Neugebauer, J. (1988) *A guide to the properties and uses of detergents in biology and biochemistry*, Calbiochem Corp., La Jolla, CA.
Neuman, R., Kauzmann, W., & Zipp, A. (1973) *J. Phys. Chem. 77*, 2687–2691.
Pal, R., Petri, W., Barenholz, Y., & Wagner, R. (1983) *Biochim. Biophys. Acta 729*, 185–192.
Paladini, A., & Weber, G. (1981) *Rev. Sci. Instrum. 52*, 419–427.
Scarlata, S. (1988) *Biophys. J. 55*, 1215–1223.
Soucaille, P., Prats, M., Tocanne, J. F., & Teissie, J. (1988) *Biochim. Biophys. Acta 939*, 289–294.

A Combination of H_2O_2 and Vanadate Concomitantly Stimulates Protein Tyrosine Phosphorylation and Polyphosphoinositide Breakdown in Different Cell Lines†

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ABSTRACT: Treatment of four cell lines [rat hepatoma (Fao), murine muscle (BC3H-1), Chinese hamster ovary (CHO), and rat basophilic leukemia (RBL)] with a combination of 3 mM H_2O_2 and 1 mM sodium orthovanadate markedly stimulates protein tyrosine phosphorylation, which is accompanied by a dramatic increase (5–15-fold) in inositol phosphate (InsP) formation. H_2O_2/vanadate stimulate best formation of inositol triphosphate while their effects on the mono and di derivatives are more moderate. In the presence of 3 mM H_2O_2, both protein tyrosine phosphorylation and InsP formation are highly correlated and manifest an identical dose–response relationship for vanadate. Half-maximal and maximal effects are obtained at 30 and 100 μM, respectively. This stimulatory effect of H_2O_2/vanadate is not mimicked by other oxidants such as spermine, spermidine, $KMnO_4$, and vitamin K_3. In RBL cells, the kinetics of inositol triphosphate formation correlate with tyrosine phosphorylation of a 67-kDa protein, while tyrosine phosphorylation of a 55-kDa protein is closely correlated with both inositol monophosphate formation and serotonin secretion from these cells. Taken together, these results suggest a causal relationship between tyrosine phosphorylation triggered in a nonhormonal manner and polyphosphoinositide breakdown. Furthermore, these results implicate protein tyrosine phosphorylation in playing a role in the stimulus–secretion coupling in RBL cells.

Tyrosine phosphorylation of proteins has been implicated both in mitogenic signal pathways and in oncogenic transformation (Hunter, 1987; Yarden & Ullrich, 1988). Recent reports have indicated the possibility of a positive cross-talk between tyrosine phosphorylation and polyphosphoinositide breakdown. For example, transformation of a mink lung epithelial cell line (CCL64) with the tyrosine kinase encoding oncogenes v-*fms* or v-*fes* results in enhanced rate of polyphosphoinositide turnover (Jackowski et al., 1986). Similarly, growth factors whose receptors comprise an intrinsic tyrosine kinase activity, such as FGF, PDGF, and EGF, stimulate inositol phosphate (InsP)[1] formation (Tilly et al., 1988) and potentiate thrombin-induced phosphoinositide breakdown in hamster fibroblasts (Paris et al., 1988). Vanadate and molybdate, which are potent inhibitors of protein tyrosine phosphatases (PTPases) (Tonks et al., 1988), increase protein tyrosine phosphorylation of a 50-kDa protein and induce the generation of InsP in electropermeabilized platelets (Lerea et al., 1989). Finally, phospholipase C-γ (PLC-γ) undergoes in vivo tyrosine phosphorylation in response to stimulation with EGF or PDGF (Wahl et al., 1988, 1989a,b; Margolis et al., 1989), and it also serves as an in vitro substrate for both the EGF and PDGF receptor kinases (Nishibe et al., 1989; Meisenhelder et al., 1989). Taken together, these observations point to a tight association between protein tyrosine phosphorylation and phosphoinositide breakdown although the molecular basis for this linkage is presently unknown.

We have recently shown (Heffetz et al., 1990) that treatment of different cell lines with a combination of H_2O_2 and vanadate leads to a 6–20-fold increase in intracellular protein tyrosine phosphorylation. The action of H_2O_2 and vanadate is mediated, at least in part, through inhibition of PTPases and through the activation of receptor tyrosine kinases (Heffetz et al., 1990). Since the combination of H_2O_2/vanadate acts as a nonhormonal stimulus, it serves as a useful tool to investigate the role of tyrosine phosphorylation in the regulation of polyphosphoinositide breakdown. This is particularly important in cells where physiological stimuli that induce tyrosine phosphorylation have not yet been identified. In the present study, H_2O_2/vanadate were employed to examine the consequences of enhanced tyrosine phosphorylation on polyphosphoinositide hydrolysis in several cell types. This approach led to the conclusions that (a) increased protein tyrosine phosphorylation and enhanced polyphosphoinositide breakdown are tightly coupled, (b) this phenomenon is general and occurs in several different cell lines, (c) in one of these lines, the rat basophilic leukemia (RBL) cells, inositol triphosphate ($InsP_3$) and inositol monophosphate ($InsP_1$) formation closely correlates with tyrosine phosphorylation of 67- and 55-kDa proteins,

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[1] Abbreviations: InsP, inositol phosphate; PTPases, protein tyrosine phosphatases; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NBT, nitro blue tetrazolium; PLC-γ, phospholipase C-γ.

