Focal exocytosis by eosinophils—compound exocytosis and cumulative fusion

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We have investigated the granule fusion events during exocytosis in horse eosinophils by time-resolved patch—clamp capacitance measurements. Stimulation with intracellular GTPγS leads to a stepwise capacitance increase by 4.0 ± 0.9 pF. At GTPγS concentrations <20 μM the step size distribution is in agreement with the granule size distribution in resting cells. Above 80 μM the number of steps is reduced and very large steps occur. The total capacitance increase, however, is unaffected. These results show that at high GTPγS concentrations granule–granule fusion occurs inside the cell forming large compound granules, which then fuse with the plasma membrane (compound exocytosis). The electrical equivalent circuit of the cell during degranulation indicates the formation of a degranulation sac by cumulative fusion events. Fusion of the first granule with the plasma membrane induces fusion of further granules with this granule directing the release of all the granular material to the first fusion pore. The physiological function of eosinophils is the killing of parasites. Compound exocytosis and cumulative fusion enable the cells to focus the release of cytotoxic proteins to well defined target regions and prevent uncontrolled diffusion of this material, which would damage intact host cells.

Key words: capacitance/compound exocytosis/eosinophil/GTP/patch—clamp

Introduction

Eosinophils play a major role in the defence against parasitic infections (Spry, 1988). The sequence of events in killing of helminths involves adherence of the eosinophil to the parasite followed by the discharge of the cytotoxic granule contents directly onto the surface of the worm (McLaren et al., 1977; Glauert et al., 1978). This occurs by an exocytotic mechanism involving fusion of the granules with the plasma membrane (Henderson et al., 1983; Nüsse et al., 1990). To ensure effective targeting of this material the release should be directed to a narrow region. The formation of degranulation sacs connected to the extracellular space through a single fusion pore (Tai and Spry, 1981; Henderson et al., 1983) could provide such a mechanism.

The formation of such structures may occur by cumulative fusion of granules with the membrane of granules previously fused with the plasma membrane (Rohlich et al., 1971; Chandler et al., 1983; Alvarez de Toledo and Fernandez, 1990). However, it has been suggested that in eosinophils and basophils, several granules may fuse among themselves upon stimulation and before the compound granule fuses with the plasma membrane (compound exocytosis) (McLaren et al., 1977; Glauert et al., 1978; Dvorak et al., 1981; Tai and Spry, 1981).

In the experiments described here we have used eosinophils from horse blood. These have particularly large granules (Henderson et al., 1983), which makes them most suitable for biophysical studies. The cells were stimulated by intracellular application of the non-hydrolysable GTP analogue GTPγS using a patch pipette with access to the cytosol. Measuring the passive electrical properties of single cells revealed that eosinophils use both mechanisms to perform focal release. Intracellular granule–granule fusion leads to the formation of compound granules and the final fusion of individual or compound granules with the plasma membrane can occur in a cumulative way such that a large degranulation sac develops directing all the secretory material to the first fusion pore.

Results

Intracellular GTPγS stimulates exocytosis

Resting horse eosinophils have an initial capacitance \( C_i = 3.0 \pm 0.5 \) pF (SD, \( n = 89 \)). When the cells were internally dialysed with a solution containing 20 μM GTPγS the cell capacitance increased by 4.0 ± 0.9 pF (SD, \( n = 89 \)) representing an increase by a factor of 2.4 ± 0.2 pF. Assuming a specific capacitance of 1 μF/cm² the measured capacitance change corresponds to an increase of plasma membrane area from 300 to 700 μm², with an insertion of 400 μm² granule membrane.

The average initial capacitance, \( C_i \), and the total capacitance increase, \( \Delta C \), varied among individual preparations obtained from different animals from \( C_i = 2.6 \pm 0.3 \) pF (SD, \( n = 10 \)) with \( \Delta C = 3.6 \pm 0.7 \) pF to \( C_i = 3.6 \pm 0.2 \) pF (SD, \( n = 6 \)) with \( \Delta C = 5.3 \pm 0.9 \) pF. The relative capacitance increase (2.4- ± 0.2-fold) was much less variable than the absolute increase, indicating that larger cells generate a larger capacitance increase. Without GTPγS the membrane capacitance did not change.

