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Characterization of the ion channel activity in planar bilayers containing IgE–Fc_ϵ receptor and the cromolyn-binding protein

A.Corcia, R.Schweitzer-Stenner[1], I.Pecht[1] and B.Rivnay

Departments of Membrane Research, and [1]Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel 76100

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Electric conductance was studied across micropipette-supported planar lipid bilayers, reconstituted with IgE–Fc_ϵ receptor and the cromolyn-binding protein (CBP) isolated from membranes of rat basophilic leukemia cells (RBL-2H3). Currents were observed following the addition of aggregating agents, specific for either of the two proteins. The results show that the two proteins are necessary and sufficient for the opening of cation channels. Both aggregation of Fc_ϵ receptor via IgE with a specific antigen and of CBP by anti-CBP induce channels with similar conductances and open-time distributions. In the presence of 1.8 mM calcium, the most frequently observed channels have a conductance of 1–2 pS. At 100 mM calcium conductance increased to 4–5 pS. Channels induced by antigen were susceptible to blocking by the anti-allergic drug cromolyn. These results suggest that CBP acts as the core of the cation channel and that the channel conductance and open-time characteristics are independent of the mode of aggregation.

Key words: basophils/mast cells/RBL-2H3 cells/stimulus-secretion coupling/calcium influx

Introduction

The initial event in the immunologically mediated triggering of secretion from normal or transformed mast cells and basophils is microaggregation of the Fc_ϵ receptor ($Fc_\epsilon R$)–IgE complexes by polyvalent antigens (Ishizaka and Ishizaka, 1984). One of the biochemical events following aggregation is a transient rise in membrane permeability to Ca^{2+} ions (Foreman *et al.*, 1973). Recent studies using the fluorescent Ca^{2+}-indicator Quin-II showed a fast transient increase in intracellular (presumably cytosolic) free Ca^{2+} concentrations (Beaven *et al.*, 1984; Sagi-Eisenberg and Pecht, 1984). This transient increase may result either from calcium influx or from intracellular sources or a combination of both. Evidence derived from $^{45}Ca^{2+}$ flux measurements (Crews *et al.*, 1981; Rivnay, unpublished results) suggests that the transient increase is predominantly due to an influx of extracellular calcium.

The anti-allergic drug cromolyn [1,3-bis(-2-carboxychromon-5-yloxy)-2-hydroxypropane] blocks both $^{45}Ca^{2+}$ influx into rat peritoneal mast cells or basophilic leukemia (RBL) cells and their subsequent degranulation (Mazurek *et al.*, 1983; Pecht and Sagi-Eisenberg, 1985). Furthermore, the membrane component binding this drug (cromolyn-binding protein, CBP) has been isolated by several procedures based on its affinity for cromolyn (Mazurek *et al.*, 1982; Hemmerich *et al.*, in preparation). The functional role of CBP has been examined, both in the intact cell and in model membranes: variants defective in cromolyn binding have been isolated from RBL-2H3 cells. These were shown to be non-responsive to IgE-mediated stimulus, although IgE binding was fully attained. Subsequent to implantation of the isolated CBP into these variants, using Sendai virus envelopes as carriers, their capacity to allow Ca^{2+} influx and to degranulate upon IgE–$Fc_\epsilon R$ aggregation has been restored (Mazurek *et al.*, 1983). Studies of reconstitution of the CBP into model phospholipid bilayers (Mazurek *et al.*, 1984; Pecht *et al.*, 1985) revealed the following features. (i) Cation fluxes display characteristics of a channel mechanism. (ii) This activity is apparently contained within the CBP since aggregation with anti-CBP antibodies could induce channel activity in membranes reconstituted with CBP alone. (iii) All the necessary components linking IgE-mediated aggregation with channel opening are found in isolated RBL plasma membrane and can be reconstituted functionally into planar lipid bilayers. (iv) Channel opening is specific, as it is only induced by ligands that aggregate the Fc_ϵ receptor or the CBP.

These findings set the stage for a more detailed examination of the channel activity. Thus, the quantitative characterization of the channel activity in terms of its amplitude and open-time was initiated. Furthermore, a comparison of these characteristics as affected by the mode of channel activation (i.e. antigen or anti-CBP) has also been attempted.

Here we show that, in model membranes co-reconstituted with the two isolated membrane elements, CBP and the IgE–$Fc_\epsilon R$ complex, ion channels open upon aggregating either element. These results corroborate the notion that the fast increase in intracellular free Ca^{2+} reflects a net influx of this ion. This most probably occurs by aggregation of the CBP via its presumed interaction with the Fc_ϵ receptor. In view of the similarity between the conductance and open-time parameters of anti-CBP- and antigen-induced channel activity, we also suggest that the mechanism of channel formation is similar in both cases. This further supports the assignment of the channel core unit to the CBP.

