# Stimulatory effect of pervanadate on calcium signals and histamine secretion of RBL-2H3 cells

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We examined the effect of pervanadate on the activation of rat basophilic leukaemia (RBL-2H3) cells. The pervanadate, generated from a combination of $H_2O_2$ and vanadate ($V_i$), induced concomitantly protein tyrosine phosphorylation, formation of inositol 1,4,5-trisphosphate ($IP_3$), an increase in $[Ca^{2+}]_i$, and histamine secretion in RBL-2H3 cells. These effects were clearly dependent on the ratio of $H_2O_2/V_i$. The secretion of histamine, $IP_3$ formation, and sustained increase in $[Ca^{2+}]_i$ were effectively induced by treatment of the cells with the pervanadate produced from 1 mM $H_2O_2$ and 1 mM $V_i$. These effects mimic the stimulatory effects of an antigen (dinitrophenylated BSA) on $Ca^{2+}$ signals, histamine secretion and morphological changes. Protein tyrosine phosphorylation, formation of $IP_3$ and transient increase in $[Ca^{2+}]_i$ were markedly induced by the pervanadate produced from 3 mM $H_2O_2$ and 1 mM $V_i$. However, histamine secretion induced by the pervanadate was very low. After the pervanadate from 3 mM $H_2O_2$ and 1 mM $V_i$ was treated with catalase, it was able to induce the $[Ca^{2+}]_i$ increase and histamine secretion as much as the antigen did. This indicates that pervanadate from a lower $H_2O_2$ concentration (1 mM $H_2O_2$/1 mM $V_i$) and catalase-treated pervanadate from a higher $H_2O_2$ concentration (3 mM $H_2O_2$/1 mM $V_i$) are able to mimic the activity that was caused by cross-linking of IgE receptors with antigen. The present results also demonstrate that protein tyrosine phosphorylation seems to have a crucial role in $Ca^{2+}$ entry from the external medium, and that a sustained $[Ca^{2+}]_i$ increase is an important step for histamine secretion in RBL-2H3 cells.

## INTRODUCTION

The high-affinity IgE receptor (FcεR1), which is expressed on the surfaces of basophils and mast cells, plays a critical role in immediate hypersensitivity reactions [1]. The role of this receptor is to bind specifically to the Fc portion of IgE and mediate the release of preformed mediators, histamine and 5-hydroxytryptamine (serotonin), in response to the receptor cross-linking induced by antigen binding to IgE–FcεR1 complexes. Results of pharmacological studies have implied that FcεR1-mediated signal transduction in RBL-2H3 cells depends on the activation of protein tyrosine kinases [2–8]. Recently we have shown, by experiments using protein tyrosine kinase inhibitors, that tyrosine phosphorylation is required for the entry of external $Ca^{2+}$ into RBL-2H3 cells [9]. One of the best ways to study the effects of tyrosine phosphorylation is to conduct an experiment in which tyrosine residues are phosphorylated directly.

It has been shown that there is a dynamic balance between phosphorylation and dephosphorylation, and that turnover of the phosphate in phosphotyrosine is very rapid [10,11]. This balance can be upset by activation of tyrosine kinases or by inhibition of phosphatase activity. Orthovanadate ($V_i$) has been shown to inhibit phosphotyrosine phosphatase activity more efficiently than phospho-serine and -threonine phosphatase activity [12,13]. The effects of orthovanadate on signal transduction have been investigated in rabbit kidney cells [14], rat liver macrophages [15] and permeabilized mast cells [16]. Recently it was shown that pervanadates produced by a combination of $H_2O_2$ and vanadate mediate a more potent insulinomimetic activity in several cell lines than does vanadate alone through phosphotryosine phosphatase inhibition [17–20]. Exposure to pervanadate has also been shown to stimulate the accumulation of phosphotryrosine-containing proteins in T lymphocytes [21,22] and RBL-2H3 cells [23,24]. In T lymphocytes, pervanadate stimulated interleukin-2 receptor expression and interleukin-2 secretion. However, in RBL-2H3 cells, the release of histamine in response to pervanadate was found to be very low. The possibility that the positive and negative biological effects of pervanadates on these cells might be caused by the difference in the $H_2O_2/V_i$ ratios employed is not excluded. Howarth and Hunt [25] reported that different chemical species are formed with different ratios.

The above situations led us to investigate the possibly different roles of different pervanadate species, and to study what ratio of $H_2O_2$/vanadate are more suitable for tyrosine phosphorylation in RBL-2H3 cells, and how $Ca^{2+}$ signalling and histamine secretion are modulated by them.

## MATERIALS AND METHODS

### Reagents

Fura-2 AM and fluo-3 AM were obtained from Dojindo (Kumamoto, Japan). An inositol 1,4,5-trisphosphate ($IP_3$) assay kit was obtained from Amersham International (Amersham, Bucks., U.K.). Herbimycin A was kindly given to us by Dr. Y. Uehara [26]. Sodium orthovanadate and $H_2O_2$ (35%) were from Wako Pure Chemicals (Osaka, Japan). Catalase was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mouse anti-dinitrophenyl (DNP) monoclonal IgE antibody (IgE-53-569) and dinitrophenylated BSA ($DNP_7$-BSA) were produced as described in our previous paper [9]. All other reagents were of the best commercial grade.

Abbreviations used: $V_i$, orthovanadate; $IP_3$, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$ concentration; DMEM, Dulbecco's modified Eagle's medium.

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### Cells

All experiments were performed with a secreting subline of rat basophilic leukaemia cells, RBL-2H3 [27]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal-calf serum.

### Pipes buffer

The following buffer was used: 140 mM NaCl, 5 mM KCl, 0.6 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.5 mM glucose, 0.1% BSA and 10 mM Pipes, pH 7.4.

