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Crosslinking of the receptors for immunoglobulin E depolarizes the plasma membrane of rat basophilic leukemia cells

(receptor crosslinking/serotonin release/tetraphenylphosphonium/calcium ions)

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ABSTRACT **Aggregation of the receptor for IgE on mast cells, basophils, and a tumor analog, rat basophilic leukemia (RBL) cells, induces a calcium-dependent degranulation of the cells. We have measured the membrane potential ($\Delta\Psi$) of RBL cells during this reaction by using the tetraphenylphosphonium ion (Ph_4P^+) equilibration technique. We observed a 20–45% reduction in ionophore-sensitive Ph_4P^+ accumulation. The phenomenon persisted under conditions expected to collapse the mitochondrial membrane potential, consistent with the effect being due to a change in $\Delta\Psi$ of the plasma membrane. We estimated that the change reflects a depolarization of 20 mV (from −90 to −70 mV, interior negative). Whereas degranulation fails to occur in the absence of external Ca^{2+}, this was not true of the depolarization, indicating that the latter was not a consequence of secretion. When aggregation of the receptor is induced by reaction of the cell-bound IgE with a multivalent antigen, the secretory reaction can be halted by adding a univalent hapten. In this case, complete repolarization occurs. Equivalent depolarization was observed in the absence of Na^+ but was diminished when both Ca^{2+} and Na^+ were absent. Together, the data suggest that aggregation of the receptor opens ion channels and that the latter disappear promptly when the receptors are disaggregated. It is plausible that formation of these channels leads to the entry of Ca^{2+} and is an early and critical consequence of the aggregation of the receptors, thereby leading to degranulation.**

The plasma membranes of mast cells, basophils, and a tumor analog, rat basophilic leukemia (RBL) cells, contain a glycoprotein that binds the Fc portion of monomeric IgE with high affinity. Combination of multivalent antigens with the Fab regions of the bound IgE, or any other procedure that aggregates the bound IgE, induces exocytotic release of mediators such as histamine and serotonin. It has been shown that aggregation of the IgE-binding glycoprotein in the plane of the membrane is the critical and sufficient signal to initiate release (reviewed in ref. 1). The glycoprotein—an oligomer of three types of polypeptide chains (2)—is therefore appropriately termed a receptor.

It is notable that, when studied, exocytosis halts abruptly when receptor aggregation is stopped (3, 4). This suggests that critical biochemical events are stoichiometrically and kinetically linked to the crosslinking of the receptors.

Noncytotoxic exocytosis can also be effected by Ca^{2+} ionophores or by other manuevers that increase the intracellular concentration of Ca^{2+}. These observations together with the Ca^{2+}-dependence of receptor-mediated release have led to the proposal that the latter process can be divided into two stages: (*a*) a receptor-mediated entry of Ca^{2+} into the cell, and (*b*) exocytosis initiated by the increase in cytoplasmic concentrations of Ca^{2+} (5).

When cells are incubated with $^{45}Ca^{2+}$ and the receptors are aggregated, an increase in cell-associated $^{45}Ca^{2+}$ is observed. Although this basic observation and related experiments are consistent with a net influx of Ca^{2+}, there are alternative explanations (5).

If the opening of ion channels is a critical consequence of the aggregation of the receptors, one could expect to be able to detect this by examining the membrane potential ($\Delta\Psi$). For this reason we have examined the plasma $\Delta\Psi$ of basophilic leukemia cells by using the tetraphenylphosphonium ion (Ph_4P^+) equilibration technique (6, 7). We have found that changes in $\Delta\Psi$ have the requisite linkage to aggregation of the receptors expected for an early receptor-mediated event. This result is consistent with the hypothesis that aggregation of the receptors opens ion channels through which Ca^{2+} may enter the cell.

