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Two Kinetically Distinct 5-Hydroxytryptamine–activated Cl^- Conductances at Retzius P-Cell Synapses of the Medicinal Leech

V. Leßmann and I. D. Dietzel

Lehrstuhl für Molekulare Neurobiochemie, Ruhr-Universität Bochum, Gebäude NC7-170, D-44780 Bochum, Germany

The properties of serotonin (5-HT)-activated Cl^- receptor/ion channel complexes in neurons of the CNS of the medicinal leech were analyzed. These channels mediate the postsynaptic response at the serotoninergic Retzius P-cell as well as Retzius-Retzius cell synapses. 5-HT–induced Cl^- currents were activated by fast superfusion of transmitter on cells dissociated from embryonic leeches including histochemically identified Retzius cells. Whole-cell currents elicited by partial superfusion of the membrane and summated single-channel currents of outside-out patches showed times to peak of 18 ± 4 msec and 10 ± 5 msec, respectively, and desensitized with time constants of 28 ± 3 msec and 20 ± 11 msec. Persistence of single-channel openings in the outside-out configuration as well as lack of effect of dialysis of the whole cell with AMP-PNP or GDP-β-S indicated that 5-HT directly gates the Cl^- channels without involving second messenger cascades. In outside-out patches, two single-channel conductances of 13 pS and 32 pS were identified. While the 13 pS conductance desensitized, the 32 pS conductance activated within several tens of msec and showed no desensitization. We postulate that two subtypes of channels are coactivated by 5-HT and that the activation of the fast desensitizing channel could be responsible for the fast decaying component of the postsynaptic response. The slow conductance explains the second slower decay time constant of the postsynaptic response and could account for the tonic component sometimes observed at Retzius P-cell synapses.

[Key words: medicinal leech, 5-HT, Cl^- currents, desensitization, serotonergic synaptic transmission, Retzius P-cell synapse]

Synaptic transmission at Retzius-P and Retzius-Retzius cell synapses of the medicinal leech is mediated by serotonin (5-HT)-activated Cl^- conductances (Henderson, 1983; Liu and Nicholls, 1989). The electrophysiological and pharmacological properties of synaptic responses of postsynaptic Retzius and P-cells are strikingly similar (Drapeau and Sanchez-Armass, 1988; Drapeau et al., 1989; Liu and Nicholls, 1989). The postsynaptic current reaches its maximum with a time to peak of about 30 msec and decays with time constants of 70 and 600 msec (Drapeau and Sanchez-Armass, 1989). This time course of synaptic transmission is slow compared, for example, to glutamatergic synaptic transmission mediated by AMPA receptors in the CNS, where rise times of less than 1.5 msec occur and the fastest decay time constants range from 2–3 msec (see, e.g., Trussell and Fischbach, 1989; Colquhoun et al., 1992; Hestrin, 1992; Leßmann et al., 1992; Yamada and Tang, 1993). It is fast compared to synapses, where the postsynaptic currents are mediated by second messenger cascades, for example, in peptidergic transmission (see, e.g., Jan et al., 1979).

In this study, we have tested whether the 5-HT–induced Cl^- currents in leech neurons are mediated by directly gated channels or whether they require the activation of second messengers. Further, we analyzed the peak and plateau components of 5-HT–induced whole-cell currents (Leßmann and Dietzel, 1991) to see whether they resulted from the gating of different single-channel conductances. Finally we related the kinetics of the single-channel conductances with the time course of 5-HT–mediated postsynaptic responses of Retzius and P-cells. We present evidence that different single-channel conductances and kinetics of channel gating underlie the peak and plateau component of the 5-HT–induced whole-cell currents during maintained transmitter application. The time constant of desensitization of the averaged, fast inactivating, 13 pS ion channel corresponds to the fast decay time constant of the postsynaptic current. The deactivation of the slower activating 32 pS ion channel could, in turn, explain the second, slower time constant of decay of the postsynaptic response. Prolonged single-channel patch-clamp recordings of embryonic cells in the outside-out configuration as well as pharmacological investigations indicated that the postsynaptic Cl^- channels are directly gated by 5-HT.

Materials and Methods

Preparation. Embryonic leeches were obtained from a breeding colony maintained in the laboratory as described in Fernández and Stent (1982). Segmental ganglia were removed from embryos between day 10 and 17 of intracocoon development at 24°C. The ganglia were rinsed two times in sterile Leibowitz 15 (L15, GIBCO) medium supplemented with 10 mM glucose, 2 mg/ml glutamine, 0.1 mg/ml gentamicin, and 2% fetal calf serum (FCS, GIBCO). Approximately 20 ganglia were then transferred into a droplet of FCS free medium on polyornithine-coated (1 mg/ml) 35 mm plastic petri dishes. Ganglia were dissociated into single cells by gentle pushing and pulling through glass pipettes with orifices of 80–120 μm diameter. When the cells had attached to the substrate, after about 1 hr, 2% FCS was added to the culture medium. Recordings were performed after 0–2 d in culture on the stage of an inverted microscope (Zeiss IM35). Only the 5–10% largest cells in the dish, which include the Retzius and P-cells, were investigated. All results reported in the following were additionally confirmed on Retzius neurons, identified by their selective uptake of the autofluores-

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Correspondence should be addressed to Irmgard D. Dietzel, Lehrstuhl für Molekulare Neurobiochemie, Ruhr-Universität Bochum, Gebäude NC7-170, Universitätsstrasse 150, D-44780 Bochum, Germany.

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cent serotonin analog 5,7-dihydroxytryptamine (5,7-DHT, Serva, Heidelberg). In brief, isolated ganglia were cultured on polyornithine-coated coverslips in L15, supplemented with 10 mM glucose and 10% FCS. After 8 hr, 300 μM 5,7-DHT, 25 μM ascorbic acid, and 40 μM iproniazid (Sigma) were added. Ganglia were then incubated for 3–4 hr at room temperature in the dark. Selectively stained Retzius cells were visualized using a fluorescence microscope with the following filter combinations: excitation, 365 nm; dichroic mirror, 390 nm; low pass, 420 nm. Cells were then dissociated following a 15 min preincubation in Collagenase/Dispase (Boehringer–Mannheim, Germany) in 2 mg/ml L15 with 2% FCS and 10 mM glucose. Illumination was minimized at all stages of the preparation to avoid oxidation of 5,7-DHT. Under these conditions, it has been shown, that 5,7 DHT is not toxic to the cells even after 2 d in culture (Leßmann and Dietzel, 1991). Cells were plated on Petriperm dishes (Bachhofer, Reutlingen, Germany), which show less UV absorption than plastic petri dishes.

To investigate the time course of Cl^- currents activated by synaptic release of 5-HT, Retzius P-cell synapses were grown in culture. We predominantly analyzed Retzius P-cell synapses because the P-cells display larger postsynaptic responses and the synapses are unidirectional. The time course of postsynaptic responses recorded from Retzius cells is, however, very similar to those observed in P-cells (Drapeau and Sanchez-Armass, 1988; Drapeau et al., 1989; Liu and Nicholls, 1989; and our own unpublished observations). To prepare Retzius P-cell synapses, cells were enzymatically removed after preincubation of the ganglia for 20 min in Collagenase/Dispase (2 mg/ml Boehringer–Mannheim in L15 with 2% FCS, 10 mM glucose, 2 mg/ml L-glutamine, 0.1 mg/ml gentamicin; Dietzel et al., 1986). Cells were maintained for 2 d in uncoated petri dishes in culture medium to allow removal of adhering cell debris from the membrane (Haydon, 1988). On the third day, R- and P-cells were paired in polyornithine-coated 3.5 cm plastic petri dishes in serum-free medium. After approximately 2 hr, as soon as cells had attached to the substrate, 6% FCS was added to the culture medium. Recordings were performed 5–10 d after pairing the cells.

