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Mechanisms of up-regulation of single calcium channels by serotonin in *Helix pomatia* neurons

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Abstract

Action of serotonin (5-HT) on single Ca^{2+} channel activity was studied in identified neurons of snail *Helix pomatia*. Only one type of Ca^{2+} channels of 5 pS unitary conductance was determined under patch-clamp cell-attached mode. Kinetic analysis have shown a monotonically declining distribution of channel open times (OT) with mean time constant of 0.2 ms. The distribution of channel closed times (CT) could be fitted by double-exponential curve with time constants 1 and 12 ms. We established that 5-HT acts on Ca^{2+} channel activity indirectly via cytoplasm. 5-HT prolonged the OT (up to 0.3 ms) and shortened the CT proportionally for both constants to 0.4 and 6 ms correspondingly. A conclusion is made that enhancement of Ca^{2+} macro-current by 5-HT is determined by kinetic changes, increase of the number of active channels, and increase of the probability of OT. At the same time the transmitter did not affect the unitary channel conductance.

Keywords: Serotonin; Single channels; Calcium channels; Snail neurons

High voltage-activated calcium channels play an important role in triggering and modulation of most metabolic processes in the nerve cells and can be subjected to significant functional regulation by a wide variety of physiological and pharmacological agents. The natural neurotransmitters are one of the classes of agents which affect Ca^{2+} channel activity. In this respect serotonin (5-HT) belongs to the phylogenetically oldest of them. Several publications were devoted to 5-HT influences on Ca^{2+} channel activity [1–9], which showed that 5-HT is capable to increase Ca^{2+} membrane conductance in neurons. Our previous data obtained from the pedal motoneurons of snail *Helix pomatia* showed that 5-HT significantly increased the magnitude of Ca^{2+} current (I_{Ca}) in these neurons, whereas other majority of neurons had no such sensitivity [1–4]. Detailed analysis of the mechanism of 5-HT stimulatory action on Ca^{2+} channels of pedal neurons has shown an involvement of cAMP-dependent phosphorylation in this process [1,5]. However, the molecular mechanisms of changes in single calcium channel activity during their phosphorylation are still unclear. In the present experiments we aimed to analyze these mechanisms on the level of functioning of single calcium channels during their 5-HT-induced and cAMP-mediated up-regulation in identified pedal neurons as model.

Materials and methods

Ca^{2+} currents were measured using the whole-cell and cell-attached configuration of single-electrode patch-clamp technique [10,11] on identified neurons from pedal ganglia of snail *Helix pomatia*. Cell isolation and identification were described previously in the details [1,12,13]. Resistance of the micropipette was measured in Ringer solution and accounted 20–40 MΩ. To decrease a spurious capacity of the recorded pipette, it was covered with hydrophobic polymer—silgard (Dow Corning, USA)—as close as possible to the tip. With the same purpose an extracellular solution was held at a lowest level in the chamber. Enzymatically dissociated cells were placed on degreased coverslip surfaces and kept in Ringer solution during 30 min before experiments that provided their adhesion to the glass. Basic Ringer solution contained (mM): NaCl, 100; $CaCl_2$, 10; $MgCl_2$, 5; Tris–HCl, 10; KCl, 5; pH, 7.6. Intracellular pipette solution contained (mM): ATP-4; CsCl, 80; $MgCl_2$, 2; Tris–aspartate, 40; Teophyline, 5; pH, 7.3. Ca-30 solution included (mM): $CaCl_2$, 20; $MgCl_2$, 4; Tris–HCl, 25; TEA–Br, 80; pH, 7.6. Ca-100 solution contained (mM): $CaCl_2$, 100; $MgCl_2$, 2; HEPES, 10; pH, 7.6.

