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Membrane Potential Changes During IgE-Mediated Histamine Release from Rat Basophilic Leukemia Cells

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Summary. The membrane potential of rat basophilic leukemia cells (RBL-2H3 cell line) has been determined by monitoring the distribution of the lipophilic [^{3}H] tetraphenylphosphonium cation between the cells and the extracellular medium. By this method, the determined potential of these cells, passively sensitized with IgE, is -93 ± 5 mV (mean $\pm$ SEM, interior negative). Almost 40% of this membrane potential is rapidly collapsed upon the addition of the proton carrier, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP). It is suggested that the FCCP-sensitive fraction of the total membrane potential results from the accumulation of this cation by the mitochondria, which mantains a negative membrane potential. Thus, the resting plasma membrane potential of these cells equals -55 ± 6 mV. During the process of immunological stimulation by antibodies directed against cell membrane bound IgE, the membrane potential decreases. Moreover, there is a correlation between the extent of degranulation of the cells and the depolarization. It is concluded that in common with other secretory systems, depolarization of the plasma membrane is involved in the stimulus-secretion coupling of the histamine secreting RBL cells.

Key Words membrane potential · depolarization · histamine secretion · rat basophilic leukemia · exocytosis · degranulation

Introduction

The immunological trigger for membranal events leading to the exocytotic secretion of immediate type hypersensitivity mediators (e.g. histamine, serotonin, etc.) from mast cells and basophils involves the cross-linking of the Fc receptors for IgE [14, 15, 18, 21, 27, 30]. This can be attained through the allergen-IgE binding where the latter molecules reside in their membrane receptors [14, 30] or indirectly by antisera raised against IgE [21] or a lectin like concanavalin A, cross-linking the Fc carbohydrates of the IgE [18]. Alternatively, antibodies raised directly against the Fc receptor can also be used to the same effect [15] or a chemical cross-linking agent leading to a covalent association [27].

Following that step, several other membranal events take place including the net influx of Ca^{2+} ions into the cells. This has been shown by direct measurements of $^{45}Ca^{2+}$ as tracer [10, 32]. Omission of extracellular calcium ions inhibits the process [23], while the introduction of calcium ionophores causes a noncytotoxic mediator release [3, 12]. On the basis of these observations, it was suggested that the cross-linking of the IgE-Fc receptors leads to the opening of putative calcium gates in the plasma membrane, giving rise to a net calcium uptake [11].

A net influx of calcium is a characteristic effect caused by many agents acting on membrane receptors [4, 7, 26, 31]. The increase in the cytosolic calcium concentration was shown to activate secondary ion channels, thereby changing the entire cellular ion balance. This alteration was suggested to play a central role in the mechanism of action of these agents, eventually leading to membrane fusion and secretion. Furthermore, since the transmembranal electric potential is crucial in the movement and distribution of ions across the plasma membrane, changes in the potential may directly mediate subsequent metabolic and physiologic responses in the cells. A considerable body of evidence suggests that changes in cell membrane potential occur in many cell types immediately after binding of ligands to transmembrane receptors. Such changes follow the binding of insulin to fat cells [6], the binding of thyrotropin to thyroid cells [13], the stimulation of lymphocytes by mitogens [19] or fertilization of eggs [33]. It was, therefore, of considerable interest to measure this parameter in the histamine-secreting system in order to elucidate potential changes during the operation of this system. We have employed a method in which the

Abbreviations: FCCP: Carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; IgE: Immunoglobulin E; RBL: Rat basophilic leukemia; TPP^+: Tetraphenylphosphonium ions.

electrical potential was determined by monitoring concentrations of a lipophilic cation (tetraphenylphosphonium) shown to be distributed between the cells and the medium in response to the potential [22]. A rat basophilic leukemia cell line which secretes histamine upon an immunological stimuli [2] and which can be grown *in vitro* to yield large numbers of cells, provided a convenient system for correlating changes in membrane potential with various phases of the secretion process in an entirely homogenous cell preparation.

