Comparison of acetylcholine receptor-controlled cation flux in membrane vesicles from *Torpedo californica* and *Electrophorus electricus*: Chemical kinetic measurements in the millisecond region

(carbamoylcholine/desensitization)

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ABSTRACT In earlier studies with the acetylcholine receptor (AcChoR) of *Electrophorus electricus* the rate and equilibrium constants for a model that relates the ligand binding to ion translocation were determined, and the dependence of these constants on the concentrations of carbamoylcholine and acetylcholine, over a 200- and 5000-fold range, respectively, could be predicted. AcChoR-controlled cation flux has now been measured in *Torpedo californica* vesicles by using a pulsed-quench-flow technique with a 2-msec time resolution. *Torpedo* vesicles on a weight basis may contain several hundred times more receptor sites than do *E. electricus* vesicles. Techniques have been developed to (i) correct for the kinetic heterogeneity of the vesicle population; (ii) use the inactivation of the receptor by its natural ligand to reduce influx rates at high ligand concentrations to a measurable level (this permitted J_A , the influx rate coefficient before the onset of inactivation, to be measured); and (iii) determine the rate coefficients of two processes that lead to successive inactivations (desensitization) of the receptor and occur in different time regions. An extension of a model proposed for the *E. electricus* receptor accommodates the ion translocation in *T. californica* vesicles. The features in common are: (i) A rapid initial flux rate [$J_A(\text{max})$ for *T. californica* is 310 sec^{-1} ; for *E. electricus* it is 7.5 sec^{-1}]. These differences in flux rates are consistent with a difference in AcChoR density. (ii) A rapid inactivation process [$\alpha(\text{max})$ for *T. californica* is 2 sec^{-1} ; for *E. electricus* it is 7 sec^{-1}]. (iii) A slow AcChoR-controlled flux that continues after the rapid inactivation [$J_I(\text{max})$ for *T. californica* is 1.3 sec^{-1} ; for *E. electricus* it is 0.015 sec^{-1}]. The main difference between the flux in the two types of vesicle is the existence of a second, slower, inactivation process in *T. californica* with a rate coefficient, β , of 0.12 sec^{-1} . The second process leads to undetectable flux activity during the time of observation (30 sec in 10 mM carbamoylcholine). These studies are also significant because fundamental differences may exist between the mechanism of AcChoR-controlled ion flux in synaptic (*Torpedo*) and conducting (*E. electricus*) membranes.

Nachmansohn suggested (1) that the binding of acetylcholine (AcCho) to the acetylcholine receptor (AcChoR) in nerve and muscle cells induces a conformational change in the protein that results in the formation of ion-conducting channels through the membrane. The important relationship between the concentration of AcCho and the rates of AcChoR-controlled ion translocation has been investigated intensively because it determines the transfer of information between cells in the nervous system.

Kasai and Changeux (2) demonstrated AcChoR-controlled ion flux in vesicles prepared from *Electrophorus electricus* electropex. Subsequently we developed methods for isolating and characterizing (3, 4) homogeneous vesicle preparations con-

taining active receptors. An adaptation of rapid reaction techniques (5) allowed four phases of the process to be measured in the 2-msec to minute time region (2, 3, 6, 7). The effect of ligand concentration [5000-fold range of AcCho and 200-fold range of carbamoylcholine (CbmCho)] (2–4) on the four phases could be predicted over the entire concentration range for each ligand we investigated, and all the rate and equilibrium constants pertaining to a minimum model (Fig. 1A) that relates the ligand-binding steps to ion translocation were determined (6–10).

AcChoR-controlled ion translocation has been studied by using vesicles prepared from the electropex of *Torpedo* (11–16). *Torpedo* vesicles may contain, on a weight basis, several hundred times more AcChoR than those from *E. electricus*, and the maximum influx rates had to be measured indirectly. Published reports differ on several important properties of the *Torpedo* AcChoR: maximal influx rates, the number of ions transported per AcChoR-formed channel per unit time, and the Hill coefficient (12, 16). Most strikingly, the rate of the CbmCho-induced inactivation of the AcChoR (17) appeared to be about 100 times slower than that observed with *E. electricus* (11, 12). Here we mention some of the problems that have not been considered in previous studies using *Torpedo* vesicles and may account for the difference in reported results. We describe methods to (i) detect and correct for populations of *Torpedo* vesicles that exhibit entirely different AcChoR-controlled flux rates, (ii) determine the flux rate constants at high concentrations of CbmCho (10 mM), and (iii) determine the rate coefficients of the two processes that occur in different time regions in 10 mM CbmCho and lead to inactivation of the AcChoR (18). We show how a simple extension of the minimal mechanism proposed on the basis of extensive experiments with *E. electricus* vesicles (3, 4) can serve as a model for further studies with *Torpedo* vesicles.