Results

Channel activity was studied by measuring electric currents across membranes reconstituted at the tip of a micropipette. These membranes contained the purified IgE–$Fc_\epsilon R$ complex and the CBP. Channel activity was induced by specific aggregating agents. Recent studies (Corcia and Babila, 1985) have shown that an electric potential across a lipid bilayer can cause reversible breakdown of the lipid matrix which appears as 'channel-like' conductance in the absence of any specific channel-forming proteins. Such currents were also observed in the present study, and the threshold voltage for such events increased almost linearly with the resistance of the membrane up to a value of 20 GOhm. To establish the validity of the subsequent results as specific, protein-mediated and ligand-activated channels, the following protocol was adopted: (i) only membranes with a resistance higher than 15 GOhm were used; (ii) once a membrane was formed at the tip of the micropipette by the procedure described in Materials and methods, it was clamped at increasing transmembrane potential. Each potential was maintained for a few seconds and if no channel activity was observed during this interval, a

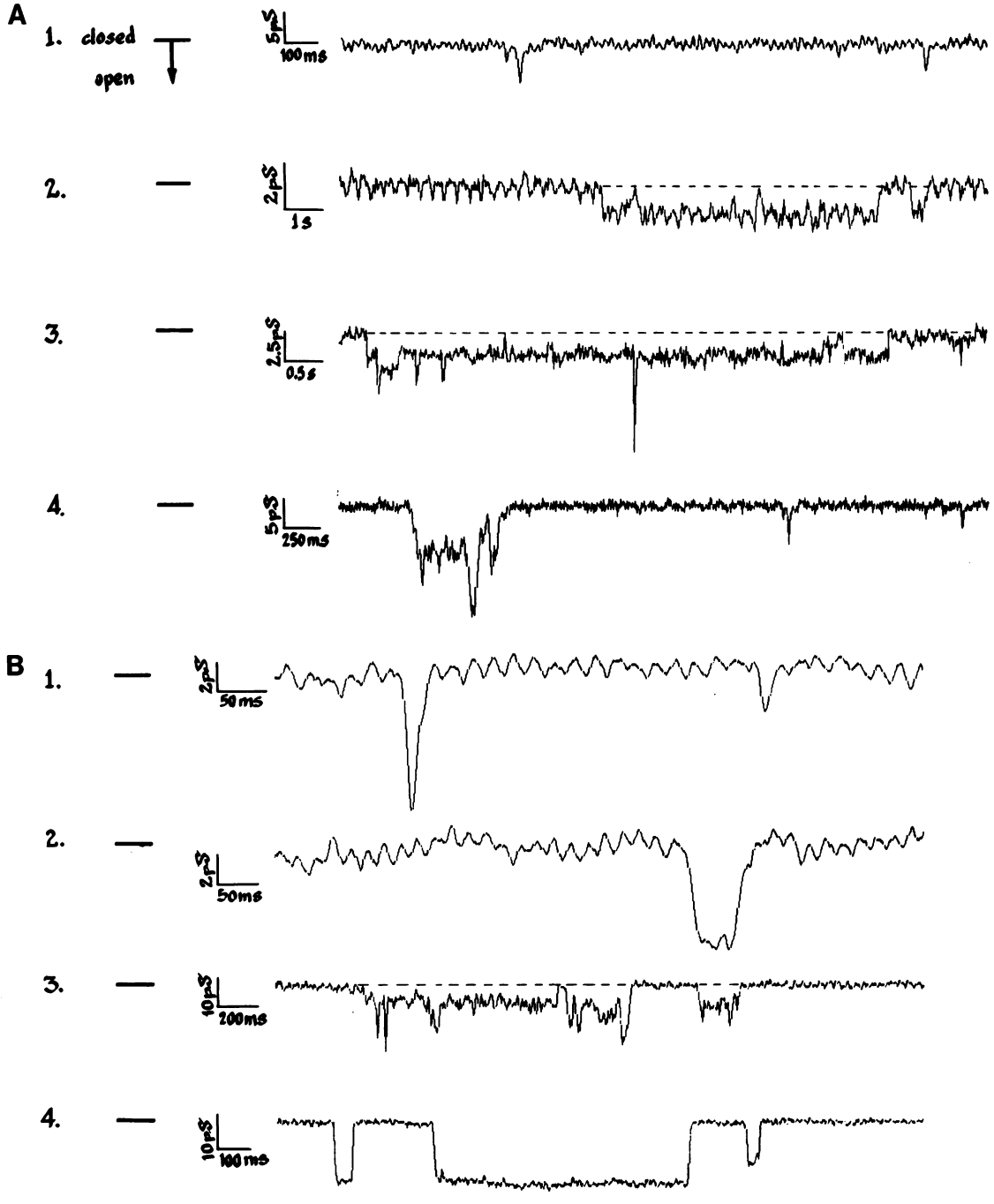

Fig. 1. Channel conductances induced in pipette-supported lipid membranes containing both the IgE–$Fc_\epsilon R$ and CBP. Channel opening appears as a downward deflection in the current trace. The bar at the left of each trace indicates the closed state level. **(A)** Channel activity induced by specific antigen (DNP_{16}–BSA). The transmembrane potential was clamped at 40 mV (pipette interior negative) in **traces 1, 2** and **4** and at 60 