During the measurement, degranulation could also be observed as a morphological change using Nomarski optics. Individual granules are clearly visible in resting eosinophils (Figure 1A). During degranulation the granules disappear and the cells become larger than resting cells (Figure 1B).

The delay preceding exocytosis is very variable (2–7 min), as is the time-course of the actual degranulation [<1 min (Figure 1C) or as much as 15 min]. The extent of the degranulation is independent of the GTPγS concentration in the range of 5–160 μM, indicating that even with 5 μM GTPγS exocytosis is complete and occurs in an all or none mode.

The fusion of a secretory granule with the plasma membrane is a discrete event associated with a single step
increase of the capacitance. The delay is thus the time between the initial penetration of the cell that allows GTP\textsubscript{y}S to diffuse into the cytoplasm and the first capacitance step. The duration of the degranulation process is taken as the time between the first and the last step indicating exocytosis of the first and last granule. Whereas the delay is independent of the GTP\textsubscript{y}S concentration (Figure 2, hatched bars), the duration diminishes as the GTP\textsubscript{y}S concentration increases (Figure 2, open bars).

**Compound exocytosis**

In cells where compound exocytosis occurs, fewer but larger capacitance steps would be expected in comparison with cells where the individual granules fuse sequentially with the plasma membrane. Following intracellular application of 20 \(\mu\text{M}\) GTP\textsubscript{y}S, stepwise capacitance changes of 50–200 \(\mu\text{F}\) can be seen (Figure 3A, left, upper trace). At 80 \(\mu\text{M}\) GTP\textsubscript{y}S much larger steps were observed in addition (Figure 3A, right, upper trace). The corresponding step size distributions (Figure 3B) were obtained by measuring the size of several hundred capacitance steps with each concentration. With increasing GTP\textsubscript{y}S concentrations, the large steps make a greater contribution to the total capacitance change. At 5 \(\mu\text{M}\) GTP\textsubscript{y}S 75\% of the total capacitance increase is due to steps of \(<100\ \mu\text{F}\), whereas at 80 \(\mu\text{M}\), the small steps contribute only 20\%, the remainder being generated by large steps of up to 2 \(\mu\text{F}\) indicating fusion of large compounds. At 160 \(\mu\text{M}\) the entire degranulation of a cell may even occur in one single fusion event indicated by a single step of \(~4\ \mu\text{F}\).

The concentration-dependent change in the duration of the degranulation (Figure 2) was analysed by measuring the time intervals between two subsequent fusion events. The frequency distributions of these time intervals decay exponentially with similar time constants at 10 \(\mu\text{M}\) GTP\textsubscript{y}S (5.6 s) and 160 \(\mu\text{M}\) GTP\textsubscript{y}S (5.0 s) (Figure 3C), indicating that the frequency of fusions with the plasma membrane does not strongly depend on the GTP\textsubscript{y}S concentration. This stands in contrast to the frequency of granule–granule fusion. As a result, at the higher concentrations of the guanine nucleotide each fusion event with the plasma membrane contributes a larger increase in capacitance.

The time resolution at which individual steps could be separated was \(~100\ \text{ms}\) since the fusion pore opening was usually faster than 100 ms. The probability that two subsequent fusion events are erroneously taken as one is thus \(<2\%\) and this would only lead to steps having twice the size of single granules and this contribution would be the same for all GTP\textsubscript{y}S concentrations.

For the above analysis we have only used those recordings where the capacitance steps could be clearly identified and where the change in the conductance trace was less than half of the change in the capacitance trace (Figure 3A). Under these conditions the error of the phase setting was small and the error of the capacitance measurement was always \(<10\%\).

However, in many cells some capacitance steps were accompanied by large changes in the conductance trace (Figure 4). These events cannot be explained by a pure change in membrane capacitance. At certain times during the degranulation the properties of the cell were apparently not well described by a simple equivalent circuit consisting of membrane capacitance \(C\), membrane conductance \(G\) and access resistance \(R_A\) (Figure 5A).