Materials and Methods

Materials

FCCP and valinomycin were purchased from Sigma (St. Louis, Mo.). [^{14}C]inulin (10 mCi/mmol) and [^{3}H]H_2O (5 mCi/ml) from Radiochemical Centre (Amersham, England). [^{3}H]TPP^+ (bromide salt 2.5 Ci/mmol) was supplied by the Nuclear Research Center-Negev, Beer-Sheva, Israel. IgE was a monoclonal anti-DNP antibody generously provided by Dr. Z. Eshhar [8] and the anti-IgE antibodies preparations were an ε-chain specific antimouse IgE produced in goat (Miles-Yeda) and a monoclonal rat IgG2b antimouse IgE provided by Dr. Z. Eshhar. All other materials used were of analytical grade.

Cells

All studies were performed on rat basophilic leukemia cells (RBL–2H3) [2]. The cells were maintained in 250 ml tissue culture flasks (Falcon Plastics, Oxnard, California) in Eagle's minimal essential medium (Gibco, Grand Island, New York) supplemented with 20% heat inactivated fetal calf serum (Bio-Lab, Jerusalem, Israel), 4 mM glutamine and antibiotics in 5% CO_2 at 36 °C. The cells were subcultured every 48 hr. The attached cells were suspended by repeated pipettation, washed and transferred to new flasks in 20 ml of medium (about 2×10^6 cells/flask). Cells for experiments were harvested the same way 48 to 72 hr after passage.

Degranulation Experiments

Cells were suspended, washed and resuspended (2×10^6 cells/ml) in Tyrode medium containing (in mM): 137 NaCl, 2.7 KCl, 0.4 NaH_2PO_4, 1.4 $CaCl_2$, 1 $MgCl_2$, 10 Hepes and 5.6 glucose, pH 7.4. The purified monoclonal IgE (4 μg/ml) was then added and the cells were incubated for 1 hr at 37 °C. The passively sensitized cells were then washed three times with Tyrode and resuspended in the same medium at 1×10^7 cells/ml. Aliquots of the cell suspension (100 μl) were then added to test tubes containing anti-IgE antibodies (final volume of the reaction mixture (250 μl). Cells were incubated at 37 °C and the reaction terminated by adding 2.25 ml of Ca^{2+}-free ice-cold Tyrode. After centrifugation the release of histamine was determined fluorometrically [29]. In some experiments the cells were incubated with 2 μCi of ^{3}H-hydroxytryptamine creatinine sulfate (10.8 Ci/mmol, Radiochemical Centre, Amersham) per 10^6 cells for 1 hr at 37 °C. After washing, release was carried out as described and hundred microliter aliquots of supernatants were counted. The viability of the cells always exceeds 95% as assessed by trypan blue exclusion.

[^{3}H]TPP^+ Distribution and Intracellular Volume

Cells suspended in Tyrode (4×10^6 cells/ml) were incubated at 37 °C in the presence of [^{3}H]TPP^+. At given times, 250 μl of the cell suspension was pipetted into a micro tube (400 μl) containing 100 μl of oil (diethylphthalate in di-*n*-butylphtalate, $d=1.045$) [1]. The tubes were then centrifuged at approximately $10{,}000\times g$ for 45 sec in a Beckman microfuge. From each tube a 100 μl sample of supernatant was taken, and the pellet was dissected off in order to avoid contamination with the supernatant. Each pellet was added to 1 ml of 10% Triton X-100 and left overnight with vigorous shaking. All the samples were then assayed for radioactivity by liquid scintillation spectrometry.