mV in **trace 3**. Data were low-pass filtered at 100 Hz in **traces 1** and **4** and at 50 Hz in **traces 2** and **3** and digitized at a sampling frequency of 3 kHz. **(B)** Channels induced by anti-CBP antibodies. Clamp potential was 100 mV in **traces 1, 3** and **4** and 40 mV in **trace 2**. Data were low-pass filtered at 100 Hz in **traces 1, 2** and **3** and at 300 Hz in **trace 4** and digitized at 3 kHz.

higher clamp-voltage was applied. Once channel-like activity appeared, the clamp potential was decreased by at least 50 mV. The actual experiment was then carried out at this new potential, well below the breakdown value. After several minutes of recording stable current values, specific triggering agents were added to the well and only channel activity observed after this addition was considered as specific to the triggering agent. It should be noted that in all these experiments continuous mixing was avoided.

Experiments in the presence of low calcium concentration (1.8 mM)

Purified Fc_ϵ receptors alone do not yield channel activity. Experiments in which the pipette-supported membrane contained only the IgE–$Fc_\epsilon R$ complex, i.e. the vesicles employed did not contain the CBP, were carried out in the presence of buffer solution containing 1.8 mM calcium. No channel activity could be detected upon aggregating the IgE–$Fc_\epsilon R$ complexes. This was the

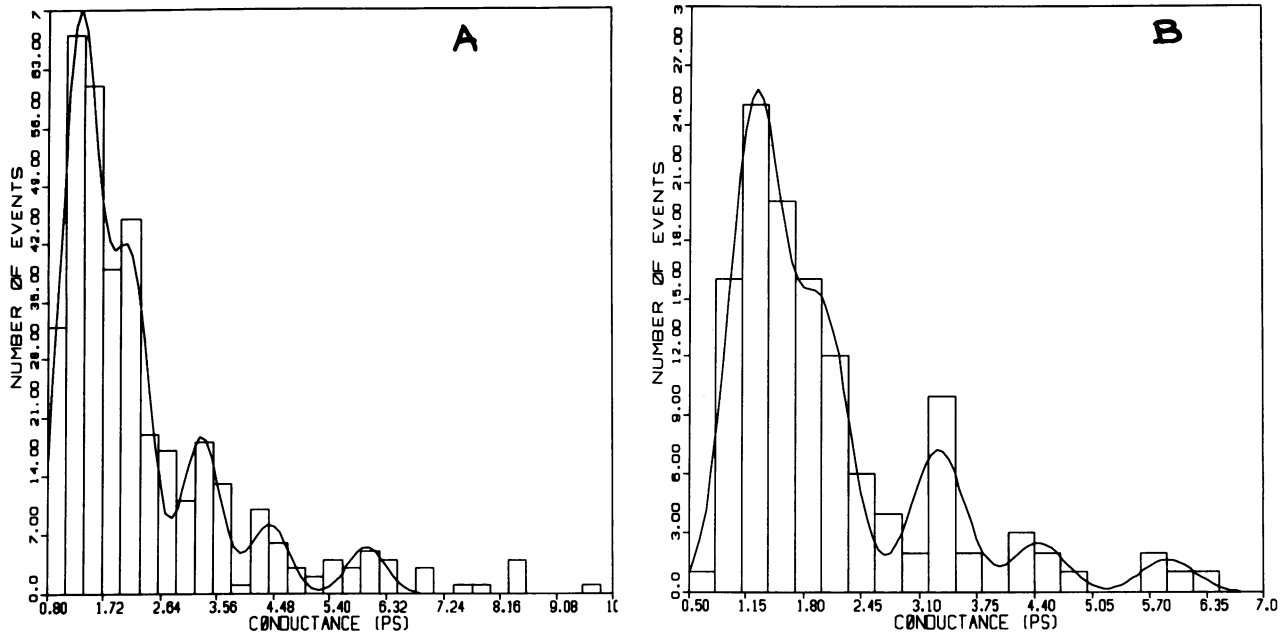

Fig. 2. Conductance histograms constructed from single channel events in the presence of 1.8 mM calcium. **(A)** induced by antigen ($DNP_{16}-BSA$), **(B)** induced by anti-CBP. Bin width 0.3 pS. Total number of events is 397 in **A** and 171 in **B**.