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respectively, and (d) tyrosine phosphorylation of pp55 correlates with serotonin secretion induced by H_2O_2/vanadate.

Materials and Methods

Cell Cultures. Rat hepatoma (Fao) cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum. Murine muscle (BC3H-1) and Chinese hamster ovary (CHO-T) cells overexpressing the human insulin receptor gene (Ellis et al., 1986; a generous gift from Dr. W. J. Rutter, University of California, San Francisco, CA) were grown in F-12 medium supplemented with 10% fetal calf serum. Rat basophilic leukemia (RBL) cells were maintained as we have previously described (Sagi-Eisenberg & Pecht, 1984) in Eagle's minimal essential medium, supplemented with 10% fetal calf serum.

Ligand Treatment of Intact Cells and Determination of Protein Tyrosine Phosphorylation. Cells grown in 60-mm plates were deprived of serum for 16 h before each treatment. One hour before treatment, the medium was replaced with fresh medium supplemented with 1 mg/mL bovine serum albumin (Sigma radioimmunoassay grade). LiCl was added to a final concentration of 10 mM, and incubation was continued for 12 min at 37 °C. H_2O_2, sodium orthovanadate, or other agents [save for antigen (vide infra)] were then added to the medium as indicated. After incubation, cells were washed twice with ice-cold PBS and frozen in liquid nitrogen. Cell extracts were prepared by the addition of buffer I (50 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mM EDTA, 2 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, and 5 μg/mL leupeptin). The extracts were centrifuged for 30 min at 4 °C at 12000*g*. Supernatants were mixed with (5 times) concentrated Laemmli sample buffer (Laemmli, 1970) containing 20 mM dithiothreitol, resolved on 7.5% SDS–PAGE under reducing conditions, and transferred to nitrocellulose.

Immunoblotting. Electrophoretic transfer of phosphoproteins from the gels to nitrocellulose papers was performed essentially as previously described (Heffetz & Zick, 1989). The transfer was carried out for 3 h at 200 mA in 50 mM glycine/50 mM Tris·HCl buffer, pH 8.8. The nitrocellulose papers were then soaked in buffer II [1% BSA, 20 mM Tris, 140 mM NaCl, and 0.05% (v/v) Tween-20, pH 7.5]. The papers were then incubated with 1 μg/mL affinity-purified anti-P-Tyr antibodies in buffer II containing 0.1% rather than 1% BSA. Following 16-h incubation at 4 °C, the papers were extensively washed in buffer III (buffer II, less BSA), and the presence of anti-P-Tyr antibodies was determined by adding 3×10^5 cpm/mL of ^{125}I-labeled goat anti-rabbit antibodies for 2 h at 22 °C. This was followed by intensive washings in buffer III. The papers were then dried and autoradiographed. When indicated, specific bands were excised from the nitrocellulose papers and counted in a γ counter.

Secretion. Secretion was determined as previously described (Sagi-Eisenberg & Pecht, 1984). Briefly, cells grown in 24-well costar plates ($\sim 1 \times 10^6$ cells/well) were serum-deprived and preloaded for 16–20 h with [^{3}H]serotonin (5 μCi/10^6 cells) in the presence of a monoclonal, DNP-specific, IgE class antibody. They were then washed 3 times and incubated at 37 °C with the desired reagents in a final volume of 0.4 mL. Following 20-min incubation, samples from the supernatants were taken for counting. Release is presented as total [^{3}H]-serotonin released by the cells.

Analysis of Inositol Phosphates. Cells grown in 24-well costar plates were preloaded for 16–20 h with [^{3}H]myoinositol (4 μCi/10^6 cells) in a serum- and inositol-free medium (medium 199). The cells were washed 3 times and, unless indicated otherwise, incubated at 37 °C for 12 min at a final volume of 0.4 mL with 10 mM LiCl before addition of the desired reagents. The reaction was terminated at the indicated times by adding 1.0 mL of methanol. The cells were scraped off the plates and the lysates transferred to polypropylene tubes. One mililiter of chloroform and 0.5 mL of H_2O were subsequently added to each tube. The upper phases were then applied onto DOWEX-1 columns (formate form) (Downes & Michell, 1981). The inositol phosphates were eluted sequentially by using 0.1 M formic acid/0.2 M ammonium formate (for $InsP_1$), 0.1 M formic acid/0.4 M ammonium formate (for $InsP_2$), and 0.1 M formic acid/1.0 M ammonium formate (for $InsP_3$).