The same methodology was used to determine intracellular volume with tritiated water and a labeled, membrane-impermeable solute ([^{14}C]inulin). After extraction, ^{3}H and ^{14}C counts were measured and corrected for spillover of ^{14}C counts into the ^{3}H channel. From the ^{3}H counts of the pellet and supernatant, the cell water volume is obtained:

$$\text{Cell water volume}=\frac{^3H_p}{^3H_s}\cdot V_s=r_{H_2O}\cdot V_s$$

where ^3H_p is the counts of tritium in pellet, and ^3H_s is the counts in supernatant. V_s is the volume of the supernatant sample. The entrapped space equals

$$\frac{^{14}C_p}{^{14}C_s}\cdot V_s=r_{inulin}\cdot V_s;$$

thus, the internal cell volume is

$$V_{internal}=V_s\left(\frac{^3H_p}{^3H_s}-\frac{^{14}C_p}{^{14}C_s}\right)=V_s(r_{H_2O}-r_{inulin}).$$

It is not necessary to know the absolute value of the internal cell volume as the fraction of internal volume is sufficient for the calculation of the distribution.

Calculation of $\Delta\psi$ from TPP^+ Distribution

The value of $\Delta\psi$, the potential difference, is calculated using the Nernst equation:

$$\Delta\psi=\frac{2.3\,RT}{F}\log\frac{[TPP^+]_{in}}{[TPP^+]_{out}}$$

where $\frac{2.3\,RT}{F}$ is equal to -61 mV at 37 °C. The concentration ratio of TPP^+ in the pellet and supernatant is:

$$\frac{TPP^+_p/H_2O_p}{TPP^+_s/H_2O_s}=\frac{r_{TPP^+}}{r_{H_2O}}$$

and the concentration ratio for the cells is:

$$\frac{[TPP^+]_{in}}{[TPP^+]_{out}}=\frac{r_{TPP^+}-r_{inulin}}{r_{H_2O}-r_{inulin}}.$$

All experiments were run in duplicate, and each one was repeated ten times.

Results

Accumulation of TPP^+

Rat basophilic leukemia cells (RBL 2H3), 1×10^7 cells/ml, were suspended in Tyrode medium in the

