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# Stimulus-secretion coupling mechanisms in mast cells

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We have investigated several aspects of the complex sequence of events, transmitting the antigen-induced signal of cross-linking immunoglobulin E (IgE) resident in the membrane surface of mast cells into the signals yielding the final process of mediator release. Already the initial phase of this cascade still requires a better understanding. Namely, we are still missing a clear physical description of the effective stimulus-producing antigen-IgE complex in terms of size and spatial requirements. We are investigating this problem on a well-defined cell line (rat basophilic leukemia-RBL-2H3) using synthetic divalent haptens and a monoclonal IgE. A subsequent phase following IgE aggregation is a transient increase in the free calcium concentration in these cells' cytosol. The source of the $Ca^{2+}$ and the way by which it enters the cytosol are studied predominantly by examining antigen-induced channel activity in the cells' membrane allowing $Ca^{2+}$ influx from the exterior medium. Finally, we have observed that under certain experimental conditions, antigen-induced degranulation can be achieved even without a rise in cytosolic free calcium. In our search for alternative second messengers, we examine the potential candidacy of the cytosolic $Na^+/H^+$ balance. So far, we have found that antigen-stimulated secretion does require extracellular sodium and involves changes in its cytosolic pH. However, further studies are required to clarify its possible role as a coupling element.

## 1. Introduction

Mast cells and basophils have repeatedly been proposed as a good paradigm for the biophysical and biochemical mechanisms which couple signals produced at membrane receptors with a biological effector function [1,2]. In these highly specialized secretory cells a relatively minor component of the immune system, immunoglobulins of the IgE class, serve as antigen receptors. A monovalent, high-affinity membrane protein complex serves to anchor the IgE via its $Fc_\epsilon$ domains. Hence, its designation is the $Fc_\epsilon$ receptor ($Fc_\epsilon R$) [3–5]. Upon binding their respective antigens, these membrane-residing IgE molecules undergo a cross-linking process which is considered to be the initial signal, starting a cascade of biochemical processes culminating in secretion of both preformed mediators stored in these cells' granules as well as agents which are being synthesized upon this stimulation, mainly arachidonic acid derivatives which further amplify the physiological effects of these cells [6,7]. Essentially most phases in this sequence of events may be considered as prototypic elements in the diversity of receptor-operated cellular processes. Furthermore, mast cells, as a specialized niche of the immune system, have at least two advantageous features: (1) The cellular activity being triggered is an immediate one. Namely, depending on the particular type of mast cell or basophil, secretion occurs within seconds to minutes following the IgE cross-linking step (Immediate-type hypersensitivity). (2) It provides a meaningful model for cell activation by an immunological stimulus, i.e., by an antibody-antigen reaction. Thus, apart from the intrinsic interest in

Dedicated to Professor Manfred Eigen on the occasion of his 60th birthday.

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understanding the activation processes of the mast cells, the biochemical and biophysical events which constitute the above cascade are a useful model system for stimulus-secretion coupling mechanisms [1–6]. One inherently early question which is also of fundamental nature is what is the physical definition of the 'signalling unit' of clustered, membrane-residing, IgE-receptor complexes? Another question which has been a subject of active study and active debate is the mechanism(s) underlying the transient rise in cytosolic free $Ca^{2+}$ level observed in these cells following stimulation. In the following, we shall try to outline some of the current insights into these problems. These two aspects of cell activation, i.e., receptor aggregation and transient rise in cytosolic free $Ca^{2+}$ are not unique to mast cells but are found to be characteristic of a wide range of other cell types. Aggregation processes have been reported to be a signalling element with receptors which are distinctly different from those for $Fc_\epsilon$, for instance, insulin [8] or epidermal growth factor receptors [9]. In these latter two cases, however, the divalent nature characteristic of IgE, which is essential for the cross-linking process, is not present. Hence, there is one further question, namely, how is the cross-linking brought about?

A transient increase in cytosolic free $Ca^{2+}$ has been found to be a very widespread event in a diverse range of cells following an initial stimulation signal [10]. The $Ca^{2+}$ may originate from the extracellular medium and enter the cell via ion channels by flowing down the immense concentration gradient existing across the plasma membrane [11]. Alternatively, $Ca^{2+}$ may be released from intracellular depots by second messengers, notably inositol 1,4,5-trisphosphate [12].