**Cumulative fusion forms degranulation sacs**

Since it is not possible to analyse more complex changes in the equivalent circuit with a lock-in amplifier operating at a single frequency, we measured the current transients evoked by \(-20\ \text{mV}\) pulses. To determine the time-course of the degranulation process a pulse was given every 2 s. If we assume that the cell and pipette are electrically represented by \(C\), \(G\) and \(R_A\) (Figure 5A), then the values of these quantities can be obtained by fits of the current responses using a single exponential plus a baseline (Lindau and Neher, 1988). The apparent time-course of the capacitance increase obtained with this method is shown in Figure 5B and contains two very large steps. The mean deviation \(\sigma\) between the fit and the measured current for each
Fig. 3. (A) (upper traces) Capacitance steps measured with a lock-in amplifier in a cell stimulated with 20 μM GTPγS (left panel) and with 80 μM (right panel). The lower traces show the ac conductance changes as indicated by the other output of the lock-in amplifier at the same scale. (B) Step size distributions measured at different GTPγS concentrations as indicated. (C) Frequency distributions of the time intervals between capacitance steps at 10 and 160 μM GTPγS. All data were obtained with the lock-in amplifier technique.

pulse is shown as the lower trace in Figure 5B. It can be seen that the single exponential fits are good only at the beginning and the end of the degranulation. σ is markedly higher than the noise level of ~20 pA throughout most of this experiment. The quality of the fits is exemplified for the three pulses labelled 1, 5 and 7 in Figure 5C. The pulse
labelled 5 cannot be described by a single exponential. Clearly the electrical representation in Figure 5A cannot explain the measured currents. A 2- to 6-fold increase of $\sigma$ was observed in 49 out of 94 cells.

So far our analysis neglects the possibility that after fusion, granules could be electrically connected to the outside through narrow fusion pores having a large electrical resistance (Figure 6A) (Breckenridge and Almers, 1987; Spruce et al., 1990). This would introduce an additional slow relaxation time constant into the current signal evoked by a voltage step. Indeed, a sum of two exponentials plus a constant fits the pulse labelled 5 perfectly well (Figure 6B).

Using the equivalent circuit of Figure 6A we obtain $C_C = 3.6 \text{pF}$, $C_G = 4.9 \text{pF}$ and $G_p = 19 \text{nS}$ for this pulse, which describes the connection of a degranulation sac with $490 \mu\text{m}^2$ membrane area to the extracellular space via a fusion pore with $19 \text{nS}$ conductance. The rest of the cell membrane has increased by only $40 \mu\text{m}^2$. More than 90% of the additional membrane capacitance is contained in the slow component of the current response.

Figure 6C shows that the total capacitance $C_C + C_G$ (top trace), which reflects the sum of the cell membrane and fused granule membranes, increases without any unusually large steps. The fits and the current signals are all superimposable and the mean deviation $\sigma$ is accordingly reduced to the noise level throughout the recording (Figure 6C). During the entire degranulation the model circuit of Figure 6A describes the properties of the cell very well.

Figure 6D provides a schematic illustration of the major events discerned by the analysis. Interestingly the area of the cell membrane remains almost constant ($320-360 \mu\text{m}^2$) for at least 8 min (Figure 6C, $C_C$ trace). However, ~2 min after the beginning of the recording a granule capacitance appears ($C_G$ trace) indicating fusion of the first granule. At label 2, a vesicle with $\sim 80 \mu\text{m}^2$ membrane area is connected to the extracellular space via a fusion pore with a conductance of $13 \text{nS}$. During the period between labels 2 and 3 the membrane of the fused vesicle increases to $\sim 300 \mu\text{m}^2$, while the fusion pore conductance remains constant indicating the development of a degranulation sac. Between labels 3 and 4 the fusion pore expands rapidly and its conductance increases to $28 \text{nS}$. Between labels 4 and 5 more granules fuse with the degranulation sac and its membrane area increases to $\sim 500 \mu\text{m}^2$. Between labels 5 and 6 the pore becomes very large attaining a conductance of $75 \text{nS}$

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**Fig. 4.** Capacitance (top) and conductance (bottom) traces measured with lock-in amplifier are shown on the same scale. Correlated step changes in both traces are indicated (arrows). At the asterisks the phase was adjusted and verified by phase tracking. The transient increase of $R_A$ by 1 M$\Omega$ generates no artefact in the capacitance trace.