MATERIALS AND METHODS

Torpedo californica was obtained from Pacific Biomarine Laboratories. Vesicle preparations labeled JW were prepared as described (19); fraction A was 15–37% sucrose, and fraction B was the bottom one. Preparations labeled EP were made essentially according to a method that has also been described (20); fraction 1 was 20–30% sucrose and fraction 3 was 38–42% sucrose. Preparations were stored in 20% sucrose in liquid N_2 . The preparation of mono[^{125}I]iodo- α -bungarotoxin and all other chemicals used have been described (21), as have the methods used to determine the concentrations of protein (22) and of toxin-binding sites (23, 24).

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; CbmCho, carbamoylcholine.

FIG. 1. The minimal mechanism to account for the rates of AcChoR-controlled cation translocation in *T. californica* membrane vesicles is based on measurements with *E. electricus* vesicles (left part of the scheme) (6, 7). On the right is an extension of the model to account for the measurements with *T. californica* vesicles. Three receptor states are involved in eel receptor; active (A), open-channel ($\bar{A}$), and inactive (I), and two microscopic ligand dissociation constants: K_1 pertaining to the A state, and K_2 pertaining to the I state. Two conformational equilibrium constants are required, two pertaining to the equilibria between the A and I states, K_c , and one, Φ , pertaining to the equilibrium between the closed (A) and open-channel ($\bar{A}$) active forms. The symbols containing A and I (representing fractions of the AcChoR) and α have been defined in terms of ligand (L) concentration and the constants of the minimal mechanism (4), in which α is the rate coefficient for the isomerization between A and I forms. The major difference between the results obtained with the AcChoR from the two organisms is that a second inactivation step occurs in the case of *T. californica*. It is assumed that in this step the concentration of the open-channel form is reduced to a low final value, which is no longer detected in the flux assay. The rate coefficient for this inactivation step is designated β . The integrated rate equation (Eq. 1) can be extended to accommodate a second inactivation reaction. When $\alpha \gg \beta$, $(\overline{AL}_2)_\infty$ (Eq. 1) can be considered to be in preequilibrium with all the other AcChoR forms; we designate this species $(\overline{AL}_2)_{PE}$ and obtain Eq. 2, the integrated rate equation for *Torpedo* AcChoR-controlled ion flux. The values of α and β can be obtained by use of Eqs. 3 and 4, respectively. The subscript T refers to the preincubation time. The subscripts t and ∞ refer to time t and the end of the experiment, respectively. M and R represent concentrations of metal ion and receptor, respectively.

The solutions were mixed consecutively, allowing incubation of vesicles with ligand before influx was measured, in a quench flow apparatus as described (5). The vesicles were first equilibrated for 12 hr at 4°C with *Torpedo* Ringer's solution (250 mM NaCl/5 mM KCl/3 mM CaCl_2 /2 mM MgCl_2 /5 mM sodium phosphate buffer, pH 7.0), and always kept in this buffer. The toxin-binding sites (pmol per mg of membrane protein) of the preparations used were: JW17A, 368; JW17B, 425; JW10B, 1280; JW8B, 1280; EP1-1, 1240; EP1-3, 1280.

Linear and nonlinear least-squares programs were used to obtain an optimal fit of the data to theory (25).

RESULTS

The time dependence of the flux of $^{86}\text{Rb}^+$ into vesicles in the presence of 1 mM CbmCho is shown in Fig. 2. The $^{86}\text{Rb}^+$ concentration reached apparent equilibrium within 50 msec and no further influx was observed during the next 250 msec. The $^{86}\text{Rb}^+$ content of the vesicles determined during this time interval was taken as M'_∞ . Even though the $t_{1/2}$ value of this influx is only 10 msec, the influx rate coefficient could be determined with some precision. The solid line was computed by using a single value for the influx rate coefficient, J_A , of $68 \pm 6 \text{ sec}^{-1}$, that satisfied all the measurements.