case irrespective of whether specific antigen or anti-IgE antibodies were used to induce aggregation. Eight separate experiments of this type were carried out. In each of these, current was recorded for a minimum of 20 min (and up to 45 min) after adding the aggregating agent. No detectable channel activity with a minimum conductance of 1 pS was observed.

Antigen-activated channels. Typical channel activity appears after adding specific antigen [$DNP_{16}-BSA$ (produced by derivatizing bovine serum albumin by fluorodinitrobenzene, yielding an average of 16 residues of 2,4-dinitrophenyl), 5 nM] to a membrane containing both the IgE$-$Fc$_\epsilon$R complex and the CBP. Four different types of events were distinguished. (i) The most frequently observed channels are of low conductance and they remain open for time periods in the range 5$-$20 ms. Trace 1 of Figure 1A shows a 2 s recording containing three such channels, detected by the screening program. Due to the filtering required to detect these small channels, the precise conductance values are difficult to determine with certainty. It will be shown below that the most frequently observed channels have a conductance between 1 and 2 pS. (ii) In a few cases these small channels remain open for significantly longer periods, in the range of seconds (Figure 1A, trace 2). (iii) Higher conductance levels, as high as 10 pS, sometimes appear superimposed on the basic low conductance of the long-lived channels (Figure 1A, trace 3). (iv) The last type, comprising ~10% of all the channels observed after addition of specific antigen, are characterized by high values of conductance, ranging between 10 and 30 pS. The higher conductance levels appear either isolated or as bursts of activity with several different conductance levels, as illustrated in Figure 1A, trace 4.

Anti-CBP activated channels. To compare quantitatively the properties of channels activated by antigen with those activated by monoclonal anti-CBP antibodies, the latter were added to membranes containing both proteins. Experiments of this kind resulted in channel activity very similar to that induced by specific antigens. Figure 1B shows several traces from such experiments. Trace 1 (Figure 1B) contains two short-lived channels (open-times of several ms), the first one with a conductance of 4.5 pS and

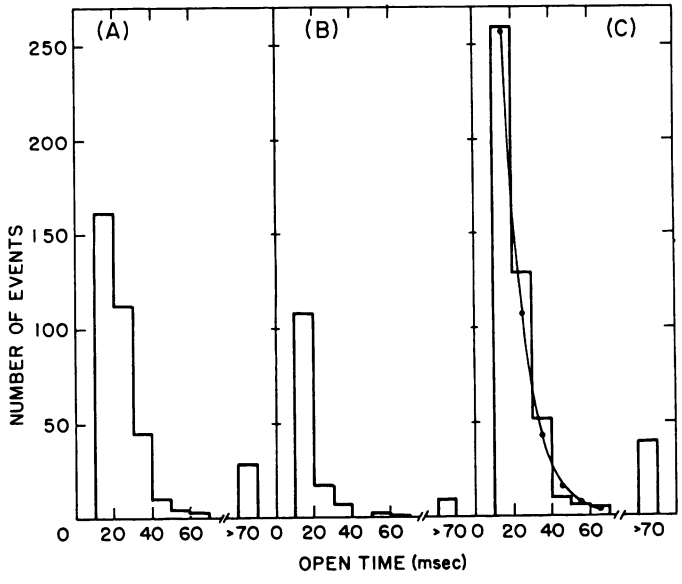

Fig. 3. Open-time histograms constructed from single channel events in the presence of 1.8 mM calcium. **(A)** induced by antigen, **(B)** induced by anti-CBP, **(C)** all results pooled together. The number of events in the histograms in **panels A** and **B** is the same as in Figure 2. The curve superimposed on the histogram of **panel C** represents the best fitting to one exponential function.

the second 1.5 pS. The channels in this trace appear distorted due to the effect of filtering on such short-lived channels. Trace 2 shows a channel of 3.5 pS with longer open-time (60 ms). Like the antigen-induced channels, these low conductance and short-lived channels induced by anti-CBP appear scattered, with intervals of up to several hundred ms. Trace 3 presents an example of a small channel with a long open-time. Several closing and re-opening levels, as well as superimposed higher conductance levels, are illustrated. In addition, trace 4 shows several channels with relatively high conductance (10$-$15 pS) and variable open-times.