MATERIALS AND METHODS

Cells, Antibodies, and Antigens. All experiments were performed on rat basophilic leukemia cells of the 2H3 subline (8) and were maintained as described (8). Rat IgE from tumor IR 162 (9) and mouse anti-dinitrophenyl (DNP) IgE from hybridoma HI-DNP-E-26.82 (10) were purified as described (11, 12). Purified antibodies to rat and mouse IgE were appropriately adsorbed on affinity columns in order to increase their species specificity. DNP-bovine IgG (DNP-IgG) was prepared as described (13) and had an average of 16 DNP groups per mol. DNP-lysine was from Sigma.

Media and Reagents. The standard medium contained 130 mM NaCl, 5 (occasionally 10) mM KCl, 10 mM Na Hepes at pH 7.3, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% glucose, and 0.1% bovine serum albumin. When Ca^{2+} was omitted, either 1 mM KEDTA or 1 mM NaEGTA, at pH 7.5, was added. When Na-free medium was used, choline chloride replaced the NaCl, KHepes replaced the NaHepes, and the KCl was omitted. A potassium medium was prepared by substituting KCl for NaCl and KHepes for NaHepes. Oligomycin, rotenone, and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were from Sigma, Ph_4PCl was from ICN, and all radiochemicals were from New England Nuclear. All other reagents were of the highest purity available commercially.

Serotonin Release. Cells were trypsinized and 10^6 cells were added to each of the six 35-mm-diameter wells of polystyrene

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Abbreviations: Ph_4P^+, tetraphenylphosphonium ion; DNP, dinitrophenyl; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; $\Delta\Psi$, membrane potential.

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plates (Costar, Cambridge, MA). The plates were incubated for 20–36 hr in a CO_2 incubator at 37°C. Then, 0.5 μg of either mouse or rat IgE was added to the monolayer together with 5 μCi (1 Ci = 3.7×10^{10} Bq) of 5-hydroxy[1,2-^{3}H(*N*)]tryptamine binoxolate (28.5 Ci/mmol). After 30–60 min of further incubation, the medium was aspirated and the cells were washed twice with standard medium. Release was initiated by addition of 1 ml of medium containing antigen (or, in some instances, anti-IgE). Usually 1.0 μg of DNP-IgG was used but 0.1 μg was equally effective. At the indicated times of incubation at 37°C, the entire supernatant was assayed in a liquid scintillation counter. The counts were corrected by subtracting a zero-time value obtained by withdrawing the supernatant from one of the wells immediately after addition of the antigen. Typically, such a sample contained 300–600 cpm compared to several thousand in experimental supernatants during release. The percentage release was calculated by dividing the corrected experimental counts by the total cell-bound counts. The latter were obtained by extracting the monolayer from which the zero-time supernatants had been withdrawn with 1 ml of 0.2 M Na_2CO_3/0.1 M NaOH. The counts in this extract were corrected for the 15–20% quenching.

Ph_4P^+ Uptake and ΔΨ. Cells were grown and incubated with IgE exactly as above but without the serotonin. After the washings, 1 ml of medium containing 1–2 μCi of [^{3}H]Ph_4PBr (4.3 Ci/mmol) and 5 μM Ph_4PCl was added and the cells were incubated further as described in specific experiments. At appropriate times, the supernatants were aspirated, and the cells were washed twice with 2 ml of ice-cold 0.15 M NaCl/10 mM NaHepes, pH 7.3. The cells were then extracted with 1 ml of the alkaline solution (above) and the extract was assayed along with an internal standard containing Ph_4P^+ in extracting buffer. Uptake into the cells amounted to 6–16% of the total Ph_4P^+. In each experiment, one or more wells were analyzed for total protein by the Lowry method (14) with bovine serum albumin as a standard.