In the presence of 10 mM CbmCho (Fig. 2 *Inset*), two influx processes were observed and interpreted to represent flux in two distinct vesicle populations (see *Discussion*). The initial phase was complete within 10 msec, and comprised 85% (EP13) and 70% (JW-17), respectively, of M_∞ , the $^{86}\text{Rb}^+$ content obtained after an influx period of 2–20 sec.

The second, slower, portion of the influx has a $t_{1/2}$ value of about 200 msec (Fig. 2 *Inset*), or about 100 times that of the

initial phase of the two preparations. Only the vesicle population that gave rise to the fast influx was analyzed in subsequent experiments.

A third major vesicle population is present. When the vesicles were equilibrated with $^{86}\text{Rb}^+$ for at least 12 hr the average final concentration was three times higher than it was after 30 sec in 10 mM CbmCho. Accordingly, about $\frac{2}{3}$ of the $^{86}\text{Rb}^+$ con-

FIG. 2. Receptor-mediated flux of $^{86}\text{Rb}^+$ into vesicles, pH 7.0, 1°C. M_t/M_∞ is fraction of influx that had occurred at the time indicated on the abscissa. Four influx measurements were made in the presence of 1 mM CbmCho, using preparations JW17B (Δ), JW10 ($\circ$), and JW8 ($\square$). The solid line was computed by using a value of $J_A = 68 \text{ sec}^{-1}$ ($\chi^2 = 0.2$) that is based on all the measurements. (*Inset*) Influx in the presence of 10 mM CbmCho, measured by using preparations JW17A ($\circ$), EP1-3 ($\square$), and JW17B ($\bullet$). Numbers above symbols indicate number of separate influx measurements in 2–20 sec used to determine M_∞ .

tent of the preparation is due to vesicles that contain few, if any, functional receptors.

To obtain the rate coefficient for the initial fast phase observed in the presence of 10 mM CbmCho, advantage was taken of the reduction in influx rate induced by exposure of the AcChoR to CbmCho (Fig. 3A). This inactivation depends on both the concentration of CbmCho used and the length of the exposure. In 10 mM CbmCho the initial activity was reduced by 50% in 300 msec, but exposure of the AcChoR to 0.25 mM CbmCho for 12 sec did not lead to a measurable reduction of activity. The influx rate coefficient, J_A , after various periods of exposure of the AcChoR to 10 mM CbmCho, $(J_A)_T$, was evaluated from the data shown. The $(J_A)_T$ values are plotted on a logarithmic scale as a function of incubation time according to Eq. 3. The inactivation follows a single exponential process. In the presence of 10 mM CbmCho the inactivation was followed for at least 90% of the reaction. The inactivation rate constant, α , obtained from the slope of the line, is $2 \pm 0.2 \text{ sec}^{-1}$. The values of α at lower concentrations of CbmCho are listed in the legend. All lines, corresponding to different concentrations of CbmCho during incubation, have a common ordinate intercept, because influx was always measured in the presence of 10 mM CbmCho; the intercept gives the value of $(J_A)_{T=0}$ as 310 sec^{-1} in the absence of inactivation of the AcChoR.

Influx measured by using preparations EP1-3 and JW17A in the presence of 10 mM CbmCho, and after various times of incubation in 10 mM CbmCho, is shown in Fig. 4. In Fig. 4A the value of M_∞ used is that obtained after 10 sec of influx in the presence of 10 mM CbmCho (Fig. 2 Inset). The value of M_∞ used to calculate J_A is the value of M obtained after influx for 10 msec in the presence of 10 mM CbmCho. This M'_∞ value was

also used in calculating the influx curves in Fig. 4B. Even though preparations EP1-3 and JW17A were made by different procedures and have different M_∞ values, a J_A value of $4.5 \pm 0.6 \text{ sec}^{-1}$ can be used to calculate a theoretical influx curve (upper curve in Fig. 4B) that fits all measurements.