## 2. $Fc_\epsilon$ receptor-mediated events

It has briefly been indicated above that a satisfactory physico-chemical definition of the signalling element produced by the $Fc_\epsilon$ receptor in mast cells is still not available. The general qualitative notion that the monovalent receptor, upon being aggregated, provides the required and sufficient message for initiating the biochemical cascade culminating in secretion has been established for some time already. This notion placed IgE in the role of fulfilling two important functions: (1) Recognizing the polyvalent antigen by means of its specific binding sites. (2) Further, because of IgE's divalency in epitope-binding capacity, cross-linking of the monovalent $Fc_\epsilon$ receptor is attained. In order to obtain a more quantitative description of the signalling oligomeric species, covalently cross-linked IgE molecules were prepared and fractionated according to size into dimers, trimers and higher oligomers. The cell-stimulation capacity of these oligomeric reagents was studied. These results led to the suggestion that already dimers can produce a sufficient signal, at least in some types of rodent mast cells and human basophils [13–15]. However, detailed examination of the secretion induced by defined small IgE oligomers from the rat mast cell line (RBL-2H3) showed a rather limited efficacy of dimers which suggested that a more complex mechanism is operative [15]. Moreover, experiments monitoring membranal mobility and distribution of $Fc_\epsilon$ receptors during aggregation, primarily by fluorescence recovery after photobleaching, suggested that the notion of the small oligomer as the triggering element may be oversimplified [16,17]. Thus, evidence that this process may involve intracellular elements, probably, cytoskeletal, also emerged from several other experimental approaches. On the one hand, McConnel and his co-workers [18] have shown that degranulation of RBL-2H3 cells can be caused by presenting RBL cells with freely mobile lipid haptens carried in vesicles. The fluid vesicle membrane cannot provide lateral forces causing IgE clustering. Hence, these authors concluded that clustering is either not a requirement for triggering or is driven by forces derived from the cell [18]. On the other hand, more recent work employing lipid haptens carried on vesicles provided evidence that clustering is indeed driven by cell-derived forces [19,20]. This could certainly provide a rationale for the observations of Holowka et al. [17] showing that cell-bound IgE oligomers coalesce to form significantly larger clusters. Hence, the interpretation of results obtained with the covalent oligomers would depend on a better understanding of the receptor-clustering process pro-

ceeding on the cells' membrane.

One possible avenue of pursuing this question is the systematic and quantitative analysis of IgE-oligomerization reactions induced by well-defined model cross-linking agents. Monoclonal antibodies of the IgE class were reacted with a family of divalent haptens. The latter group of reagents, which provide the simplest antigen models [21], are expected to cross-link the antigen-binding sites of IgE and form either linear, open-chain aggregates or closed rings (mono- or oligomeric ones). In synthesizing such divalent haptens, one can introduce spacers of different length and flexibility, hence imposing geometric constraints on the reaction products with IgE and eventually also on the $Fc_\epsilon$ complexes. The homogeneity of the employed reagents and the structural constraints imposed by use of designed divalent haptens are therefore the informative and significant features of this experimental approach [22].

Relatively short and flexible spacers providing a root mean square distance between the centers of the dinitrophenyl (DNP) rings ($\Gamma$) of about 14–21 Å (e.g., $\alpha,\epsilon$-di(dinitrophenyl)-L-lysine and bis(dinitrophenyl-$\beta$-alanyl)mesodiamino succinate) were reacted in homogeneous aqueous solutions with a monoclonal, DNP-specific IgE. They were found to induce aggregation of the examined IgE, predominantly into closed-ring dimers. The trend toward oligomer formation increases on extending the spacer, yet the ring-closure process becomes less favorable. For example, on increasing the length of the former haptens (e.g., up to $\Gamma \approx 45$ Å in bis($\alpha$-*N*-DNP-tri-D-alanyl) (*N*,*N*′-1,7-diaminoheptane)), one finds that while for the former shorter divalent haptens the equilibrium constant for the aggregation step to form an open dimer, $K_{agg}$, equals $2.6 \times 10^4$ and $9.2 \times 10^4$ $M^{-1}$, respectively, it amounts to $3.4 \times 10^5$ $M^{-1}$ for the latter. The equilibrium constant for the dimeric ring-closure process, which is highest for the short ($\alpha$-,$\epsilon$-di(dinitrophenyl)-L-lysine) hapten and equals 139, decreases to 60 and 26 for the other two divalent haptens with increased length [22].

Longer divalent haptens with rigid oligoproline spacers were synthesized by Dr. I. Lüscher and their interactions with the same monoclonal IgE were also studied. The trend toward oligomer formation seems to be saturating with length, one reason for this probably being the rigidity of the spacer. Also significant is the trend of a decrease in the oligomeric ring-closure reaction. However, with sufficiently long spacers ($\Gamma > 140$ Å) evidence for the intramolecular ring-closure process is emerging [22]. All these observations were, however, made in homogeneous aqueous medium. The implications of such oligomerization processes with respect to events taking place in the two dimensions of a membrane surface have still to be studied.