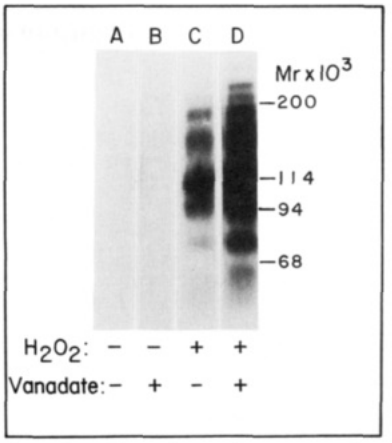

FIGURE 1: Effect of H_2O_2 and vanadate on protein tyrosine phosphorylation. Fao cells were incubated at 37 °C with buffer (A), 1 mM vanadate (B), 3 mM H_2O_2 (C), or their combination (D). The incubation was terminated after 20 min by washing the cells with ice-cold PBS and freezing in liquid nitrogen. Solubilization of the cells and blotting of the extracts with affinity-purified anti-P-Tyr antibodies were carried out as described under Materials and Methods.

Data Presentation. Unless otherwise indicated, the results presented are the mean ± SE of duplicated measurements of a representative experiment, repeated at least 3 times with essentially identical results.

Materials. [^{3}H]Myoinositol (13.8 Ci/mmol) and [^{3}H]-serotonin (10.8 Ci/mmol) were purchased from Amersham. Monoclonal, DNP-specific, IgE was a generous gift from Dr. Z. Eshhar (Weizmann Institute), and DNP-BSA was synthesized according to the procedure described by Fara et al. (1960). Affinity-purified anti-P-Tyr antibodies were generated as previously described (Heffetz & Zick, 1989). All other reagents used were from Sigma.

Results

Effects of H_2O_2 and Vanadate on Protein Tyrosine Phosphorylation. We have previously shown (Heffetz & Zick, 1989; Heffetz et al., 1990) that H_2O_2 added to intact cells enhances protein tyrosine phosphorylation and that inclusion of vanadate markedly potentiates this effect. In the example depicted in Figure 1, we could demonstrate that incubation of intact rat hepatoma (Fao) cells with 2 mM H_2O_2 enhanced tyrosine phosphorylation of four major proteins, pp180, -150, -114, and -100. Inclusion of 0.1 mM vanadate together with 2 mM H_2O_2 augmented tyrosine phosphorylation of these and additional proteins (pp80, pp68, and pp56; Figure 1). Vanadate, added alone, had no effect on protein tyrosine phosphorylation under these conditions. The combination of H_2O_2/vanadate exerted similar effects on other cells although the proteins most heavily phosphorylated varied depending on the cell type (Heffetz et al., 1990). All phosphorylations occurred exclusively on tyrosine residues, as immunoblotting,

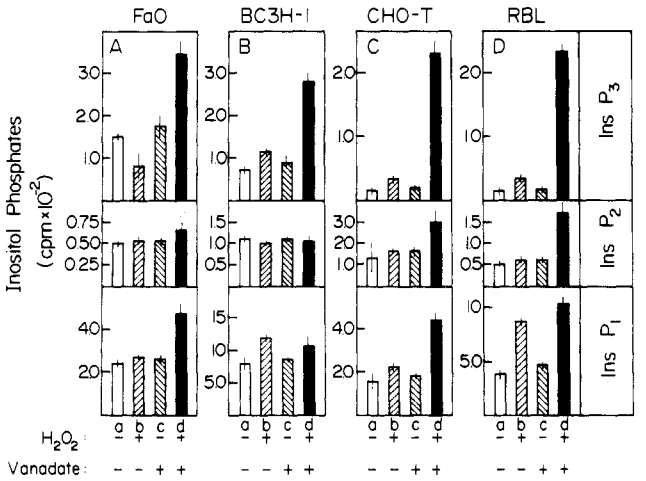

FIGURE 2: Effects of H_2O_2 and vanadate on phosphoinositide metabolism in different cell lines. Fao (A), BC3H-1 (B), CHO-T (C), and RBL (D) cells, loaded with [^{3}H]inositol as described under Materials and Methods, were incubated for 20 min at 37 °C in the absence (a) or presence of 3 mM H_2O_2 (b), 1 mM vanadate (c), or their combination (d). At the end of incubation, inositol phosphates formed were determined as described under Materials and Methods.

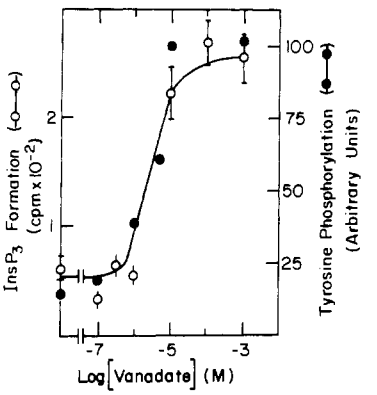

FIGURE 3: Effect of vanadate on protein tyrosine phosphorylation and $InsP_3$ formation by H_2O_2-treated Fao cells. Fao cells were incubated in duplicate plates for 20 min at 37 °C with 3 mM H_2O_2 and the indicated vanadate concentrations. At the end of incubation, protein tyrosine phosphorylation (●) and $InsP_3$ formed (○) were determined as described under Materials and Methods. For determination of protein tyrosine phosporylation, the radioactive lanes were excised from the blotted nitrocellulose papers and counted in a γ-counter. Maximal response (100%) corresponds to 10390 cpm.

with anti-P-Tyr antibodies, could be inhibited with 10 μM P-Tyr but not with 250 μM P-Ser or P-Thr (not shown).