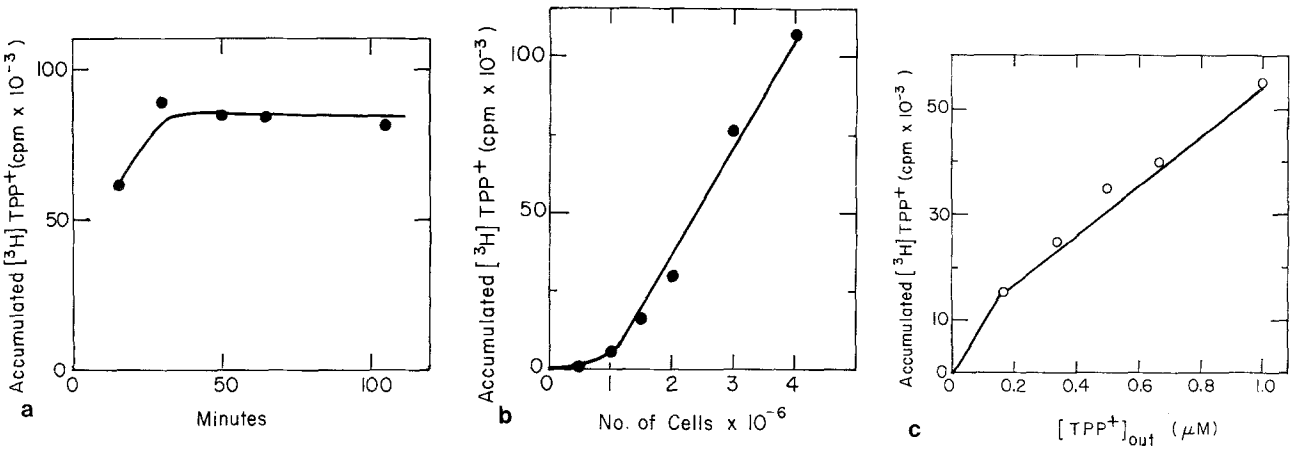

Fig. 1. (*a*): Time course of [^{3}H]TPP$^+$ accumulation. 40×10^6 cells suspended in Tyrode at 10×10^6 cells/ml were incubated at 37 °C in the presence of [^{3}H]TPP$^+$ (0.5 μM). At indicated times, 150 μl samples were transferred to microfuge tubes and assayed for [^{3}H]TPP$^+$ uptake as described in Materials and Methods. (*b*): Effect of cell concentration of [^{3}H]TPP$^+$ accumulation. Cells were incubated at 37 °C in the presence of 0.5 μM [^{3}H]TPP$^+$ for 1 hr and transferred to microfuge tubes, centrifuged and assayed for [^{3}H]TPP$^+$ uptake as described in Materials and Methods. (*c*): Effect of TPP$^+$ concentration on its uptake. Samples of 1×10^6 cells suspended in 250 μl of Tyrode were incubated at 37 °C in the presence of [^{3}H]TPP$^+$. After 1 hr of incubation, the cells were transferred to microfuge tubes and assayed for [^{3}H)TPP$^+$ uptake. The [^{3}H]TPP$^+$ concentration varied from 0.16 μM to 1.0 μM

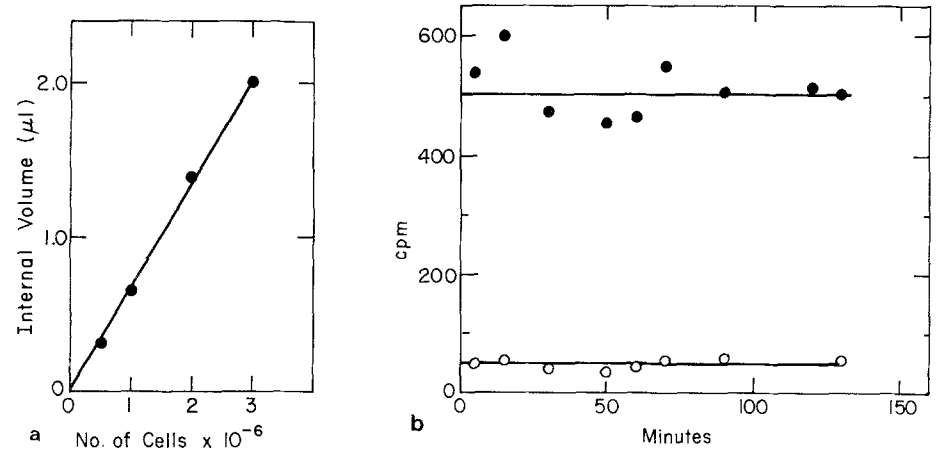

Fig. 2. (*a*): Effect of cell number on cell water volume. One ml of cell suspension containing 14×10^6 cells was incubated at 37 °C for 1 hr in the presence of 7 μCi [^{3}H]H_2O. Samples containing 0.5, 1.0, 2.0 and 3.0×10^6 cells were then transferred to microfuge tubes containing Tyrode to a final volume of 250 μl, centrifuged and the cell water volume determined as described in Materials and Methods. (*b*): Effect of duration of incubation on the trapped and water cell spaces. 20×10^6 cells suspended in Tyrode at 10×10^6 cells/ml were incubated at 37 °C with 0.6 μCi of [^{14}C]inulin and 2 μCi of [^{3}H]H_2O. At given times, samples of 150 μl were transferred to microfuge tubes, centrifuged and the [^{14}C]inulin (o–o) and [^{3}H]H_2O (•–•) associated with the cells were determined as described in Materials and Methods

presence of 0.5 μM TPP$^+$. Maximal accumulation of TPP$^+$ by the cells was observed at about 30 min. This steady-state accumulation level was maintained for at least 110 min (Fig. 1*a*). To elucidate whether this level of accumulation represents true equilibration with the electrical membrane potential, the dependence of the TPP$^+$ uptake process on the number of cells and external TPP$^+$ concentration was studied. It was found to be linearly related to the number of cells in the range of 1 to 4×10^6 cells in the reaction mixture (Fig. 1*b*) and to the TPP$^+$ concentration from 0.2 to 1.0 μM (Fig. 1*c*). Thus, experimental conditions, where the accumulation of TPP$^+$ by the cells may be described by a Nernstian behavior were used throughout this study.