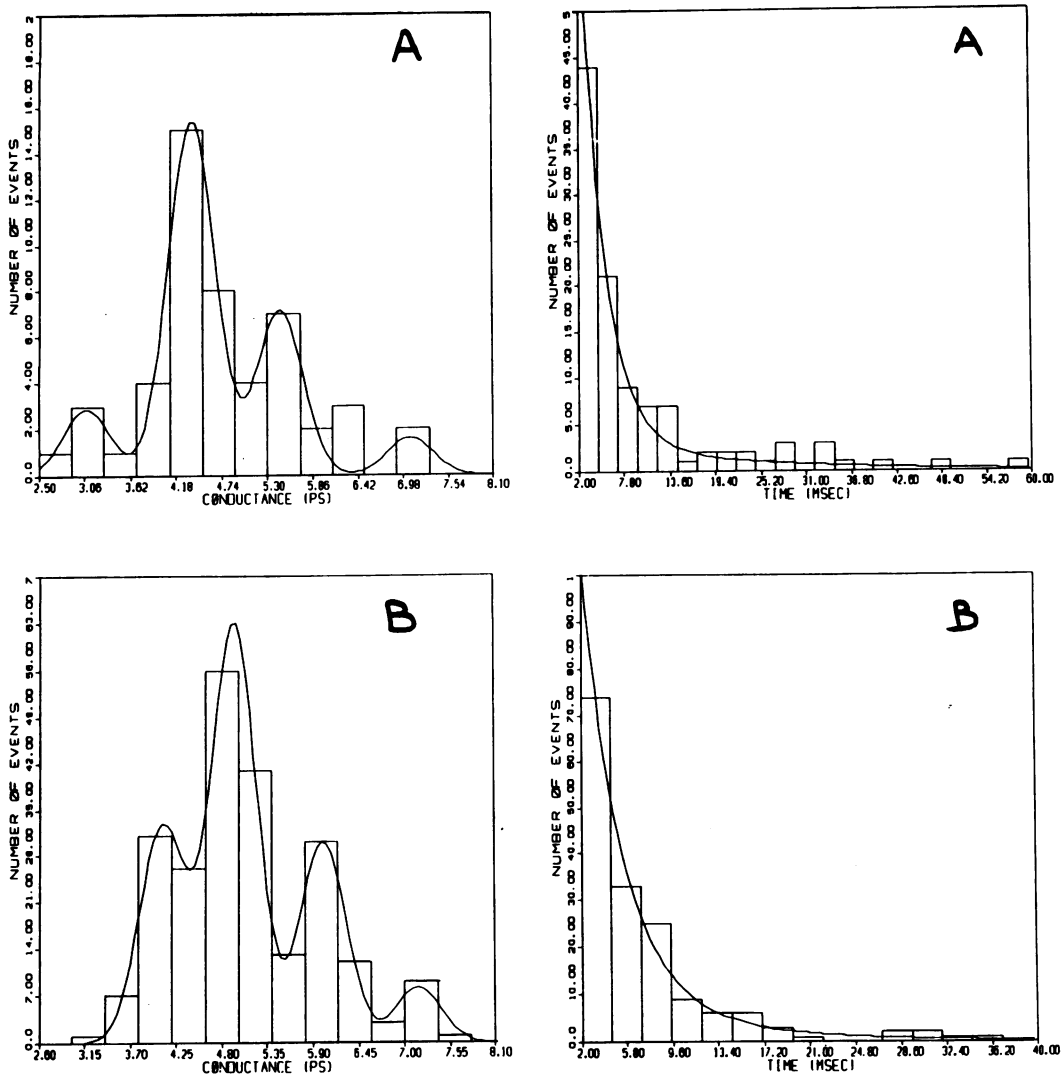

Fig. 4. Conductance and open-time histograms constructed from single channel events in the presence of 100 mM calcium. **(A)** induced by antigen, **(B)** induced by anti-CBP. Bin widths are 0.4 pS and 2.5 ms, respectively. Records filtered at 500 Hz. Applied potential was 80 mV in **A** and 50 mV in **B**, pipette interior negative.

Sensitivity to cromolyn. All four different types of channel activity induced by antigen disappeared after adding cromolyn (final concentration 100 μM) to the bath. The complete blocking effect of cromolyn is usually attained in <5 min. In contrast, the activity induced by anti-CBP antibodies was not blocked by cromolyn. Channel activity was still observable 20 min after adding up to 1 mM cromolyn. In all the experiments the drug was added to already activated channels.

