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Biochimica et Biophysica Acta 1590 (2002) 109–122

BIOCHIMICA ET BIOPHYSICA ACTA

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A calcium-permeable channel activated by muscarinic acetylcholine receptors and InsP3 in developing chick ciliary ganglion neurons

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Received 16 November 2001; received in revised form 13 March 2002; accepted 4 April 2002

Abstract

The electrical responses elicited by the muscarinic cholinergic pathway have been studied in cultured embryonic chick ciliary ganglion (CG) neurons. Neurons obtained from E7–E8 ganglia were maintained in serum-free medium for 1 to 3 days. Stimulation with 50 μM muscarine induced depolarizing responses in about 30% of the cells tested. In voltage clamp experiments at a holding potential of − 50 mV, an inward current could be recorded in the same percentage of cells in response to muscarinic stimulation. In single channel experiments, with standard physiological solution in the pipette, muscarine transiently activated an inward conducting channel. Cell-attached recordings with 100 mM $CaCl_2$ in the pipette provided evidence that muscarinic agonists can activate a cationic calcium-permeable channel. Two main conductance levels could be detected, of 2.3 ± 0.6 and 5.6 ± 0.6 pS, respectively. In excised patches, addition of 5–20 μM inositol 1,4,5-trisphosphate ($InsP_3$) to the bath reactivated a channel that could be blocked by heparin and whose characteristics were very similar to those of the channel seen in response to muscarinic stimulation. A channel with similar properties has been previously shown to be activated by basic fibroblast growth factor (bFGF) and $InsP_3$ in the same preparation.

Keywords: Muscarinic acetylcholine receptor; Calcium-permeable channel; InsP3; Chick embryo neuron; Ciliary ganglion

1. Introduction

In the adult nervous system neurotransmitters are the main chemical mediators of intercellular communication. In addition to this classical and established function, experimental evidence suggests that they play, in the developing nervous system, a broad spectrum of actions including regulation of proliferation, differentiation, survival, neurite outgrowth, motility and target innervation [1–7]. Moreover, the hypothesis that neurotransmitters can exert a trophic and differentiative role is consistent with the evidence that some classes or subtypes of neurotransmitter receptors are expressed during specific developmental periods [8–11] and that the naturally occurring neuronal death is dependent on interactions between neurons and both their targets and afferents [12,13].

The neuronal population of the chick ciliary ganglion (CG) is a model well suited for studying the neurotrophic role of neurotransmitters. It is composed of two homogeneous populations of neurons: choroid and ciliary cells. Afferent nerve terminals from the accessory oculomotor nucleus reach the ganglion by embryonic days 4–5 (E4–E5) and by the stage E8, all the neurons receive a functional synaptic input [14]. Between E8–E9 and E14–E15, the number of CG neurons is reduced approximately by half. Both removal of the preganglionic input [15] and blockade of the ganglionic transmission [16,17] increase cell death in the CG, as does blockade of transmission to the target tissues, at least for ciliary neurons [16].

The major mode of chemical synaptic transmission in the CG of chick embryos is cholinergic, and both nicotinic [14,18] and muscarinic [19–23] acetylcholine (ACh) receptors are involved. Regarding the former, Meriney et al. [16] have shown that the muscarinic antagonist atropine blocks synaptic transmission in ciliary ganglia of stage 38 (E12)

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0167-4889/02/$ - see front matter
PII: S0167-4889(02)00206-9

chick embryos (complete block for choroid neurons; partial block for ciliary ones); moreover, synaptic fatigue in choroid neurons [18] is a muscarinic receptor-mediated process occurring approximately during the same developmental period (E10–E16) as cell death. In vitro cytofluorimetric studies have provided additional evidence that E10–E15 embryonic chick CG neurons have muscarinic acetylcholine receptors (MAChRs) capable of increasing intracellular [Ca^{2+}] by means of a mechanism that involves both calcium release and calcium influx [21–23]. Regarding influx, both voltage-dependent and -independent pathways have been proposed [22,23]. On the other hand, electrophysiological evidence for the activation of calcium currents in response to MAChRs stimulation is still lacking.

We have previously reported [24] that basic fibroblast growth factor (bFGF), which has a trophic role on these cells, can elicit an inward current carried through calcium-permeable channels. We have also shown that this calcium influx is not dependent on either L- or N-type voltage-dependent channels, and that the same channels opened by bFGF can be reactivated in excised patches by the application of inositol 1,4,5-trisphosphate ($InsP_3$) in the micromolar range. These data were obtained at stage E7–E8, i.e. at an earlier stage than the one for which muscarine-activated calcium influx has been reported. In this study, using CG neurons from the same developmental stage (that corresponds to the onset of the cell-death period), we extend our investigation to the electrical responses induced by the muscarinic pathway: we provide evidence for the activation of a calcium-permeable channel, and for a role of $InsP_3$ in this process. A preliminary account of some of the data presented in this paper was given at the Nato Advanced Research Workshop on Calcium Signaling, Lucca, Italy, April 2000 [25].