Examination of the capacity of the divalent haptens discussed above to induce secretion from the line of rat basophils (RBL-2H3) carrying the same DNP-specific IgE have consistently yielded interesting yet disappointing results; namely, that essentially all these divalent haptens are rather poor secretion inducers (B. Reck and I. Pecht, to be published). It should be stressed, however, that all these divalent haptens were found to be effective inhibitors of antigen-induced stimulus. Hence, they are capable of binding to the respective cell-residing IgE molecules and competing with the antigen. One rationale for the very limited triggering capacity of this family of divalent haptens is interesting insofar as it may reflect some steric requirement of $Fc_\epsilon$-receptor aggregation which cannot be met by these cross-linking reagents. It should be recalled that the divalent haptens could form linear oligomers or small closed rings. By contrast, the routinely employed antigen, which is an effective degranulating agent for this RBL-2H3 cell line, carries its epitopes randomly spaced on its surface (the antigen usually employed is a protein multiply derivatized with DNP, e.g., $DNP_8$-bovine serum albumin). Hence, it can easily impose different steric constraints on the $Fc_\epsilon R$ aggregate. However, this is in conflict with the results obtained with haptens carried on lipid vesicles. Thus, interaction between such vesicles carrying a DNP-lipid conjugated hapten and the RBL-2H3 cells carrying DNP-specific IgE caused, as mentioned above, significant secretion [18]. More recent studies with such lipid-hapten-carrying vesicles have shown a marked lateral redistribution of IgE molecules on RBL cell membranes leading to their accumulation at the site of cell-

vesicle contact [20]. These findings suggest quite clearly that the clustering is driven by forces derived from the cells. Still we are left with the need for a better definition of the activating cluster state in terms of size and proximity requirements among $Fc_\epsilon$ receptors essential for a secretion stimulus. It could well be that the limitation is defined not only by oligomer size and its configuration but also by its capacity to interact with other membranal or cytoskeletal elements involved in propagating the signal for secretion. This point may indeed be very pertinent, since all the above results were obtained with one particular cell line. Early studies of the stimulation capacity of divalent haptens performed with polyclonal IgE and rabbit leukocytes clearly showed that divalent haptens do provide an effective simulus to these cells [21].

### 3. Second messengers for an immunological stimulus

The universality of the second messenger role of $Ca^{2+}$ has also been well established for mast cells and basophils [23–25]. It is the source of $Ca^{2+}$ and the possibility of circumventing it by alternative messengers which constitute current research topics.

In mast cells, the dependence of immunologically stimulated release on external calcium was shown to vary markedly with the particular type and source of cells. Thus, the alternative possibility of $Ca^{2+}$ being released from cellular depots such as the endoplasmic reticulum has also to be considered. Receptor-induced phosphoinositide hydrolysis yielding inositol polyphosphates [12], as $Ca^{2+}$ releasing agents from the above pool, could well be an operative alternative or a parallel coupling mechanism. It is the rat basophilic leukemia cell (RBL-2H3 line), which provides a widely used model system for both cellular and biochemical studies of IgE-mediated secretion, which was shown to have an essentially absolute dependence on extracellular $Ca^{2+}$ for this process [24]. Furthermore, the transient rise in cytosolic free $Ca^{2+}$ induced by antigen was shown to be primarily derived from the extracellular medium as demonstrated by meticulous tracer studies and other methods [26] (see also below).

A more general mechanistic scheme that is now emerging would therefore favor the availability of parallel pathways for providing the required increase in cytosolic free $Ca^{2+}$, from cellular pools as well as from external medium. Models trying to exclude one or the other pathway are probably unrealistic for such a complex system as a mammalian cell.

Our studies of the mechanism of antigen-induced $Ca^{2+}$ influx into RBL-2H3 cells have centered on trying to understand the conductance properties of their plasma membranes. Since this influx of extracellular $Ca^{2+}$ is an absolute requirement for antigen-induced release, we investigated the possibility that a $Ca^{2+}$-specific channel opens upon antigen cross-linking of the $Fc_\epsilon$ receptor. The main experimental approach employed micropipette-supported bilayers [27] made either from RBL cells' unfractionated plasma membranes or from proteins isolated from these membranes and incorporated into liposomes of a lipid composition of the appropriate choice. The experiments employing liposomes made from unfractionated cells' plasma membranes indeed showed the induction of ion channels by antigen [28]. More revealing results came from reconstitution experiments in which purified proteins were used. We were able to show [29] that two components isolated from RBL-2H3 plasma membranes are the necessary and sufficient membrane elements for $Fc_\epsilon$R-operated ion channels. These are the $Fc_\epsilon$ receptor isolated as its IgE complex [30] and another membrane protein, most probably the channel-forming component itself. The latter is being isolated by an affinity chromatography procedure based on the anti-allergic asthma drug cromolyn [31]. Hence, it has so far been named the cromolyn-binding protein (CBP). Bilayers containing the $Fc_\epsilon$-receptor complex alone never showed channel activity in response to the triggering agents. However, in membranes containing CBP alone, channel activity can be induced by anti-CBP antibodies. Moreover, in bilayers containing both components channels triggered by antigen binding to $Fc_\epsilon$ receptors and by antibodies to CBP have remarkably similar characteristics of conductance, open

times and closed times, supporting the notion that the same channel entity is induced to open in both cases [29].