Recordings. Transmitter-induced currents were investigated using the patch-clamp method in the whole-cell and outside-out recording configuration (Hamill et al., 1981). Electrodes were fabricated from thick-walled (0.3 mm) borosilicate glass tubings with outer diameters of 1.5 mm (Science Products, Frankfurt). Glass capillaries were pulled to tip diameters of 1.5 μm (Narishige PP83) and fire polished with a heated platinum-iridium wire on a microforge immediately prior to recording. Resistances of 3–8 MΩ were obtained after filling with a recording solution of the following composition (in mM): Na^+ 4, Cs^+ 150, Ca^{2+} 1, EGTA 10, Cl^- 141, HEPES 10, Mg^{2+}-ATP 2; the external solution contained (in mM) *N*-methyl-D-glucamine (NMDG) 165, Ca^{2+} 5, Mg^{2+} 2, Cl^- 170, HEPES 10, glucose 10. pH was adjusted to 7.3 with CsOH, HCl or NaOH. Resulting concentration changes of Na^+, K^+, and Cl^- have been accounted for in the concentrations given above. This choice of solution allowed us to selectively investigate 5-HT–activated Cl^- channels excluding additional 5-HT–activated K^+ and Na^+ channels (Leßmann and Dietzel, 1991). Recordings were performed using a List EPC7 Patch-clamp amplifier. Series resistances were compensated to 40–70%. Synapses were investigated using a conventional amplifier for stimulating the presynaptic cell and a patch-clamp amplifier to record the postsynaptic currents. Data were digitized using a Labmaster interface (TL-125) and P-Clamp software (Axon Instruments) and were further processed using the universal data evaluation software AUTES P, written by H. Zucker, Max Planck-Institute for Psychiatry, Martinsried. Single-channel recordings were filtered with 3 kHz prior to digitization (10 kHz), if necessary, with 1.5 kHz during further data evaluation. Where necessary, data were analyzed for significance of differences utilizing the paired Student's *t* test (*p*-level as individually indicated).

Fast application of transmitter. After equilibration of the pipette solution with the interior of the cell (ca. 5 min, see, e.g., Pusch and Neher, 1988), transmitter application was started. Several variants of fast superfusion systems for single-channel and whole-cell recordings have been described (Franke et al., 1987; Maconochie and Knight, 1989; Mayer and Vyklický, 1989; Smith et al., 1991). Here, we employed an application system allowing fast superfusion of outside-out patches and whole cells with an identical experimental arrangement, leading to complete solution exchanges at the tip of a patch pipette within 0.5 msec (Leßmann et al., 1992). In addition, our application system enabled us to apply transmitter solutions to a limited area of the soma of a cell to minimize desensitization. Driven by hydrostatic pressure, the different transmitter-containing solutions were applied through a pipette assembled from up to nine hypodermic needles (diameters 0.4 mm) as described by Carbone and Lux (1987). A glass pipette with an opening diameter of 40 μm surrounded the needles to create a common outlet of the superfusion solutions. The tip of the outlet pipette was positioned at a distance of 10 μm from the bottom of the petri dish at a lateral distance of 20 μm and a frontal distance of 50–100 μm from the cell (Fig. 1). By positioning a drain pipette (the flow rate of the suction was hydrostatically adjusted by the vertical position of a drain reservoir), a laminar flow of solution containing the neurotransmitter was installed laterally from the cell. Fast transmitter application was achieved by triggering the magnetically operated valve of a second channel containing identical transmitter solution, driven by a higher hydrostatic pressure (Fig. 1*B*; Leßmann et al., 1992). This increased stream of transmitter solution was reflected from the bottom of the dish leading to a sudden lateral extension of the laminar flow of transmitter to cover the cell or part of its membrane. The procedure of using two transmitter-containing perfusion channels avoided the turbulences which otherwise accompany transmitter application when switching from a control channel to a transmitter-containing channel. Fast removal of transmitter from the membrane (see Fig. 5) was achieved by simultaneously closing the high pressure transmitter inflow channel and opening an internal drain channel driven by negative pressure. The velocity of removal of the transmitter solution was estimated after termination of the record. For this purpose, diffusion currents induced by application of a low Cl^- solution to the tip of the patch pipette after removal of the cell were monitored. The time course of application was identical to the protocol used for

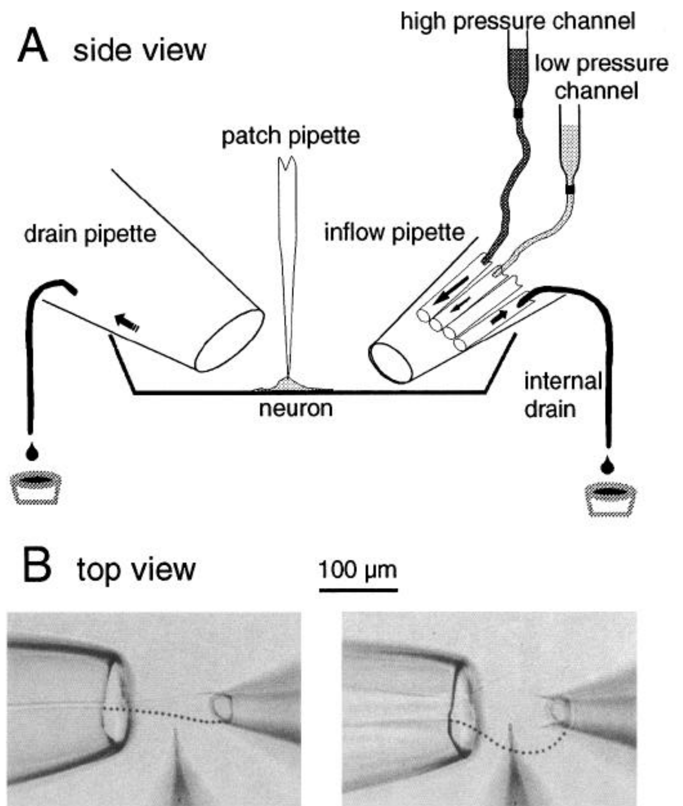

Figure 1. Drawing of the fast transmitter application system. *A*, Arrangement of the perfusion, as seen from the side (not drawn to scale). Fluid pressure in the different inlets and outlets is adjusted by vertical positioning of the different fluid reservoirs (10 ml syringes). Note the internal drain channel of the inflow pipette for the fast removal of transmitter solutions (see Materials and Methods). *B*, Photographs show positions of glass pipettes directing the flow of solutions relative to the position of the recording electrode. *Left*, Flow of solution adjusted by low-pressure inflow and drain pipette. *Right*, The additional opening of a high-pressure channel in the inflow pipette containing the same solution leads to horizontal extension of the laminar flow of solution, now covering the cell membrane/the patch pipette. *Stippled line* indicates border of the laminar flow.