Effects of H_2O_2 and Vanadate on Phosphoinositide Metabolism in Different Cell Lines. To correlate the enhanced protein tyrosine phosphorylation with polyphosphoinositide breakdown, we studied the effects of H_2O_2, vanadate, and their combination on the generation of inositol mono- ($InsP_1$), di- ($InsP_2$), and triphosphates ($InsP_3$) in four different cell lines. As seen in Figure 2, H_2O_2 (3 mM) or vanadate (1 mM) had only trivial effects on inositol phosphate (InsP) formation. By contrast, in their combined presence, InsP formation was stimulated severalfold. H_2O_2/vanadate stimulated best formation of $InsP_3$ while their effects on $InsP_2$ and $InsP_1$ were more moderate. Out of the four cell lines studied, CHO and RBL cells responded best to H_2O_2/vanadate which caused a ~10–15-fold increase in their $InsP_3$ content.

The effects of H_2O_2/vanadate on polyphosphoinositide breakdown were specific. They were not mimicked by other oxidants such as phenylarsinine oxide, spermine, spermidine, vitamin K_3, or $KMnO_4$ (Table I), added alone or together with 0.1 mM vanadate. Stimulation of $InsP_3$ formation by vanadate

Table I: Effect of Different Oxidants on Phosphoinositide Metabolism[a]

oxidant (3 mM)	vanadate (0.1 mM)	cpm $InsP_1$	cpm $InsP_2$	cpm $InsP_3$
expt 1				
none	–	4600 ± 400	400 ± 4	430 ± 40
	+	3200 ± 200	500 ± 100	620 ± 20
H_2O_2	–	3000 ± 500	600 ± 100	800 ± 100
	+	4000 ± 200	700 ± 100	2600 ± 500
phenylarsine oxide	–	3200 ± 100	500 ± 100	800 ± 100
	+	2800 ± 100	520 ± 10	700 ± 100
spermine	–	2690 ± 10	550 ± 30	360 ± 10
	+	2700 ± 200	440 ± 10	400 ± 10
spermidine	–	3300 ± 1000	700 ± 50	460 ± 20
	+	3200 ± 500	580 ± 50	570 ± 80
expt 2				
none	–	1400	100	290
	+	1700	120	198
H_2O_2	–	1500 ± 100	100 ± 10	210 ± 30
	+	2700 ± 400	265 ± 65	1500 ± 100
NBT	–	1800 ± 100	123 ± 5	210 ± 30
	+	1900 ± 300	144 ± 4	280 ± 4
$KMnO_4$	–	1100 ± 100	119 ± 1	170 ± 60
	+	1200 ± 40	140 ± 30	290 ± 50
Vit K_3	–	1216 ± 2	160 ± 20	180 ± 10
	+	1600 ± 500	148 ± 4	190 ± 20

[a] CHO-T cells were incubated for 20 min at 37 °C with a 3 mM sample of the indicated oxidants and in the absence or presence of 0.1 mM vanadate. At the end of incubation, the levels of InsP formed were determined as described under Materials and Methods.

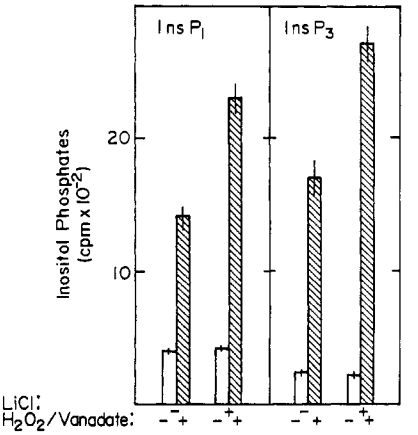

FIGURE 4: Effect of Li^+ on H_2O_2/vanadate-induced InsP formation. [^{3}H]Inositol-loaded RBL cells were preincubated for 12 min at 37 °C with or without 10 mM LiCl before the addition of a combination of 3 mM H_2O_2 and 1 mM vanadate. At the end of 20 min of further incubation, $InsP_1$ and $InsP_3$ levels were determined as described under Materials and Methods.

in H_2O_2-treated Fao cells was dose-dependent; half-maximal effects were obtained at 30 μM vanadate, and maximal stimulation occurred at 100 μM (Figure 3). This effect highly correlated with vanadate-stimulated total protein tyrosine phosphorylation (Figure 3).