### Pervanadate and catalase treatment

An orthovanadate stock solution was prepared by adding sodium orthovanadate to the Pipes buffer to a concentration of 20 mM. An $H_2O_2$ stock solution was prepared by adding 35% $H_2O_2$ to the buffer to a concentration of 31 mM. $H_2O_2$ and orthovanadate (10 mM) were mixed at molar ratios ranging from 0.2:1 to 3:1 and incubated at room temperature for 15 min. One-tenth volumes of the pervanadate solutions thus made were added to the cell suspensions. For catalase treatment, catalase solution was added to the pervanadate solution to a concentration of 1 $\mu$g/ml and the mixture was incubated at room temperature for 15 min.

### Analysis of protein tyrosine phosphorylation

RBL-2H3 cells ($2 \times 10^6$/ml) were stimulated with pervanadate and catalase-treated pervanadate. The reaction was terminated 2 min later by adding reaction-stop buffer (0.25 M sucrose, 50 mM NaF, 2 mM EDTA, 20 mM $Na_4P_2O_7$, 1 mM phenylmethanesulphonyl fluoride, 2 mM $Na_3VO_4$, 10 mM Tris/HCl, pH 7.4). After centrifugation, 80 $\mu$l of Laemmli buffer [28] was added to the cell pellets and the suspension was boiled for 3 min. Solubilized samples (15 $\mu$l) were analysed by electrophoresis on Tris/glycine-buffered 7.5–15%-polyacrylamide gels in SDS. The separated proteins were transferred to 0.22 $\mu$m-pore nitrocellulose membranes with an electroblotter (Bio-Rad, Mini Trans-Blot Cell). After transfer, the membranes were treated with 1000-fold-diluted anti-phosphotyrosine antibody (PY20, ICN) [29] for 2 h at room temperature. After being washed 3 times with 0.05% Tween in Tris-buffered saline, the membranes were treated with $^{125}$I-labelled anti-mouse Ig [$F(ab')_2$ fragment, Amersham; $5 \times 10^6$ c.p.m.] for 1 h at 37 °C. The membranes were washed as described above and exposed to Kodak X-AR films. If necessary, the autoradiograms were scanned with a densitometer (Atto AE6900).

### Histamine secretion

The degranulation process was monitored by measuring histamine release. Cells were incubated for 24 h at 37 °C in a 24-well flat-bottom microtitre plate (Falcon, no. 3047) in 1 ml of DMEM containing 10% fetal-calf serum per well. The supernatants were discarded, and the cells ($2 \times 10^5$/well) were washed 3 times with Pipes buffer and then incubated at 37 °C for 30 min with pervanadate or catalase-treated pervanadate. The supernatants were withdrawn from each well, and the amount of histamine was determined by h.p.l.c. by the post-column derivative-formation method using *o*-phthalaldehyde. To quantify the histamine remaining in the cells, they were then treated with 500 $\mu$l of 2% $HClO_4$. The extract was analysed for histamine as described above.

### Cell morphology

RBL-2H3 cells were cultured on 25 mm × 25 mm glass coverslips in tissue-culture dishes for 16 h. The dish was set on the stage of an inverted epifluorescence microscope (Nikon TMD-EFQ). The morphology of cells in representative fields before and after pervanadate treatment were checked on a phase-contrast apparatus and photographed.

### Measurement of $IP_3$ formation

$IP_3$ formation in RBL-2H3 cells after antigen stimulation was measured as described previously [30]. RBL-2H3 cells ($8 \times 10^6$ cells/ml) were stimulated with pervanadate or catalase-treated pervanadate. The reaction was terminated by adding 160 $\mu$l of cold 20% $HClO_4$ and the cells were then left at 4 °C for 20 min. After centrifugation, the supernatant (equivalent to $1.3 \times 10^6$ cells) was adjusted to pH 7.4 with 5 M KOH. After centrifugation, the $IP_3$ concentration in the supernatant was measured with an $IP_3$ assay kit (Amersham).

### Measurement of cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$)

RBL-2H3 cells ($6 \times 10^5$ cells/ml) were loaded with fura-2 AM (6 $\mu$M) as described previously [30]. After removal of free dye by centrifugation, the cells were resuspended in 1.5 ml of Pipes buffer. Fluorescence was measured in a 1 cm quartz cuvette with a Shimazu RF-5000 spectrophotometer (excitation, 335 or 362 nm; emission, 495 nm) with stirring at 37 °C. During fluorescence monitoring, the cells were stimulated by adding pervanadate or catalase-treated pervanadate.

### $[Ca^{2+}]_i$ imaging experiments

Single-cell observation was done by the following procedures. RBL-2H3 cells were harvested from culture dishes and were transferred to an observation chamber. After incubation for 16 h, the cells were treated with the culture medium containing 10 $\mu$M fura-2 AM for 30 min at 37 °C. They were then washed with Pipes buffer three times. Fluorescence-microscopic images of the fura-2 loaded RBL-2H3 cells were taken with an inverted epifluorescence microscope (Nikon TMD-EFQ) as described previously [31,32]. The fluorescence images (excitation at 340 and 380 nm, emission at 500 nm) were analysed with a digital image processor (Hamamatsu Photonics, Argus 50).

## RESULTS

### Effect of pervanadates on histamine secretion

We first investigated the effect of pervanadates generated with various $H_2O_2/V_i$ ratios on histamine secretion from RBL-2H3 cells. Table 1 shows that pervanadate (3 mM $H_2O_2$/1 mM $V_i$ and 2 mM $H_2O_2$/1 mM $V_i$) induced release of twice as much histamine as did the control. The level was much lower than that induced by antigen stimulation. These results agree well with those reported by Santini and Beaven [24]. However, catalase-treated pervanadates (3 mM $H_2O_2$/1 mM $V_i$ and 2 mM $H_2O_2$/1 mM $V_i$) and pervanadate from a lower $H_2O_2$ concentration (1 mM $H_2O_2$/1 mM $V_i$) induced the release of as much histamine as antigen stimulation did. The histamine release by the catalase-treated pervanadate (1 mM $H_2O_2$/1 mM $V_i$) was slightly decreased. The pervanadate-induced histamine release was also dependent on external $Ca^{2+}$ (results not shown).