More recently (ref. 32 and unpublished results) we have shown that these antigen-induced channels are indeed rather specific for $Ca^{2+}$ against monovalent cations. Micropipette-supported bilayers containing both the $Fc_\epsilon$-IgE receptor complex and CBP were immersed in solutions that had identical composition of monovalent ions (150 mM of either NaCl or KCl) yet had a gradient of $Ca^{2+}$ of over four orders of magnitude (1.8 mM $CaCl_2$ in the well vs. 0.1 $\mu$M $CaCl_2$, buffered with EGTA, in the pipette). Addition of antigen to the well induced opening of channels that could be recorded at different transmembrane potentials. Fig. 1 shows the current-voltage relationships of the channels observed in one experiment of this kind in the presence of 150 mM KCl. About 90% of the detected events (open symbols) had low conductance, in agreement with previous results obtained under different experimental conditions

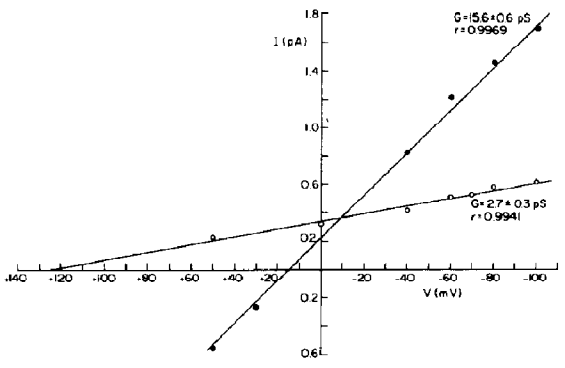


Fig. 1. Current-voltage relationships for antigen-induced channels in the presence of a $Ca^{2+}$ gradient in a micropipette-supported bilayer containing both the $Fc_\epsilon$-IgE receptor complex and CBP. 150 mM KCl, 5 mM Hepes (pH 7.0) in both the well and the micropipette; $CaCl_2$ at 1.8 mM in the well and 0.1 $\mu$M (buffered with EGTA) in the pipette. No channels were observed before addition of antigen. Antigen- ($BSA\text{-}DNP_{16}$) induced opening of channels with two conductance levels. (○) Average current of the low-conductance channel that constituted 90% of the observed events. (●) Average current of the less frequently observed, high-conductance channel. (———) Best fit to experimental points by linear regression. Each potential value was maintained for about 3 min.

[28,29]. The reversal potential for these small channels was 124 mV, practically equal to the Nernst equilibrium potential for $Ca^{2+}$ in this experiment. This result indicates that these low-conductance channels are selective for calcium. Less than 10% of the channel events detected in this experiment (closed symbols in fig. 1) had higher conductance values and a lower selectivity for calcium (reversal potential of 16 mV). The observation that antigen-induced ion channels in reconstituted membranes have two levels of conductance is quite general in our experiments. The question still open is whether in intact RBL-2H3 cells antigen-induced channels have a similar distribution of conductance levels.

Significantly, a receptor-operated, voltage-independent $Ca^{2+}$ channel has very recently been suggested to exist in another rather different cell type. Thus, in the A431 epidermoid cell line, epidermal growth factor was shown to induce $Ca^{2+}$ influx in a mode also suggesting the presence of a potential independent, ligand-activated channel [33].

These results lead us to propose a two-component system enabling $Ca^{2+}$ influx in immunological activation of RBL cells. In this model, the $Fc_\epsilon$ receptor-IgE complex serves as a ligand sensor by its specific binding of antigen, while CBP constitutes a channel (or a fundamental part of it) that allows influx of extracellular calcium. Modulation of this apparently oversimplified channel system would most probably be attained by a manifold of cellular components. Thus, one control would be by inducing changes in the kinetics of opening and closing of the channels. This could be one role of the protein kinase C activated by diacylglycerol, produced upon hydrolysis of phosphatidylinositol bisphosphate [12,34]. Indeed, the antigen-induced cytosolic rise of $[Ca^{2+}]_i$ was shown to be drastically attenuated by adding to RBL-2H3 cells the phorbol ester analogues of diacylglycerol [35,36]. Part of this modulation of $[Ca^{2+}]_i$ could reflect such a control mechanism of the channel gating (see also below).

Our conductance results with reconstituted bilayers clearly indicate that the two membrane proteins we incorporated are sufficient for expression of antigen-induced $Ca^{2+}$ channels. The majority of these channels have relatively low

conductance (2–5 pS, depending on experimental conditions) and dwell in the open state for short periods of time (milliseconds). These results would require aggregation of practically every existing receptor on the plasma membrane in order to allow for the $Ca^{2+}$ fluxes observed with other techniques. However, it is known that maximal stimulation of the cells can be obtained with a limited extent of $Fc_\epsilon R$ aggregation. This apparent difficulty may be resolved by the possible role of other cell components in modulating the channel kinetics. Another alternative is suggested by our observation that in cell-free reconstituted membranes, a small percentage of the detected events have a higher conductance than the value of 2–5 pS regularly observed while retaining part of their selectivity for $Ca^{2+}$. These higher conductance levels may be due to poly-aggregation of receptors, a situation which more probably prevails in intact cells than in an artificial membrane which contains a small number of active receptor components. Further research is clearly needed to resolve this question.