The lower curves in Fig. 4B represent influx measurements made after incubation for more than 2 sec. After incubation for 3 sec the influx rate decreased with incubation time, at about 6% of the rate at which the influx rate decreased during the first 2 sec of incubation. A considerable amount of flux activity, characterized by the rate coefficient J_1 , is therefore still associated with the AcChoR after the first inactivation is complete (Fig. 4B). The value of the rate coefficient, J_1 , is plotted on a logarithmic scale as a function of incubation time in Fig. 4C according to Eq. 4. The slower inactivation process, characterized by the rate coefficient β , also appears to follow a first-order rate law. The value (0.12 sec^{-1}) of β , obtained from the slope of the line, is about 1/15th the value of α at the same concentration of CbmCho. The ordinate intercept gives the value of $(J_1)_{T=0}$ as $1.3 \pm 0.2 \text{ sec}^{-1}$, which is smaller by a factor of $\approx 1/300$ than the value of $(J_A)_{T=0}$.

DISCUSSION

In the determination of $(J_A)_{T=0}$ in 10 mM CbmCho advantage was taken of the natural inactivation of the AcChoR by its own ligand (Fig. 3A). Thus not only the influx rate coefficient but also the rate of AcChoR inactivation could be determined. As was also observed with *E. electricus* receptor (26), the inactivation follows a single-exponential rate law for over 90% of the reaction (Fig. 3B). From these measurements the rate coefficient, $(J_A)_{T=0}$, associated with the active state of the AcChoR

FIG. 3. Rapid inactivation of $^{86}\text{Rb}^+$ flux into vesicles by different CbmCho concentrations, pH 7.0, 1°C . (A) Percent AcChoR activity remaining plotted versus time of incubation with CbmCho prior to 10-msec influx measurement. Percent activity is defined as $(M_{t=10 \text{ msec}})_T / (M'_\infty)_{T=0} \times 100$. $(M_{t=10 \text{ msec}})_T$ and $(M'_\infty)_{T=0}$ refer to the concentration of $^{86}\text{Rb}^+$ inside the vesicles 10 msec or 10–40 msec, respectively, after influx was initiated by addition of $^{86}\text{Rb}^+$ and 10 mM CbmCho. The subscript T refers to the length of incubation with CbmCho before influx was measured. (B) Evaluation of the inactivation rate coefficient, α , at various concentrations of CbmCho and of the influx rate coefficient before inactivation, $(J_A)_{T=0}$, in 10 mM CbmCho. The values of $(J_A)_T$ were calculated from the measurements in A by using Eq. 3. α values at different concentrations of CbmCho are: $2 \pm 0.2 \text{ sec}^{-1}$ [10 mM ($\bullet$, $\oplus$, $\square$, $\diamond$)]; $0.5 \pm 0.04 \text{ sec}^{-1}$ [3 mM ($\blacksquare$)]; $0.14 \text{ sec}^{-1} \pm 0.02 \text{ sec}^{-1}$ [0.5 mM ($\triangle$)]; an estimated value of 0.03 sec^{-1} is obtained at 0.25 mM CbmCho. The mean value of $(J_A)_{T=0}$ obtained from the ordinate intercept of the solid lines is $310 \pm 66 \text{ sec}^{-1}$. Solid lines were computed by using the same value of $(J_A)_{T=0}$ and values of α obtained with different concentrations of CbmCho in the incubation solutions. Values of χ^2 were less than 1.0 in all determinations. In A, values indicated by $\diamond$, $\bullet$, and $\oplus$ were obtained from influx measurements in 10 mM CbmCho conducted over a 10- to 20-sec time period with two preparations, EP1-3 ($\diamond$) and JW17A ($\oplus$, $\bullet$). In all other 10-msec influxes, preparation EP1-1 was used.