Characterization of H_2O_2/Vanadate Effects on InsP Formation in RBL Cells. Since H_2O_2/vanadate stimulated best $InsP_3$ formation in RBL cells, we studied this effect in somewhat more detail. To rule out the possibility that H_2O_2/vanadate acts by inhibiting InsP breakdown, the dependency of H_2O_2/vanadate effects on the presence of LiCl, a known inhibitor of InsP phosphatase (Berridge et al., 1982), was evaluated. As seen in Figure 4, H_2O_2/vanadate stimulated by 3.4- and 14-fold $InsP_1$ and $InsP_3$ formation in the absence of LiCl. These effects were further enhanced 1.6-fold when the cells were preincubated in the presence of 10 mM LiCl. These results suggest that the effects of H_2O_2/vanadate are

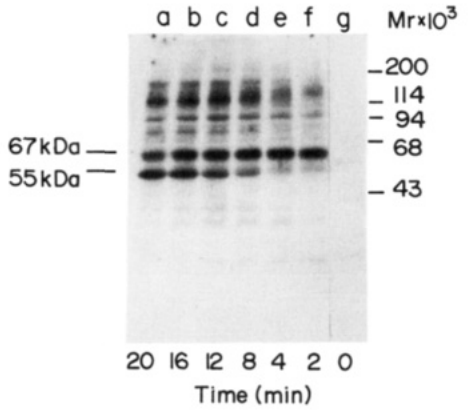

FIGURE 5: Effect of H_2O_2/vanadate on protein tyrosine phosphorylation in RBL cells. RBL cells were incubated for the indicated times with a combination of 3 mM H_2O_2 and 1 mM vanadate. At the end of incubation, protein tyrosine phosphorylation was determined as described under Materials and Methods.

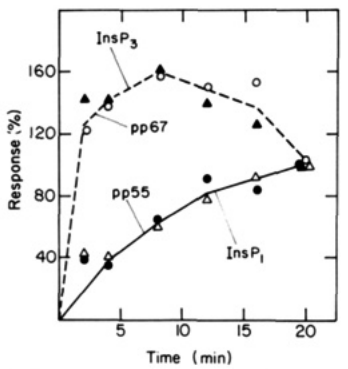

FIGURE 6: Correlation between protein tyrosine phosphorylation and InsP formation in RBL cells. Duplicate plates of RBL cells were incubated with a combination of 3 mM H_2O_2 and 1 mM vanadate for the indicated time periods. At the end of incubation, protein tyrosine phosphorylation and InsP formation were determined as described under Materials and Methods. The lanes corresponding to pp67 (○) and pp55 (△) were excised from the blotted nitrocellulose paper and counted in a γ-counter. One hundred percent signal was assigned to the radioactivity incorporated following 20-min incubation. This corresponded to 1900 and 800 cpm for pp67 and pp55, respectively. $InsP_3$ (▲) and $InsP_1$ (●) levels were determined as described in the legend to Figure 4. One hundred percent signal was assigned to the InsP levels formed at the end of 20-min incubation, which corresponded to 2100 and 1200 cpm incorporated into $InsP_3$ and $InsP_1$, respectively.

not largely mediated by inhibition of InsP phosphatase.

Inclusion of 3 mM EGTA in the incubation medium (in the presence of 10 mM LiCl) did not alter the effects of H_2O_2/vanadate on $InsP_1$ and $InsP_3$ formation (data not shown). We can therefore conclude that InsP formation induced by H_2O_2/vanadate is not the result of enhanced Ca^{2+} influx into the cells, thereby leading to activation of PLC (Eberhard & Holz, 1988).

Time Course of Tyrosine Phosphorylation in RBL Cells. As seen in Figure 5, a 2-min treatment of cells with H_2O_2/vanadate stimulated tyrosine phosphorylation of a major protein of M_r 67K (pp67). A second major protein, pp55, appeared phosphorylated but at a slower rate. Additional tyrosine-phosphorylated proteins (mainly pp150 and pp114) were also evident under these conditions, although their intensity was much lower when compared with the phosphorylation of pp67 and pp55.

Correlation between Tyrosine Phosphorylation and InsP Formation in RBL Cells. The correlation between the elevated cellular content of $InsP_3$ and $InsP_1$ and the increase in P-Tyr content of pp67 and pp55 was next evaluated. We assigned

Table II: Serotonin Release by RBL Cells Induced by Antigen or H_2O_2/Vanadate[a]

addition	serotonin released (cpm)
none	7000 ± 400
antigen (DNP_8-BSA, 150 ng/mL)	50000 ± 200
H_2O_2 (3 mM)	6200 ± 500
vanadate (1 mM)	4900 ± 800
H_2O_2/vanadate	18000 ± 650

[a] IgE-bearing RBL cells, loaded with [^{3}H]serotonin, were incubated for 20 min at 37 °C with the indicated reagents. At the end of incubation, 100-μL samples from the supernatant were taken for counting. Values presented are the mean ± SE of total [^{3}H]serotonin released by the cells.