In most cases we express the results simply as nmol of Ph_4P^+ per mg of protein, a procedure that involves no assumptions. Because the probe equilibrates rather slowly (see Fig. 1), we generally did not monitor the kinetics of changes in ΔΨ but focused on the extent of the changes. Thus, in a sense the time points represent quadruple measurements of the effect. From these as well as from the duplicate samples taken prior to addition of antigen, the variability can be seen to be <10% and often was much less. The plasma membrane potential was estimated by using this value—obtained in the presence of the inhibitors rotenone and oligomycin which abolish the mitochondrial ΔΨ—and substracting the value obtained in the presence of the proton ionophore CCCP. As expected, addition of CCCP inhibited serotonin release. In order to estimate the concentration of intracellular Ph_4P^+, we used an assumed value of 1.5 μl/10^6 cells (15) and a measured value of 0.2 mg of protein per 10^6 cells. Although these values were obtained for cells in suspension, the estimate of 7.5 μl/mg of protein is in good agreement with that obtained for other cells grown in monolayers (16). Then, from the Nernst equation

$$\Delta\Psi = -\frac{2.3RT}{F}\log\frac{[Ph_4P^+]_{in}}{[Ph_4P^+]_{out}}$$

in which $-2.3RT/F$ is −61 mV at 37°C, the potential could be estimated.

RESULTS

IgE-Mediated Release Causes Depolarization. Addition of the polyvalent DNP-IgG to cells bearing mouse IgE antibodies

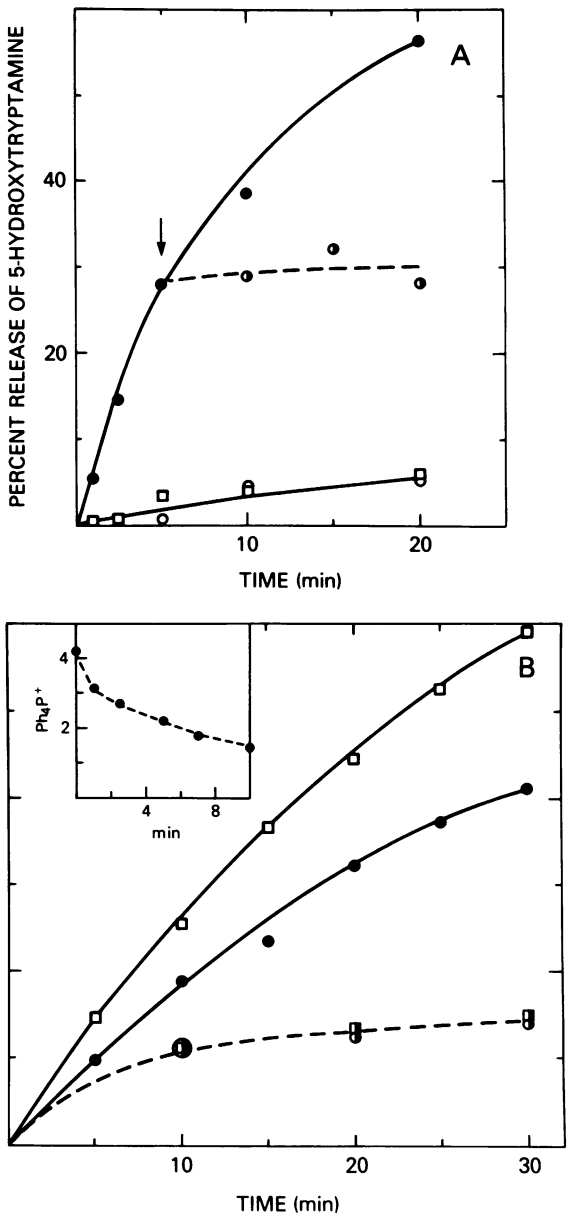

FIG. 1. Effect of DNP-IgG on cells reacting with mouse IgE anti-DNP and rat IgE lacking anti-DNP activity. (*A*) Release of incorporated 5-hydroxy[^{3}H]tryptamine. ●, ○, ◑, Cells loaded with mouse IgE: ●, control; ○, without Ca^{2+}; ◑, addition of DNP-lysine 5 min after addition of DNP IgG. □, Cells loaded with rat IgE. (*B*) Uptake of [^{3}H]Ph_4P^+. ●, ◑, Cells loaded with mouse IgE: ●, control; ◑, with CCCP added. □, ◨, Cells loaded with rat IgE: □, control; ◨, with CCCP added. (*Inset*) Control with CCCP (final concentration, 5 μM) added at 30 min.