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**Fig. 5.** A. The minimal equivalent circuit of the patch-clamp whole-cell configuration consists of access resistance $R_A$, membrane conductance $G$ and membrane capacitance $C$. $R_A$, $G$ and $C$ were obtained by fitting the current transients evoked by voltage pulses with a single exponential. B. (upper trace) Apparent time-course of $C$; (lower trace) the mean deviation $\sigma$ between the current signals and the fits. C. Three individual current signals together with the single exponential fits. The pulses correspond to the time-points labelled 1, 5 and 7 in panel B. Whereas the signals 1 and 7 are rather well described by a single exponential, signal 5 is clearly nonexponential.
and then continues to expand until the two membrane domains cannot accurately be distinguished any more on the basis of electrical time constants. It should be noted that the large ‘capacitance steps’ appearing in Figure 5B do not indicate fusion events. They were due to sudden increases in the conductance of the fusion pore making the granule membrane more accessible.

**Individual granule – plasma membrane fusion events cannot explain the observed changes**

It may be argued that a large slow component in the current response could also be due to many individual granules of similar size each being connected to the extracellular space by a fusion pore of similar conductance. However, this possibility can be excluded when the changes of the individual parameters during the degranulation process are considered. During the 24 s between pulses 5 and 6 (Figure 6B), a large change occurs simultaneously in the amplitude and time constant of the slow component, while the integral of the current transient, and thus the total capacitance, remains almost unchanged. With the model of Figure 6A this can be fully explained by a conductance increase of one single fusion pore (Gp) to 75 nS (Figure 6C, bottom trace). If the slow component was due to the sum of many granules individually fused with the plasma membrane, then the conductance of all the individual fusion pores must have simultaneously increased by a factor of four, which is extremely unlikely. The same considerations apply for the 2-fold increase of pore conductance between labels 3 and 4. Furthermore, the individual fusion of more and more granules with the plasma membrane would generate an increase of the amplitude of the slow component of the current transients while degranulation proceeds. In contrast, about half of the capacitance increase occurs while the amplitude of the slow component remains constant (between labels 2 and 3 in Figure 6C). During this period the capacitance of the degranulation sac increases from 0.8 to 2.9 pF while the pore conductance is constant. These independent changes of Cg and Gp confirm that there is indeed only one major degranulation sac and this grows due to cumulative fusion of subsequent granules.

The question arises why, in about half of the cells, the currents show only small deviations from a single exponential. The reason could be either that in these cells the granules fuse individually with the plasma membrane or that the conductance of the connection between the degranulation sac and the exterior is comparable with or larger than the conductance of the pipette—cell connection. When we tried double exponential fits on cells where the single exponential showed only small deviations from the current, two exponentials with similar time constants were obtained that were not very well separated. The analysis with the equivalent circuit of Figure 6A thus led to rather noisy traces for the equivalent circuit elements. However, in these cells a developing degranulation sac could still be detected, the main difference being a larger pore conductance.

**Discussion**

**GTPγS stimulates degranulation and granule – granule fusion**

We show here that introduction of GTPγS into single horse eosinophils induces a 2- to 3-fold capacitance increase indicating a corresponding expansion of the plasma membrane area. This increase is associated with visible degranulation and the disappearance of the granules confirming an exocytotic mechanism.
The variability of the initial plasma membrane area of the cells is characteristic of the individual animals. It does not reflect different activated states of the cells, since we observed no correlation between the initial or final capacitance and the percentage of eosinophils among the granulocytes. The cells having a larger initial capacitance are not partially degranulated since partially degranulated cells would generate a smaller capacitance increase during exocytosis of the remaining granules than undegranulated cells. In contrast to this expectation, the cells having a larger initial capacitance actually generate the same relative capacitance increase indicating that larger cells contain more and/or larger granules.