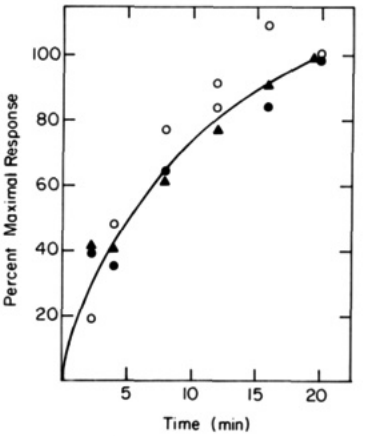

FIGURE 7: Effect of H_2O_2/vanadate on pp55 phosphorylation, $InsP_1$ formation, and serotonin release by RBL cells. RBL cells, loaded either with [^{3}H]inositol or with [^{3}H]serotonin, were treated with a combination of 3 mM H_2O_2 and 1 mM vanadate for the indicated time periods. At the end of incubation, serotonin release (○), p55 phosphorylation (▲), and $InsP_1$ formation (●) were determined. Maximal response (100%) corresponds to the values obtained following 20-min treatment.

as 100% the changes detected following a 20-min stimulation of the cells with H_2O_2/vanadate. As seen in Figure 6, tyrosine phosphorylation of pp67 correlated with $InsP_3$ formation whereas the elevated levels of $InsP_1$ correlated with the increased content of pp55. These findings suggest that tyrosine phosphorylation of pp67 and pp55 is regulated by the same rate-limiting step responsible for the accumulation of $InsP_3$ and $InsP_1$, respectively.

Correlation between pp55 Phosphorylation, $InsP_1$ Formation, and Serotonin Release by RBL Cells. RBL cells can be triggered to release their granular contents (e.g., serotonin, histamine) by aggregation of their cell surface receptors for immunoglobulin E (IgE). This is normally achieved by binding of an antigen, against which IgE is directed, to IgE-bearing RBL cells. Antigen-induced serotonin release is accompanied by polyphosphoinositide breakdown (Beaven et al., 1984). We therefore studied whether the nonhormonal stimulation of tyrosine phosphorylation and of InsP breakdown induced by H_2O_2/vanadate also results in the induction of the secretory response. As seen in Table II, treatment of RBL cells with a combination of 3 mM H_2O_2 and 1 mM vanadate induced serotonin release which was 24% that induced by optimal concentrations of antigen. The rate of release induced by H_2O_2/vanadate highly correlated with the rate of phosphorylation of pp55 and accumulation of $InsP_1$ (Figure 7).

Discussion

In the present study, we have demonstrated that enhanced protein tyrosine phosphorylation, induced in a hormone-independent manner, is associated with a marked stimulation

of polyphosphoinositide breakdown, leading to a severalfold increase in the intracellular content of inositol mono-, di-, and triphosphates. The tight coupling between the two processes appears to be a general phenomenon as it occurs in several cell types from different origins.

The agents which induce these effects, a combination of H_2O_2 and vanadate, act both as in vivo activators of protein tyrosine kinases (Kadota et al., 1987; Koshio et al., 1988; Heffetz & Zick, 1989; Heffetz et al., 1990) and as in vivo inhibitors of protein tyrosine phosphatases (Klarlund, 1985; Bernier et al., 1988; Heffetz et al., 1990). As such, they induce continuous supraphysiological levels of tyrosine phosphorylation as opposed to the transient and much smaller effects induced by growth factors such as insulin, EGF, or PDGF. Therefore, the use of H_2O_2/vanadate enables detection of tyrosine-phosphorylated proteins that are otherwise missed either due to their low P-Tyr content following hormonal stimuli or due to the failure to identify the physiological ligands that induce their phosphorylation.

Two lines of evidence support the notion that enhanced tyrosine phosphorylation is the cause, rather than the result, of polyphosphoinositide breakdown. First, H_2O_2/vanadate stimulate 6–20-fold protein tyrosine phosphorylation in Fao and BC3H-1 cells, where their effects on inositol phosphate (InsP) levels are rather modest (i.e., 2–3-fold increase). Second, agents known as potent inducers of InsP formation such as antigens in RBL cells (Beaven et al., 1984) fail to reproduce the dramatic effects of H_2O_2/vanadate on protein tyrosine phosphorylation.

The mechanism which couples tyrosine-phosphorylated proteins with InsP formation is presently unknown. We can, however, rule out the possibility that H_2O_2/vanadate acts by inhibiting InsP phosphatases, since all experiments were carried out in the presence of 10 mM LiCl, which by itself acts as a potent inhibitor of these enzymes (Berridge et al., 1982). Similarly, the independence of H_2O_2/vanadate effects on external Ca^{2+} suggests that these agents do not act distal to PLC, by stimulating Ca^{2+} influx, thereby leading to Ca^{2+}-dependent activation of PLC. Hence, tyrosine phosphorylation of the 145-kDa PLC-γ is a possible link between these two signalling pathways (Wahl et al., 1989a,b). Indeed, a 145–150-kDa minor tyrosine-phosphorylated protein is readily detected both in Fao (Figure 1) and in RBL cells (Figure 5), suggesting that tyrosine phosphorylation of PLC-γ could account for the H_2O_2/vanadate effects on InsP formation in these cells. However, such a conclusion should be made rather cautiously in view of the fact that it has not yet been demonstrated that tyrosine phosphorylation of PLC-γ either enhances its enzymatic activity or changes its substrate specificity.