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The indefinite value of the cell membrane potential during cell-attached patch-clamp measurements makes difficult the estimation of the membrane potential at the clamped membrane fragment. To overcome this difficulty we used direct microelectrode measurements which showed the mean value of resting potential of isolated neurons about −100 to −90 mV (Fig. 1A) in Ca-30 and −30 to −30 in Ca-100 solutions. These values were used for corrections of real value membrane potential. The recording of Ca^{2+} single channel activity was carried out in frequency range 1–2 kHz, when the background noise accounted for 0.05–0.06 pA. Measurements were conducted with amplifier EPC-9, with software "Pulse," "HEKA-Electronik," Germany. Data processing as well as their acquisition were materialized by computer Power PC Macintosh 7100/66, "Pulse" and special software developed by EAL written in macros language of "IgorPro" program, "WaveMetrics," USA. According to chosen algorithm of data processing, the identification of channel opening (idealization of current sweeps) was derived according to threshold line adjusted on half-level between line of zero current and the line which corresponded to mean magnitude of unitary current magnitude during channel opening [14]. It was considered that in those moments when the values of current in the pulse were equal or exceeded the half value, the channel was in open state, and in another case it was closed. Approximations of experimental histograms by mono- or double-exponential functions were made by the method of a least-square value with the assistance of the above-mentioned program "IgorPro". Data presented as mean ± SEM.

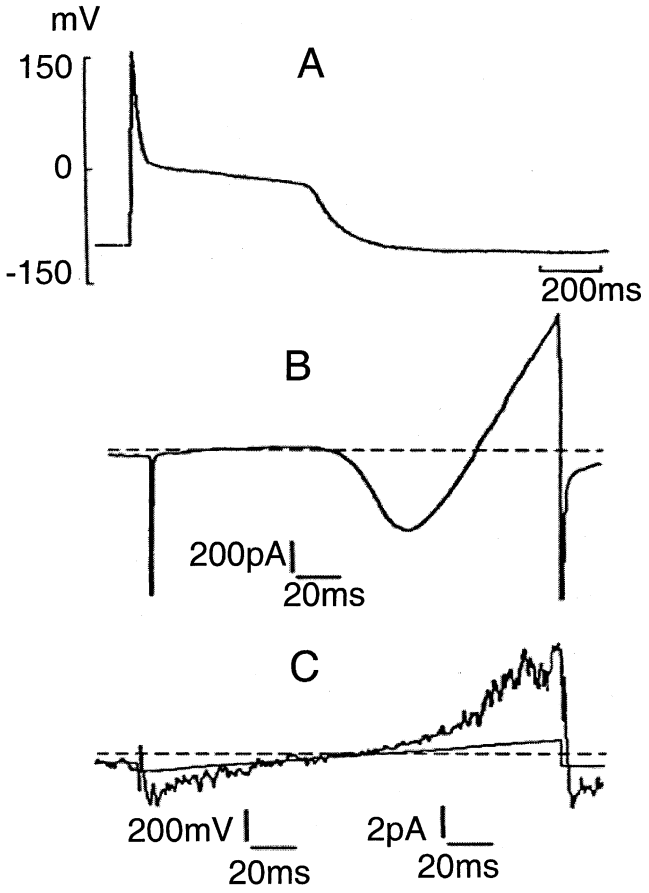

Fig. 1. Calcium conductance in isolated neuron of *Helix pomatia*. Calcium action potential (A), original records of Ca^{2+} channel activity obtained in response to ramp stimulation of membrane fragment from potential −100 to +100 mV in whole-cell (B) and cell-attached (C) configurations measured in the same neuron.

Serotonin was from SERVA, Germany. All other reagents were from Sigma, USA.

Results

Calcium conductance in molluscan neurons

Studies of Ca^{2+} membrane conductance on the level of single channel activity were performed on freshly isolated identified molluscan neurons from pedal ganglia; these preparations were chosen due to detailed exploration of their Ca^{2+} currents on macro-level [1–4] by using the intracellular dialysis method [10]. Background electrical activity of isolated neurons was usually absent or was marked by rare spontaneous action potentials (AP), Fig. 1A.