to DNP led to substantial release of incorporated tritiated serotonin (Fig. 1*A*). Cells that had been exposed instead to rat IgE lacking anti-DNP activity showed minimal release—equivalent to that obtained with the cells bearing the mouse IgE but incubated without Ca^{2+}. Fig. 1*A* also shows the effect of adding 20 μM DNP-lysine. This concentration of the hapten has no effect on IgE-mediated release in general [e.g., that induced by anti-IgE (data not shown)] but is sufficient to disrupt the binding of the antigen (DNP ≤ 10 nM) to the IgE antibody which has an intrinsic binding constant of 7×10^7 M^{-1} at 37°C (10). The release of the hydroxytryptamine stopped abruptly, a result previously obtained with this system (4) as well as with an analogous one (3).

Fig. 1*B* shows a release experiment carried out under con-

ditions similar to those in Fig. 1A except that the tritiated serotonin was omitted and [^{3}H]Ph_4P^+ was added at the same time as the antigen. (In control experiments, Ph_4P^+ up to 5 μM had no effect on the release of serotonin.) Rapid uptake of the ion was seen with the cells bearing the non-antibody rat IgE. Under conditions that trigger degranulation (Fig. 1A), the cells bearing the mouse IgE showed considerably decreased uptake. In the absence of the DNP-IgG antigen, the cells bearing the alternative IgEs showed equivalent uptake of Ph_4P^+ at 30 min (mean ± SD): cells with rat IgE, 6.07 ± 0.01 nmol/mg of protein; cells with mouse IgE, 6.04 ± 0.08 nmol/mg of protein. The proton ionophore CCCP, which at 5 μM is expected to depolarize the cells fully, strongly inhibited the uptake of the Ph_4P^+ and to equivalent levels with the cells bearing either type of IgE. When the ionophore was added to cells that had accumulated Ph_4P^+ over 30 min, efflux of the cation was observed (Fig. 1B *Inset*), and after 10 min, the level was similar to that observed with cells exposed to the ionophore throughout (Fig. 1B). A similar low level of bound Ph_4P^+ was observed when the cells were incubated in a medium in which Na^+ was replaced with K^+—a medium that depolarizes a wide variety of cells. It is likely, therefore, that this residual uptake represents simple binding of Ph_4P^+ rather than voltage-dependent accumulation. In the subsequent experiments, the uptake of Ph_4P^+ in controls incubated with CCCP was subtracted from the experimental values.

The addition of DNP-IgG to cells that bore the rat IgE and had accumulated Ph_4P^+ was without effect. In contrast, those cells bearing the anti-DNP mouse IgE released a considerable amount of the accumulated Ph_4P^+ (Fig. 2, upper curves). Release varied between 20% and 40% in different experiments (Table 1).

Studies with other cells (6, 7) and with synaptosomes (17, 18) indicate that the uptake of Ph_4P^+ reflects both equilibration across the plasma membrane, governed by $\Delta\Psi$, and accumulation into mitochondria (which are negative inside compared to the cytoplasm) (17, 18). This also seems to be the case with