Proteins that differ in their molecular weight from PLC-γ are strongly phosphorylated in cells treated with H_2O_2/vanadate. For example, in RBL cells which are highly responsive to such treatment, there is a tight correlation between tyrosine phosphorylation of pp67 and pp55 and the induced accumulation of $InsP_3$ and $InsP_1$, respectively. These findings raise the intriguing possibility that at least in RBL cells pp67 and pp55 may be causally linked to InsP formation although we cannot exclude the possibility that other minor bands that parallel pp67 and pp55 in kinetics are involved. The identity and function of these two tyrosine-phosphorylated proteins remain, however, to be established. pp55 is of special interest as its tyrosine phosphorylation also correlates with serotonin secretion from RBL cells induced by H_2O_2/vanadate.

Stimulus–secretion coupling in RBL cells was previously shown to be associated with InsP formation (Beaven et al., 1984). Our results provide evidence that the latter process could be mediated, at least in part, through protein tyrosine phosphorylation. The role of tyrosine phosphorylation in the induction of serotonin secretion from RBL cells needs further elucidation. H_2O_2/vanadate-induced secretion is only 24% that obtained when antigen is bound to the IgE–Fc_ϵ receptor complex, indicating that antigen-induced release could be mediated by signalling pathways that do not involve tyrosine phosphorylation. Furthermore, antigen-induced tyrosine phosphorylation has not been documented as yet, in RBL cells. Thus, H_2O_2/vanadate-induced release could be mediated by a signalling pathway independent of that induced by the IgE receptor, or, alternatively, antigen binding induces low and transient tyrosine phosphorylation of proteins that escaped notice so far. That tyrosine phosphorylation indeed forms part of a physiological release process is supported by the recent observations of Lerea et al. (1989), who demonstrated that tyrosine phosphorylation of a 50-kDa protein might be part of the platelet activation cascade which involves secretion of serotonin and PDGF. It will be of interest to determine whether the 50-kDa protein and pp55 described here share common features.

Added in Proof

A recent report (Benhamou et al., 1990) indicates that tyrosine phosphorylation coupled to IgE receptor mediates signal transduction and histamine release.

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Registry No. PTPase, 79747-53-8; $InsP_1$, 15421-51-9; $InsP_2$, 27216-57-5; $InsP_3$, 27121-73-9; H_2O_2, 7722-84-1; orthovanadate, 14333-18-7; protein tyrosine kinase, 80449-02-1; serotonin, 50-67-9.

References

Beaven, M. A., Moore, I. P., Smith, G. A., Hesketh, R. T., & Metcalfe, J. C. (1984) *J. Biol. Chem. 259*, 7137–7142.

Benhamou, M., Gutkind, J. S., Robbins, K. C., & Siraganian, R. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5327–5330.

Bernier, M., Laird, D. M., & Lane, M. D. (1988) *J. Biol. Chem. 263*, 13626–13634.

Berridge, M. J., Downes, C. P., & Hantley, M. R. (1982) *Biochem. J. 206*, 587–595.

Downes, P. C., & Michell, R. H. (1981) *Biochem. J. 198*, 133–140.

Eberhard, D. A., & Holz, R. W. (1988) *Trends Neurosci. 11*, 517–520.

Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., & Rutter, W. J. (1986) *Cell 45*, 721–732.

Farah, F. S., Kern, M., & Eisen, H. N. (1960) *J. Exp. Med. 112*, 1195–1210.

Heffetz, D., & Zick, Y. (1989) *J. Biol. Chem. 264*, 10126–10132.

Heffetz, D., Bushkin, I., Dror, R., & Zick, Y. (1990) *J. Biol. Chem. 265*, 2896–2902.

Hunter, T. (1987) *Cell 50*, 823–829.

Jackowski, S., Rettenmier, C. W., Sherr, C. J., & Rock, C. O. (1986) *J. Biol. Chem. 261*, 4978–4985.

Kadota, S., Fantus, I. G., Deragon, G., Guyda, H. J., & Posner, B. I. (1987) *J. Biol. Chem. 262*, 8252–8256.

Klarlund, J. K. (1985) *Cell 41*, 707–717.