2. Materials and methods

2.1. Cell culture

Chick CG neurons were obtained from 7–8-day embryos and maintained for 1–3 days in a chemically defined N2 medium as previously described [24]. Briefly, ganglia were incubated in divalent cation-free phosphate-buffered saline (PBS) containing 0.06% trypsin at 37 °C for 5 min before a gentle trituration, plated on poly-D-lysine- (100 μg/ml) and laminin (2 $\mu g/cm^2$)-treated dishes and then cultured, in order to improve cell viability, in the presence of bFGF (10 ng/ml).

2.2. Electrophysiology

Whole-cell recordings were performed with an Axopatch 1-C patch-clamp amplifier (Axon Instruments, USA) and filtered at 1–2 kHz. Electrodes (Clark Electromedical Instruments, UK) with a resistance of 2–5 MΩ were used. Data were stored on a PCM video recorder system and subsequently digitized, usually at 25- or 100-ms intervals, and analysed using pClamp 6 (Axon Instruments) and Origin (Microcal Software, USA) software. Capacitance measurements were performed by means of analog compensation.

Single-channel recordings were obtained by means of electrodes with a resistance of 5–10 MΩ coated with Sylgard (Dow Corning, Belgium). Unitary currents were filtered at 1–2 kHz and digitized at 5–10 kHz. Since the channels were of rather low conductance and did not exhibit marked flickering behaviour, traces were further low-pass filtered at 300–500 Hz for illustrations as indicated in the figure legends. Single channel amplitude was measured by fitting amplitude histograms by a sum of two or more Gaussian distributions. Single channel $I–V$ relationships were obtained by applying a voltage ramp (1 V s^{-1}) from −100 to 50 mV during channel openings and then subtracting for the leakage current.

Values are given as mean ± S.D. All experiments were performed at 22–24 °C.

2.3. Solutions and drugs

During whole cell experiments, a standard Tyrode solution (Sol. A) of the following composition, in millimolar (mM), was perfused by means of a peristaltic pump: NaCl, 154; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; *N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid (HEPES), 5; glucose, 5.5; NaOH to pH 7.4; when indicated, TTX 10^{-6} M was added. In some experiments NaCl and KCl were replaced with a solution (Sol. B) containing 150 mM tetraethylammonium chloride (TEACl) and the pH was adjusted to 7.4 with CsOH. The standard electrode solution (Sol. C) consisted of: K^+-aspartate, 118; KCl, 15; $MgCl_2$, 3; HEPES, 5; Na_2GTP, 0.4; Na_2ATP 5; Na_2phosphocreatine, 5; ethylene glycol-bis(β-aminoethyl Ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5; pH 7.3 with KOH. Experiments with TEACl in the external solution were performed with an electrode solution (Sol. D) of the following composition: CsCl, 20; $MgCl_2$, 3; HEPES, 5; Na_2GTP, 0.4; Na_2ATP 5; Na_2phosphocreatine, 5; EGTA, 5; CsOH 113; aspartic acid to pH 7.3. After seal formation, cells were also perfused, by a gravity microperfusion system, with the external solution to which 50 μM muscarine had been added.

In cell-attached and inside-out experiments electrodes were filled with the solution A or with a solution (Sol. E) containing (in mM): $CaCl_2$, 100; TEACl, 20; 4-aminopyridine (4AP), 4; HEPES, 5; CsOH to pH 7.3. In some experiments, 10 μm ω-conotoxin GVIA (Alomone Labs, Israel) and/or 2 μm nifedipine were added. After seal formation, the Tyrode solution in the bath and in the microperfusion pipette was replaced by a solution (Sol. F) of the following composition: KCl, 150; $MgCl_2$, 0.55 $CaCl_2$, 1; EGTA, 1.1; KOH to pH 7.3, in order to set the membrane potential near 0 mV. Drugs (in cell-attached configuration, muscarine and carbachol: 10–100 μM) were added to the microperfusion pipette solution; in inside-out recordings,

10–20 μM $InsP_3$ (Alomone Labs) was added to the high-K^+ external solution in which 130 mM of KCl was isomolarly substituted with K^+-aspartate (Sol. G).

If not otherwise specified, all chemicals and drugs were purchased from Sigma Chemical Co., USA.