FIG. 4. The second, slower, phase of AcChoR inactivation. AcChoR-mediated flux of $^{86}\text{Rb}^+$ into vesicles in presence of 10 mM CbmCho, pH 7.0, 1°C , after various relatively long periods of incubation with 10 mM CbmCho. (A) Influx after 2 sec of incubation. M_t/M_{∞} is the fraction of influx that has occurred at the time indicated on the abscissa, and M_{∞} is the value of M after 10 sec of $^{86}\text{Rb}^+$ influx in the presence of 10 mM CbmCho. Preparation EP1-3 ($\square$): the broken line was computed by using the values $J_A = 4.7 \pm 0.5 \text{ sec}^{-1}$, $\alpha = 2.0$, and M_{∞} (calculated) = 0.9; $\chi^2 = 0.5$. Preparation JW17A ($\circ$, $\bullet$): the broken line was computed by using the values $J_A = 4.5 \pm 0.3 \text{ sec}^{-1}$, $\alpha = 2.0$, and M_{∞} (calculated) = 0.60; $\chi^2 = 0.4$. (B) Influx after 2 sec ($\circ$, $\bullet$, $\square$), 3 sec ($\diamond$), 5 sec ($\triangle$), 10 sec ($\oplus$), 15 sec ($\square$), and 20 sec ($\times$) of incubation. M_t/M_{∞} is the fraction of influx that had occurred at the time indicated on the abscissa, and M_{∞} is the observed value after 2-sec incubation with 10 mM CbmCho and 20-sec influx. Experiments were done using preparation JW17A, except one with preparation EP1-3 ($\square$). Coordinates of influx curves after 2-sec incubation were computed by using the values $J_A = 4.5 \pm 0.6 \text{ sec}^{-1}$, $\alpha = 2.0$; $\chi^2 = 0.7$. Coordinates of the other curves were computed by using $\beta = 0.12 \text{ sec}^{-1}$ and influx rate coefficients given in C as a function of incubation time. (C) Evaluation of the inactivation rate coefficient, β , and the influx rate coefficient, $(J_I)_{T=0}$ (see the text). $(J_I)_T$ values, obtained from the experiments in B, are given on the ordinate (logarithmic scale); the abscissa gives the incubation time with 10 mM CbmCho prior to influx measurement. See Eq. 4. Vertical bars give the standard deviations. The ordinate intercept gives $(J_I)_{T=0} = 1.3 \pm 0.2 \text{ sec}^{-1}$, and the slope gives $\beta = 0.12 \pm 0.03 \text{ sec}^{-1}$; $\chi^2 = 0.8$.

before the onset of inactivation can be evaluated (Fig. 2B). The value we obtained, 310 sec^{-1} in 10 mM CbmCho, can be compared with the $J_A(\text{max})$ value of 1500 sec^{-1} obtained by using a stopped-flow spectroscopic technique in which thalium flux is measured (16) and with the value of 65 sec^{-1} (13) obtained by using similar vesicle preparations and a quench-flow approach similar to one used with *E. electricus* vesicles (27, 28).

Some factors that may contribute to discrepancies in the values of $J_A(\text{max})$ that have been reported are: (i) Differences in AcChoR concentrations and internal volume of the vesicle preparations exist. The flux rates depend on R , the moles of AcChoR per liter internal volume of the vesicles (29). As judged by measuring flux rates, the preparations contain at least three vesicle populations, each with a characteristic ion flux rate. Therefore, the populations differ from each other in the ratio of active AcChoR to internal volume. The noncorrespondence between AcChoR per mg of membrane protein and flux rates is demonstrated experimentally. In eight different preparations the J_A values were found to vary by a factor of 15, while the AcChoR concentration differed by only a factor of 3. (ii) Except in our studies, toxins were used to reduce influx rates to a measurable level. This involved comparing influx rates at low CbmCho concentrations in the presence and absence of toxin and determining the ratio of the rate constants. This ratio was then used to multiply the flux rate obtained at high CbmCho concentrations in the presence of toxin to obtain the $J_A(\text{max})$ value in the absence of toxin. This gives the correct $J_A(\text{max})$ value only when the binding constants of toxin and CbmCho are completely independent of each other. There is also a more subtle problem in the use of inhibitors. Toxin can affect the ratio of influx rates to inactivation rates by decreasing the number of functional AcChoR. This ratio determines whether the influx curves reflect J_A before, and J_I after, addition of toxin, or both (7).

Determination of the Inactivation Rate Constant α . In *Torpedo* vesicles the influx rate coefficients (J_A), which depend on

AcChoR concentration, are much larger than the inactivation rates (α), which are expected to be independent of the AcChoR concentration. Consequently influx will appear to follow a single-exponential rate law and the effect of α will not be seen in influx measurements until the values of J_A and α become comparable. The initial rapid inactivation that occurs in *Torpedo* vesicles has escaped detection (11, 12, 13, 16). Consecutive mixing of solutions (Fig. 2) allowed us to measure the rate coefficient, α , of the rapid inactivation and to determine α and $(J_A)_{T=0}$ in the same experiment (Fig. 2B).

