the presence of subsets of cells which respond only to one agonist. That these results are not an artefact of the radioactive precursor used can be seen from the results shown in Table 3. Disappearance of [³²P]_i-labelled PI is partially additive in the presence of both agonists, as is disappearance of ³H-labelled phosphoinositide.

Effect of agonists on turnover of [³²P]_i-labelled PI

The ability of each agonist alone to turn over more than 50 % of the phosphoinositide suggests that both ligands are accessing

Table 2. Effect of hormones on [³²P]P_i incorporation into PI

Confluent cultures were incubated in serum-free minimal essential medium containing no unlabelled phosphate and 10 μ Ci of carrier-free [³²P]P_i/ml with the additions indicated for 60 min. Lipids were extracted and quantified as described in the text. The concentration of vasopressin (VP) was 0.1 μ M, and of bradykinin (BK) was 1 μ M. Results are the means of triplicate determinations $\pm$ S.D.

Addition	[³² P]PI (c.p.m./dish)
Expt. 1:	
None	1604 $\pm$ 97
VP	3934 $\pm$ 45
BK	10724 $\pm$ 538
VP + BK	10436 $\pm$ 739
Expt. 2:	
None	593 $\pm$ 35
VP	5177 $\pm$ 861
BK	8056 $\pm$ 617
VP + BK	8187 $\pm$ 1030

the same lipid pools. We have previously demonstrated that incubation of cells with radioactive phosphate for 2 h or less results in labelling of a pool of PI which will not subsequently turn over in response to vasopressin [6,9]. However, incubation of the cells with [³²P]P_i in the presence of vasopressin resulted in the labelling of a vasopressin-sensitive PI pool. We tested this pool for bradykinin sensitivity, as well as testing a pool labelled in the presence of bradykinin for vasopressin sensitivity (Table 4). As previously demonstrated, incubation of the cells for less than 2 h (in this case 30 min) with radioactive phosphate did not result in the labelling of a vasopressin-sensitive pool. However, the pool labelled in the presence of either agonist was subsequently responsive to the other agonist, suggesting the existence of a common hormone-sensitive pool. Interestingly, although the pool labelled in the absence of agonist was not responsive to vasopressin, it was responsive to bradykinin. In the three experiments shown, bradykinin was able to turn over 52, 26 and 35% of the radioactive PI in cells labelled under non-stimulatory conditions. A time-course experiment was then carried out (Fig. 6). Bradykinin-responsive lipid was labelled at much earlier times than was vasopressin-responsive lipid. These data suggest that at least a small portion of the hormone-sensitive lipid is specific for bradykinin, and that part of the PI pool that was previously considered to be hormone-insensitive was merely vasopressin-insensitive.

Table 3. Effect of hormones on turnover of [³²P]P_i-labelled PI

Cells were incubated in growth medium for 24 h with 10 μ Ci of [³²P]P_i/ml. The medium was then changed to serum-free minimal essential medium containing 10 mM-LiCl and the additions indicated (VP, vasopressin; BK, bradykinin). Lipids were extracted and quantified as described in the text. Values represent the means $\pm$ S.D. of triplicate determinations. The effects of VP + BK were significantly less than those of BK alone, $P < 0.001$.

Addition	[³² P]PI (c.p.m./dish)
None	8073 $\pm$ 1150
VP	5994 $\pm$ 580
BK	5717 $\pm$ 543
BP + BK	3960 $\pm$ 379

Table 4. Turnover of [³²P]PI in cells labelled under various conditions

Cells were preincubated in serum-free phosphate-free minimal essential medium for 30 min under the conditions indicated, followed by a second incubation in minimal essential medium containing 10 mM-LiCl and indicated additions for 60 min. Lipid radioactivity was quantified as described in the text. Values represent the means of triplicate determinations $\pm$ S.D. The effects of vasopressin (VP) and bradykinin (BK) on cells prelabelled in the presence of agonist were all highly significant. The effect of BK on cells prelabelled in control medium was significant: Expt. 1, $P < 0.001$; Expt. 2, $P < 0.05$; Expt. 3, $P < 0.05$.

Pre-incubation addition	Incubation addition	[³² P]PI (c.p.m./dish)		
		Expt. 1	Expt. 2	Expt. 3
None	None	911 $\pm$ 121	666 $\pm$ 69	593 $\pm$ 144
	VP	872 $\pm$ 121	883 $\pm$ 97	534 $\pm$ 56
	BK	435 $\pm$ 86	494 $\pm$ 100	386 $\pm$ 43
VP	None	3833 $\pm$ 785	4887 $\pm$ 385	1709 $\pm$ 153
	VP	2005 $\pm$ 127	2530 $\pm$ 208	932 $\pm$ 80
	BK	957 $\pm$ 90	934 $\pm$ 128	644 $\pm$ 85
BK	None	3850 $\pm$ 554	4862 $\pm$ 201	1803 $\pm$ 254
	VP	1423 $\pm$ 254	1988 $\pm$ 256	979 $\pm$ 105
	BK	929 $\pm$ 110	1100 $\pm$ 112	691 $\pm$ 46

DISCUSSION

Compartmentation of metabolic phenomena within the cell is a well-recognized entity, especially with respect to specific subcellular localization of a metabolic event to a particular organelle, such as endoplasmic reticulum or mitochondria. However, many data exist to suggest an even greater degree of organization [10]. A close linkage between sequential metabolic enzymes, for example, has been inferred from a variety of biochemical, biophysical and genetic studies. With respect to hormone action and signal transduction, the generation of cyclic AMP appears to be compartmentalized within the cell based on evidence from dose-response relationships [11], differential activation of protein kinases [12] and morphological studies in patch-clamped cells [13].