### Protein tyrosine phosphorylation by pervanadate

Next, the effect of pervanadates on tyrosine phosphorylation was

**Table 1 Histamine secretion from RBL-2H3 cells induced by pervanadate, catalase-treated pervanadate or antigen**

RBL-2H3 cells were incubated for 30 min at 37 °C with the indicated reagents. Values presented are means ± S.D. of triplicate measurements. Similar results were obtained in two other experiments.

| Addition | Histamine release (%) |
|---|---|
| None | 6.5 ± 0.5 |
| 3 mM $H_2O_2$/1 mM $V_i$ | 11.9 ± 0.1 |
| 2 mM $H_2O_2$/1 mM $V_i$ | 12.3 ± 0.3 |
| 1 mM $H_2O_2$/1 mM $V_i$ | 28.9 ± 0.5 |
| 3 mM $H_2O_2$/1 mM $V_i$ with catalase | 30.2 ± 2.0 |
| 2 mM $H_2O_2$/1 mM $V_i$ with catalase | 28.2 ± 2.1 |
| 1 mM $H_2O_2$/1 mM $V_i$ with catalase | 23.6 ± 0.1 |
| Antigen | 35.2 ± 2.2 |

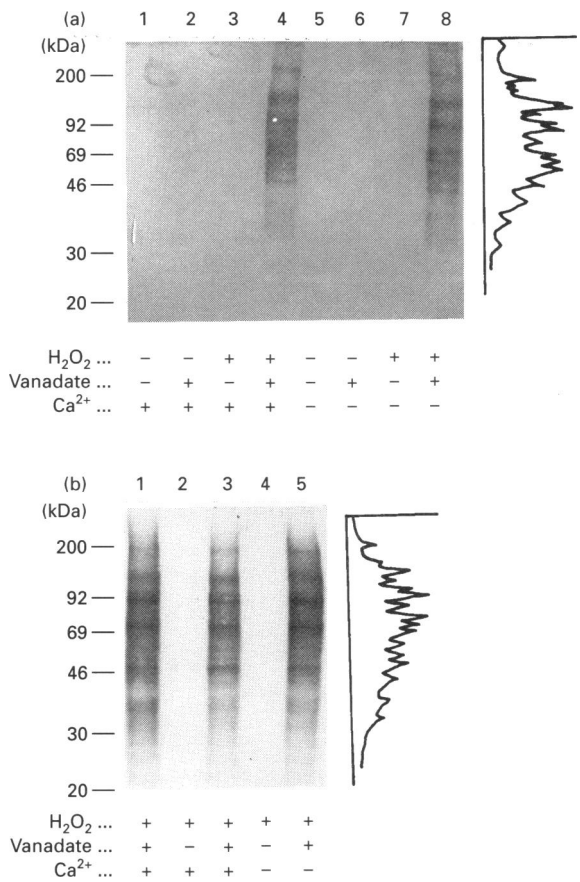


**Figure 1 Effect of $H_2O_2$, vanadate and pervanadate on protein tyrosine phosphorylation**

(**a**) RBL-2H3 cells were incubated at 37 °C in complete Pipes buffer (lanes 1–4) or in $Ca^{2+}$-depleted Pipes buffer (lanes 5–8) without (lanes 1 and 5) or with 1 mM vanadate (lanes 2 and 6), 3 mM $H_2O_2$ (lanes 3 and 7), or 1 mM vanadate plus 3 mM $H_2O_2$ (lanes 4 and 8). The densitometric pattern of lane 8 is shown at the right side of the autoradiogram. (**b**) RBL-2H3 cells were incubated at 37 °C in complete Pipes buffer (lanes 1–3) or in $Ca^{2+}$-depleted Pipes buffer (lanes 4, 5) with 1 mM vanadate plus 3 mM $H_2O_2$ (lane 1), 1 mM $H_2O_2$ (lanes 2 and 4), or 1 mM vanadate plus 1 mM $H_2O_2$ (lanes 3 and 5). The densitometric pattern of lane 5 is shown at the right side of the autoradiogram. The incubation was terminated after 2 min by adding ice-cold Pipes buffer. The washed cells were solubilized, and the extracts were subjected to immunoblotting with anti-phosphotyrosine antibodies as described in the Materials and methods section. The molecular mass (kDa) of the phosphorylated protein was calculated from a calibration curve using standard markers.

investigated. As shown in Figure 1(a), RBL-2H3 cells were treated with 3 mM $H_2O_2$, 1 mM orthovanadate ($V_i$) or the pervanadate (3 mM $H_2O_2$/1 mM $V_i$). $V_i$ or $H_2O_2$ alone did not cause any increase in protein tyrosine phosphorylation (lanes 2 and 3). However, the pervanadate with 3 mM $H_2O_2$ and 1 mM $V_i$ produced numerous tyrosine-phosphorylated proteins (180, 150, 125, 110, 98, 86, 72, 68, 56, 41, 34 kDa) (lane 4). The increase in tyrosine phosphorylation of these proteins by pervanadate was also observed in the absence of extracellular $Ca^{2+}$ (lane 8). Therefore, it seemed that this tyrosine phosphorylation did not require $Ca^{2+}$ influx from the external medium. The extent of protein tyrosine phosphorylation by catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) was almost the same as that by untreated pervanadate (results not shown). The increase in protein tyrosine phosphorylation with 1 mM $H_2O_2$ or 1 mM $H_2O_2$/1 mM $V_i$ are shown in Figure 1(b). The autoradiogram of the tyrosine-phosphorylated protein induced by pervanadate (3 mM $H_2O_2$/1 mM $V_i$) running on the same gel was also shown for comparison (lane 1). $H_2O_2$ (1 mM) alone did not cause any increase in protein tyrosine phosphorylation (lane 2). However, the extent and pattern of phosphorylation induced by the pervanadate from 1 mM $H_2O_2$ and 1 mM $V_i$ were almost the same as those induced by pervanadate (3 mM $H_2O_2$/1 mM $V_i$) (lane 3). The increase in protein tyrosine phosphorylation was also observed without extracellular $Ca^{2+}$ (lane 5).