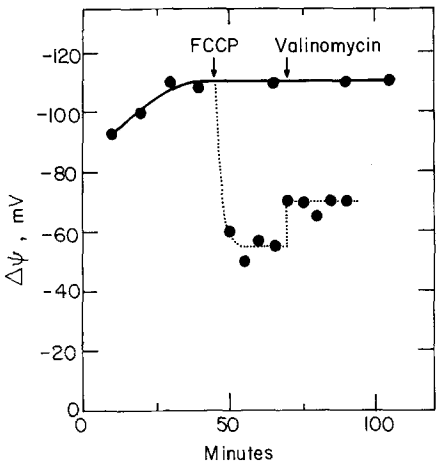

Fig. 3. Effect of ionophores on $\Delta\psi$. Two test tubes, each containing 22×10^6 cells/ml were incubated at 37 °C with 0.4 μM [^{3}H]TPP$^+$. After 45 min of incubation, FCCP was added to test tube 1 yielding a final concentration of 2.5 μM. After 70 min, valinomycin was added to test tube 1 to a final concentration of 4 μM. Test tube 2 was kept as control. [^{3}H]TPP$^+$ uptake was assayed at the given times by transferring 150 μl samples from both test tubes to microfuge tubes. The corresponding values of the membrane potential were calculated as described in Materials and Methods, and using the cell water and entrapped spaces obtained from data shown in Fig. 2*b*. The solid line is the control experiment (without any additions) and the dashed line represents the changes in $\Delta\psi$ in the presence of the added ionophores

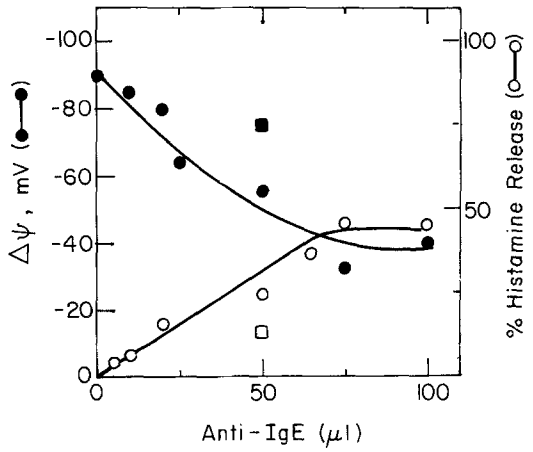

Fig. 4. Effect of immunological stimuli on histamine release and $\Delta\psi$. Cells were passively sensitized with IgE as described in Materials and Methods. After washing, samples of 1×10^6 cells were incubated at 37 °C with increasing anti-IgE concentrations in a final volume of 250 μl in the presence of: (a) 0.4 μM [^{3}H]TPP$^+$; (b) 2 μCi [^{3}H]H_2O and 0.06 μCi [^{14}C]inulin; (c) no radioactive isotope. After 1 hr of incubation, the [^{3}H]TPP$^+$ containing samples and the [^{3}H]H_2O+[^{14}C]inulin containing samples were transferred to microfuge tubes, centrifuged and assayed for radioactivity. $\Delta\psi$ was calculated as described in Materials and Methods. The nonradioactive samples were assayed for histamine release as described in Materials and Methods. (●—●) $\Delta\psi$, ((o—o) histamine release, (■) $\Delta\psi$ determined in the presence of 50 μl of anti-IgE and in Ca^{2+}-free Tyrode, (□) histamine release in Ca^{2+}-free Tyrode

Internal Volume of the Cells

In order to calculate the TPP$^+$ concentration gradient, the intracellular volume was determined. The cells' water space was a linear function of the number of cells giving an average value of 0.66 μl per 1×10^6 cells (Fig. 2*a*). The trapped space given by the impermeable marker inulin for the extracellular fluid carried down by the cells was 0.14 μl for 1×10^6 cells, which represents 21% of the total pellets' volume. Both the trapped and the cell water spaces were independent of the duration of incubation with the radioisotopes (Fig. 2*b*).