The investigated neurons had a resting potential about −100 mV in Ca-30 solution (Fig. 1A), that was very close to its physiological values. Mean magnitude and duration of AP in Ca-30 solution accounted for 221 ± 34 mV ($n = 4$) and 0.9 ± 0.1 s ($n = 4$). The AP revealed a plateau with magnitude and duration 80 ± 23 mV ($n = 4$) and 0.4 ± 0.2 s ($n = 4$). The AP plateau was of calcium nature as it was changed following the changes in external Ca^{2+} concentration. Ionic membrane conductance of isolated neurons that was measured under voltage clamp mode, confirmed the presence of Ca^{2+} channel activity. Fig. 1B demonstrates a current–voltage relation (I–V) of integral Ca^{2+} current evoked by ramp protocol (−100 to +100 mV) from holding potential −80 mV with 200 ms pulse in whole-cell configurations from the same neuron. In these experiments the recordings were performed with "intracellular" pipette solution. Fig. 1B shows a clear peak corresponding to the maximum of I–V relationship of integral Ca^{2+} current (I_{Ca}).

Single calcium channels activity

Despite numerous attempts to use different combinations of enzymatic regimes for treatment of snail neurons, it was very problematic for many studies to obtain the stable gigaohm contacts on freshly isolated *Helix* neurons [15]. These difficulties could be connected with significant asperity of *Helix* neuronal surface caused by the remaining of connective tissue. However, we avoided such difficulties without particular problem. Positive adhesion of the cells to the coverslips in vast majority of cases guaranteed a formation of stable gigaseal between the membrane of neurons and the tip of the recording pipette in cell-attached configuration. In this series of the experiments we used Ca-100 pipette solution. Stimulation of the membrane fragment by the ramp-pulses allowed to detect the Ca^{2+} channel "hit" in the tested patch in cell-attached mode. Stimulation was

executed from −100 mV to maximal depolarization of +100 mV (Fig. 1C). An example of single calcium channel activity recorded from the same cell as in Figs. 1A and B shows single channel currents evoked by ramp depolarization of membrane fragment. We were under necessity to use pipettes with quite small diameter of the tip, and the resistance of such pipettes accounted for 20–40 MΩ. A significant number of channels were active in the tested membrane fragment (10–15 per fragment and more) if the pipettes with lower resistance were used. Still we were frequently under the necessity to work with membrane fragments containing several active channels (Fig. 4A). In these cases the number of channels could be easily determined from the amplitude histograms, since during synchronous opening of several channels the total amplitude was always a multiple to the value of the unitary event with smallest amplitude. Fig. 2C shows the original records of current flowing through single calcium channel of isolated pedal neuron that were obtained at membrane potential −30 mV in Ca-100 patch pipette solution in configuration cell-attached. As it could be seen from the figure, the activity of channel could have also a burst pattern that is usually characteristic for Ca^{2+} channel activity.

Despite using the neurons which we identified as 'pedal' by their location in pedal ganglia and their morphological properties, we also tested calcium current sensitivity of chosen cell to 5-HT. Usually cell-attached recordings preceded the whole cell recordings from the same neuron. Fig. 2A shows an example of whole cell recording of Ca^{2+} current in pedal neuron. The current traces obtained in control (trace marked by open circle) and during action of 10 μM 5-HT (dark circle) are superimposed. As it could be seen 5-HT evoked an increase of Ca^{2+} current in a wide range of depolarization potentials (Fig. 2B) and this effect was reversible and persistent during prolonged period of recordings (up to 2 h) Fig. 2A. Analysis of this effect on macrocurrent was described previously in details [1,5].

I–V relationships of single Ca channels obtained in control (open circles) and during application of 5-HT (closed circles) are presented in Fig. 3. In these experiments we stimulated the membrane fragment by holding the membrane potential on necessary depolarization level. Recordings were fulfilled with 300 ms stimuli at 0.33 Hz frequency, 200 stimulations for every voltage points were applied. Fig. 3 shows that conductance of the channel, determined as the slope of *I–V* relationship proved to be very low and was about 5 pS. As it can be seen, application of 5-HT did not change the channel conductance (dark circles).