Electron microscopic investigations have led to the suggestion that eosinophil granules may discharge their contents into large vacuoles before final release (McLaren et al., 1977; Glauer et al., 1978; Tai and Spry, 1981; Henderson et al., 1983) and the formation of degranulation sacs has previously been observed in guinea-pig basophils (Dvorak et al., 1981). If individual granules fused one by one with the plasma membrane, the capacitance increase should comprise a series of step changes, each step reflecting a single fusion event. If, on the other hand, many granules fuse with each other to form large compounds, which then fuse with the plasma membrane, the capacitance should increase in a few large steps (compound exocytosis), while the total increase would be the same. When we stimulated the cells with intracellular GTP\(_\gamma\)S, the step size distributions were found to depend on the GTP\(_\gamma\)S concentration. It was recently shown that stimulation of guinea-pig peritoneal eosinophils or human blood eosinophils with 20 \(\mu\)M GTP\(_\gamma\)S leads to a step size distribution which is in very good agreement with the granule size distribution in resting cells (Lindau et al., 1993; Hartmann et al., in preparation). The step size distributions that we obtained for horse cells stimulated with GTP\(_\gamma\)S between 5 and 20 \(\mu\)M are very similar to each other. The corresponding granule diameter distribution has a peak at 1.4 \(\mu\)m and a half width of 0.7 \(\mu\)m, which agrees very well with granule sizes seen in electron micrographs (Henderson et al., 1983). At low GTP\(_\gamma\)S concentrations the individual granules are thus exocytosed sequentially one by one.

When the GTP\(_\gamma\)S concentration is elevated to \(\geq 80 \, \mu\)M, then the step size distribution changes markedly. Although the total capacitance increase remains the same, now only a small percentage occurs in normal steps. Under these conditions granule—granule fusion occurs inside the cell before the compound granule fuses with the plasma membrane, as indicated by very large capacitance steps. On occasion this may even lead to release of all the granular material in one single fusion event.

Granule—granule fusion has been stimulated with very high calcium concentrations between isolated cortical granules from sea urchin eggs (Vogel and Zimmerberg, 1992) and fusion of chromaffin granules was obtained in the presence of high calcium, annexin and arachidonic acid (Drust and Creutz, 1988). Here we have shown that G protein-mediated fusion can occur between secretory granules and not only between secretory granules and plasma membrane. GTP-binding proteins have been implicated in regulated exocytosis (Gomperts, 1990) and in many steps of vesicle traffic and fusion events (Balch, 1990) including a ‘like—like’ fusion between early endosomes (Gorvel et al., 1991).

Our results indicate that the rate of fusion events between granules and the plasma membrane is independent of the GTP\(_\gamma\)S concentration between 5 and 160 \(\mu\)M. However, the frequency of fusions among granules leading to the formation of larger compounds increases with increasing GTP\(_\gamma\)S concentration. The GTP\(_\gamma\)S sensitivity of granule—granule fusion is thus different from that of granule—plasma membrane fusion indicating the possibility that two different GTP-binding proteins are involved.

**Cumulative fusion directs release to a focal site**

We found that even the sequential fusion of individual granules is not a random process occurring everywhere at the plasma membrane. Our results indicate that after the first granule fused with the plasma membrane, the next granule fuses with the membrane of the first and this is followed by the fusion of all subsequent granules with the membrane of previously fused granules. This leads to the cumulative formation of a degranulation sac and the focal release of all the granular material at the site of the first fusion event. There is thus an increased probability for cytoplasmic granules to fuse with the membrane of the fused granule. In mast cells an increased fusion probability between fused and unfused granules has recently been suggested to lead to multigranular compounds at the periphery of the cell (Alvarez de Toledano and Fernandez, 1990) and was the explanation for the cooperativity of fusion among cortical granules from sea urchin eggs (Vogel and Zimmerberg, 1992). The mechanism responsible for this phenomenon is not yet clear. After opening of the fusion pore the granule membrane potential may change or the membrane tension in consequence of a possible change in granular volume by solubilization of the granular matrix. Alternatively, exposure of the intragranular membrane surface to a different environment could induce changes in transmembrane proteins involved in the fusion process. Whatever the underlying processes is, the promotion of fusion by fusion provides a mechanism allowing for directed exocytosis.

The main physiological function of eosinophils is to destroy large extracellular targets. The adherence of the eosinophils to Ig-coated parasites is followed by the release of granule contents onto the intact surface (McLaren et al., 1977; Glaert et al., 1978). Material released in the wrong direction is not only ineffective but may also damage host tissue. In mast cells, stimulation with agonist-coated beads induces local degranulation (Lawson et al., 1978). The stimulation of eosinophils also occurs locally, in the area of contact with an Ig-coated surface (Glaert et al., 1978). An increased probability of granule fusion with the granules fused at the stimulation site leads to release of all the secretory material onto the target. The combination of compound exocytosis and cumulative exocytosis provides a safe, rapid, effective and flexible mechanism to perform focal release.