## Effect of pervanadates on $Ca^{2+}$ signalling

Next we examined the effect of pervanadates on $Ca^{2+}$ signalling. Pervanadate solutions made from $H_2O_2$ and $V_i$ at various molar ratios from 0.2:1 to 3:1 were added to Fura-2-loaded RBL-2H3 cells. As shown in Figure 2(c), the time course of the $[Ca^{2+}]_i$ increase induced by pervanadates from 1 mM $H_2O_2$/1 mM $V_i$ was not transient. The increase in $[Ca^{2+}]_i$ started within 150 s, reached a maximum at 230 s, and the elevated $[Ca^{2+}]_i$ level was sustained for more than 150 s. As shown in Figures 2(b) and 2(a), the $[Ca^{2+}]_i$ increase caused by pervanadates (0.5 mM $H_2O_2$/1 mM $V_i$ or 0.2 mM $H_2O_2$/1 mM $V_i$, respectively) was lower than that with 1 mM $H_2O_2$/1 mM $V_i$. As shown in Figures 2(d) and 2(e), the addition of pervanadates (3 mM $H_2O_2$/1 mM $V_i$ or 2 mM $H_2O_2$/1 mM $V_i$) caused an increase in $[Ca^{2+}]_i$ in RBL-2H3 cells within 100 s, and the $[Ca^{2+}]_i$ decreased again to nearly the initial level by 200 s.

These results indicate that the level and kinetics of $[Ca^{2+}]_i$ increase are dependent on the molar ratio of $H_2O_2$ used for generation of pervanadates, and probably on the chemical differences in pervanadate species formed.

We then investigated whether or not the $[Ca^{2+}]_i$ increase caused by pervanadate from higher $H_2O_2$ concentrations might be changed after catalase treatment. As shown in Figures 3(a) and 3(b), the $Ca^{2+}$ response obtained by addition of catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) was markedly different from that obtained by pervanadate without catalase treatment. The kinetics was very similar to that obtained with pervanadate from 1 mM $H_2O_2$/1 mM $V_i$ (Figure 2c). The $Ca^{2+}$ responses by catalase-treated pervanadate from 2 mM $H_2O_2$/1 mM $V_i$ and 1 mM $H_2O_2$/1 mM $V_i$ were also similar to that obtained with pervanadate from 1 mM $H_2O_2$/1 mM $V_i$, although the maximal increase in $[Ca^{2+}]_i$ by catalase-treated pervanadate from 1 mM $H_2O_2$/1 mM $V_i$ was slightly decreased (results not shown).

To determine the requirement for external $Ca^{2+}$ in the pervanadate-induced $[Ca^{2+}]_i$ increase, we compared the $Ca^{2+}$ responses with and without external $Ca^{2+}$. As shown in Figure 3(a), $[Ca^{2+}]_i$ was increased by pervanadate (3 mM $H_2O_2$/1 mM $V_i$), even in

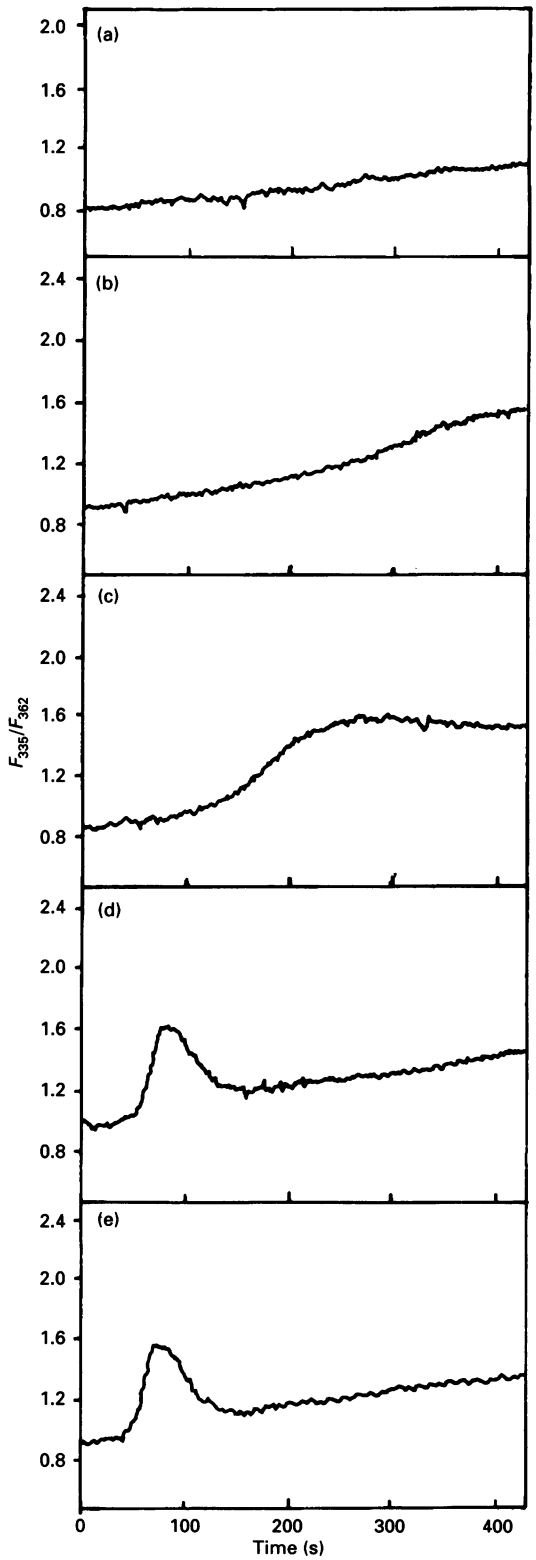


**Figure 2 Effect of pervanadate on $[Ca^{2+}]_i$ of RBL-2H3 cells**

Fura-2-loaded RBL-2H3 cells were treated with the pervanadate formed at 0.2 mM $H_2O_2$/1 mM vanadate (**a**), 0.5 mM $H_2O_2$/1 mM vanadate (**b**), 1 mM $H_2O_2$/1 mM vanadate (**c**), 2 mM $H_2O_2$/1 mM vanadate (**d**) and 3 mM $H_2O_2$/1 mM vanadate (**e**). The fluorescence (excitation, 335 and 362 nm; emission, 500 nm) of fura-2-loaded cells was measured. These traces are representative of three experiments.