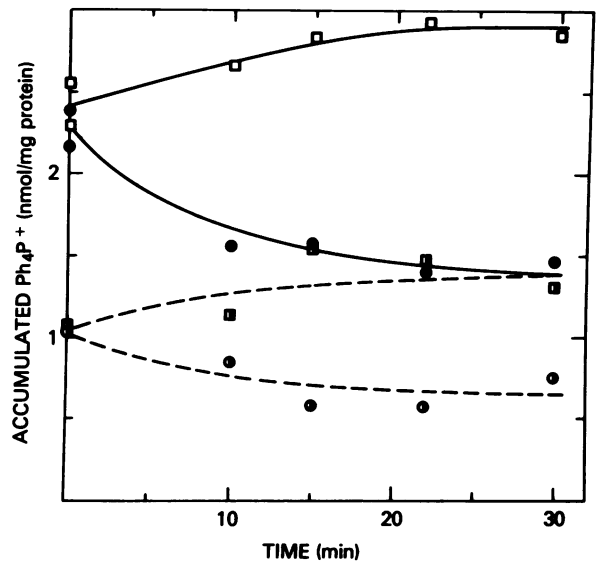

FIG. 2. Effect of DNP-IgG on that part of the uptake of Ph_4P^+ that is sensitive to uncoupling reagents, in the presence and absence of mitochondrial inhibitors. The cells were incubated with 5 μM Ph_4P^+ (2 μCi per well) for 30 min in the presence or absence of 6 μM oligomycin and 2 μM rotenone, with the appropriate IgE. At zero time, DNP-IgG was added at 1 μg/ml. ●, ◑, Cells loaded with mouse anti-DNP IgE: ●, control; ◑, with inhibitors. □, ◨, Cells loaded with rat nonspecific IgE: □, control; ◨, with inhibitors.

Table 1. Effect of Na^+ and Ca^{2+} on receptor-mediated depolarization

Ion		Depolarization,	Experiments,
Na^+	Ca^{2+}	%*	no.
+	+	30.9 ± 10.2	13
+	−	25.0 ± 7.7	4
−	+	38.5 ± 4.8	4
−	−	8.2 ± 2.0	3

*Cells were preincubated with Ph_4P^+ and rat or mouse IgE. The percentage depolarization was calculated by comparing the residual Ph_4P^+ in the cells after addition of DNP-IgG

$$\% \text{ depolarization} = \left(1 - \frac{Ph_4P^+ \text{ (mouse)}}{Ph_4P^+ \text{ (rat)}}\right) \times 100.$$

In virtually all experiments the comparison was made between samples taken at several times during the 30-min incubation. Results are shown as means ± SD.

RBL cells: blocking of the respiratory chain, which generates the mitochondrial $\Delta\Psi$ with rotenone and blocking of the proton-translocating ATPase with oligomycin caused a 60% decrease of Ph_4P^+ uptake (Fig. 2, zero time point, lower curves). Nevertheless, crosslinking of the receptors for IgE induced depolarization of such cells (Fig. 2, lower curves). These results suggest that the depolarization occurs at the level of the plasma membrane. Using the data from this experiment and an estimated volume of 7.5 μl/mg of cell protein, one can calculate that crosslinking of the receptors induced a change in the plasma $\Delta\Psi$ from −94 mV to −73 mV. Upon addition of increasing amounts of unlabeled Ph_4P^+ (to 100 μM), which preferentially collapses the mitochondrial $\Delta\Psi$ (17), the effect of crosslinking the receptors persisted (data not shown). This provides additional evidence that it is the plasma membrane that is depolarized.

That the depolarization was dependent upon aggregation of the receptors could also be demonstrated by the use of antibodies directed to the IgE. Fig. 3 illustrates such an experiment. Addition of the appropriate anti-IgE to cells bearing rat or mouse IgE caused substantial release, whereas with the heterologous combinations there was substantially less secretion (Fig. 3A). The corresponding data on release of accumulated Ph_4P^+, shown in Fig. 3B, show the expected correlation of depolarization with receptor-mediated exocytosis. Reaction of antibodies that can agglutinate the tumor cells but do not stimulate exocytosis failed to induce depolarization (data not shown).

Depolarization Is Not a Consequence of Exocytosis. It was of interest to determine if the observed depolarization preceded or was a consequence of the exocytosis. There is no release of 5-hydroxytryptamine in the absence of Ca^{2+} in the medium (Fig. 1A, open circles). Nevertheless, depolarization was observed under these conditions (Fig. 4). Although in the experiment illustrated, the percentage depolarization was less than that usually observed when Ca^{2+} was present, our aggregate data (Table 1) show no statistically significant difference in the extents of depolarization in the presence and absence of the divalent cation. It should be noted, however, that the absolute magnitude of $\Delta\Psi$ appeared to be reproducibly lower in the absence of Ca^{2+}. We have no explanation for this finding.