An example of behavior and statistical analysis of single Ca channel activity recorded at membrane potential −30 mV is shown in Fig. 4. The histogram certifies the activity in only one type of channel. The location of histogram peak was about 0.5 pA at −30 mV, that corresponded to the amplitude of unitary current for the chosen value of membrane potential.

For analysis of kinetic properties of single Ca^{2+} channels, the histograms of open and closed times of the channel were constructed. Fig. 5A presents the histogram of open times of Ca^{2+} channels for membrane potential −30 mV and its approximation with a monoexponential curve with the least-square method. Time constant (τ_o) of the exponent had the mean value of 0.2 ms (Fig. 5A). As it can be seen from the figure, the

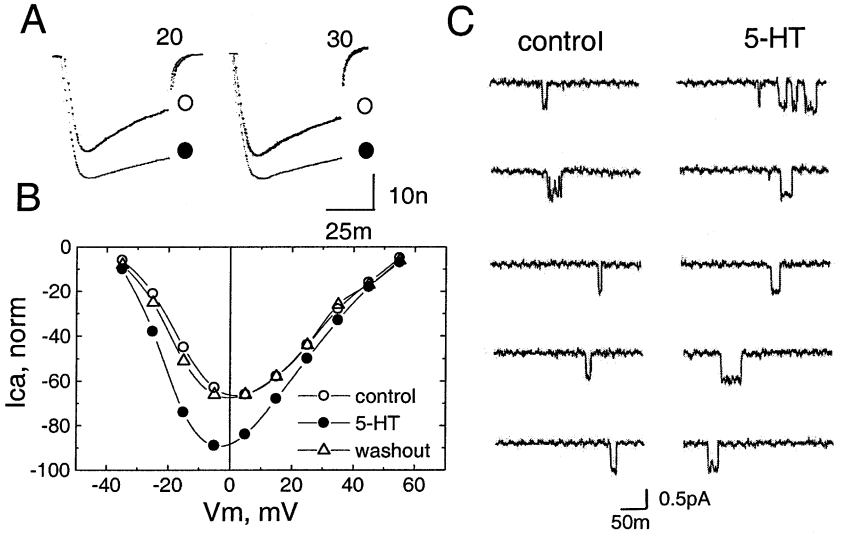

Fig. 2. Stimulatory effect of 5-HT on calcium current in identified pedal neuron. (A) Superimposed current traces recorded in control (open circle near traces) and after application of 10 μM 5-HT (dark circles) at different time of the experiment (pointed near traces). (B) *I–V* relation obtained in whole-cell configuration in control and after application of 10 μM 5-HT in the pedal neuron. (C) Single calcium channel activity of pedal neuron in control (left column) and after application 5-HT (right column).

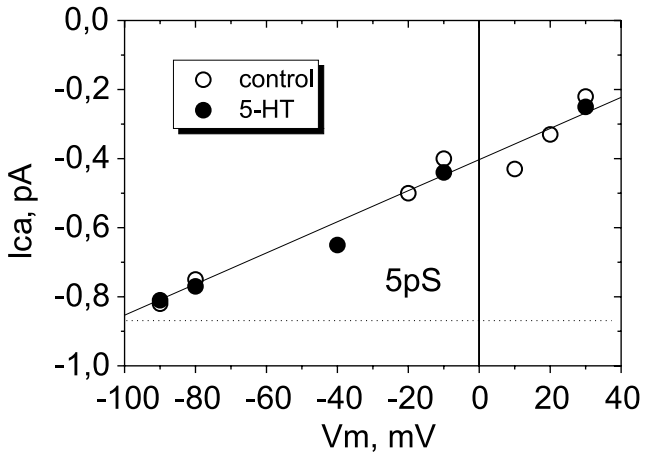

Fig. 3. Current–voltage relationship of single Ca^{2+} current of pedal neuron. 100 mM Ca^{2+} in patch pipette was used as charge carrier. Data obtained in control (open circles) and after 5-HT (dark circles) application. The value of conductance is presented near the *I–V* curves.