Recently, it has been reported that antigen-induced degranulation of rat mast cells may proceed without observing $Ca^{2+}$ channel opening [37] and that degranulation, as assessed by microscopical observation of morphological cell membrane and capacitance changes, has no dependence on extracellular calcium [38]. These observations may indicate that in rat peritoneal mast cells, in contrast to RBL cells, mouse mast cells and human basophils, the contribution of released intracellular calcium suffices to induce degranulation. An alternative explanation may reside in the rather small conductance changes that, according to our results, would be expected to occur upon antigen stimulation (a 100–200 pS increase in whole-cell conductance). Conductance changes of this order of magnitude were indeed also observed in the above-mentioned experiments on rat mast cells [37]. It is also important to take into consideration that at least in RBL cells, extensive morphological changes in membrane architecture (ruffling) take place upon antigen binding to the receptor. These changes were shown to occur also in the absence of extracellular calcium and do not indicate cell degranulation. Clearly, further analysis of both the response of rat peritoneal mast cells to antigen as well as of whole-cell conductance measurements on the RBL-2H3 is necessary before concluding that these cells are indeed different from other mast cells with respect to their calcium requirements.

## 4. Alternatives to $Ca^{2+}$ as second messenger?

The antigen-induced transient rise in cytosolic free $Ca^{2+}$ was shown to be swiftly and effectively attenuated in several ways. One rather simple and interesting procedure is the abrogation of the IgE-antigen aggregates. This can be achieved by adding an appropriate excess of monovalent hapten [25,36] which causes a commensurate decrease in amplitude of the transient rise in $[Ca^{2+}]_i$. Another experimental protocol found to decrease both antigen (fig. 2) or even an ionophore-induced rise in $[Ca^{2+}]_i$ is the addition of the tumor-promoting phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) [35,36]. This is a diacylglycerol analog known to activate protein kinase C [34]. This kinase most probably acts on the cytosolic calcium level via a complex manifold of cell components. Monitoring the $[Ca^{2+}]_i$ with quin2 we have found that TPA exerts its effect probably by both blocking the $Ca^{2+}$ influx into the cytosol and accelerating its clearance (fig. 2) [35,36].

A rather significant difference between the two above-described protocols employed for attenuation of the calcium signal is that while in the first, addition of increasing concentrations of monovalent hapten causes proportional inhibition of secretion [36], the second gives rise to both enhancement at low doses and only partial inhibition even at high doses of TPA. Thus, using an optimal dose of antigen one can use TPA to effect complete suppression of the rise in $[Ca^{2+}]_i$ and yet still allow a substantial extent of mediator release to be observed [36]. These findings prompted us to search for alternative second messengers which could provide coupling between the membrane IgE-provided signal and other cell components which propagate the degranulation process. One of the first cellular targets identified as being affected by kinase C is the $Na^+/H^+$

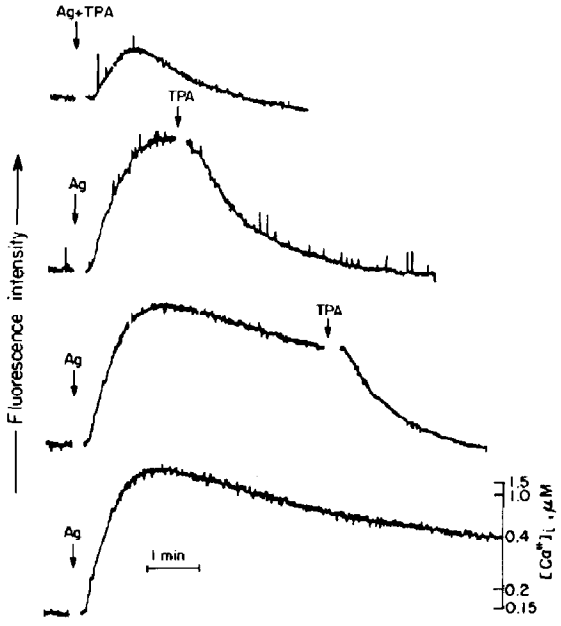


Fig. 2. Effect of TPA on antigen-induced rise in $[Ca^{2+}]_i$ of RBL-2H3 cells as monitored by quin2 emission at 492 nm (excitation at 339 nm). 81 nM TPA was added at different times relative to antigen (Ag) addition (5 ng BSA $DNP_8$/ml) to a stirred cell suspension ($10^6$ cells/ml tyrode buffer, pH 7.4) thermostatted at 37° C. In the experiment without TPA (lowest trace) the scale for $[Ca^{2+}]_i$ is shown calculated by a calibration procedure which uses $E_{max}$ for total quin2 concentration (after cell lysis with 0.05% Triton X-100) and $E_{min}$ (after addition of $Mn^{2+}$ to 0.5 mM to the lysed cells, cf. ref 25).