Repolarization When Crosslinking of Receptors Is Stopped. We have already shown the effect on exocytosis when crosslinking of receptors induced by the multivalent DNP-IgG was abrogated by adding monovalent DNP-lysine 5 min later (Fig. 1A). The corresponding effects on accumulation of Ph_4P^+ are shown in Fig. 5. When added to the control cells bearing rat IgE there was a minimal effect; if anything the hapten caused

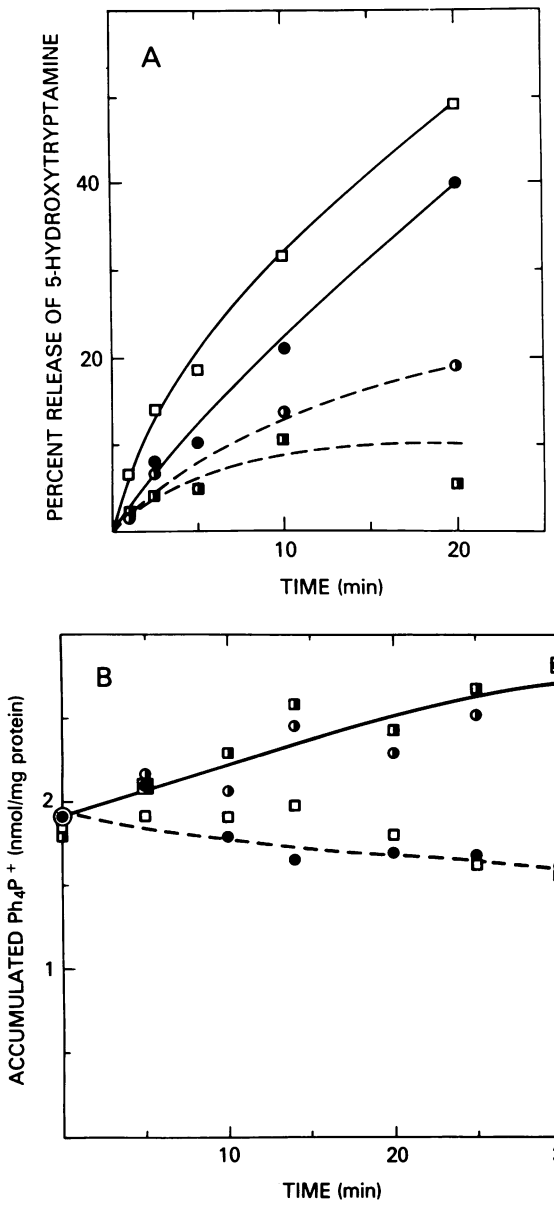

FIG. 3. Effect of anti-IgE-induced exocytosis and uncoupler-sensitive uptake of Ph_4P^+. (*A*) 5-Hydroxytryptamine release. (*B*) Ph_4P^+ accumulation. The cells had been preincubated for 30 min with the probe prior to the addition of the anti-IgE. In these experiments, 20–25% additional uptake of the probe was observed in the controls. This amount of additional uptake after the preincubation was seen variably and is not unexpected from the kinetics shown in Fig. 1. ●, ◑, Cells loaded with mouse IgE and exposed to anti-mouse IgE (●) or anti-rat IgE (◑). □, ◨, Cells loaded with rat IgE and exposed to anti-rat IgE (□) or anti-mouse IgE (◨).

a small release of Ph_4P^+. However, when added to the cells bearing the mouse IgE the effect was dramatic: instead of the progressive depolarization which reached 40% at 30 min, the cells repolarized and reached control levels within 10 min after addition of the hapten. A similar effect was observed when the hapten was added even 30 min after addition of DNP-IgG (data not shown). These results indicate that the depolarization of the plasma membrane is maintained only so long as there are crosslinked receptors on the membrane. In preliminary experiments, no substantial repolarization was seen when receptor aggregates were not disrupted with hapten, even after 100 min—i.e., after the secretion had plateaued.