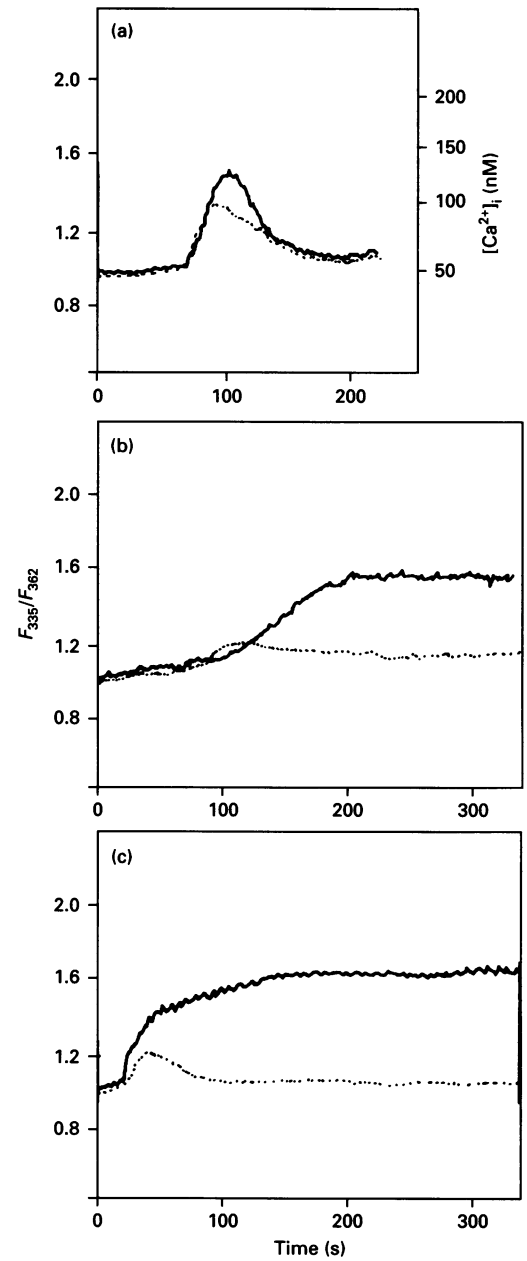


**Figure 3 Effect of catalase treatment of pervanadate on $[Ca^{2+}]_i$ increase in RBL-2H3 cells**

(**a**) Fura-2-loaded RBL-2H3 cells were treated with pervanadate (3 mM $H_2O_2$/1 mM $V_i$). (**b**) Fura-2-loaded RBL-2H3 cells were treated with catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$). (**c**) Fura-2-loaded and IgE-primed RBL-2H3 cells were stimulated with antigen. Fluorescence (excitation, 335 and 362 nm; emission, 500 nm) of fura-2-loaded cells was measured in complete (——) or $Ca^{2+}$-depleted (· · · · · ·) Pipes buffer. These traces are representative of three experiments.

the absence of external $Ca^{2+}$ (dotted line). The maximal $[Ca^{2+}]_i$ level (105 nM) was about 80% of that (131 nM) in the presence of external $Ca^{2+}$ (continuous line). This indicates that the pervanadate from 3 mM $H_2O_2$/1 mM $V_i$ caused mainly the release of $Ca^{2+}$ from the internal stores. As shown in Figure 3(b), the $[Ca^{2+}]_i$ increase (70 nM) induced by catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) in the absence of external $Ca^{2+}$ was much less than that (138 nM) in the presence of external $Ca^{2+}$.