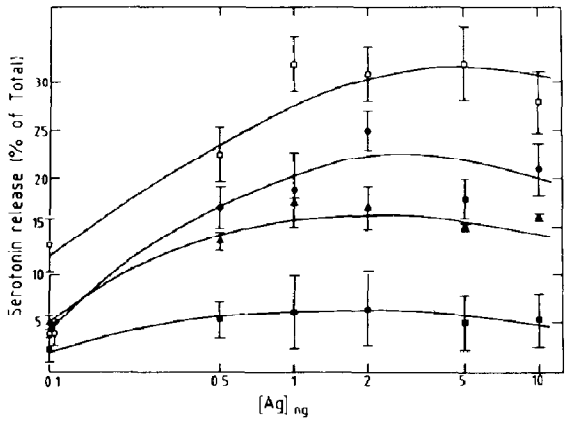


Fig. 3. Antigen-induced serotonin release from RBL-2H3 cells as a function of different $Na^+$ concentrations in the medium: 10 mM (■), 25 mM (▲), 100 mM (●) and 140 mM (○). Released secretion is presented as a percentage of total cell content. Cells were incubated overnight with 5 μCi/ml 5-[$^3$H]hydroxytryptamine creatinine sulfate (11 Ci/mmol, Radiochemical Centre, Amersham). During the following morning, cells were suspended, washed and after counting and adjusting their density, resuspended ($1\times10^6$ cells/ml) in Tyrode medium containing 137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Hepes and 5.6 mM glucose (pH 7.4) and an appropriate amount of monoclonal, DNP-specific IgE antibody. After incubation for 90 min to allow binding of IgE, cells were washed again with Tyrode buffer and degranulation was assayed in a total volume of 150 μl cell suspension aliquots, each in a 96-well flat-bottomed microtiter plate (Nunclon) containing $10^5$ cells and the indicated reagents per well. After 35 min incubation at 37° C, cells were spun down and 100-μl aliquots of the supernatants counted. The total radiolabeled 5-hydroxytryptamine incorporated into cells was determined by dissolving cells in 50 μl of 2 N NaOH and counting the radioactivity in the whole sample.

exchanger [39]. This membrane protein provides a control element for cytosolic pH and $Na^+$ concentrations [40]. In thymocytes, the activity of the $Na^+/H^+$ exchanger was shown to undergo a marked shift in pH dependence upon kinase C activation [39]. This and other results led us to examine the possible mutual relationship between cytosolic pH, $Na^+$ and $Ca^{2+}$ in the context of the stimulus-secretion coupling process.

The dependence of antigen-induced secretion on $Na^+$ concentration in the medium of the RBL-2H3 cells is illustrated in fig. 3. Evidently, secretion requires $Na^+$ in the medium and is completely blocked in its absence. Several other different experimental protocols which affect the cytosolic $Na^+/H^+$ balance were therefore employed and their correlation with the extent of mediator secretion was studied. First, the effects of antigen and of TPA on cytosolic pH of RBL-2H3 cells were examined by monitoring the emission of the intracellular fluorescent pH indicator 2′,7-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF) (fig. 4) [41]. No change could be resolved throughout the range of TPA concentrations employed (5–80 nM). In contrast, addition of specific antigen caused a small but consistent acidification of the RBL-2H3 cytosol. This could be promptly reversed upon addition of TPA, supporting the idea that the antigen-induced acidification is indeed a controlled cellular event and not an artifact

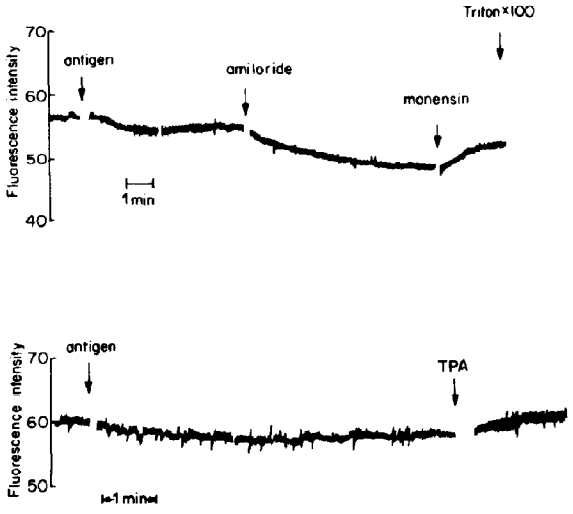


Fig. 4. Traces of the emission intensity of the cytosolic pH indicator BCECF monitored at 526 nm ($\lambda_{ex}$ = 505 nm) demonstrating intracellular pH changes of RBL-2H3 cells. (Upper trace) Cells were triggered by subsequent addition of antigen (5 ng BSA $DNP_{15}/10^6$ cells per ml), amiloride (final concentration 100 $\mu$M) and monensin (final concentration 0.5 $\mu$M). (Lower trace) Cells were triggered with antigen (1 ng/$10^6$ cells per ml) and subsequent addition of TPA. For further details see the legend to fig. 5.

due to secretion of granular acidic components. We are currently continuing our investigation of this process.