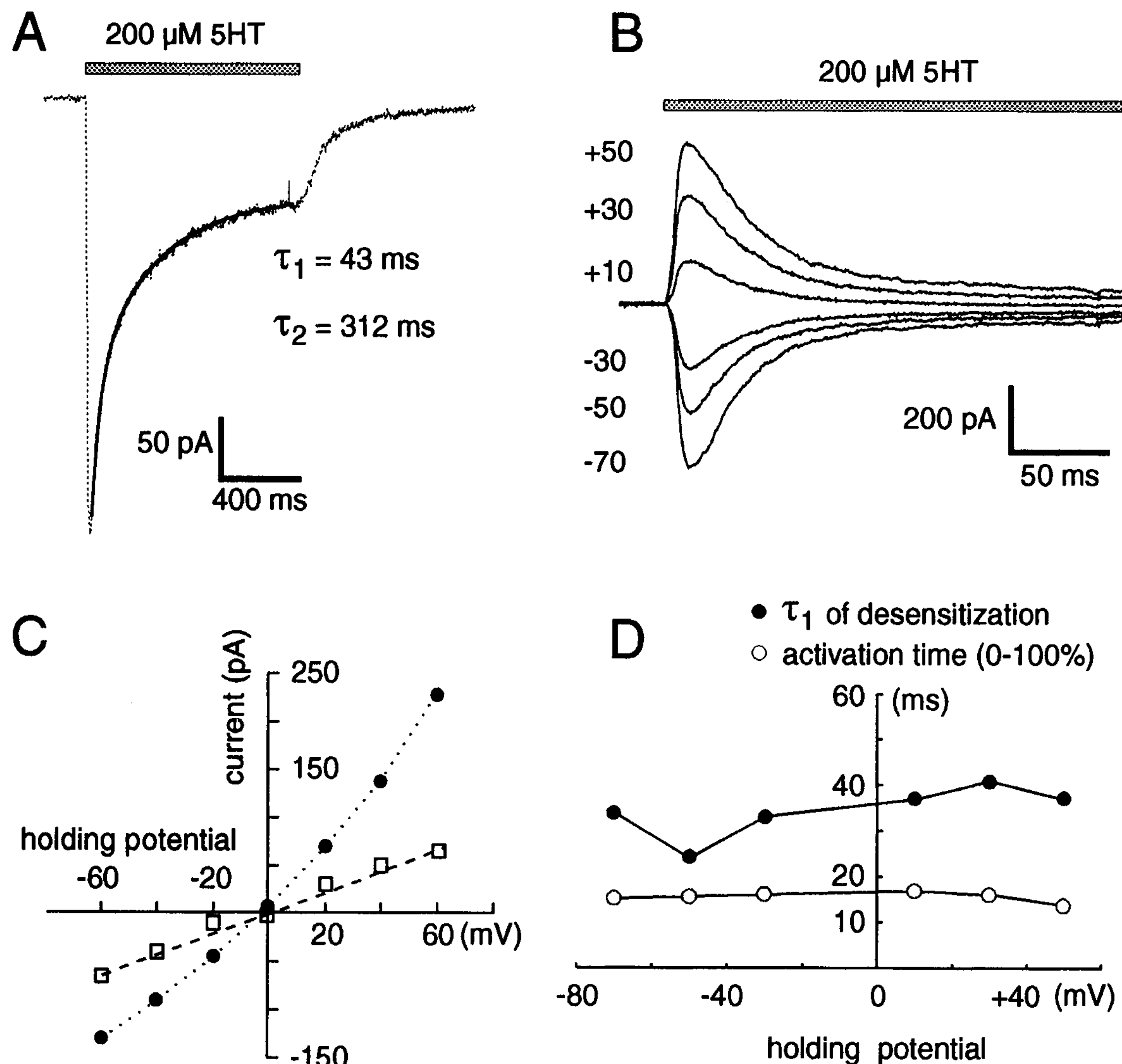

Figure 2. Desensitization and voltage dependence of 5-HT–activated Cl^- currents in a large embryonic cell. *A, dotted line* is the average current of seven successive applications of 200 µM 5-HT for 1.1 sec (indicated by *bar*) to the same cell. Cell isolated at E12 and maintained in culture for 2 d. Holding potential: −70 mV. *Solid line* is the sum of two exponentials adjusted to the recorded trace (fast time constant τ_1 = 43 msec, relative contribution: 41%; slow time constant τ_2 = 312 msec, relative contribution: 59%; nonlinear least squares fit). *B,* Family of Cl^- currents measured in response to the application of 200 µM 5-HT at the indicated holding potentials (in mV). *C,* Current–voltage relation of the 5-HT response shows a slight outward rectification of the peak component (*filled circles*). *Open squares,* plateau component. *D,* Activation time and the fast time constant of desensitization were independent of the holding potential; investigated and confirmed in three cells. Recordings were only evaluated, when control records taken at a holding potential of −70 mV at the beginning and the end of the experiment deviated by less than 5%.

the preceding transmitter experiment. Since the opening of the electrode tip was, however, smaller than the superfused membrane area, the resulting diffusion currents give only the lower limit of the true time course of the transmitter at the membrane.

Results

Characterization of 5-HT–induced whole-cell currents

Complete superfusion of cell somata with 5-HT (200 µM, ~90% of the saturating concentration, see below) evoked Cl^- currents, which reached their maxima 32 ± 17 msec following the onset of the transmitter response (n = 102). From the peak values, the currents declined during maintained presence of transmitter (Fig. 2*A*). In most recordings, this desensitization was incomplete and could be best described by the sum of two exponentials with a fast time constant of 20–60 msec (mean 47 ± 28 msec, n = 84) and a second slower time constant that was more variable and could be as large as 1077 msec. The relative contribution of the fast component amounted to 51% ± 22% (n = 84), but could vary between 6 and 92%.

Figure 2*B–D* shows the influence of the holding potential on the 5-HT evoked currents. The time constant of desensitization as well as the activation time of the current were independent of the holding potential (Fig. 2*D*). In 12 of 16 cells investigated, the peak component of the current showed a slight outward rectification while the "plateau" component (measured after 500 msec) displayed a linear dependence in symmetrical Cl^- solutions (see also Leßmann and Dietzel, 1991). The rectification of each component was calculated from the ratio of the amplitudes at +60 mV and −60 mV. The peak component showed a rectification ratio of 1.7 ± 0.6 and was significantly different from the corresponding value for the plateau component, which amounted to 1.2 ± 0.5 (mean ± SD, n = 12, $p < 0.05$, paired Student's t test). In the four remaining cells peak, as well as

plateau components displayed a linear current to voltage relationship for both, plateau, and peak component. Our predominant observation of outwardly rectifying peak currents upon 5-HT application is in accordance with the results obtained for adult Retzius and P-cells (Drapeau et al., 1989) as well as the 5-HT–mediated postsynaptic responses of P-cells (Drapeau and Sanchez-Armass, 1988).

The relative contribution of the two components was independent of the 5-HT concentration, as can be inferred from the almost identical dose–response relationships obtained for the peak component of the current and the value measured after 500 msec (Fig. 3*B,C*). In addition, the time constants of desensitization were also independent of the 5-HT concentration (data analysis not shown).

To test for second-messenger effects on 5-HT activated Cl^- currents, we investigated activators of protein kinase A (PKA). This kinase has been shown to participate in the activation of Cl^- currents in P-cells of adult leeches (Drapeau and Sanchez-Armass, 1989; Sanchez-Armass et al., 1991; see Discussion for details). Reagents elevating the intracellular level of cAMP were added to the superfusion solution while 5-HT evoked currents were monitored every 30 sec. The application of 120 μM 8-Br-cAMP (for 1–5 min) caused a reduction of the amplitudes of the 5-HT activated currents to 71 ± 21%, $n = 4$ (Fig. 4*A*, difference to control not significant with $p < 0.05$). Likewise, superfusion of the cells with 200 μM IBMX, which inhibits the cAMP cleaving enzyme phosphodiesterase, reduced the peak current to 81 ± 17% of control values ($n = 8$; significant with $p < 0.05$). In some cases, the application of both agents induced continuous inward currents of less than 20% of the amplitudes of the 5-HT–induced currents. At membrane potentials of −70 mV and symmetrical Cl^- concentration on both sides of the membrane this current could be interpreted as opening of Cl^- channels. Secondly, we tested whether phosphorylation of the Cl^- channel by kinases other than PKA mediates the channel gating or modulates the amplitudes of 5-HT–evoked currents. Cells were dialyzed with 7 mM of the nonhydrolyzable ATP analog AMP-PNP in the patch pipette solution. After waiting more than 5 min following opening of the seal to achieve a complete equilibration of the cytoplasm with the pipette solution (see Pusch and Neher, 1988), ATP should have been replaced by nonhydrolyzable ATP dialyzed into the cell, blocking all phosphorylation processes. In all 10 cells investigated, even after 30 min of perfusion with AMP-PNP, 5-HT still evoked Cl^- currents comparable in amplitude to currents at the start of the recording. Mean amplitudes of currents recorded 30 min after opening the seal with AMP-PNP in the pipette did not differ from the currents observed with the control intracellular solution which, on the average, showed a "run-down" of about 30% in longer lasting recordings (Fig. 4*B*). Additionally, we investigated the involvement of GTP-hydrolyzing proteins in the gating or modulation of the 5-HT–activated channels. Cells were dialyzed with the G-protein blocking GDP analog GDP-β-S (200–400 μM), which binds to G-proteins, preventing their activation by binding of GTP. As was the case in the experiments with AMP-PNP, no changes in the amplitudes of the currents were observed compared to control recordings, even in experiments lasting for up to 1 hr (Fig. 4*B*). In an additional series of experiments, where transmitter application started immediately after obtaining the whole-cell configuration, it was assured that neither AMP-PNP nor GDP-β-S had short-term effects during the 5 min of equilibration between the cell interior and the pipette solution. In the control records, 30% of the cells showed a decline of the amplitudes of 5-HT–activated currents when transmitter pulses were repetitively applied for times lasting for up to 1 hr. Using GDP-β-S or AMP-PNP in the pipette solution, neither the relative number of cells showing a depression of the 5-HT–activated currents