Fig. 6. Time course of labelling of hormone-sensitive PI

Cells were incubated for the times indicated in serum-free phosphate-free minimal essential medium containing 10 μ Ci of [³²P]P_i/ml. Following this, cells were washed and reincubated in serum-free minimal essential medium containing 10 mM-LiCl for 60 min. PI was extracted and quantified as described in the text. The amount of radioactivity which disappeared from PI as a result of hormonal treatment was considered as hormone-sensitive. Results are the means of quadruplicate determinations. At 30 min, and 1 and 2 h, levels of PI in samples treated with vasopressin ($\blacktriangle$) were not significantly lower than those in controls; however, levels of PI in samples treated with bradykinin ($\triangle$) were significantly lower than those in controls ($P < 0.01$).

Data exist to support compartmentalization of the phosphoinositide cycle as well. As early as 1963, Hokin & Hokin reported that phosphoinositide turnover involved only a minor fraction of the total mass of PI in the avian salt gland [14]. In 1979, Fain & Berridge demonstrated in the blowfly salivary gland that recently labelled PI was hydrolysed preferentially in response to 5-hydroxytryptamine stimulation [15]. More recently, multiple phosphoinositide metabolic pools have been suggested in pancreas [16,17], platelets [18–20], rat liver [21], brain [22–24], kidney [25], neutrophils [26] and human erythrocytes [27,28]. On the other hand, some investigators have reported lack of evidence for such pools.

The situation in platelets is particularly controversial. Vickers & Mustard [20] reported that the various phosphoinositides present in platelets have different specific activities when radiolabelled, suggesting multiple metabolic pools. Wilson *et al.* [29] noted that the specific activity of ^{32}P -labelled phosphatidylinositol monophosphate (PIP) is less than that of phosphatidylinositol biphosphate (PIP_2), and interpret this to mean that PI is turning over directly, without first being converted to polyphosphoinositides. It could just as well be indicative of multiple metabolic pools of PIP. Billah & Lapetina [18] invoke different metabolic pools to explain the differential effects of thrombin and ionophore A23187 on phospholipases C and A. Tynes *et al.* [30], on the other hand, found no differences in the specific activities of labelled platelet phosphoinositides either at rest or when stimulated with thrombin. They suggest a single homogeneous metabolic pool; however, they do say that there may be different functional pools, since only 70–80% of the phosphoinositides are subject to agonist-induced hydrolysis [31]. Augert *et al.* [32] suggest the existence of a single homogeneous metabolic pool of PIP_2 in hepatocytes based on the findings that (1) the fatty acid composition of PIP_2 is the same before and after stimulation with vasopressin, and (2) the values obtained for turnover of $[\text{H}]$ inositol-labelled phosphoinositides agreed with that obtained when mass of the lipids was measured. However, neither of these findings rules out the existence of functionally heterogeneous pools. Furthermore, the data describing the localization of PIP and the fatty acid composition of PI strongly suggest the existence of multiple metabolic pools in hepatocytes.

We have reported such evidence with respect to the vasopressin-stimulated phosphoinositide cycle in WRK-1 rat mammary tumour cells. Using $[\text{P}^{32}]\text{P}_i$, we were able to differentiate vasopressin-sensitive from vasopressin-insensitive phosphoinositides [6,9]. However, similar results were not observed when radioactive inositol incorporation was analysed. The same percentage of inositol-labelled PI turns over regardless of labelling times or the presence of hormone [7]. Michell *et al.* [33] have confirmed this finding in studies using doubly labelled inositol.

In the present study, we examined the effect of two different agonists on the turnover of labelled phosphoinositides in WRK-1 cells in an attempt to determine whether or not the same PI pool was accessed by both stimulants. From the results presented here, the following conclusions were drawn. (1) Partial additivity is observed in the presence of both agonists when either disappearance of prelabelled PI or accumulation of IPs is monitored; (2) This additivity is not observed when stimulation of $[\text{P}^{32}]\text{P}_i$ incorporation into PI is the end-point monitored; (3) heterologous desensitization is taking place; and (4) vasopressin and bradykinin access a common pool of PI, with the exception of a small fraction of PI which responds only to bradykinin.

With respect to the absolute amount of PI which is sensitive to each agonist, it must be mentioned that this varies with the

passage of WRK-1 cells used. One often notes a decreasing responsiveness to agonists with increasing time in culture, and it is clear that individual clones can vary greatly in their responsiveness [5,9]. It is also possible to obtain clones which respond to one and not the other agonist (results not shown). With this variability in mind, it is easy to imagine situations under which the extent of additivity also varies, e.g. as a function of the number of receptors and/or the number of guanine-nucleotide-binding proteins (G-proteins) and/or numbers of phosphoinositidases in a given cell.

This work was supported by a grant from the National Science Foundation (DCB-8901476) and a Merit Review Award from the Veterans' Administration.

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