An effective though artificial procedure available for examination of the role of $Na^+/H^+$ balance in these cells' function involves the use of ionophores. The $Na^+/H^+$ exchanging ionophore monensin was shown to induce effectively noncytotoxic serotonin release. It causes release of more than 40% of these cells' serotonin content at 1 $\mu$M, with a half-maximal dose of 0.2 $\mu$M at pH 7.4. This release was found to be independent of extracellular calcium, since an essentially identical dose-response curve is obtained in the presence of 3 mM EGTA. The monensin-induced release is, however, dependent on both pH and $Na^+$ concentration of the extracellular medium. Monensin-induced serotonin release markedly increases with raising of the external pH and is suppressed by lowering the external $Na^+$ concentrations. To investigate possible cytolytic serotonin release, the extent of released cytosolic enzyme – lactate dehydrogenase (LDH) – activity in supernatants of the cells was measured. RBL-2H3 cells treated with monensin at up to 5 $\mu$M were found to have LDH activity in their supernatants similar to that of the

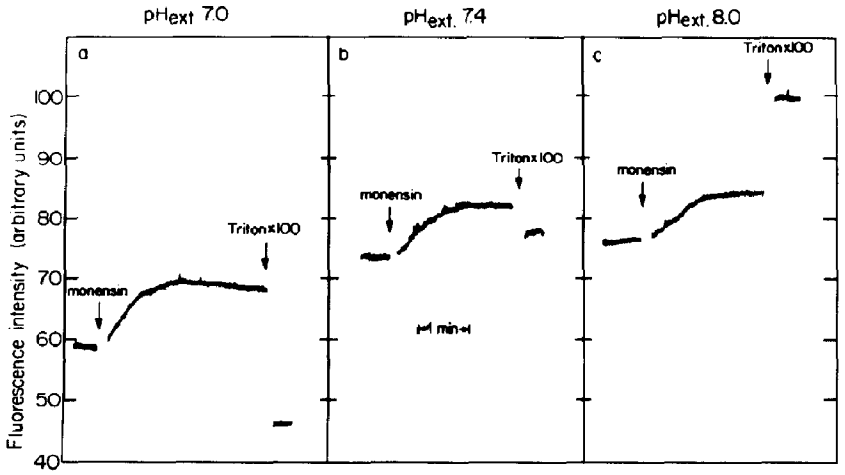


Fig. 5. Changes in cytosolic pH of RBL-2H3 cells effected by the addition of monensin (1 $\mu$M) and monitored by the emission of the fluorescent indicator BCECF ($\lambda_{ex}$ = 505 nm, $\lambda_{em}$ = 526 nm). Cells were incubated with the acetoxy methyl ester of BCECF (1 $\mu$M) to allow its uptake and intracellular hydrolysis (37 °C, 1 h). After washing off excess indicator, they were resuspended at a density of $10^6$ cells/ml in a stirred and thermostatted cuvette in Tyrode buffers adjusted to the three pH values indicated.

control cells' supernatants which were not exposed to this ionophore or that were triggered by antigen.

The change in $pH_i$ caused by addition of monensin to cells is illustrated in fig. 5 which shows traces of the fluorescence intensity of the trapped pH indicator BCECF in cells suspended in buffer at different pH values. The alkalinization brought about by monensin and its dependence on extracellular pH are evident. In separate experiments, the concomitant changes induced by monensin in $[Ca^{2+}]_i$ were monitored via the emission intensity of quin2. A transient rise in $[Ca^{2+}]_i$ is also observed as a result of monensin addition. However, when external free calcium is practically eliminated by excess EGTA (3 mM), no significant rise in $[Ca^{2+}]_i$ can be resolved yet the alkalinization is maintained.

Several different ionophores known to transport preferentially $Na^+$ were then examined for their capacity to cause serotonin release from RBL-2H3 cells. Significantly, however, none of these four ionophores employed: 4-crown-12; gramicidin; eniatin B and beauvericin cause any release. The most probable rationale for this behavior lies in the $Na^+/H^+$ exchange capacity of monensin. The other ionophores examined would carry $Na^+$ but do not undergo protonation [42]. Hence, they cannot effect the $Na^+/H^+$ exchange reaction. This observation was significant in as much as it suggested that monensin-induced serotonin release depends on an $Na^+/H^+$ exchange process and not on just the cells' $Na^+$ concentration only.