Statistical analysis. The above data indicate that the system reconstituted with both IgE$-$Fc$_\epsilon$R and CBP exhibits channel activity upon aggregation of either one of the two components and thus confirm earlier observations obtained with Teflon-supported planar bilayers (Mazurek *et al.*, 1984, 1986; Pecht *et al.*, 1985). The properties of channels obtained by either of the two different modes of activation were analyzed statistically and compared. This required the accumulation and processing of a statistically significant number of events. We have analyzed the characteristics of 568 separate single channel events, 397 of which were detected in experiments using antigen and the rest with anti-CBP monoclonal antibodies.

Single channel amplitudes. Histograms of channel conductances (Figure 2) were constructed from data obtained either by antigen-mediated aggregation (A) or by anti-CBP-mediated aggregation (B). Although each of the experiments was carried out at a certain potential in the 40$-$100 mV range, the conductance values were similar in all experiments. In this analysis only conductance values of single channel events are included, namely burst behavior with different conductance levels as illustrated in Figure 1A, trace 3; trace 4 and Figure 1B, trace 3 were not included. Under our experimental conditions the confidence limit for detection of conductances was 0.8 pS. The histograms were fitted to Gaussian distributions, shown superimposed in the figure. In both panels, the main proportion of detected events (70%) have conductances in the range 1$-$2 pS. The number of channels with larger conductances decreases drastically and only a few events have been detected with conductances of 7 pS or more. Both histograms show at least three additional conductance peaks (3.3, 4.4 and 6.0 pS, respectively) that can be detected with the fitting procedure.

Single channel open-times. Figure 3 shows open-time histograms for the channels recorded in experiments using antigen (A), or anti-CBP (B) as agonists and for data of both types of experiments pooled together (C). Values <10 ms, although detectable, were not included in these histograms since the distortion induced by

filtration, particularly in channels of very short open-times, render them less reliable. This limitation imposed by the necessity to filter the data in order to resolve channels with small amplitudes, introduces an uncertainty in the calculated values of the average open-times discussed below. With this reservation in mind, the marked similarity of the open-time histograms shown in the panels of Figure 3 is evident, regardless of the channel triggering mode. In both cases the channels tend to open for short periods and only a small fraction of the events have open-times of 70 ms or more. The probability of finding a long-lived event is higher in the experiments using antigen as a trigger than in those using anti-CBP.

The best fitting of the experimental data to a single exponential curve is shown superimposed on the histogram in panel C of Figure 3, for those events with open-times up to 70 ms. The fitting of each histogram has been performed by linear regression to a logarithmic function. In such a way, the slope of the best linear fit is the reciprocal of the average open-time of the distribution. The three sets of data in Figure 3 (antigen-induced events, anti-CBP-induced events and both pooled together), present good linear correlations with coefficients of 0.982, 0.972 and 0.982, respectively. Moreover, the three regression lines are practically parallel, yielding average open-times of 11.1, 11.6 and 11.0 ms, respectively. This is a good indication that the channels analyzed, irrespective of whether triggered by antigen or by anti-CBP, have the same open-time characteristics. However, since the records of these experiments have been heavily filtered before the analysis and events with short open-times have been discarded, these time constants must, at this stage, be considered as indicative, non-conclusive values.

Experiments in the presence of high calcium concentration (100 mM)

The filtering of the electrical signal, required for obtaining a reasonable signal-to-noise ratio in the above experiments performed at 1.8 mM calcium, weakens the claim that both antigen and anti-CBP trigger similar channel activity. Hence, in order to increase resolution, experiments were carried out at a calcium concentration of 100 mM in both bath and pipette (see Materials and methods). Figure 4 shows conductance and open-time histograms derived from two such experiments in which channel activity was induced either by antigen (panels A) or by anti-CBP (panels B). The better signal-to-noise ratio in these experiments allowed the signal to be filtered at 500 Hz before analyzing and to detect events of up to 2 ms duration. As in the previous histograms, only single channel events are included in these histograms.

Both conductance histograms show four peaks corresponding to four different conductance levels. The most frequent amplitude value is in the range 4−5 pS, rather than the 1−2 pS range observed in the presence of 1.8 mM calcium. The values of the four conductance levels are quite similar for the two triggering modes and reinforce the earlier claim that we are dealing with the same channel despite the difference in the mode of triggering. The open-time histograms are also quite similar and can be fitted to the sum of two exponential curves. The time constants are practically identical for the two modes of activation: 3.0 and 20.9 ms for antigen and 3.4 and 20.8 ms for anti-CBP.