maximal time when the channel was open corresponds to 12 ms. The histogram of closed state of channel had a more complicated pattern (Fig. 5B) reflecting the presence at least two characteristic times. The main amount of events fell into the initial part of the histogram (0–4 ms), after which a long low amplitude sector was observed up to times of the order of 40 ms. This initial "fast" component characterized all closing of the channel within the bursts and could be described by an exponent with time constant (τ_{c1}) 1 ms (Fig. 5B). The "slow" component of the distribution (τ_{c2}) which describes interburst channel closing was equal to 11.5 ms. Insignificant contribution of the "slow" component to the summary histogram of closed channel times and their significant stretch certified that the amount of channel closing within the burst significantly exceeds the interburst intervals. The ratio of open and closed channel times points out on the pattern of intrabursting "blinking" of the channel, namely that after short opening the channel stands in shut state for time that exceeded the channel open time by about five times.

Action of serotonin on single calcium channels

Serotonin, a natural neurotransmitter which is famed due to its possibility to influence calcium membrane conductance of molluscan pedal neurons [1–3,5] was chosen to estimate the influence of cAMP-mediated phosphorylation on single calcium channels. Previously we have shown the capability of 5-HT to increase Ca^{2+} macro-current in pedal neurons through involving of cAMP-dependent cascade of phosphorylation [1,5]. Fig. 2C presents the original records of the single Ca^{2+} channel activity in cell-attached configuration before and after 5-HT application in the bath solution. During such application 5-HT could not directly contact the tested membrane fragment, as the last was tightly restricted from chamber solution by the walls of recording micro-

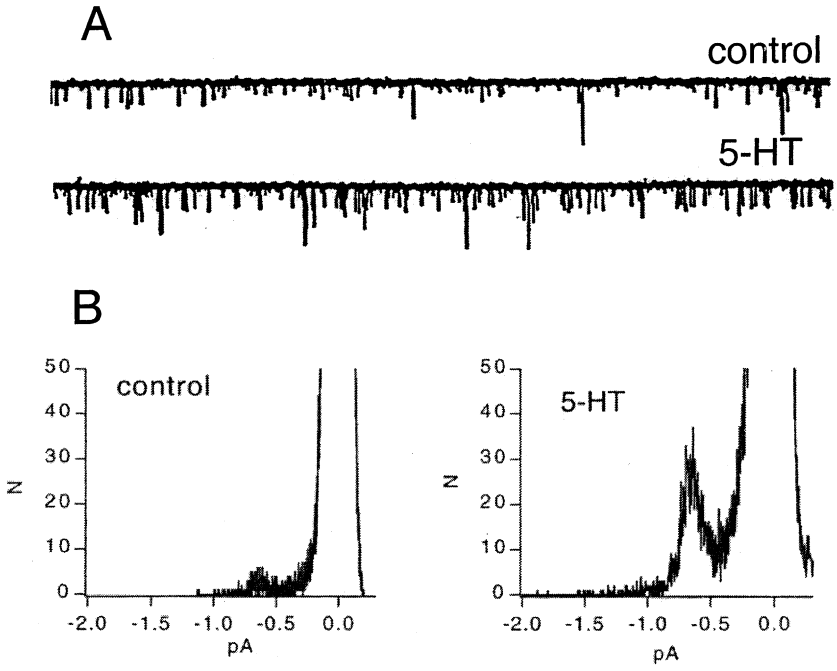

Fig. 4. Increase of frequency of Ca^{2+} channel opening induced by 5-HT. (A) Current traces induced by 300 ms membrane depolarizations from resting potential to −30 mV in control and after application of 10 μM 5-HT into external solution. 200 records are presented in time order corresponding to the consequence of their registration. (B) Amplitude histogram of single channel recordings obtained in control and after 10 μM 5-HT application into the extracellular solution. Depolarizing potential −30 mV.