In order to resolve further whether the observed monensin-induced serotonin release is distinct from antigen-induced mediator release we examined, in parallel experiments, both the activity of β-hexoseaminidase, a granule-stored enzyme secreted into the cells' medium, and the amounts of [$^3$H]serotonin released. The interesting outcome of these experiments was that only as a result of antigen stimulation did we observe the concurrent presence, in RBL-2H3 supernatants, of both the macromolecular enzyme, β-hexoseaminidase and serotonin. Monensin consistently failed in releasing β-hexoseaminidase activity in the cells' medium. Thus, serotonin is most probably released by monensin through a different mechanism. We assume that monensin acts by a non-physiological process allowing efflux of the low-molecular-weight mediator serotonin while maintaining unperturbed the granule-stored macromolecular ones, such as β-hexoseaminidase. Monensin also penetrates into the cells' internal compartments and, being capable of causing $Na^+/H^+$ exchange, perturbs the balance of these ions across the granular membrane as well. This would lead to a change in protonation state of stored amines such as serotonin and facilitate their release by diffusion as the uncharged species. This mechanism is supported by essentially all the experimental observations described above: dependence on extracellular pH and $[Na^+]$ and independence from extracellular $[Ca^{2+}]_i$, the effect being limited to the $Na^+/H^+$ ionophore monensin.

The above-described ionophore-induced effect made us return to the physiological process of antigen-induced degranulation and investigate whether specific modulators of cellular $Na^+$ do indeed affect it. Amiloride and its analogs are well-established inhibitors of the cellular $Na^+/H^+$ antiporter [43]. We have determined whether it affects the antigen-induced release. As shown in table 1, it causes pronounced inhibition of secretion. Control experiments showed that amiloride alone, even at the highest concentration employed (100 μM), did not cause any significant change of the basal level of release (≈ 3%). The inhibition exhibited some dependence on the period of

Table 1

Inhibition by amiloride of antigen-induced serotonin release from RBL-2H3 cells

Release by 1.0 ng antigen per $10^5$ cells (150 μl total volume) was 60% of the serotonin taken up by the cells. All results are averages of triplicate experiments.

| [Amiloride] (μM) | Percent inhibition of release | |
|---|---|---|
| | 5 min preincubation | 10 min preincubation |
| 1 | 7.6 | 3.5 |
| 2 | 7.6 | 17.5 |
| 5 | 15.3 | 26.3 |
| 10 | 24.6 | 26.3 |
| 20 | 38.4 | 36.8 |
| 50 | 40.0 | 38.5 |
| 100 | 56.9 | 43.8 |

incubation of cells with amiloride. Clearly, however, it is an effective inhibitor, especially at the higher end of the reported concentration range for its inhibition of $Na^+/H^+$ activity in other cell types [43].

A different control of intracellular $Na^+$ concentration is attained by the $(Na^+ + K^+)$-ATPase pump [44]. A potent inhibitor of this enzyme is ouabain. In contrast to the inhibitory effect on secretion found for amiloride, application of ouabain to RBL-2H3 cells caused already by itself a significant amount of secretion, e.g., about 20% of the cells' serotonin contents were secreted by treatment with 1.0 mM ouabain.

In conclusion, antigen stimulation of mast cells of the type exemplified by the RBL-2H3 line causes several transient changes in cytosolic ion composition: the transient rise in free calcium shown to couple stimulus to function in a wider range of cells is also observed in this type of mast cell. The calcium signal is dramatically suppressed by TPA, most probably because activation of protein kinase C causes blockage of $Ca^{2+}$ influx and its enhanced extrusion. In pursuing the possibility that the $Na^+/H^+$ exchanger of RBL-2H3 cells is activated by this kinase and serves as an alternative coupling mechanism following TPA treatment, we observed that antigen stimulation by itself causes a slight decrease in $pH_i$ of these cells. However, TPA does not induce any change. Nevertheless, the sensitivity of antigen-induced degranulation to changes in cytosol $Na^+/H^+$ was demonstrated by the inhibition exerted with amiloride and the secretion enhanced by ouabain. In this context it is of interest that the $Na^+/H^+$ ionophore monensin, which causes release of low-molecular-weight mediators such as serotonin, does not induce real degranulation. However, it should also be noted that opposite changes in $pH_i$ are caused by antigen and monensin: The former induces acidification, the latter causing alkalinization. Hence, one can exclude the possibility that the monensin-induced increase in $pH_i$ per se is a cause of degranulation and it now remains to be critically examined whether acidification has any messenger role. Thus, the mechanism by which antigen-stimulated secretion is partly maintained although the calcium signal is eliminated by TPA is still unresolved and requires further study.

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