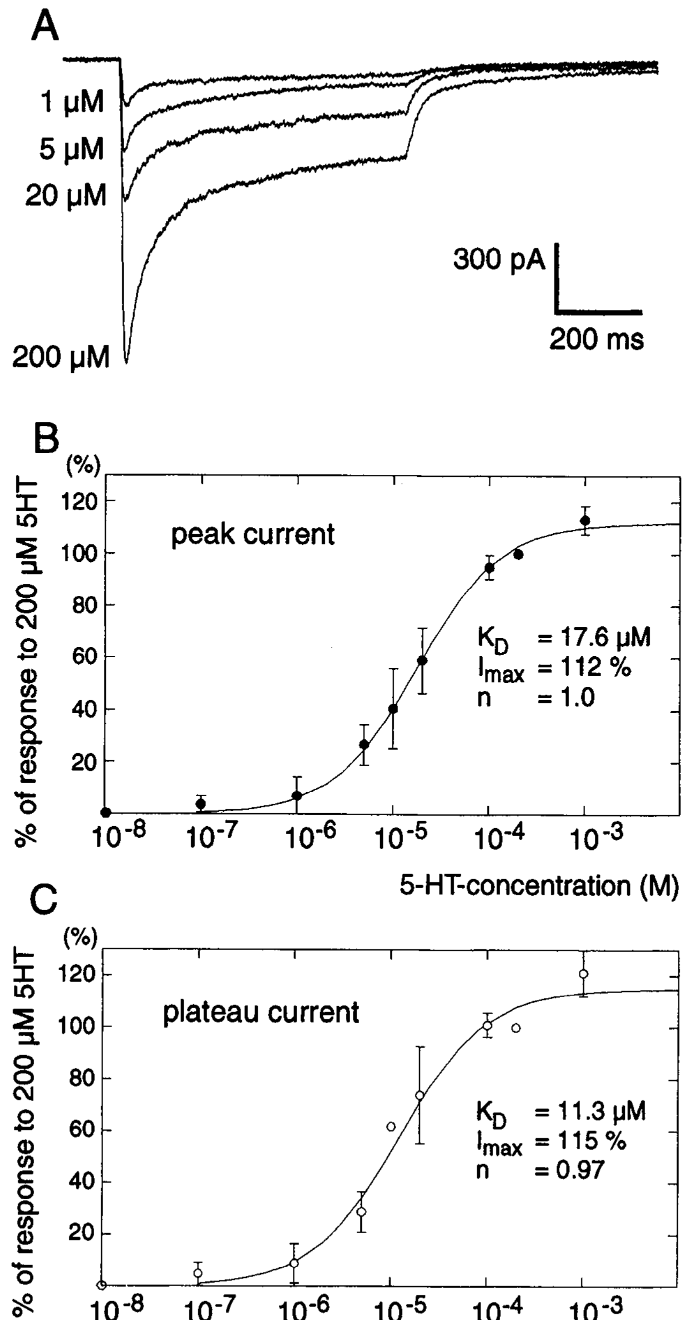

Figure 3. Dependence of 5-HT-induced whole-cell currents on the 5-HT concentration. *A*, Family of Cl^- currents elicited by different 5-HT concentrations applied to the same neuron (perfusion pipette with four different solutions). Peak current (*B*) and current after 500 msec (*C*) versus 5-HT concentration. Currents normalized to amplitude of current upon application of 200 μM 5-HT to the same cell. Measurements were only included when recovery to control values of 200 μM 5-HT showed a deviation of less than 10% from the initial value. The solid curve was fitted to the Hill equation $I = I_{max}/(1+(K_D/[\text{5-HT}])^n$, with K_D, binding constant; n, Hill-coefficient; I_{max}, fitted maximal current (least squares fit). Each point represents the average of measurements from at least four large embryonic cells. Error bars represent standard deviations.