Discussion

One main aim of the present study was to substantiate the evidence that the CBP is a functional calcium channel involved in the cascade of events coupling the IgE−receptor aggregation to histamine release from mast cells (Pecht *et al.*, 1985). Hence, a sufficient number of single channel events was accumulated enabling a statistical analysis of both the channel conductance and its characteristic open-times, under different triggering conditions. Another objective was to begin examining the specificity of the reconstituted channel events in terms of activating and blocking agents.

The present data confirm that the isolated IgE−Fc$_\epsilon$R complex and CBP are both necessary and sufficient for antigen-induced channel opening, and that the IgE−Fc$_\epsilon$R complex by itself is insufficient (Pecht *et al.*, 1985; Mazurek *et al.*, 1986). The statistical analysis of channel conductances shows that in the presence of 1.8 mM calcium, its maximum is in the range of 1−2 pS, a value similar to the 2 pS reported earlier (Mazurek *et al.*, 1984, 1986). However, the analysis reveals the existence of at least three further conductance levels appearing with much lower frequency. Also, in the presence of 100 mM calcium, single channel events appear with four different conductance levels. The most frequent level under these conditions has a value of 4−5 pS. These results support the notion that the CBP is the channel-forming protein and that the actual opening mechanism of this channel (not of the overall aggregation) is very similar in both cases: antigen-induced channel through receptor aggregation and anti-CBP-induced channel through CBP aggregation. The origin and significance of the different levels observed in the single channel conductance is under investigation. They may be due to different states of protein aggregation induced by the polyvalent triggering agents used, e.g. a decavalent IgM anti-CBP and a randomly derivatized DNP$_{16}$−BSA.

That both antigen and anti-CBP trigger essentially the same channel activity is further supported by the similarity in the open-time distributions of single channel events. Under both experimental conditions employed in this study, open-time histograms have similar shapes and similar distribution parameters. The fact that the open-times of events observed in the presence of low calcium can be fitted by one exponential (independent of the triggering agent) while those observed in the presence of high calcium must be fitted by the sum of two exponentials, may be due to two reasons: the limit of experimental resolution of our measurements in the first case may mask the existence of a second exponential with a time constant below 10 ms; alternatively, the presence of high calcium concentration in the second case may induce the appearance of a blocked state of the channel that was not observable with low calcium. It is clear from our results that, under any given experimental conditions, the channel events induced by antigen are statistically indistinguishable from the events induced by anti-CBP.

We are exploring systematically the parameters that control the kinetic characteristics of the channels. Whether the difference in open-time distribution (the fact that relatively long events appear more frequently in the presence of antigen than in the presence of anti-CBP) reflects intramolecular structural differences or is a result of different intermolecular organization requires a detailed stoichiometric examination. Another question under investigation is the ion selectivity of these channels and the interplay between calcium and other physiologically relevant cations. The two exponential functions needed in order to fit the open-time histograms of events detected at high calcium suggests that the process is more complicated than a first order reaction with one closed and one open state. An appropriate kinetic scheme needs also to be elucidated.

The effects of the anti-allergic drug cromolyn are informative in several ways. First, the fact that cromolyn blocks antigen-

induced channel activity provides evidence that the proteins involved in channel formation maintain their specific response to the drug throughout the experimental procedure. Second, since the inhibition by cromolyn has so far only been examined after introduction of the aggregating agent, the lack of susceptibility of the anti-CBP-induced channel activity to cromolyn may be helpful in approaching the structure–function analysis of the CBP. It may indicate that cromolyn binds to the very same epitope recognized by the antibody or to a site which is sterically or conformationally affected by it.

Materials and methods

Reagents

Mouse monoclonal 2,4-dinitrophenyl (DNP_ϵ–26)-specific antibodies (IgE class) secreted by hybridoma cells (HI-DNP-e-26.82) (Liu *et al.*, 1980) were isolated as detailed elsewhere (Holowka and Metzger, 1982). Iodination (^{125}I) of the IgE was performed for quantitation as indicated earlier (Rivnay and Metzger, 1982), yielding routinely a specific activity of $1-10 \times 10^5$ c.p.m./μg. 3-[3(cholamidopropyl)- dimethylammonio]-1-propanesulfonate (CHAPS), cholesterol, egg phosphatidylcholine and soybean lecithin (Asolectin, Type IV-S) were all purchased from Sigma Co. (St. Louis, MO). Asolectin was further processed as described by Kagawa and Racker (1971). Crude lipid extract from rat basophilic leukemia cells (RBL- 2H3) was prepared as described previously (Rivnay and Metzger, 1982).

Channel opening was induced by either of two different reagents (DNP_{16}–BSA) or an IgM class monoclonal anti-CBP (supernatant of cultured hybridoma, line 25/94) (Mazurek, 1983). Cromolyn was a gift from Fisons Pharmaceutical Division Ltd (Loughborough, UK).