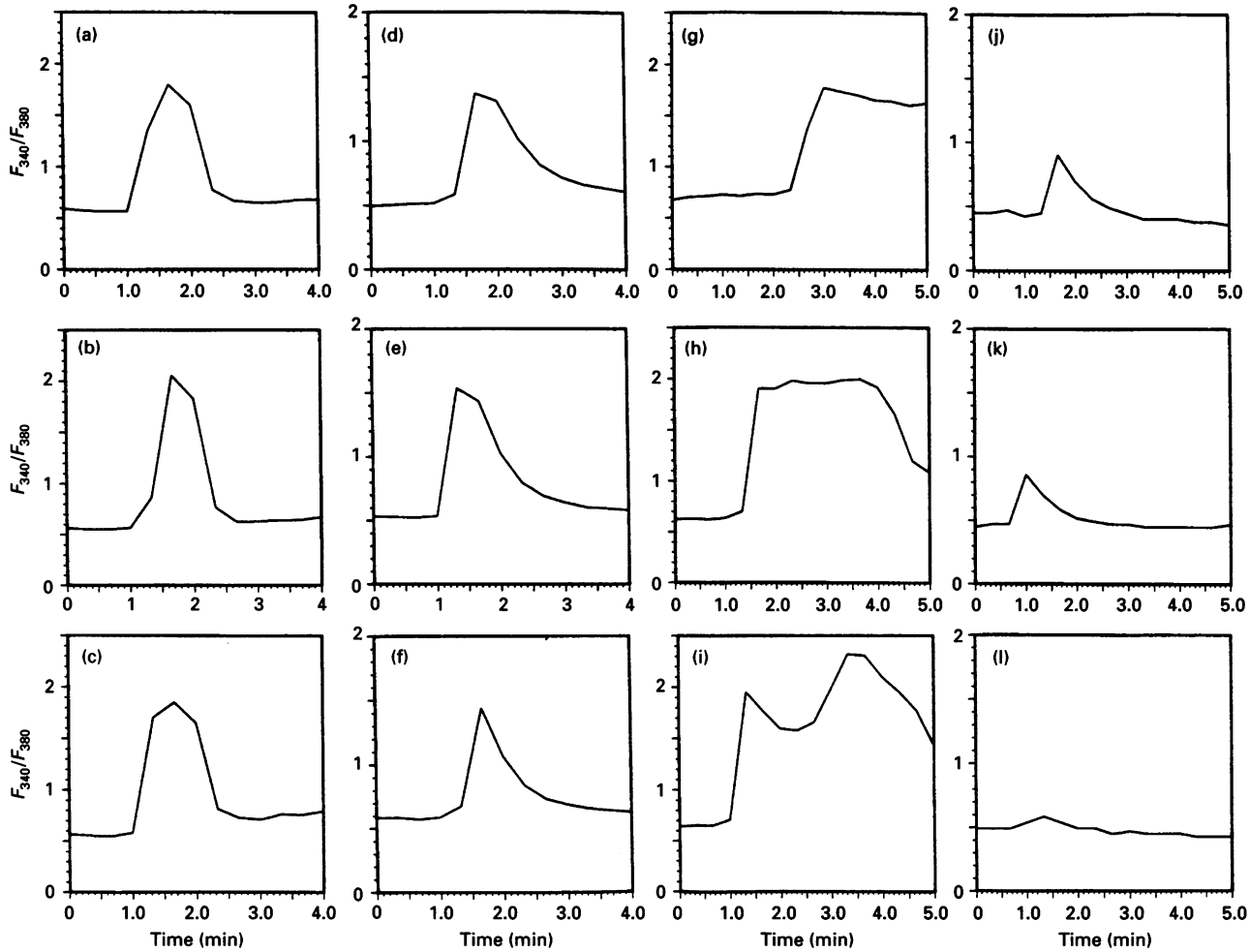


**Figure 4 Single-cell observation of $[Ca^{2+}]_i$ increase caused by catalase-treated or untreated pervanadates**

Fura-2-loaded RBL-2H3 cells were stimulated with pervanadate (3 mM $H_2O_2$/1 mM $V_i$) in Pipes complete buffer (**a–c**) or $Ca^{2+}$-deleted buffer (**d–f**), or the cells were stimulated with catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) in Pipes complete buffer (**g–i**) or $Ca^{2+}$-depleted buffer (**j–l**). Fluorescence images were obtained at 20 s intervals at 37 °C. Data from three cells showing typical sequential fura-2 fluorescence after each stimulation are shown.

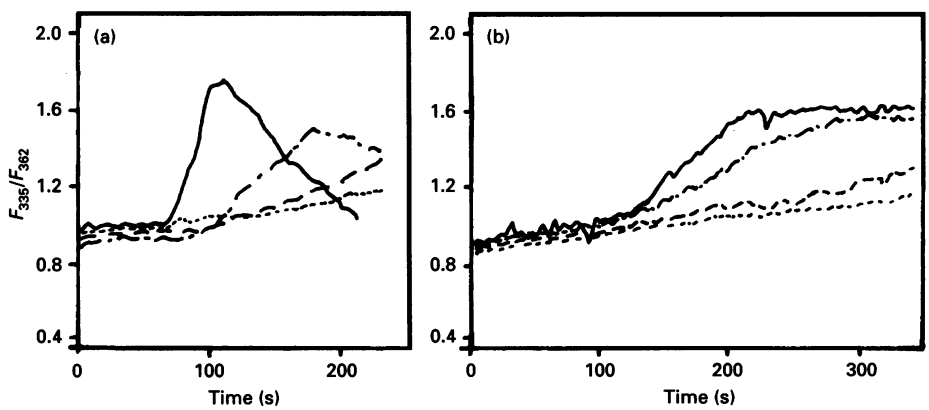


**Figure 5 Effect of herbimycin A on the $[Ca^{2+}]_i$ increase stimulated by catalase-treated or untreated pervanadate**

Cells were incubated for 16 h without (——) or with different doses of herbimycin A (·—·—·, 0.2 μg/ml; – – –, 0.4 μg/ml; · · · · · ·, 0.8 μg/ml), then stimulated with 3 mM $H_2O_2$/1 mM $V_i$ (**a**) or catalase-treated 3 mM $H_2O_2$/1 mM $V_i$ (**b**). The fluorescence of fura-2-loaded cells was measured as described in the legend for Figure 3. Representative results of two experiments are shown.

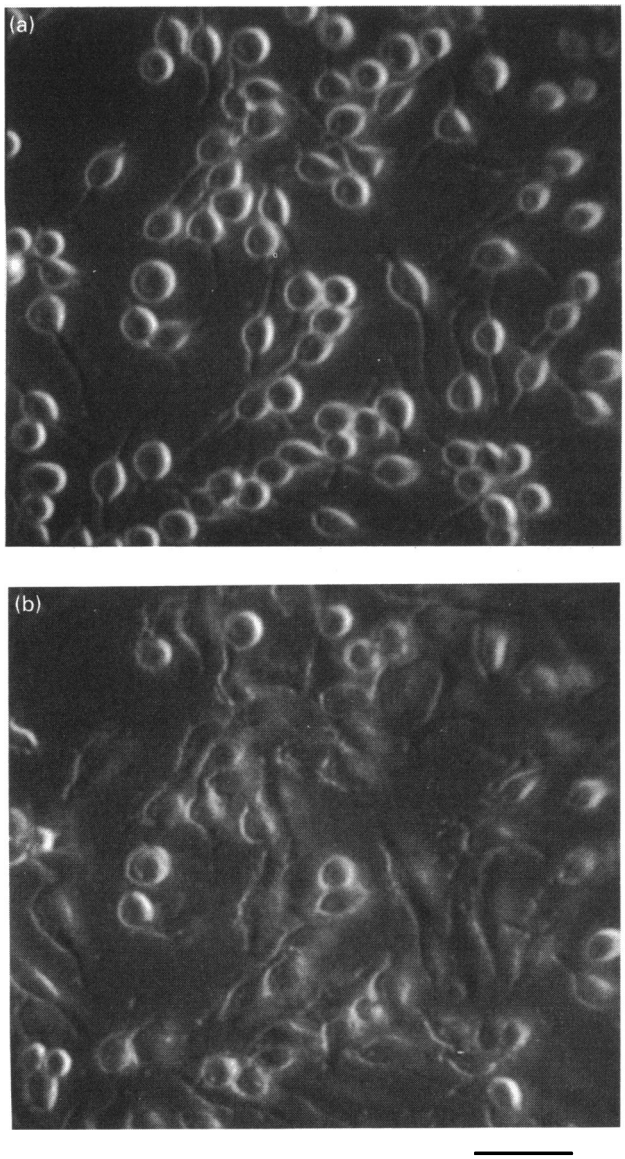


**Figure 6 Morphological changes in RBL-2H3 cells caused by pervanadate**

The morphology of representative fields of cells before (**a**) and after (**b**) 30 min stimulation with 1 mM $H_2O_2$/1 mM $V_i$ was observed with a phase-contrast microscope and photographed. Bar, 50 $\mu$m.