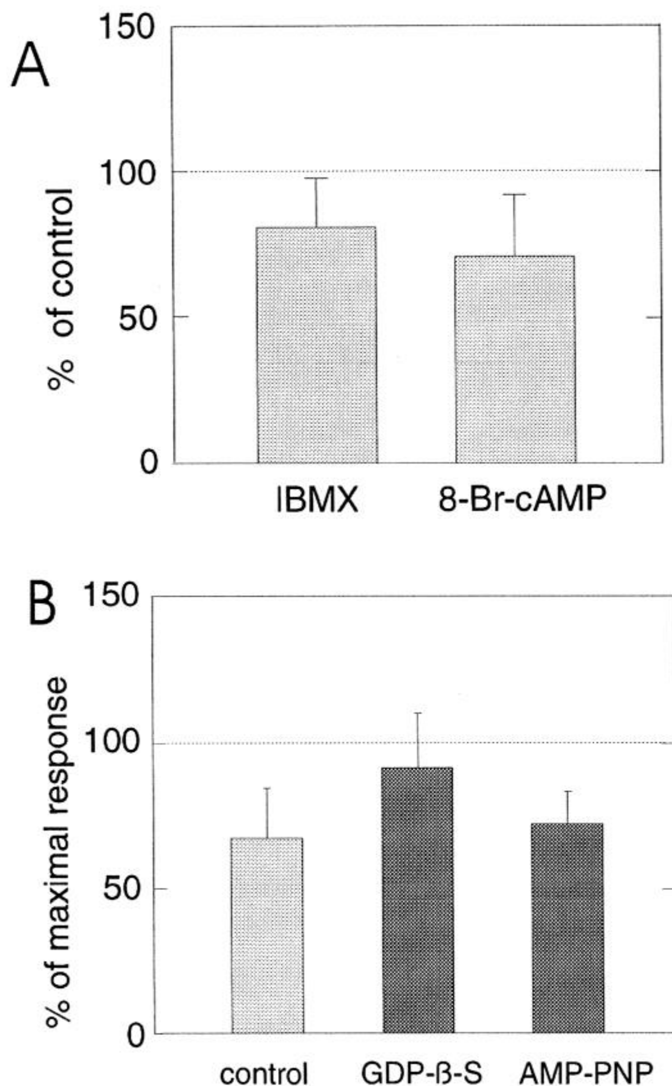

Figure 4. Effect of intracellular messengers on the activation of 5-HT–dependent Cl^- channels. *A,* Modulation of the amplitude of the 5-HT–induced Cl^- currents by cAMP. *IBMX*, relative change in amplitude of the peak current after superfusion of the cell with 200 μM IBMX for 1–5 min (n = 8). *8-Br-cAMP*, relative change in amplitude of the peak current after superfusion with 120 μM 8-bromo- cAMP for 1–5 min (n = 4). Columns represent mean values; error bars, standard deviations; holding potential, −70 mV. For IBMX, mean values are significantly different from control (paired Student's t test, $p < 0.05$). Intra- and extracellular solutions as given in Materials and Methods. *B,* Lack of influence of G-proteins and phosphorylation on the amplitude of 5-HT–induced Cl^- currents in long-term recordings. Amplitudes of peak currents were normalized to the maximal current amplitude observed in the respective recording session on the same cell. *Control*, average of minimal currents measured during a recording session of at least 0.5 and up to 2 hr (n = 61 cells) using the standard pipette solution (see Materials and Methods). *GDP-β-S*, average of relative minimal currents (normalized to maximum value during the record) measured in five cells where 200–400 μM GDP-β-S had been added to the pipette solution. *AMP-PNP*, average minimal amplitudes of currents after addition of 7 mM AMP-PNP to the pipette solution (10 large embryonic cells investigated). *Columns*, mean values; error bars, standard deviations. Mean values of currents recorded in both test solutions not significantly different from control ($p < 0.05$, Student's t test).

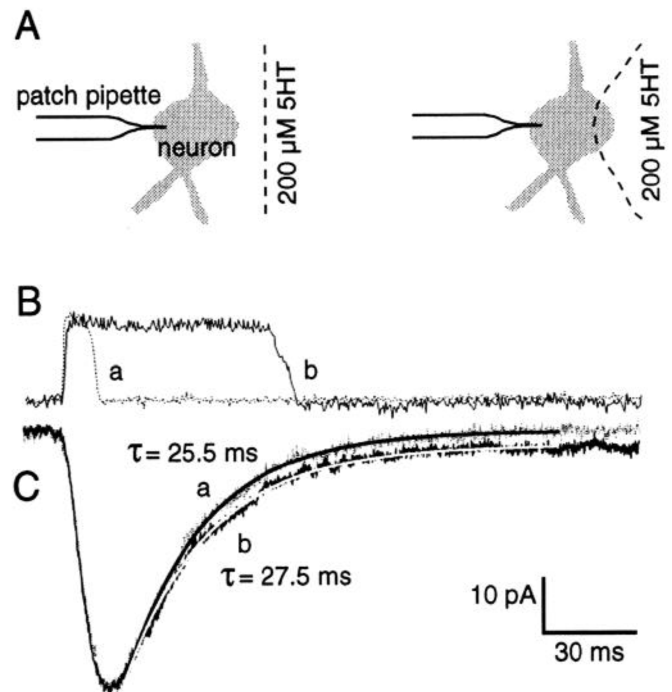

Figure 5. Fast application of 5-HT to a small area of the somatic membrane of a large embryonic cell and fast removal of transmitter. *A,* Schematic drawing of the experimental arrangement allowing only a partial superfusion of the cell. *Broken line* indicates the border between transmitter-containing solution and bath solution. *B,* Duration of transmitter application indicated by measuring the diffusion currents induced at the tip of the open patch pipette. *C,* Cl^- currents induced by application of 5-HT (200 μM) for duration indicated by the diffusion current registered in *B*. *Bb,* Diffusion current drawn with a *solid line* (transmitter application for 70 msec) corresponds to 5-HT–activated current shown as *solid trace* (*Cb*). *White line*, exponential function fitted to the desensitizing phase of the current (time constant, 27.5 msec). *Ba,* Diffusion current drawn with *dotted line* (transmitter application terminated after 12 msec prior to full activation of peak current) corresponds to 5-HT–induced current shown as *dotted trace* (*Ca*). *Black line*, exponential function fitted to the deactivating phase of the current (time constant, 25.5 msec). Amplitude of the current elicited with short transmitter pulse normalized to the amplitude of the current evoked by longer application (amplification factor, 1.45).

during long-term recording, nor the average reduction of amplitudes significantly exceeded control values. Thus, the activation of G-proteins or protein phosphorylation is not required for the activation of Cl^- channels by 5-HT.

Partial membrane superfusion

The movement of the laminar border of the superfusion solution may lead to the successive activation of ion channels distributed over the surface of the whole cell, causing a slowing of the activation and desensitization time constants recorded upon superfusion of the whole cell. To find out, whether the spatial distribution of the 5-HT receptors over the membrane leads to a significant slowing of the response, additional experiments were performed in which only part of the membrane was superfused. This procedure resulted in the activation of a current of smaller amplitude from a limited membrane area (Fig. 5). As expected, the time to peak of these currents (0–100%: 18 ± 4 msec, n = 7) and the time constant of desensitization (τ = 28 ± 2 msec, n = 5) were reduced, compared to the values obtained when superfusing the whole cell membrane (time to peak:

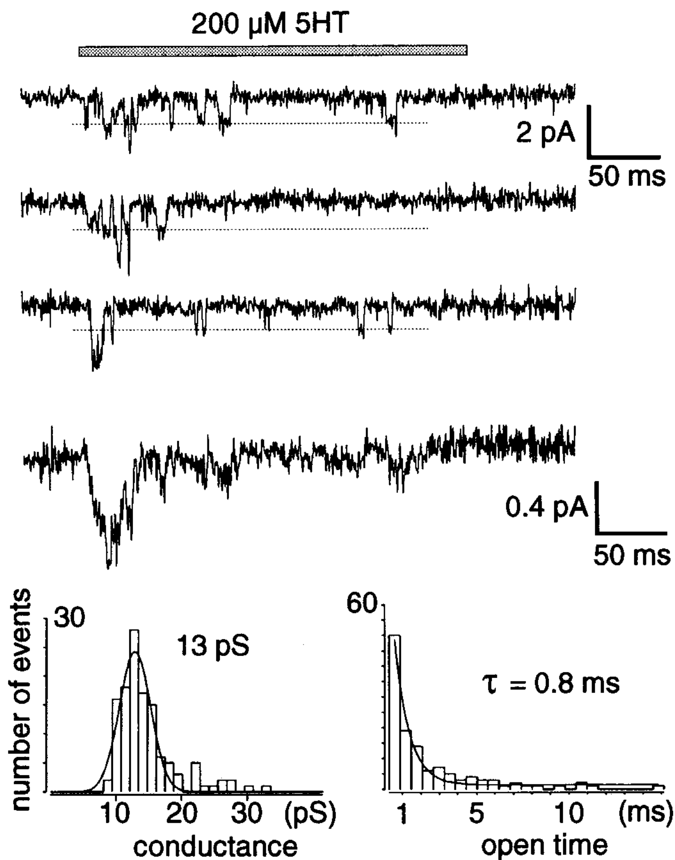

Figure 6. 5-HT–activated 13 pS conductance. *Top three traces*, single-channel currents elicited by application of 200 μM 5-HT to an outside-out patch. *Stippled lines* indicate a calculated chord conductance of 13 pS. *Forth trace* shows an average of 12 5-HT applications onto the same patch. Histograms show conductances (*left*) and open times (*right*) of all single openings from this patch; mean single-channel conductance, 13 pS; open time constant, 0.8 msec. Membrane potential, −70 mV; intra- and extracellular solution is given in Materials and Methods; reversal potential for Cl^-, 0 mV. Cells prepared at E15, 2 d in culture, large embryonic neuron.