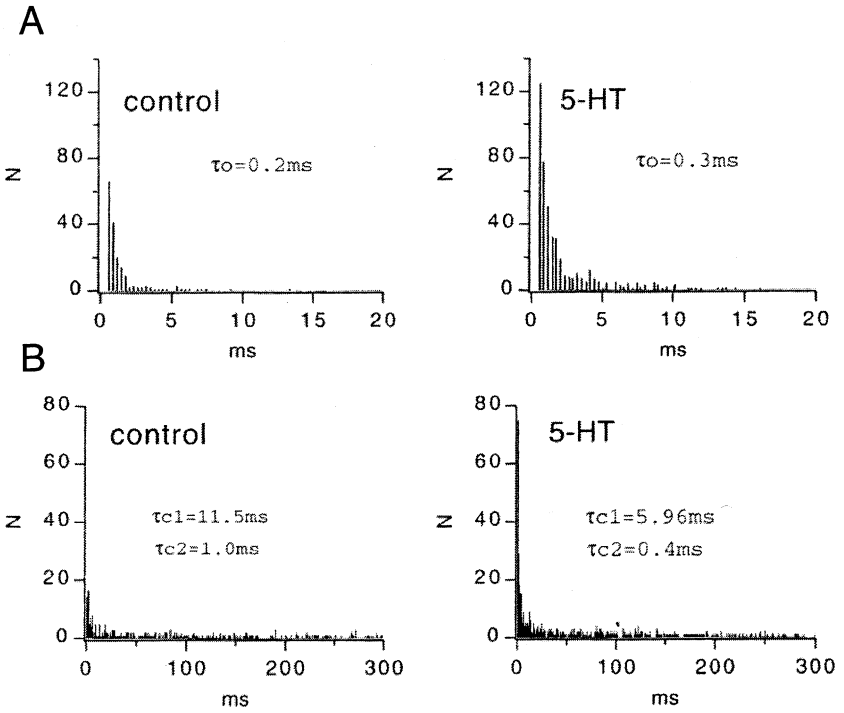

Fig. 5. Open (A) and closed (B) time histograms of single calcium channel activity in pedal neuron. Histograms were obtained in control (left parts) and after 10 μM 5-HT application (right parts).

pipette. In this case, the effect could be mediated only through cellular cytoplasm. The figure shows, that unitary current before and after agonist application in bath solution had practically the same magnitude (Figs. 2C and 4B). A significant increase of number of channel's openings was obvious (Fig. 4A). The latest is reflected by a significant increase of the magnitude of the peak of the amplitude histogram—in the presence of 5-HT (peak was increased by sevenfold). The amplitude histograms clearly testify that, firstly, the number of channel openings or number of active channels was changed by 5-HT. This could be observed when several active channels hit into the same patch. In this case, 5-HT induced about twice increasing of the number of channel openings (Fig. 4A). Secondly, 5-HT did not induce the appearance of activity of a different type of Ca^{2+} channels (Fig. 4B). Finally, the amplitude histogram points out the unchanged unitary conductance of the channel, as the localization of its peak was not changed after 5-HT application at given potentials (Figs. 3 and 4B).

To characterize the changes in kinetic properties of Ca^{2+} channel after 5-HT application, we constructed the histograms of living times of the channel in open and closed states. The particularities of the histogram are distinctly notable on Fig. 5. Similarly to control conditions, the histogram of open time distribution could be satisfactory fitted with a single exponent. However, its time constant τ_o was increased after 5-HT application by one and half times in comparison to control value and accounted for 0.3 ms (Fig. 5A). The fitting of the histogram of closed time distribution, similarly to control, could be better achieved by using two exponents with time constants $\tau_{c1} = 0.4$ ms and $\tau_{c2} = 5.9$ ms, that is about twice less as compared with similar values obtained in control conditions (Fig. 5B). In this case, intrabursting "blinking" of the channel occurred with approximately similar durations of channel being in open and closed states.