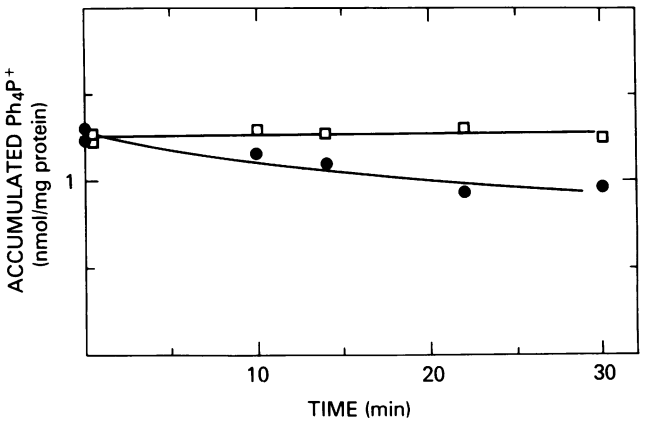

FIG. 4. Uncoupler-sensitive accumulation of Ph_4P^+ in the absence of Ca^{2+}. The cells were incubated for 30 min with Ph_4P^+ in a calcium-free medium. At zero time, DNP-IgG was added. ●, Cells loaded with mouse anti-DNP IgE; □, cells loaded with rat nonspecific IgE.

Effect of Na^+ and Ca^{2+} on Depolarization. The depolarization induced by crosslinking of the receptors was observed in the absence of Ca^{2+} (Fig. 4; Table 1). This indicates that, if depolarization results from the formation of ion channels, the latter cannot be completely specific for Ca^{2+}. When Ca^{2+} was present but all the Na^+ was replaced by choline, the effect was still observed. However, when both Na^+ and Ca^{2+} were omitted, the depolarization was not seen (Table 1).

DISCUSSION

We have observed that RBL cells will accumulate lipophilic Ph_4P^+ in amounts substantially in excess of those seen in the presence of the proton ionophore CCCP. When the mitochondrial respiratory chain and the ATPase that translocates protons were blocked with rotenone and oligomycin, respectively, a residual uptake of Ph_4P^+ was observed. This residual uptake likely reflects the plasma $\Delta\Psi$. With reasonable estimates of the cell

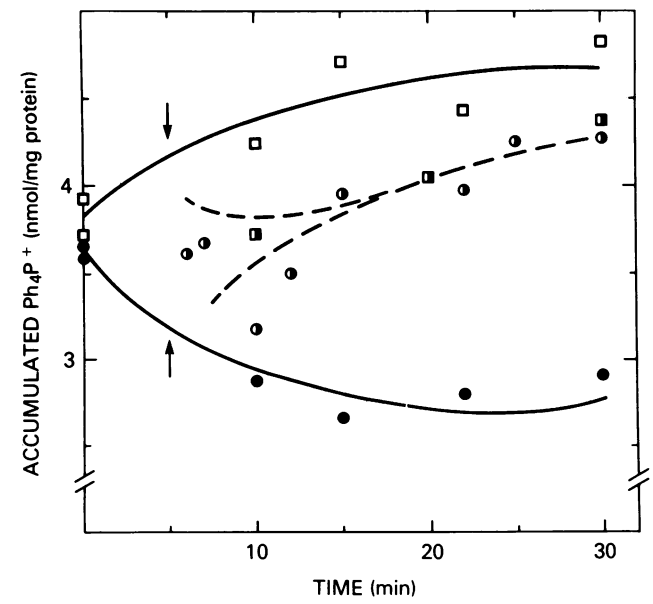

FIG. 5. Effect of disrupting receptor aggregates on Ph_4P^+ accumulation. Cells were loaded with mouse anti-DNP IgE (circles) or rat nonspecific IgE (squares) and incubated with Ph_4P^+ for 30 min. At time zero, DNP IgG was added. ●, □, Controls; ◑, ◨, addition of 20 μM DNP-lysine at 5 min.

volume, the calculated value for this potential (−94 mV) is similar to that observed with other cells (6).