Effect of Ionophores on the Accumulation of TPP$^+$

Cells were preincubated with TPP$^+$ until a steady-state level of accumulation had been reached. The final levels of accumulation corresponded to a transmembranal electrical potential of -102 ± 7 (mean value of 4 experiments) (Fig. 3).

At this point, the proton carrier FCCP was added to part of the cells, and the rest of the cells were left as a control. The FCCP-treated cells were rapidly (within 5 min) depolarized reaching a new membrane potential of -55 ± 6 mV. The subsequent addition of valinomycin was followed by a rapid hyperpolarization to a value of -70 mV (Fig. 3).

When the total membrane potential of the cells collapsed upon addition of gramicidin (20 μM) which permeabilizes the membrane to Na^+, K^+ as well as H^+ ions, only 2% of the original TPP$^+$ counts were maintained by the cells. Thus, the potential-independent solubility of TPP$^+$ in the membrane under the selected experimental conditions is small and represents merely an overestimation of about 10 mV of the determined transmembranal electrical potential.

Effect on $\Delta\psi$ of Immunological Stimulation of Histamine Release

Release of histamine was evoked by adding antibodies specific for IgE to a suspension of cells which was previously reacted with IgE. Interest-

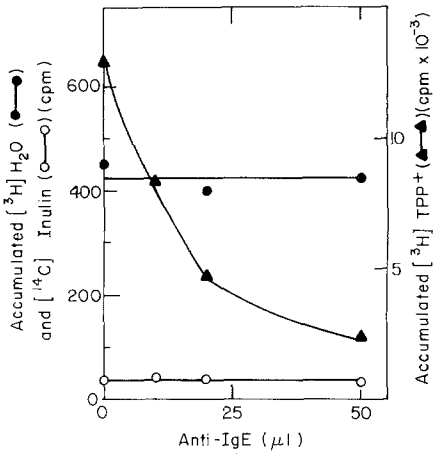

Fig. 5. Effect of anti-IgE on [^{3}H]TPP$^+$ accumulation and on cell water and entrapped spaces. The experimental procedure is the same as described in the legend to Fig. 4. (o–o) Cell-associated [^{14}C]inulin (cpm); (●–●) Cell-associated [^{3}H]H_2O (cpm); (▲–▲) Cell-associated [^{3}H]TPP$^+$ (cpm)