Discussion

The presented investigation of high threshold Ca^{2+} channels in isolated identified pedal neurons of the snail *Helix pomatia*, conducted on the level of single channel activity, showed the presence of only one type of Ca^{2+} channels with 5 pS conductance under using of 100 mM Ca^{2+} as charge carrier in the pipette solution. This value coincides with the value obtained on the same object earlier by Lux and co-authors [15]; however it is close to the value of low voltage-activated channels and markedly less than the conductance of high voltage-activated channels in mammalian neurons [14]. At the same time, kinetic characteristics of the channel activity proved to be more close to characteristics of high voltage-activated Ca^{2+} channels in mammalian neurons. Thus, time constant of channel living in the open state obtained in our experiments accounted for 0.2 ms, while the same constant for mammalian neurons was in the range of 0.16–1.2 ms [16]. The initial "fast" component of time of

channel living in closed state that characterizes channel closings within the bursts ($\tau_{c1} = 1$ ms), was also close to mammalian τ_{c1} (0.9–1.3). The slow time constant describing interbursting closing ($\tau_{c2} = 12$) also displayed properties similar to corresponding constants in mammalians (7–10.2 ms) [14,16]. Thus, we can state that the functional properties of single high voltage-activated Ca^{2+} channels of molluscan neurons are enough close to the properties of mammalian single Ca^{2+} channels with the exception that molluscan channels posses lower unitary conductance.

As it was mentioned in Introduction, the determination of the molecular mechanism that mediates the increase of Ca^{2+} conductance of the neuronal membrane during the process of 5-HT-induced and cAMP-mediated phosphorylation deserves a special interest [17]. Several variants seem to be possible in this mechanism:

1. 5-HT induces an increase of the number of active channels.
2. 5-HT increases the probability of opening of active channels.
3. 5-HT changes the kinetic properties of the active channel.
4. 5-HT activates channels of different type—"specific" for regulation by phosphorylation.

To make a distinction between these possibilities is possible only on the level of single channel activity. Our experiments showed that 5-HT acts on the channel molecule indirectly, as it was effective during application into the bathing solution, which had no contact with the tested membrane fragment located in patch pipette. This finding excludes the possibility of direct binding of 5-HT with the channel molecule and testifies that the effect is really mediated through a cytoplasmic messenger—cAMP-dependent cascade of phosphorylation—the participation of which was in detail investigated previously on the level of macro-currents in these cells [1,2,5]. The presented data also conclusively confirm that the increase of Ca^{2+} macro-current by 5-HT is not due to the appearance of another type of Ca^{2+} channels. The presence of single peak on amplitude histogram before and after 5-HT application (Fig. 4B) testifies this assumption. The same histogram and measured current conductance also showed the second property of the effect, namely unchanged unitary conductance of the active channel during 5-HT action—the peak of the histogram corresponded to the same value of current in control and after 5-HT application as well as I–V relationships. Finally, the third conclusion following from this histogram is more than the sevenfold increase of the probability of channel to stay in open state under the action of 5-HT. The time of single channel opening stay was prolonged by one and half times and channel stay in closed state was reduced about twice. In addition, there is a substantial increase of the number of active channels of the same type.

Obtained data are in agreement with previously published data about the mechanism of increase of Ca^{2+} macro-current in muscle cells induced by cAMP-dependent phosphorylation [6,18,19]. In particular Reuter [18] explained the cAMP-dependent increase of Ca^{2+} macro-current by isoprenaline through moderate lengthening of channel openings and shortening of channel shutting. On the other hand, several authors explained cAMP-induced increase of I_{Ca} by increased probability of channel to stay in open state without marked changes in channel kinetics [6,20–22]. Our data certify the instantaneous participation in the implementation of cAMP-dependent regulation of Ca^{2+} channel in molluscan neurons of the several mechanisms, which might be called—"probability," "kinetics," and "number." However the question about what of these mechanisms is more important and why is still to be studied.

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