The Properties of Carbachol-Activated Nonselective Cation Channels at the Single Channel Level in Guinea Pig Gastric Myocytes

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ABSTRACT—We investigated the properties of carbachol (CCh)-activated nonselective cation channels (NSC_CCh) at the single channel level in the gastric myocytes of guinea pigs using a magnified whole-cell mode or an outside-out mode. The channel activity (NPo) recorded in a magnified whole-cell mode increased with depolarization (from −120 to −20 mV) and had the half activation potential of −81 mV under the symmetrical 140 mM Cs⁻ condition. The single channel conductance depended upon the extracellular monovalent cations with the order of Cs⁻ (35 pS) > Na⁺ (25 pS) > Li⁺ (21 pS). The channel activities markedly diminished or disappeared when external Cs⁻ was replaced with Na⁺ or N-methyl-D-glucamate (NMDG⁺).

With Cs⁻ and Na⁺ as external cations, the channel showed a monotonic increase in NPo with the increased mole fraction of Cs⁻ over Na⁺, and it had an intermediate conductance value in solution containing 67% Cs⁻ with 33% Na⁺. These data suggested that the extracellular monovalent cations regulate the whole-cell current of NSC_CCh by modulating both the open state probability and the unitary conductance, and there is one binding site for the extracellular cations within the pore.

Keywords: Nonselective cation channel, Single channel, Carbachol, Gastric smooth muscle

It is well known that acetylcholine (ACh) and carbachol (CCh) induce depolarization and consequently cause the contraction of mammalian gastrointestinal smooth muscle. In mammalian gastrointestinal smooth muscle cells, muscarinic agonists bind to M2 and M3 muscarinic receptors (1, 2) and then activate a non-selective, voltage-sensitive inward current (3). On the other hand, muscarinic agonists inhibit the outward current in the toad stomach (4). Since the first report by Benham et al. (3), the characteristics of non-selective cationic channels activated by ACh or CCh (NSC_ACh or NSC_CCh) have been reported. Firstly, the channel is voltage-dependent. Initial reports of the voltage-dependent activation indicated a half maximal activation potential (V₀/2) of −50 mV and slope factor (k) of −15 mV (3, 5). However, the V₀/2 value depended upon the concentrations of agonists and the type of extracellular cations used for current recordings (6, 7). Secondly, the channel shows inward rectification. Thirdly, its activation depends on G-protein activity (8, 9). We also showed that the Go type among GTP-binding proteins is responsible for activating the channel (10). Fourthly, the unitary conductance was about 25 – 30 pS (1 – 3).

Inoue et al. (11) recorded single channel currents with low concentrations of ACh (<10 μM) in the guinea pig ileum, and Vogalis and Sanders (12) recorded them in the canine stomach. We also reported that single channel activities could be resolved in whole cell recordings after largely suppressing the gross inward current by repeated CCh applications leading to desensitization (13). In this paper, we used this method to investigate the properties of NSC_CCh at the single channel level in gastric myocytes of guinea pigs and compared them with those at the whole-cell level, which we previously reported (6).

MATERIALS AND METHODS

Single cell dissociation

Gastric myocytes were isolated enzymatically from the antral circular layer of the guinea pig stomach as described previously (6). Briefly, guinea pigs of either sex weighing 300 – 350 g were exsanguinated after stunning. The antral
part of the stomach was cut and the circular muscle layer was dissected from the longitudinal layer using fine scissors and was cut into small segments (2 – 3 mm). The tissue chunks were then incubated for 20 – 25 min at 37°C in a digestion medium that was a Ca²⁺-free Tyrode’s solution (see below) containing 0.15% collagenase (type IA; Wako, Osaka or Sigma, St. Louis, MO, USA), 0.05% dithiothreitol, 0.1% trypsin inhibitor and 0.2% bovine serum albumin. Single myocytes were dispersed by gentle agitation of the digested segments with a wide-bored glass pipette. Isolated myocytes were kept at 4°C until use. All experiments were carried out within 10 h of harvesting cells and at room temperature.