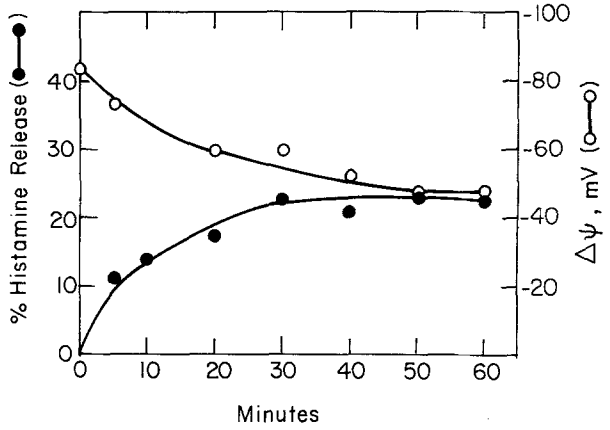

Fig. 6. Kinetics of histamine release and membrane depolarization. After sensitization with IgE (4 μg/2 × 10^6 cells), samples of 1 × 10^6 cells were incubated at 37 °C ($t=0$) with (a) 0.4 μg [^{3}H]TPP$^+$; (b) 2 μCi [^{3}H]H_2O + 0.06 μCi [^{14}C]inulin, and (c) no radioisotope, in a final volume of 200 μl. At $t=0$, 10, 20, 30, 40, 50, 55 and 60 min, fifty μl of anti-IgE were added to the corresponding test tubes. After 1 hr of incubation ($t=60$), cell samples were assayed for histamine release and $\Delta\psi$ as described under Materials and Methods. (●–●) release of histamine; (o–o) $\Delta\psi$

Table 1. The effect of monoclonal anti-IgE antibodies on the accumulation of TPP$^+$, the membrane potential and the release of ^{3}H-serotonin[a]

	TPP^+_p (cpm)	$\Delta\psi$ (mV)	% Release of serotonin
Cells saturated with IgE	9240 ± 1290	−88	8
Cells saturated with IgE in the presence of monoclonal anti-IgE[b]	4967 ± 795	−72	21
Control cells (nonsensitized)	6587 ± 400	−80	10
Control cells in the presence of monoclonal anti-IgE	7001 ± 180	−81	9

[a] The percentage of serotonin release and the membrane potential were determined as described in Materials and Methods.
[b] The supernatants of an anti-IgE-producing hybridoma were collected and used in a dilution of 1:5 to degranulate the cells.

ingly, the membrane potential determined for cells saturated with IgE, was always greater than the one determined for control cells in the same experiment. However, since this difference never exceded 10 mV, its significance still remains to be examined in more detail. The addition of the anti-mouse IgE to these cells caused the release of histamine reaching a value of 48% of the cell content compared with a basal level of release of 3%, and a depolarization of the membrane potential from an initial value of −93 ± 5 mV to −40 ± 2 mV, mean ± SE of 10 experiments (Fig. 4). The dose-response relationships of these two processes (histamine release and depolarization) were similar. Furthermore, when calcium was omitted from the extracellular medium, the release of histamine induced by 50 μl of anti-IgE serum was inhibited from a net release of 25 to 14%. [That amount of anti-IgE antibodies has been employed so as to avoid the saturation range (*cf.* Fig. 4)]. The membrane potential under these experimental conditions was reduced to a value of −75 mV rather than to −55 mV determined in the presence of calcium. It can be seen in Fig. 4 that in the presence of calcium, 20 μl of anti-IgE which induce release of about 14% of its histamine, also depolarize the membrane to the same value of −75 mV.

The addition of the anti-IgE antibodies caused the loss of most of the accumulated TPP$^+$ from the cells while their water volume and the entrapped space remained unchanged (Fig. 5). The addition of the anti-IgE serum to cells which were not previously saturated with IgE did not stimulate histamine release, nor did it affect the resting membrane potential. Thus, neither the goat IgG, nor any of the components, other than the IgG, affect the RBL cells in the absence of IgE. Still, in order

to exclude the possibility of a component different from the anti-IgE antibody, being responsible for the observed membrane depolarization, monoclonal rat anti-mouse IgE antibodies were used to trigger release. Cells which were passively sensitized with the monoclonal IgE molecules were incubated further with monoclonal anti-IgE antibodies produced by a rat hybridoma. The release under those conditions was also accompanied by depolarization of the membrane potential (Table 1). The addition of the monoclonal antibody to cells which were not previously saturated with IgE did not increase the basal release, nor did it affect the membrane potential (Table 1).

Kinetics of the Anti-IgE-Induced $\Delta\psi$ Change

The kinetic pattern of the depolarization process induced by the addition of a constant concentration of anti-IgE antibodies to passively sensitized cells was investigated. The depolarization occurred concomitantly with the secretion of histamine (Fig. 6). The half-life of the secretion process being about 10 min and the half-life of the depolarization process 12 min.