The data in the *Inset* to Fig. 2 can be used to correct for the heterogeneity of the vesicle population. This treatment of the data is justified by: (i) The value of M_{∞} obtained after 10 msec of influx in the presence of 10 mM CbmCho is essentially the same as the value obtained after incubation for 2 sec with CbmCho and influx for 20 sec (Fig. 4A). (ii) The vesicle population that gives rise to the slower AcChoR-controlled influx rates (Fig. 2 *Inset*) is not expected to take up a significant amount of $^{86}\text{Rb}^+$ during the 20-sec period of observation in the experiments in Fig. 4. (iii) The use of M_{∞} gave consistent values for all four rate constants evaluated, whereas the use of M_{∞} did not. (iv) The changes in amplitude (Fig. 4B) are accounted for by the ratio J_I/β , as it should be (Eq. 2) if we are observing a property of the AcChoR rather than of a heterogeneous vesicle population.

Our working model for further measurements of AcChoR-controlled ion translocation in *Torpedo* vesicles (Fig. 1) is based on the following considerations. The experiments in Figs. 3 and 4 show that in 10 mM CbmCho the flux rate decreased initially with a rate of 2 sec^{-1} , and did so exponentially over 90% of the reaction. After incubation with 10 mM CbmCho for 5 sec, we would expect a flux rate of 0.02 sec^{-1} , but we observed a flux rate that was higher by a factor of 30. In addition, the flux rate after incubation for 3–20 sec decreased but at a rate approximately 1/15th of that during the first 2 sec. This indicates that, after the first inactivation process has gone to completion, the

resulting quasi-equilibrium between active and inactive AcChoR forms is associated with a measurable flux rate (J_1).

The lack of correlation between toxin site concentration and ion flux rates indicates differences between preparations in the value of R and makes direct comparison of results obtained with different preparations tenuous. However, the ratio of constants obtained with the same preparation is expected to be independent of the value of R . For example, the ratio of the values of $(J_A)_{T=0}$ in 10 mM CbmCho to $(J_A)_{T=0}$ in 1 mM CbmCho is 4.3; the corresponding ratio in *E. electricus* is 5. This indicates that the constants for binding of CbmCho to the active form of the receptor are similar in the two organisms. This is of interest because it was discovered with *E. electricus* vesicles that the initial rapid flux, characteristic of the active state of the AcChoR, can be seen only at mM concentrations of CbmCho (30) rather than in the μ M region expected from electrophysiological measurements (31). The ratio of $(J_A)_{T=0}$ to $(J_I)_{T=0}$ in 10 mM CbmCho is about 300 in *Torpedo* and about 400 in *E. electricus*. The inactivation rate coefficient, α , which reflects isomerization rate constants, is 3.5 times more in *E. electricus* than in *Torpedo*.

Rapid mixing experiments with *Torpedo* vesicles had indicated (13) rather large differences between that AcChoR and the *E. electricus* one. The methods that we have introduced here, consecutive mixing experiments in which J_A and α values can be determined simultaneously at high ligand concentration, and detection of and correction for vesicle populations with quite different influx rate coefficients, lead to a different view. As with the *E. electricus* AcChoR, the active form of the AcChoR is saturated at approximately 10 mM CbmCho and rapid inactivation is followed by a quasi-equilibrium of AcChoR forms that mediates AcChoR-controlled flux with a rate constant of 1.3 sec^{-1} . The only major difference detected between the two organisms is the second, much slower, inactivation reaction observed in *Torpedo* vesicles and also in some muscle cell AcChoR (32).

The model (Fig. 1) can be treated quantitatively if one uses caution. Because α and β may have different ligand dependencies, interpretation of influx measurements requires an independent knowledge of the constants. The pulsed-flow-quench technique and the approach described to determine J_A , J_I , α , and β are capable of providing the desired information. These studies are also significant because fundamental differences may exist between the mechanism of AcChoR-controlled ion flux in synaptic (*Torpedo*) and conducting (*E. electricus*) membranes.

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