Therefore, catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) seems to cause an influx from the external medium, in addition to the release of $Ca^{2+}$ from the internal stores.

For comparison, Figure 3(c) shows the time courses of $[Ca^{2+}]_i$ changes after antigen stimulation in the presence or absence of external $Ca^{2+}$. Here, $[Ca^{2+}]_i$ increased within 20 s and reached a maximum at 100 s, and the level was sustained for more than 150 s. The $[Ca^{2+}]_i$ increase was transient when external $Ca^{2+}$ was omitted. The lag time of the $Ca^{2+}$ response was shorter in antigen stimulation than in pervanadate stimulation. However, the maximum levels of $[Ca^{2+}]_i$ were almost the same in both cases.

To study the single-cell variation masked by the results in mass analysis, $[Ca^{2+}]_i$ changes at the single-cell level were monitored after pervanadate treatment. Representative time courses of the $[Ca^{2+}]_i$ changes are shown in Figure 4. The transient $[Ca^{2+}]_i$ increase was induced in a similar time course by pervanadate (3 mM $H_2O_2$/1 mM $V_i$) either with or without external $Ca^{2+}$ (Figures 4a–4c and 4d–4f), whereas the lag time for the $[Ca^{2+}]_i$ increase by catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) differed with the cell (Figures 4g–4i). This difference in the lag time seems to result in the gradual $[Ca^{2+}]_i$ increases in the cell suspensions shown in Figure 3(b). As shown in Figures 4(j)–4(l), the increase in $[Ca^{2+}]_i$ is transient and small in the absence of $Ca^{2+}$. This indicates that the $Ca^{2+}$ responses to the catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) were mainly attributable to $Ca^{2+}$ entry into the cells. This type of $Ca^{2+}$ entry prevails also in the stimulation of RBL-2H3 cells with antigen, or with pervanadate from lower $H_2O_2$ concentration (1 mM $H_2O_2$/1 mM $V_i$).

To understand more precisely the mechanism of pervanadate in the tyrosine phosphorylation and the $Ca^{2+}$ responses in RBL-2H3 cells, we examined the effect of a protein kinase inhibitor, herbimycin A. As shown in Figures 5(a) and 5(b), the $[Ca^{2+}]_i$ increases induced by the catalase-treated and untreated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) solutions were diminished in herbimycin A-treated RBL-2H3 cells in a dose-dependent manner.

This suggests that the increases in $[Ca^{2+}]_i$ caused by pervanadates, either sustained or transient, are events resulting from protein tyrosine phosphorylation.

### Effect of pervanadates on $IP_3$ formation

To investigate the effect of pervanadate on phosphatidylinositol hydrolysis, the $IP_3$ level was determined after pervanadate stimulation. As shown in Table 2, pervanadate (3 mM $H_2O_2$/1 mM $V_i$) brought about an increase in the $IP_3$ level in 2 min, which was 10 times that of the control. Catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) and pervanadate with a lower $H_2O_2$ concentration (1 mM $H_2O_2$/1 mM $V_i$) increased $IP_3$ to levels similar to that obtained by antigen stimulation, which was about 3 times that of the control.

### Induction of morphological changes in RBL-2H3 cells caused by pervanadate

In RBL-2H3 cells, morphological changes are caused by antigen stimulation [33]. Therefore, we also examined the effect of pervanadate on the morphology of the cells. RBL-2H3 cells attached to a glass coverslip have a fibroblastic appearance (Figure 6a). Upon activation with pervanadate (1 mM $H_2O_2$/1 mM $V_i$), more than 80% of the cells underwent striking morphological changes after 30 min (Figure 6b). The cells lost their spindle-shaped appearance, gave an increased ruffling or fluffy impression, and spread over the glass surface, causing out-of-focus pictures when photographed with a phase-contrast microscope. Almost the same morphological changes were induced by catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) or antigen stimulation, but not by untreated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) (results not shown).

Thus pervanadate (1 mM $H_2O_2$/1 mM $V_i$) and catalase-treated pervanadate (3 mM $H_2O_2$/1 mM $V_i$) mimicked the antigen in terms of effects on cell morphology.

## DISCUSSION

The cross-linking of FcεR1 of RBL-2H3 cells leads to rapid phosphorylation of the $\beta$-chain (on tyrosine and serine) and $\gamma$-chain (on tyrosine and threonine) of IgE receptors. Thereafter tyrosine residues of several proteins (42, 56, 66, 72, 92, 110, 150 kDa) are also phosphorylated [9].