**Electrophysiological recordings**

Membrane currents were measured with an Axopatch-1D patch-clamp amplifier (Axon Instrument, Foster City, CA, USA) filtered at 5 kHz. Glass pipettes with a resistance of 2 – 4 MΩ were used to make a giga-seal. The pClamp v.6.0.2 and Digidata-1200 (both from Axon Instrument) were used for the acquisition of data and applying command pulses. Openings of single channels were discernible under magnified whole-cell recording condition as described previously (11, 13). In the whole-cell configuration, a long-lasting application of 50 – 100 μM (20 – 30 min) CCh induced a marked desensitization of I_C, the low channel activity permitting single channel recordings. After achieving the whole-cell configuration, pulling away the pipette resulted in the formation of outside-out membrane patches. In this configuration, unitary currents were amplified and filtered at 1 kHz with the Axopatch 1-D amplifier. The data were stored on a digital tape recorder (DTR 1204; Biological, Clai, France) for later analysis. Recorded data were played back and digitized using Digidata 1200 at 1 or 5 kHz and filtered low-pass at 0.5 or 1 kHz for illustration. Using the back and digitized using Digidata 1200 at 1 or 5 kHz and filtered low-pass at 0.5 or 1 kHz for illustration. Using the back and digitized using Digidata 1200 at 1 or 5 kHz and filtered low-pass at 0.5 or 1 kHz for illustration.

**Results**

In a magnified whole-cell mode under the symmetrical 140 mM Cs⁺ solution, the single channel activities could be easily recorded (Fig. 1). We usually used a magnified whole-cell mode because we could record single channel activities more easily in a magnified whole-cell mode than in an outside-out mode. In the magnified whole-cell mode, the open probability of the channels increased as the membrane potential was depolarized (from −120 to −20 mV) and was fully activated at around −40 mV. The mean amplitude of the single channel current increased at more negative potentials, but the absolute number of openings decreased. At the calculated reversal potential (approximately 0 mV), no detectable channel activity was recorded. The outward currents at above 0 mV are not as large as inward currents at the hyperpolarized region (inward rectification). However, the mechanism of inward rectification is still unknown. We have tried to record outward currents in the absence of intracellular Mg²⁺ or polyamines, but we could see the inward rectification at single channel currents or whole-cell currents even under these conditions. Over 0 mV, we could not resolve the open and closed state of the channels. Thus it was excluded from the analysis (Fig. 1A). The calculated unitary conductance obtained between −120 and −40 mV was 34 pS and reversed at +1.7 mV (included in Fig. 2D). The channel activities (NPo) between −120 and −40 mV were calculated from the all-point histogram and plotted against membrane potential (Fig. 1B). When we fitted data with a Boltzmann equation, the line had the V1/2 of −81.1 ± 2.5 mV with the k of −13.8 ± 2.6 mV (n = 3). This result shows that NSC_Cch is a voltage-dependent cationic channel. Such a result is consistent with those of whole cell currents in the guinea pig stomach (−77 mV at 50 μM.
CCh) (6) and the guinea pig ileum (~70 mV at 3 μM CCh and ~106 mV at 300 μM CCh) (7) under Cs⁺ external solution, although \( V_{1/2} \) has a more depolarized value in the rabbit jejunum (3) and the guinea pig ileum (5) under Na⁺-rich external solution.

With 140 mM KCl bathing solution and 140 mM CsCl pipette solution containing 1 μM CCh, we could record single channel currents in the cell-attached mode in only 2 out of 121 cells. The probability of channel opening decreased in the hyperpolarized range, and the calculated conductances were 32 and 37 pS, respectively (data not shown). When we plotted the open probability of the channels obtained in a cell-attached mode, the value of \( V_{1/2} \) was ~52 mV (data not shown).

In Fig. 2, the single channel activities were recorded in an outside-out or a magnified whole-cell mode in different external cationic solutions. Before the application of CCh, there was no channel activity. When CCh was applied, it induced the opening of the channel. Figure 2A shows the single channel activity recorded in the outside-out mode with symmetrical 140 mM Cs⁺ as a charge carrier. The channel clearly showed bursting activities. The calculated values of the single channel conductance and the reversal potential were \( 34.8 \pm 0.4 \) pS and \( -1.5 \pm 2.6 \) mV (n = 3) (Fig. 2D). These values were similar to those obtained from the magnified whole-cell mode (34.3 ± 0.3 pS and 4.6 ± 1.4 mV, n = 4) (Fig. 2D). These results give evidence that there was no difference between the modes used (an outside-out and a magnified whole-cell mode) to record single channel currents. When we used Na⁺ or Li⁺ as an external charge carrier, in the magnified whole-cell mode, the single channel conductance and reversal potential were \( 25.3 \pm 2.4 \) pS and \( 6.3 \pm 2.7 \) mV for Na⁺ (n = 3) and \( 20.8 \pm 0.9 \) pS and \( 0.3 \pm 0.1 \) mV for Li⁺ (n = 3), respectively (Fig. 2: B–D). These results clearly show that the channel conductance depends on the permeating cations.