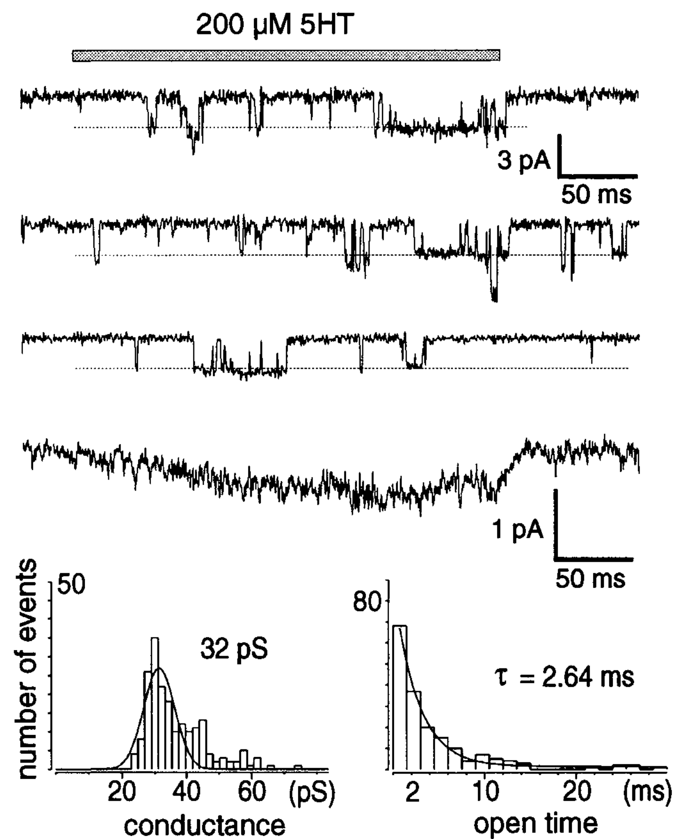

Figure 7. 5-HT–activated 32 pS conductance. *Top three traces*, single-channel currents elicited by application of 200 μM 5-HT to an outside-out patch. *Stippled lines* indicate a calculated chord conductance of 32 pS. *Fourth trace* shows an average over 30 5-HT applications onto the same patch. Histograms show conductances (*left*) and open times (*right*) of all single-channel openings from this patch; mean single-channel conductance, 32 pS; open time constant, 2.6 msec. Membrane potential, −70 mV; intra- and extracellular solution as given in Materials and Methods; reversal potential for Cl^-, 0 mV. Cells prepared at E15, 1 d in culture, large embryonic neuron.

32 ± 17 msec, n = 102; τ = 47 ± 28 msec, n = 84). Hence, the whole-cell superfusion does not permit a solution exchange fast enough to evoke a simultaneous activation of all ion channels, even on the membranes of the relatively small embryonic cells. To compare the activation and desensitization kinetics to those obtained at the synapse measurements using partial superfusion are required.

A postsynaptic current can be terminated by either the decay of the current upon removal, by diffusion of transmitter, or by desensitization. Using partial superfusion of the membrane, we also tested whether fast removal of 5-HT causes a decay of the transmitter-induced current significantly faster than the time constant of desensitization. Therefore, fast removal of transmitter solution was performed as described in Materials and Methods. In cases where the transmitter was removed prior to the beginning of desensitization, the deactivation time constant was only insignificantly smaller (25 ± 1 msec, n = 5, $p < 0.05$, compared to 28 ± 2 msec, paired Student's t test). From these data we cannot conclude, however, that deactivation cannot occur faster than desensitization at 5-HT receptors, since the measured diffusion currents at the open patch pipette give only a lower limit for the time needed for removal of transmitter from the membrane. Nevertheless, from these experiments it can be ruled out that deactivation at these channels occurs dramatically faster than desensitization.

Single-channel recordings

To analyze the conductances underlying the 5-HT–induced whole-cell responses, single-channel openings were investigated in outside-out patches. From 20 cells that showed 5-HT–activated whole-cell currents, single-channel currents were recorded. Application of 200 μM 5-HT for 100–400 msec activated conductances of either 13 or 32 pS. In seven patches, only the 13 pS conductance was found (Fig. 6); 3 patches showed exclusively the 32 pS conductance (Fig. 7) and the remaining 10 patches displayed openings of both conductances at various proportions (Fig. 8). The mean open time of a single-channel opening of the 13 pS conductance was 1.1 ± 0.4 msec (seven different patches) and 3.1 and 2.9 msec for the two patches analyzed that only contained the 32 pS conductance (Figs. 6, 7). The sum over the multiple transmitter applications on the same patch revealed that patches containing only the 13 pS conductance showed a fast rising averaged current that displayed marked desensitization (seven patches). The average activation time (0–100%) of the summated single-channel currents was 10 ± 5 msec (n = 11). In all patches, in which enough records

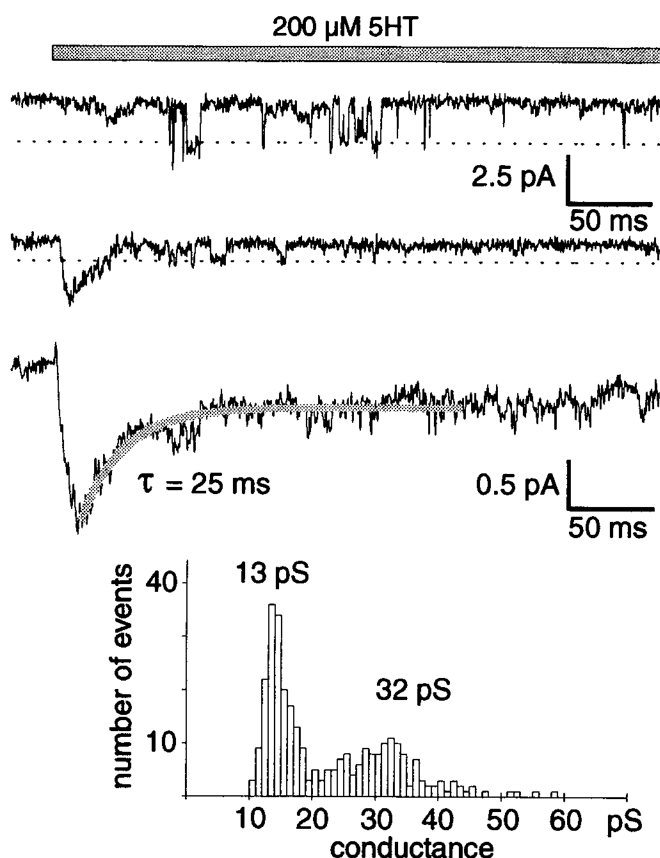

Figure 8. 5-HT–activated 13 and 32 pS conductances in the same patch. *Top two traces*, single-channel currents elicited by application of 200 μM 5-HT to the same outside-out patch. *Stippled line* in top record indicates a conductance of 32 pS; *stippled line* in second record, conductance of 13 pS. *Third trace* shows an average over 22 5-HT applications onto the same patch. *Hatched line* is the result of an exponential fit to the averaged single-channel current. Histogram reveals openings with mean single-channel conductances of 13 and 32 pS, respectively. Membrane potential, −70 mV; intra- and extracellular solution as given in Materials and Methods; reversal potential for Cl^-, 0 mV. Cells prepared at E15, 1 d in culture, large embryonic neuron.