Experiments were performed in isosmotic, buffered solutions containing either 145 mM NaCl, 1.8 mM $CaCl_2$ and 10 mM Hepes, or 100 mM $CaCl_2$ and 10 mM Hepes, pH 7.6. The buffer solutions were filtered through a 0.45 μm filter (Millipore) prior to use. Salts and solvents were all of analytical grade.

Isolation of IgE–Fc_ϵR complexes and CBP

The IgE–Fc_ϵR has been purified by two steps of affinity chromatography. Throughout the procedure the buffer contained 2 mM phospholipids from RBL and 10 mM CHAPS (Rivnay *et al.*, 1984). Receptors thus obtained are >80% intact as assessed by sucrose gradient analysis following liposomes incorporation (Rivnay and Metzger, 1982).

The CBP was isolated under non-denaturing conditions from RBL-2H3 cells grown in culture, essentially as described elsewhere (Mazurek *et al.*, 1982). The main modifications are in the preparation of the cromolyn conjugates of macromolecular carrier. The earlier procedure yielded low covalent derivatization. Hence an alternative derivative of the 5-hydroxy chromone-2-carboxylate was prepared. All CBP preparations were analyzed by SDS-electrophoresis (Mazurek, 1983). The gels were stained with silver nitrate (Mazurek *et al.*, 1982). All preparations showed bands at 68, 47 and 24 kd. The yields of the active components are still variable and improvement of the preparative procedure is in progress.

Preparation of lipid–protein vesicles

2 mg of a mixture of carrier lipids (soybean lecithin, egg phosphatidylcholine and cholesterol in weight ratio 8:11:1) from a solution of 3.5 mg/ml lipids in chloroform/methanol 2:1 were dried, first under a flow of nitrogen and then under vacuum overnight, to remove the solvents. The dry lipid film was resuspended in a volume of 770 μl of one of the buffer solutions described above. This also contained 50 μg of RBL-2H3 phospholipids, 15 nM of IgE–Fc_ϵR complex and, when appropriate, CBP. This mixture was dialyzed for at least 4 h before adding it to the lipid layer. The suspension was sonicated for 30 s. After sonication, 75 μl of this suspension were added to a plastic well (6 mm diameter, Microtiter plates, Nunclone) containing 100 μl of the buffer alone, to give a final concentration of ~7 nM for each protein.

Bilayer formation

Glass micropipettes were pulled by the two-step method (Hamill *et al.*, 1981) from borosilicate glass capillary tubing (o.d. 1.5 mm; i.d. 0.86 mm; A-M Systems, Everett, WA) in a vertical pipette puller (Model 700C, David Kopf Inst., Tujunga, CA) and backfilled with the same buffer used to resuspend the lipid film. These micropipettes had tip diameters between 0.8 and 1.5 μm and tip resistances from 10 to 50 MOhm when immersed in the buffer solution. No polishing or coating of the pipettes was necessary.

The method for lipid bilayer formation at the tip of micropipettes is a variation of a previously published procedure (Coronado and Latorre, 1983). Briefly, a micropipette was introduced into 100 μl of the buffer solution contained in a plastic well maintained at room temperature (22 ± 2°C). The resistance of the pipette was monitored continuously by applying pulses of 100 μV. 75 μl of the solution containing the lipid–protein vesicles were then added to the well and enough time allowed (normally 1–2 min, but up to 20 min) for a lipid monolayer to form at the air–liquid interface). At this stage, the pipette was pulled out of the solution and slowly reintroduced with the help of a three-dimensional hydraulic micromanipulator (Model MO-103, Narishiga, Japan). Formation of a bilayer was monitored by measuring the changes in the micropipette resistance with an EPC-5 patch-clamp system (List Electronics, FRG).

Once a bilayer was formed, it was clamped at different potentials with the patch-clamp system. The current necessary to clamp the transmembrane voltage was low-pass filtered at 1 kHz by the clamp system, monitored with an oscilloscope and recorded on FM tape (Hewlett Packard, Model 3694A).

Analysis of data

The experimental data obtained were analyzed off-line. The data were replayed from the tape recorder, filtered if necessary with a variable low-pass filter (Model 3202, Krohn-Hite, MA) and digitized by a PC-compatible microcomputer equipped with a 12-bit analog-to-digital conversion interface (Model DT2801A, Data Translation, MA). A program written in compiled BASIC scanned the digitized data and detected changes in current in excess of a given threshold value (usually four times the standard deviation of the baseline values), plotted hardcopies of the stretches with channel activity and, with operator feedback, determined the values of the amplitude and open-times of the detected channels.

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