The extracellular monovalent cation determined open probability as well as the unitary conductance. After the current was activated by 10 μM CCh, different extracellular cationic solutions were applied (Fig. 3). When we replaced external Cs⁺ solution with NMDG⁺ solution, both in a whole-cell mode and an outside-out mode, the channel
activity disappeared completely (Fig. 3: A and C). When we replaced external Cs\(^+\)/G2b solution with Na\(^+\)/G2b solution, the channel opened with a low opening frequency and brief open time. In two outside-out patches (at G2d 60 mV), NP\(_o\) decreased from 0.42 and 0.49 for Cs\(^+\)/G2b solution to 0.009 and 0.011 for Na\(^+\)/G2b solution. The single channel activities were recorded in a magnified whole-cell mode and compared in Fig. 3B. The NP\(_o\) obtained from a magnified whole-cell mode were 0.74 ± 0.03 (n = 10) for Cs\(^+\), 0.12 ± 0.02 (n = 5) for Na\(^+\), and 0.61 ± 0.07 (n = 4) for Li\(^+\) solution, and no detectable channel activities were recorded in NMDG\(^-\)/G2b solution (Fig. 3B). The NP\(_o\) significantly (P<0.05) decreased in Na\(^+\) solution, while it was not significantly changed in Li\(^+\) solution. The same results were obtained when we reversed the order of application of Cs\(^+\) and Na\(^+\). During the application of different extracellular cationic solutions in a whole-cell mode, the amplitudes of the current were compared (Fig. 3D). The amplitude order of current at −60 mV was Cs\(^+\) (100%) > K\(^+\) (66.8 ± 6.0%) > Li\(^+\) (28.3 ± 0.8%) > Na\(^+\) (5.1 ± 1.3%) (n = 5). We also obtained the relative permeability ratio from the reversal potentials under the bi-ionic conditions, by applying ramp pulses to get current-voltage curves. The relative permeability ratio was similar to that obtained from a previous study (9), that is, Rb\(^+\)/K\(^+\)/Cs\(^+\)/Na\(^+\)/Li\(^+\) = 1.1 : 1.1 : 1.0 : 0.98 : 0.8. There was a difference between the permeability and selectivity among cations.

We tested whether the channel shows an anomalous mole fraction behavior. The single channel activities were measured at −60 mV by using a magnified whole-cell mode in the solutions containing different proportions of Cs\(^+\) and Na\(^+\) with a total concentration of 140 mM. In 100% external Cs\(^+\), NP\(_o\) of the channel was 0.41 ± 0.08 (n = 3). As the mole fraction of Cs\(^+\) decreased, the NP\(_o\) decreased; i.e., 0.052 ± 0.004 in 67% Cs\(^+\) (n = 3), 0.042 ± 0.002 in 50% Cs\(^+\) (n = 3), 0.043 ± 0.005 in 33% Cs\(^+\) (n = 3), and 0.017 ± 0.002 in Cs\(^+\)-free, 100% Na\(^+\) solution (n = 3) (Fig. 4: B and C (>).). The concave relationship between the mole fraction of Cs\(^+\) and NP\(_o\) with no nadir indicates that NSC\(_{CCh}\) has only one binding site within the pore (14). However, the whole-cell currents in external Na\(^+\) or Li\(^+\) solution showed more linear dependence on the mole fraction of Cs\(^+\) (Fig. 4: A and C (●, ■)). The normalized amplitudes of
the whole-cell currents at 67%, 50%, 33% and 0% Cs\(^+\) were
46.7 ± 5.4%, 35.2 ± 4.6%, 24.1 ± 3.2% and 3.3 ± 1.0% for
Na\(^+\) (n = 3) and 65.3 ± 3.1%, 54.2 ± 4.8%, 42.0 ± 2.9% and
24.1% ± 1.2% for Li\(^+\) (n = 3), respectively. The calculated
unitary conductance in the solution containing 67% Cs\(^+\) with
33% Na\(^+\) was 30.0 ± 2.1 pS (n = 3), which is an intermediate
value compared with those obtained from the 140 mM Cs\(^+\)
(approximately 35 pS) and 140 mM Na\(^+\) (approximately 25 pS)
solutions.