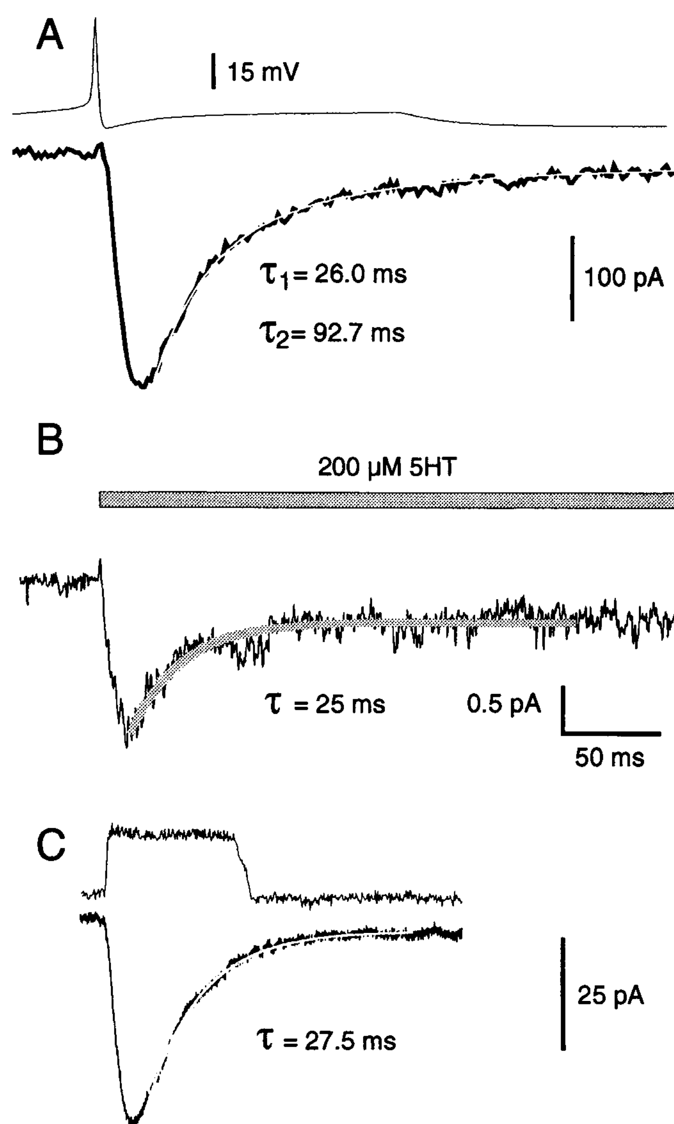

Figure 9. Close correspondence of the desensitization of 5-HT–activated Cl^- currents and the initial decay of the postsynaptic response. *A*, Postsynaptic current in P-cell from an adult leech (*lower trace*) elicited by an action potential in the Retzius cell (*upper trace*). Retzius P-cell synapse for 5 d in culture. Intracellular solution in mM: K^+ 155, Na^+ 4, Ca^{2+} 1, EGTA 10, Cl^- 141, HEPES 10, Mg^{2+}-ATP 2. Extracellular solution in mM: K^+ 4, Na^+ 151, Ca^{2+} 5, Mg^{2+} 1, Cl^- 164, HEPES 10, glucose 20. Membrane potentials, −50 mV. *White line* superimposed on record, double exponential fit to decay of the postsynaptic current (relative amplitudes of both time constants: 68% for τ_1, 38% for τ_2). *B*, Averaged single-channel currents as shown in Figure 8. *C*, Whole-cell current after partial superfusion of the cell as shown in Figure 5. *A–C* drawn to same time scale.

were obtained to reconstruct an average macroscopic current, a time constant of desensitization of 20 ± 11 msec (n = 12) was obtained. All three patches containing only the 32 pS conductance showed no signs of desensitization during 100 to 400 msec of application.

Kinetics of postsynaptic currents

Synaptically induced currents were elicited by action potentials evoked in the presynaptic Retzius cell (Fig. 9*A*). Postsynaptic currents were recorded under whole-cell patch-clamp conditions. The delay time between the peak of the action potential and the beginning of the rise of the postsynaptic current was about 3 msec. The activation time (0–100%) of the postsynaptic current was 25 ± 6 msec (n = 5). Its decay was best fitted with two time constants of equal weight: 27 ± 9 msec and 227 ± 242 msec (n = 5). A comparison of the decay of the synaptic current with the desensitization of the 5-HT–induced current obtained after partial membrane superfusion or with the averaged single-channel recordings shows a close correspondence of the kinetics of decay of all three currents (see Fig. 9) with mean values for fast decay time constants of 27 ± 9 msec (n = 5, fast time constant of decay of the synaptic current), 28 ± 2.4 msec (n = 5, partial superfusion of the soma), and 20 ± 11 msec (n = 11, averaged single-channel currents). The postsynaptic response displays both a fast and a slow time constant of decay. This suggests that both conductances measured in the outside-out patches contribute to the postsynaptic current, the desensitizing 13 pS conductance being responsible for the fast declining phase of the synaptic response, the 32 pS conductance for the slower time constant of decay.

Discussion

The aim of the present study was to find out whether 5-HT–activated ion channels in leech neurons are directly gated. Sec-

ondly, we analyzed 5-HT–evoked single-channel currents and compared the kinetics of their averages with the time course of the postsynaptic current.

Gating of 5-HT–activated ion currents

First, we investigated whether the relatively slow kinetics of the synaptic response with an average time to peak of 25 msec and a fast decay time constant of 27 msec could result from an activation of postsynaptic channels via second-messenger cascades as, for instance, known for muscarinic acetylcholine-receptors or $GABA_B$-receptors (see, e.g., Nicoll et al., 1990, for review).

Our results suggest that the 5-HT–activated channels are not gated by second-messenger cascades, since (1) 5-HT–activated Cl^- currents were observed in 20 outside-out patches for more than 30 min. If second messengers had been involved, they should have been washed out in an outside-out patch, where only a small membrane area faces the artificial pipette solution. (2) Dialysis of the cell with the G-protein inhibitor GDP-β-S or the phosphorylation inhibitor AMP-PNP for up to 2 hr did not result in reduction of the 5-HT–induced Cl^- currents exceeding control values.

Drapeau and Sanchez-Armass (1989) and Sanchez-Armass et al. (1991) suggested an activation of the 5-HT–induced Cl currents by protein kinase A (PKA). This assumption was favored by the observation that an increase in the level of intracellular cAMP-induced Cl^- currents in P-cells, which resembled the 5-HT–induced Cl^- currents with respect to their modulation by tolbutamide and H-8. In our present study, we also observed inward currents upon an intracellular rise of cAMP, which could be carried by Cl^- at the holding potential and transmembrane Cl^- distribution used in our experiments. Furthermore, 5-HT activated Cl^- currents were reduced if neurons had been superfused with IBMX (to 81%) or 8-Br-cAMP (71%) (see Fig. 4*A*). This observation could be interpreted as a competition of cAMP (or PKA) and 5-HT for the same Cl^- channels, such that some of the channels could be directly activated by 5-HT as well as by an increase in cAMP (or phosphorylation mediated by PKA).

Activation and deactivation kinetics of whole-cell currents

The time to peak for the 5-HT–activated current seems relatively slow for a directly gated ion channel as compared to the fast activation times of about 1 msec known, for instance, for averaged single-channel currents of AMPA receptors. However, other directly gated ion channels show similarly slow activation times. For instance, NMDA-induced currents in the vertebrate CNS system display activation times in the range of 15 msec in outside-out patches (Lester et al., 1990). Likewise, relatively slow desensitization time constants were observed for NMDA-activated currents in the whole-cell configuration (100–400 msec; Benveniste et al., 1990; Lerma, 1992) and GABA- activated Cl^- channels of CNS neurons (see, for review, Sivilotti and Nistri, 1991).

The time to peak of the whole-cell current generally showed only a weak dependence on the 5-HT concentration (Fig. 3*A*). This finding and the slow absolute activation time obtained using a nearly saturating 5-HT concentration is, at least in part, due to the sequential activation of Cl^- channels distributed over the cell soma. However, the same experimental setup employed in this study was used to investigate glutamate activated whole-cell currents mediated by AMPA/kainate receptors of cultured rat thalamic neurons comparable in soma size to embryonic leech neurons. In this preparation, the activation times of glutamate-induced whole-cell currents displayed a steep concentration dependence and were below 10 msec with half saturating glutamate concentrations (Leßmann et al., 1992). Thus, it seems very unlikely that the rather slow activation and the weak concentration dependence of the rise times of 5-HT–activated currents simply reflect the rate-limiting soma superfusion. An approximate change in absolute rise time by 10 msec was observed for a 100-fold change in 5-HT concentration in the present experiments (not explicitly shown, but compare Fig. 3*A*). A kinetic scheme explaining the small relative dependence of the rise time on the concentration remains to be worked out.