**Materials and methods**

**Cell preparation**

Eosinophils were isolated from 50 ml fresh blood from the jugular vein of horses and purified by centrifugation over discontinuous Percoll gradients.
During spontaneous sedimentation of the red cells in tubes with small diameter, the leucocyte containing plasma was rapidly collected and washed twice in Hanks’ solution containing 30 mg/l DNase (Biochrom) at pH 7.2 by centrifugation at 4°C for 10 min at 300 g. The total number of leucocytes and percentage of eosinophils was then determined using Kimura stain. The cell density was adjusted to 10−20 × 106 per ml. 4 ml of the cell suspension were layered onto a discontinuous gradient in 15 ml conical tubes, containing 4 ml 1.09 g/ml Percoll (Biochrom, with physiological values of osmolality and pH), delivered to the bottom and overlaid with 3 ml of 1.089 g/ml Percoll. After centrifuging for 30 min at 25°C and 400 g, the pellet contained 95% eosinophils with neutrophils as the main contaminating cell type and a yield of 50%. The cells were transferred to Medium 199 (Biochrom) containing 4 mM glutamine (Biochrom), penicillin, streptomycin and 4.2 mM NaHCO3 at pH 7.2 and centrifuged twice at 300 g and 4°C for 10 min. The purified eosinophils were stored in the described medium at room temperature. The pH of all solutions was adjusted with NaOH and HCl. The cells were used within 48 h after isolation.

**Patch – clamp experiments**

For the experiments −200 µl of our standard external solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM HEPES–NaOH and 15–20 mM glucose at pH 7.2–7.3) were filled into a chamber with a glass bottom (coverslip). 50 µl of cell suspension were then added. The cells were dialysed in the whole-cell configuration with a pipette solution containing 125 mM potassium gluconate, 10 mM NaCl, 10 mM HEPES–NaOH, 7 mM MgCl2, 5 mM EGTA, 4.5 mM CaCl2 and 1 mM Na2ATP, pH 7.2–7.3. GTP·S concentration was varied as described in the text. The resulting intracellular free calcium concentration was estimated to be in the range of 1–2 µM.

**Capacitance measurements**

For high resolution capacitance recordings, a continuous 800 Hz, 20 mV rms sine wave was given as the command voltage signal of the patch–clamp amplifier (EPC 9, List Electronics) operating in the voltage clamp mode (holding potential −10 mV). The current output signal was analysed by a two-phase lock-in amplifier (PAR 5210, EG&G). The capacitive currents were determined by a computer using automatic phase tracking (Fidler and Fernandez, 1989; Lindau, 1991). This method could be used only while cells could be described with a simple equivalent circuit consisting of capacitance C and conductance G of the membrane in series with the access resistance Ra.

To analyse more complex equivalent circuits, square voltage pulses (amplitude V = −20 mV, 1.4 ms duration) were given every 2 s as the command voltage (EPC-7, List Electronics). The current responses are the sum of a constant current flowing through ion channels in the plasma membrane and a transient current charging the membrane capacitance. Fits of each current relaxation using a single exponential plus baseline yield the access resistance Ra, membrane conductance G and membrane capacitance C if the equivalent circuit contains only these three elements (Lindau and Neher, 1988).

Current signals showing significant deviations from a single exponential were fitted by a sum of two exponentials plus baseline: \( I(t) = I_1 \exp(-k_1t) + I_2 \exp(-k_2t) + I_{SS} \). The five parameters \( I_1, I_2, k_1, k_2 \) and \( I_{SS} \) were determined by a least squares fit using standard methods. The equivalent circuit elements \( C_C, C_C, R_A, C_F \) and \( G_P \) were then calculated using the following equations: \( R_A = V/(I_1 + I_2 + I_{SS}); \ C_C = I_{SS}(V-R_A I_{SS}); \ B = 1 + R_A C_C; \ C = C_C + C = (I_1/V) + (I_2/V) \); \( \tau = (k_1 + k_2)/k_1 k_2 - R_A C/B; \ C_C = B(R_A C/k_2); \ C_C = C-C; \ G_P = G_P \).

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**References**


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