**Table 2 $IP_3$ formation in RBL-2H3 cells induced by pervanadate, catalase-treated pervanadate or antigen**

$IP_3$ was extracted and its concentration was measured with an $IP_3$ assay kit after 120 s stimulation with the reagents. Values presented are means ± S.D. of duplicate measurements. Similar results were obtained in two other experiments.

| Addition | $IP_3$ formation (pmol/1.3 × $10^6$ cells) |
|---|---|
| None | 1.7 ± 0.3 |
| 3 mM $H_2O_2$/1 mM $V_i$ | 15.0 ± 1.7 |
| 1 mM $H_2O_2$/1 mM $V_i$ | 3.8 ± 0.3 |
| 3 mM $H_2O_2$/1 mM $V_i$ with catalase | 4.2 ± 0.6 |
| Antigen | 4.1 ± 1.2 |

In the present study, we used the tyrosine phosphatase inhibitor pervanadate to upset the balance between phosphorylation and dephosphorylation in the direction of increased protein tyrosine phosphorylation in the cells. As shown in Figure 1(a) (lane 2), orthovanadate ($V_i$) alone did not augment protein tyrosine phosphorylation, probably because of its insufficient entry into the cell [34]. Once orthovanadate was oxidized to pervanadates by addition of $H_2O_2$ [25], probably because of facilitation of their entry into the cells, protein tyrosine phosphorylation was markedly increased (Figure 1a, lane 4; Figure 1b, lane 2). At least 11 proteins (180, 150, 125, 110, 98, 86, 72, 68, 56, 41 and 34 kDa) in RBL-2H3 cells underwent enhanced tyrosine phosphorylation in response to pervanadate. Although it is difficult to relate any of these phosphorylated proteins to individual events required for histamine release, their presence suggests the involvement of multiple tyrosine-phosphorylated proteins in mediation of the biological effect of pervanadates. Since the pervanadate-induced $Ca^{2+}$ responses were phosphorylation-dependent (Figure 5), some of these proteins will be necessary at an early stage preceding phosphatidylinositol hydrolysis (Table 2) and $Ca^{2+}$ entry (Figure 3). Santini and Beaven [24] recently reported tyrosine phosphorylation of a 41 kDa protein, mitogen-activated protein (MAP) kinase in antigen-stimulated RBL-2H3 cells. In our experiment, phosphorylation of a 42 kDa protein was also observed (Figure 1). It is likely that the phosphorylation of MAP kinase works at a later stage in the histamine secretion.

As stated in the Results section, histamine secretion, $Ca^{2+}$ signals and the quantity of $IP_3$ formed were different with catalase-treated and untreated pervanadate (3 mM $H_2O_2$/1 mM $V_i$). According to the $^{51}V$ n.m.r. study by Howarth and Hunt [25], several peroxo-complexes of vanadium (V) are formed when $V_i$ is oxidized with $H_2O_2$. The pervanadate species formed were different, depending on the molar ratio of $H_2O_2$ to $V_i$. When more than two equivalents of $H_2O_2$ were added, the main species formed at neutral pH were $[HVO_2(OO)_2]^{2-}$, $[H\{VO(OO)_2\}_2O]^{3-}$ and $[H_2VO_2(OO)_3]^{2-}$, and the yield of total pervanadate was very high. When one equivalent of $H_2O_2$ was used, $[HVO_3(OO)]^{2-}$ and $[HVO_2(OO)_2]^{2-}$ were mainly formed. We examined the effect of various types of pervanadate on $Ca^{2+}$ signals in RBL-2H3 cells by changing the molar ratio of $H_2O_2/V_i$ (Figure 2). Interestingly, the catalase-treated pervanadates formed with three equivalents of $H_2O_2$ caused $Ca^{2+}$ responses similar to those obtained by the pervanadate (1 mM $H_2O_2$/1 mM $V_i$). Therefore, $[HVO_3(OO)]^{2-}$ or $[HVO_2(OO)_2]^{2-}$ seems to cause the sustained $[Ca^{2+}]_i$ increase pattern as well as histamine secretion. Kadota et al. [20] reported that the pervanadate formed was stable for at least 2 h after catalase treatment, and Secrist et al. [22] successfully used catalase-treated pervanadate for T-cell activation. The concentration of $H_2O_2$ remaining in the pervanadate solutions without catalase are estimated to be below 1 mM in both 1 mM $H_2O_2$/1 mM $V_i$ and 3 mM $H_2O_2$/1 mM $V_i$. Under the conditions that we used for antigen-induced histamine release, $H_2O_2$ below 1 mM did not show any inhibitory effect on $IP_3$ formation, $Ca^{2+}$ responses or histamine release. Therefore, we assume that the highly peroxidized vanadate complexes are readily changed by catalase into a pervanadate of lower peroxide content with decreasing concentration of $H_2O_2$, and this form was stable to catalase treatment. In other words, the decrease in the concentration of $H_2O_2$ seems to disturb the equilibrium between $V_i$ and pervanadate.

$IP_3$ formation caused by pervanadate formed from 3 mM $H_2O_2$ and 1 mM $V_i$ was marked (Table 2). This finding is consistent with the data reported by Zick and Sagi-Eisenberg [23]. As $H_2O_2$ at 1–3 mM does not increase $IP_3$ formation, the reason why $IP_3$ production is so marked without catalase treatment is not clear. It is possible that the pervanadate species produced with three equivalents of $H_2O_2$ more effectively induces the tyrosine phosphorylation of phospholipase C. The marked increase in $IP_3$ led to the transient increase in $[Ca^{2+}]_i$ (Figure 2c). However, this transient increase in $[Ca^{2+}]_i$ was not accompanied by an influx of $Ca^{2+}$. It is likely that some feedback mechanism inhibited the $Ca^{2+}$ influx because $IP_3$ formation was too high [35], or that the intracellular oxidative conditions resulting from pervanadate resulted in inhibition of the $Ca^{2+}$ influx.

In conclusion, (1) pervanadate formed at a lower $H_2O_2$ concentration (1 mM $H_2O_2$/1 mM vanadate) and catalase-treated pervanadate formed at a higher $H_2O_2$ concentration (3 mM $H_2O_2$/1 mM vanadate) is a very useful tool to mimic the signal transduction stimulated by antigen stimulation in RBL-2H3 cells, (2) the increase in protein tyrosine phosphorylation itself can induce histamine secretion in RBL-2H3 cells, which is comparable with that induced with antigens, (3) treatment of cells with pervanadate formed with 3 mM $H_2O_2$/1 mM vanadate has direct effects on the $IP_3$-releasable $Ca^{2+}$ store, and this is also mediated through an effect on tyrosine phosphorylation.

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