Another property of NSC\(_{CCb}\) is the dependence upon exter-
cellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)). In a magnified whole-

channel mode with symmetrical 140 mM CsCl solution, the \(N_{P_o}\) decreased as the [Ca\(^{2+}\)]\(_o\) decreased from 1.8 to 0.1 mM. When we plotted \(N_{P_o}\) against [Ca\(^{2+}\)], with the log-log

graph, a linear relationship was obtained. The slopes of the lines in the plot (n = 2) were 0.6 and 0.72, showing that the activating effect of [Ca\(^{2+}\)]\(_o\) on open probability is propor-
tional to [Ca\(^{2+}\)]\(_o\)\(^{0.66}\) (Fig. 5A). Increasing [Ca\(^{2+}\)]\(_o\) (from 2 to
14 mM), however, did not increase the open probabilities of the channel further, while it slightly decreased the unitary current size (Fig. 5B). As [Ca\(^{2+}\)]\(_o\) increased from 2 to 14 mM, the \(N_{P_o}\) decreased; i.e., 0.65 ± 0.02 in 2 mM [Ca\(^{2+}\)]\(_o\)
(n = 3), 0.34 ± 0.09 in 4 mM [Ca\(^{2+}\)]\(_o\) (n = 3), 0.35 ± 0.09 in
6 mM [Ca\(^{2+}\)]\(_o\) (n = 3), 0.28 ± 0.15 in 8 mM [Ca\(^{2+}\)]\(_o\) (n = 3),
and 0.35 ± 0.09 in 14 mM [Ca\(^{2+}\)]\(_o\) (n = 3). The unitary cur-
rent sizes at −100 mV were 3.1 ± 0.2, 3.1 ± 0.2, 3.1 ± 0.1,
3.0 ± 0.1 and 2.9 ± 0.1 pA at 2, 4, 6, 8, 14 mM [Ca\(^{2+}\)]\(_o\),
respectively (n = 3).

DISCUSSION

Single channel currents of ACh-activated nonselective
cation channels have been recorded in smooth muscle
cells of the guinea pig ileum (11), rabbit portal vein (15),
and canine pyloric circular muscle (12). Under physiological
conditions (bath: normal Kreb’s, pipette: high-K), cat-

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**Fig. 3.** The activity of NSC\(_{CCb}\) depends on extracellular cations. Currents were activated by the application of 10 \(\mu\)M CCh. Single channel activities were recorded in the outside-out mode (A). Single channel activities in the magnified whole-cell mode were markedly decreased when extracellular Cs\(^+\) was substituted with NMDG\(^+\) or Na\(^+\) (B). C, D: Amplitudes of the whole-cell current were compared by changing extracellular cations. At each cationic condition, voltage dependence and reversal potential were evaluated by applying a ramp pulse (marked by vertical bars from +40 to −120 mV for 2 s).
NSCCs did not show anomalous mole fraction behavior. A: The whole-cell current in response to 10 μM CCh (I_CCh) was measured at a holding potential of −60 mV in solutions containing different proportions of Cs⁺ and Na⁺ (total 140 mM). Pipette solution contained 140 mM CsCl. Vertical bars indicate ramp pulses applied during the current activation (from +40 to −120 mV for 2 s). B: Single channel currents in response to 10 μM CCh were recorded in magnified whole-cell mode in solutions containing indicated proportions of Cs⁺ and Na⁺. Ascending mole fraction of Na⁺ decreased channel activities. C: Amplitudes of the whole-cell current, and the open probabilities of the channel (P_o), which were obtained from the magnified whole-cell mode, were normalized and plotted against mole fraction of external Cs⁺. Cationic solutions (Na⁺ or Li⁺ solutions) used are shown within the figure.