An average 30% reduction in $\Delta\Psi$ was observed when the receptors for IgE were aggregated. This depolarization appears to be a consequence of receptor aggregation *per se* rather than a consequence of the exocytosis that is thereby induced. Thus, in the absence of Ca^{2+} in the medium—which prevents the exocytosis (Fig. 1A)—the depolarization was not substantially diminished (Table 1). The fact that the cells promptly repolarized when receptor aggregation was abrogated early or late in the course of mediator release also suggests that the cells have not simply been made "leaky" during the process of degranulation.

When the cells were depolarized with the ionophore CCCP after having accumulated Ph_4P^+ (Fig. 1*B Inset*), the Ph_4P^+ probe reequilibrated slowly compared to the rate at which the membrane potential is likely to have been discharged. This prevented a precise assessment of the kinetics of depolarization by using this ion. Nevertheless, the evidence is good that receptor aggregation and depolarization are linked kinetically. This was most clearly shown by the experiments in which the aggregation of the receptors induced by the reaction of the multivalent DNP-IgG with the receptor-bound IgE was interrupted by hapten. That secretion was also promptly abrogated indicates that maintenance of aggregation of the receptors is required throughout the secretory process. Interrupting the aggregation caused the Ph_4P^+ probe to reequilibrate at a rate as rapidly as could be anticipated if prompt repolarization had occurred simultaneously with the interruption of receptor aggregation. The increase in the percentage depolarization throughout the course of the secretory reaction, as well as the repolarization experiments, also demonstrates a close stoichiometric linkage between receptor aggregation and the discharge of the membrane potential.

Various other biochemical perturbations have been implicated as early events in secretion—e.g., methylation of phospholipids (13, 19), increases in cAMP (20, 21), and activation of a type I protein kinase (22). It would be interesting to analyze whether these show similar kinetic and stoichiometric linkages with aggregation of the receptors for IgE.

The depolarization we have observed is likely due to the formation of ion channels. Although the data in Table 1 indicate that these channels do not have an absolute specificity for Ca^{2+}, they could be partially selective because the Na^+ was present at a 140-fold higher concentration than Ca^{2+} in these experiments. In the absence of both of these ions, depolarization was not observed, so that the channels appear to be able to transport other cations less well. It is reasonable to postulate that it is these channels that transport Ca^{2+} into the cytoplasm to initiate the exocytotic pathway. An alternative possibility is that, as with nerve terminals, the depolarization secondarily opens voltage-dependent calcium channels (23, 24). However, at present there are no experimental data that require one to invoke such a more complex mechanism.

The prompt repolarization of the plasma membrane upon abrogation of receptor aggregation is of interest. It is possible that the receptors themselves serve as ion channels as long as they remain aggregated. It is also possible that the channels are generated by receptor aggregation but decay "spontaneously." The effect of receptor disaggregation (Figs. 1A and 5) could then be due to the failure to generate new channels. Still other models can be envisioned but it is likely that further experimentation will be more fruitful than additional speculation.

Our results show many parallels to the recent studies by Young *et al.* on the receptors for IgG present on a cell line of mouse macrophages (25, 26). They observed that bridging of these receptors resulted in depolarization of the membrane potential—apparently by opening relatively unspecific channels for cations. In preliminary experiments with isolated receptors incorporated into liposomes they obtained evidence for aggregation-induced ion channels (26). Recent success in isolating and reincorporating apparently intact receptors for IgE (ref. 27; unpublished data) now permits a similar approach for that system.

Dr. Chaviva Isersky provided antiserum to nonreceptor components of RBL membranes. We acknowledge helpful discussions with Drs. Art J. Blume and Evelyn F. Grollman and we thank Dr. H. Ronald Kaback for sending us preprints of the work on the Fc receptors of macrophages.

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