Discussion

The experimental results presented here provide clear evidence that the transmembranal electric potential of the RBL cells can be determined from TPP^+ distribution measurements. Maximum accumulation of TPP^+ by these cells is obtained within 30 min and is maintained for at least 110 min. The fast response to ionophores shows that the accumulation is reversible with the plateau level representing a steady-state condition rather than a conversion to a cellular stable product. Furthermore, the distribution ratio of TPP^+ between the cells and the extracellular medium responded in the predicted direction upon the alteration of the membrane potential with ionophores. Both the protonophore FCCP, and the ionophore gramicidin decreased the TPP^+ accumulation ratio. Valinomycin, which increases the K^+ permeability of the membrane, thereby hyperpolarizing it, increased this ratio. Thus, under the conditions chosen, the accumulation of TPP^+ truly represents an equilibrium distribution and reflects the electrical potential across the membrane. The level of the potential-independent accumulated TPP^+ (measured in the presence of gramicidin which brings about a total collapse of the electrical potential) is small, suggesting that the solubility of TPP^+ in the membrane is low.

The resting potential calculated from the steady-state level of TPP^+ accumulation is relatively high and equals -93 ± 5 mV (mean value of 10 experiments). Nevertheless, this value was not corrected for the solubility of TPP^+ in the membrane, since under those conditions (high gramicidin concentration) serious deformations of the cells took place (*unpublished results*). We therefore preferred to present the uncorrected value. This calculated potential is, however, of the same magnitude as the potentials reported for human and guinea pig granulocyte membranes (-102 ± 5 and -70 mV, respectively) [17, 34]. It was found to be unaffected by the absence or presence of 1.4 mM Ca^{2+}. Yet, it increased slightly upon saturation of the IgE receptors. Part of the accumulated TPP^+ undoubtedly represents that due to internal organelles, predominantly by the mitochondria, which maintains a high negative membrane potential and is therefore likely to accumulate the positively charged TPP^+ ions. Indeed, the rapid loss of TPP^+ from the cells upon addition of FCCP suggests that a fraction of the TPP^+ taken up by the cells is accumulated across the mitochondrial membrane. The secretory granules were also reported to maintain a proton gradient [16] suggesting that a proton pump might be operating in their membranes building up an electrical potential. Still, since they were reported to maintain an acidic ΔpH, it is most likely that in common with other secretory organelles [5, 25] those of the RBL cells also generate a positive membrane potential and will therefore not contribute significantly to the accumulation of TPP^+. This point, however, still remains to be clarified by direct potential measurements on isolated RBL granules. On the basis of these considerations, the plasma membrane potential of the cells is the one calculated from the steady-state level of TPP^+ accumulation obtained immediately after the addition of FCCP [9]. It equals -55 ± 6 mV (mean value of 4 experiments).

The immunological degranulation is associated with changes in the membrane potential of the RBL cells. Exposure of the cells to an IgE cross-linking agent, in this case both polyclonal and monoclonal antibodies raised against IgE, leads to histamine secretion and membrane depolarization. Furthermore, the extent of depolarization depended on the concentration of anti-IgE and could be well correlated with the amount of histamine released. It is apparent that, in common with other secretory systems (neutrophils [28], platelets [20] as well as lymphocyte activation [24]), the trans-

membranal electrical potential is involved in stimulus-secretion coupling in the histamine-secreting RBL cells and the process of secretion is closely associated with depolarization of the cell membrane. Still, further work is required in order to elucidate the generality of this phenomenon, namely the effect of other triggering reagents on the membrane potential. In addition, the ionic nature of these membrane potential changes has to be resolved. They could be associated with the increased Ca^{2+} influx as well as with an increased Na^{+} influx. In experiments which are currently in progress, an effort is made to evaluate the role of these ions in the processes of secretion and depolarization by fusing the RBL cells with calcium and sodium containing liposomes and measuring the effect on the release of histamine and on the transmembranal electrical potential.

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