Koshio, O., Akanuma, Y., & Kasuga, M. (1988) *Biochem. J. 250*, 95–101.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lerea, K. M., Tonks, N. K., Krebs, E. G., Fischer, E. H., & Glomset, J. A. (1989) *Biochemistry 28*, 9286–9292.
Margolis, B., Rhee, S. G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A., & Schlessinger, J. (1989) *Cell 57*, 1101–1107.
Meisenhelder, J., Suh, P. G., Rhee, S. G., & Hunter, T. (1989) *Cell 57*, 1109–1122.
Nishibe, S., Wahl, M. I., Rhee, S. G., & Carpenter, G. (1989) *J. Biol. Chem. 264*, 10335–10338.
Paris, S., Chambard, J. C., & Pouyssegur, J. (1988) *J. Biol. Chem. 263*, 12893–12900.
Sagi-Eisenberg, R., & Pecht, I. (1984) *Immunol. Lett. 8*, 237–241.
Tilly, B. C., Van Paridon, P. A., Verlaan, I., De Laat, S. W., & Moolenaar, W. H. (1988) *Biochem. J. 252*, 857–863.
Tonks, N. K., Diltz, C. D., & Fischer, E. H. (1988) *J. Biol. Chem. 263*, 6731–6737.
Wahl, M. I., Daniel, T. O., & Carpenter, G. (1988) *Science 241*, 968–970.
Wahl, M. I., Olashow, N. E., Nishibe, S., Rhee, S. G., Pledger, W. J., & Carpenter, G. E. (1989a) *Mol. Cell. Biol. 9*, 2934–2343.
Wahl, M. I., Nishibe, S., Suh, P. G., Rhee, S. G., & Carpenter, G. (1989b) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1568–1572.
Yarden, Y., & Ullrich, A. (1988) *Annu. Rev. Biochem. 57*, 443–478.

Chorismate Mutase–Prephenate Dehydrogenase from *Escherichia coli*. 1. Kinetic Characterization of the Dehydrogenase Reaction by Use of Alternative Substrates

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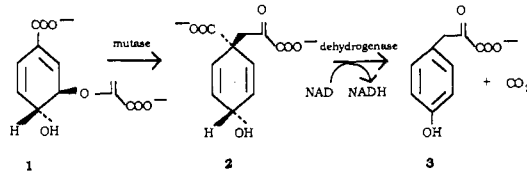
ABSTRACT: The bifunctional enzyme involved in tyrosine biosynthesis, chorismate mutase–prephenate dehydrogenase, has been isolated from extracts of a plasmid-containing strain of *Escherichia coli* K12 and purified to homogeneity by a modified procedure that involves chromatography on both Matrex Blue A and Sepharose–AMP. Detailed studies of the dehydrogenase reaction have been undertaken with analogues of prephenate that act as substrates. The analogues, which included two of the four possible diastereoisomers of 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate (deoxodihydroprephenate) as well as D- and L-arogenate, were synthesized chemically. As judged by their V/K values, all analogues were poorer substrates than prephenate. The order of their effectiveness as substrates is prephenate > one isomer of 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate > L-arogenate > other isomer of 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate > D-arogenate. Thus the dehydrogenase activity is dependent on the degree and position of unsaturation in the ring structure of prephenate as well as on the type of substitution on the pyruvyl side chain. With prephenate as a substrate, the reaction is irreversible because it involves oxidative decarboxylation. By contrast, 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate undergoes only a simple oxidation, and thus, with this substrate, the reaction is reversible. Steady-state velocity data, obtained by varying substrates over a range of higher concentrations, suggest that the dehydrogenase reaction conforms to a rapid equilibrium, random mechanism with 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate as a substrate in the forward reaction or with the corresponding ketone derivative as a substrate in the reverse direction. The initial velocity patterns obtained by varying prephenate or 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate over a range of lower concentrations, at different fixed concentrations of NAD, were nonlinear and consistent with a unique model that is described by a velocity equation which is the ratio of quadratic polynomials. An equilibrium constant of 1.4×10^{-7} M for the reaction in the presence of 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate indicates that the equilibrium lies very much in favor of ketone production.

Chorismate mutase–prephenate dehydrogenase [chorismate pyruvatemutase (EC 5.4.99.5)–prephenate:NAD oxidoreductase (decarboxylating) (EC 1.3.1.12)] is a bifunctional enzyme that catalyzes two sequential reactions in the tyrosine biosynthetic pathway of *Escherichia coli* and other organisms (Cotton & Gibson, 1965; Koch et al., 1971a). These reactions (Scheme I) involve the rearrangement of chorismate (**1**) to prephenate (**2**) and, in the presence of NAD,[1] the oxidative decarboxylation of prephenate to (4-hydroxyphenyl)pyruvate (**3**). Through the action of an aromatic aminotransferase, the product (**3**) undergoes conversion to tyrosine, which acts as an end-product inhibitor of chorismate mutase–prephenate dehydrogenase (Koch et al., 1971a).

Scheme I



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[1] Abbreviations: AMP, adenosine monophosphate; CHCP, 1-carboxy-4-hydroxy-2-cyclohexene-1-propanoate (deoxodihydroprephenate); COCP, 1-carboxy-4-oxo-2-cyclohexene-1-propanoate; DTT, dithiothreitol; MES, (*N*-morpholino)ethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; NADH, reduced form of NAD; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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