The experiments comparing the decay of the 5-HT–induced current after fast removal of the transmitter with the time constant of desensitization during continued presence of 5-HT (see Fig. 5) suggest another parallel with what is known from NMDA-activated ion currents: upon fast removal of transmitter the NMDA-induced current shows kinetics of deactivation that are slow compared to the kinetics of the removal of the transmitter. As suggested by Lester et al. (1990), the bound transmitter remains attached to the receptor-channel complex for a time that cannot be influenced by the fast removal of the ligand. Hence, the channel opens multiple times before the slow dissociation of the ligand from the receptor occurs. This finding could be explained by the time-limiting closure of the channels, which either react to the desensitized state or—in the case of deactivation—to the dissociation of the ligand from the receptor (Vyklický et al., 1991). The similarity of the channel closing kinetics of the 5-HT and NMDA-receptor/channel complexes suggested by our present whole-cell recordings needs further confirmation with fast removal of 5-HT from outside-out patches.

Single-channel conductances

As shown in Figures 6–8, the 5-HT–activated currents are composed of two different single-channel conductances in the range of 13 pS and 32 pS. The finding of two different single-channel conductances rises the question whether the observed conductances represent different states of the same ion channel or are due to the gating of different channel proteins. Several indirect observations favor the interpretation that two distinct types of channels are involved: (1) no direct transitions between both conductance levels were obvious. In 1863 channel openings visually inspected, no events were detected, where both types of channels opened simultaneously but closed independently or vice versa. Such events would have been a strong indication for a channel with both conductance levels, since the synchronous opening and closing of two independent channels is extremely unlikely (Jahr and Stevens, 1987). (2) Both amplitudes of openings occur independently in different patches. From 20 patches investigated, 10 showed only one of both conductances. (3) Both conductances showed different kinetic properties. (4) In whole-cell recordings, the peak amplitudes showed a slight inward rectification, which was not observed for the plateau component.

For other transmitter-gated ion channels, such as the nicotinic acetylcholine receptors, different subunit compositions display different single-channel conductances and gating kinetics (Mishina et al., 1986). Different glutamate-receptor subunit compositions have been shown to cause differences in desensitization (Sommer at al., 1990), Ca^{2+} permeability (Hollmann et al., 1991), and voltage dependence of the whole-cell currents (Verdoorn et al., 1991). It remains to be shown, whether two different subunit compositions of 5-HT receptors or alternatively

spliced variants of the same channel gene may explain the two different conductances observed in the present investigation.

Role of the two different single-channel conductances for the dynamics of the synaptic response

Since no fluorescent label for postsynaptic 5-HT receptors is presently available and the postsynaptic ion channels are predominantly located at inaccessible membrane invaginations (see, e.g., Kuffler et al., 1987), we could not directly investigate patches of identified postsynaptic membrane areas. In order to record from cells smaller than 20 μm that allowed us to monitor fast times of activation and desensitization, we used embryonic neurons. To select for Retzius and P-cells, only the 10 largest cells in the dish were recorded from. All results were additionally confirmed on Retzius cells identified by uptake of the autofluorescent 5,7-DHT (see Materials and Methods). The striking similarity between 5-HT–evoked Cl^- currents of adult Retzius and P-cells (Drapeau et al., 1989), postsynaptic responses of Retzius and P-cells (Liu and Nicholls, 1989; own unpublished observations), and the homogeneous population of 5-HT–induced whole-cell currents recorded from unidentified embryonic neurons justifies a comparison of the results obtained from embryonic cells with those of postsynaptic P-cells.

Since at neuromuscular junctions the same types of channels are present at subsynaptic and extrasynaptic sites, however, at different relative densities (Brehm and Henderson, 1988), it is most likely that our extrasynaptic records also contain the channel subtypes expressed in the subsynaptic membrane.

The comparison of the times to peak and time constants of desensitization yielded striking parallels between the whole-cell currents measured using partial superfusion of the cell, averaged single-channel currents, and postsynaptic responses (Fig. 9). In addition to the comparable times to peak and decay time constants, the peak component of the synaptic response shows the same outward rectification as the peak component of the transmitter-induced current (compare Drapeau and Sanchez-Armass, 1988, and Fig. 2*C*). As shown in Figures 6–8, the activity of the 13 pS conductance could explain the fast time to peak and time constant of desensitization of the whole-cell current, while the opening of the 32 pS conductance could account for the slow component of the transmitter-induced current. One could interpret this result such that the 13 pS conductance is responsible for the fast activation and its desensitization for the initial decline of the synaptic responses while the deactivation of the second 32 pS conductance underlies the slow time constant of decay seen in postsynaptic recordings. Apart from differences in presynaptic release parameters, the variable expression of both types of receptors could explain why some synapses show more tonic postsynaptic responses upon Retzius cell depolarization (see, e.g., Dietzel et al., 1986; Stewart et al., 1989), while other synapses display more dominant "phasic" responses. Interestingly, the time to peak of the synaptic response was slower than the rise times that can be reached with fast superfusion of single channels (10 ± 5 msec), or partial cell surfaces (18 ± 4 msec). One explanation for this finding could be that the slower rising phase of the synaptic current is caused by inadequate clamping of the larger postsynaptic membrane of the adult cells. On the other hand, the postsynaptic current could have been delayed due to conduction of the action potentials across a presynaptic fiber network with release sites distributed over the postsynaptic cell (see Fuchs et al., 1982; Kuffler et al., 1987), since we did not attempt to synchronize release by voltage clamping the presynaptic cell. Due to asynchronous transmitter release, in this case, the miniature postsynaptic currents should be significantly faster than the ensemble postsynaptic current. Since miniature postsynaptic potentials show, however, the same kinetics as the postsynaptic response (Liu and Nicholls, 1989), this explanation seems less likely.

Desensitization and termination of the synaptic response

The close correspondence of the fast decay time constant of the synaptic response with the fast time constants of desensitization in whole-cell and single-channel recordings raises the question whether the desensitization of the postsynaptic current could contribute to the termination of synaptic transmission at this synapse. Channel closure due to removal of transmitter dominates the decay of the postsynaptic response at the neuromuscular endplate (see, e.g., Magleby and Stevens, 1972; Katz and Miledi, 1975; Franke et al., 1991) and the same seems to be true for GABAergic synapses (see, e.g., Segal and Barker, 1984; Thompson and Gähwiler, 1992). For central glutamatergic synapses, however, the picture is still less clear: although at some synapses desensitization is slower than the decay of the synaptic response (Colquhoun et al., 1992) in other preparations transmitter-activated channels desensitize with the same time constant as the decay of the synaptic current (see, e.g., Trussell and Fischbach, 1989; Leßmann and Dietzel, 1994) and substances that prolong the desensitization also prolong synaptic responses (see, e.g., Tang et al., 1991; Vyklický et al., 1991; Leßmann et al., 1992; Yamada and Rothman, 1992). However, these substances also influence the decay of the current after transmitter removal. To definitely answer whether desensitization contributes to the decay of the postsynaptic current at the Retzius P-synapse, drugs are needed that selectively influence desensitization without disturbing deactivation. Our present results show that the time constant of desensitization is close to the decay time constant of the fast decaying component of the postsynaptic current. It is an intriguing finding that at several types of synapses both time constants closely match. Whether fast desensitization could be used as a safety factor to limit postsynaptic currents in certain cells remains to be shown.

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