Fig. 4. NSCCs did not show anomalous mole fraction behavior. A: The whole-cell current in response to 10 μM CCh (I_CCh) was measured at a holding potential of −60 mV in solutions containing different proportions of Cs⁺ and Na⁺ (total 140 mM). Pipette solution contained 140 mM CsCl. Vertical bars indicate ramp pulses applied during the current activation (from +40 to −120 mV for 2 s). B: Single channel currents in response to 10 μM CCh were recorded in magnified whole-cell mode in solutions containing indicated proportions of Cs⁺ and Na⁺. Ascending mole fraction of Na⁺ decreased channel activities. C: Amplitudes of the whole-cell current, and the open probabilities of the channel (P_o), which were obtained from the magnified whole-cell mode, were normalized and plotted against mole fraction of external Cs⁺. Cationic solutions (Na⁺ or Li⁺ solutions) used are shown within the figure.
burger and Ascher (17) showed that there is a coupling of permeation and gating, and ions bound to a site in the permeation pathway influence the gating of the NMDA-channel pore mutant. External and internal cations regulated the unitary conductance of a substate and the rate of transition between the substate and the main state. The unitary conductance of a substate was smaller in Na\(^{+}\)/G2b than in Cs\(^{+}\)/G2b like our results.

Like our results, it has been reported that extracellular divalent cations strongly modulate the cationic current under whole-cell voltage clamped conditions (18, 19). When the sum of [Ca\(^{2+}\)]\(_{o}\) and [Mg\(^{2+}\)]\(_{o}\) was kept constant, increasing [Ca\(^{2+}\)]\(_{o}\) from 0 to 2 mM enhanced whole-cell currents in guinea pig ileal smooth muscle cells (18), whereas increasing [Ca\(^{2+}\)]\(_{o}\) up to 10 mM reduced the maximum conductance of the current (19). In their reports, a direct interaction of Ca\(^{2+}\)/G2b on or near the channels was interpreted as an important modulation mechanism, and the screening of negative surface charges by Ca\(^{2+}\) was not ruled out. Although we did not pursue the precise mechanisms of Ca\(^{2+}\) modulation, it was clearly shown that, at the single channel level, external Ca\(^{2+}\) modulates NSC\(_{CCh}\) by changing both the open probability and single channel conductance of the channels.

However, the decrease in open probability and unitary conductance do not seem to be enough to explain a large reduction in maximum conductance of the currents in the previous report (19).

We used both a magnified whole-cell mode and outside-out mode for our experiments assuming that there is no large difference between the results obtained from both methods. We must admit that there were some quantitative differences between the results obtained with the two methods, although the results were similar qualitatively. Especially, there was a difference between the degree of reduction in open probability in the outside-out mode (Fig. 3A) and that in the magnified whole-cell mode (Fig. 3B). In addition, there was a difference between the relationship of the mole fraction of Cs\(^{+}\) with NP\(_{o}\) and that of the mole fraction with whole cell current (Fig. 4C). Such a difference might occur because there is either a difference of intracellular environment between the outside-out mode and the magnified whole-cell mode or we recorded single channel activities under the desensitized condition in a magnified whole-cell mode. The mechanism of desensitization is yet not clear, although we suggested PKC might be involved with the desensitization (20). We think the functional chan-

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**Fig. 5.** Effects of extracellular Ca\(^{2+}\) on the activity of NSC\(_{CCh}\). A: Decreasing concentration of extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{o}\)) decreased NP\(_{o}\) of the channel. Upper panel: original traces and lower panel: relationship between NP\(_{o}\) and [Ca\(^{2+}\)]\(_{o}\). NP\(_{o}\) against [Ca\(^{2+}\)]\(_{o}\) with log-log scale was included as an inset. B: Increasing [Ca\(^{2+}\)], decreased the unitary current size at -100 mV. In both in A and B, channel activities in response to 10 μM CCh were recorded in the magnified whole-cell mode.
nels decrease during the desensitization with little change in channel properties. The blockage by quinidine was the same in both whole cell recordings and single channel recordings after repeated application of CCh (6, 13). The unitary conductance in magnified whole cell recordings was similar to that in outside-out patches or inside-out patches. Thus, we think the desensitization decreases the density of functional channels and the suppressed component would be the same as the component remaining after repeated application of CCh.

In conclusion, permeating monovalent cations regulate NSC CCh activity by changing the open probability and unitary conductance. The NSC CCh has one binding site within the pore. The external divalent cation, Ca$^{2+}$, also regulates NSC CCh activity.

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