3. Results

3.1. Stimulation of MAChRs induces changes in the membrane resting potential

Whole-cell experiments were performed on cells maintained in culture for 1–3 days. During the experiments, cells were bathed in a standard Tyrode solution (Sol. A); the electrode was filled with solution C. In 92 neurons, the resting membrane potential V_m was rather stable and its mean value was -58.9 ± 10 mV (range: from -39 to -83 mV). Cell capacitance was measured in 48 cells, giving a mean of 6.1 ± 1.3 pF.

A first set of recordings was performed in the current clamp mode, in order to check if muscarine could elicit changes in membrane potential. In 48 experiments, application of muscarine (50 μM) to the bath induced a reversible depolarization in 29% of the cells tested ($n = 14$). An example is shown in Fig. 1A. As compared to the responses to bFGF [24], those to muscarine showed more complexity and variability: in six cells, the depolarizing phase was preceded by a fast, transient hyperpolarization, and six additional cells gave only a hyperpolarizing response (not shown). Since we were interested in studying ionic events potentially involving activation of calcium-permeable channels, only depolarizing responses, and the associated inward currents (see below), have been the object of analysis in this paper. The depolarization developed with a delay from a few seconds to tens of seconds; the mean ΔV_m from the resting value was 6.9 ± 3.4 mV ($n = 14$). Washing out the agonist abolished all responses. In three experiments, the cell was challenged with a second stimulation with muscarine, and a comparable change in V_m could be observed (Fig 1B).

3.2. Activation of inward currents in response to muscarinic stimulation

In order to identify the ionic currents involved in the depolarizing responses described above, voltage clamp

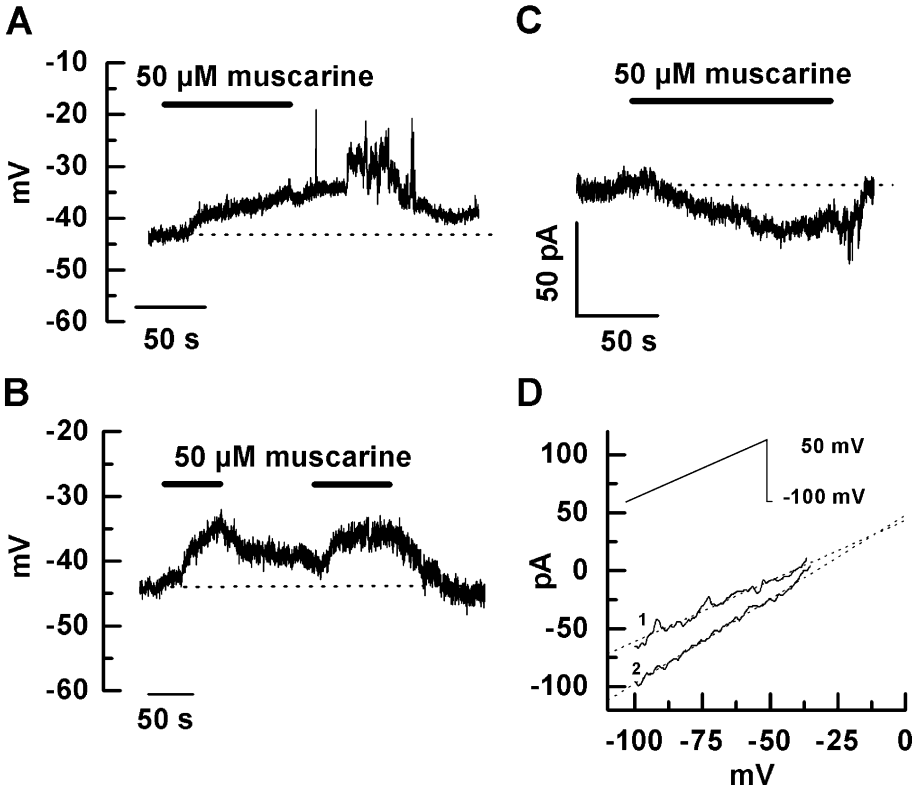


Fig. 1. Depolarizing response and activation of a cationic inward current induced by muscarinic stimulation in chick CG neurons. (A) Muscarine, 50 μM, induced a reversible depolarization of the neuron. (B) In this experiment, comparable responses to repeated stimulations with 50 μM muscarine were obtained. (C) In response to muscarinic stimulation, an inward current developed slowly in a voltage clamped cell and was abolished by washout of the agonist. $V_h = -50$ mV; low-pass filter 1 kHz. (D) Current–voltage relationships obtained from a different cell by applying voltage ramps from -100 to $+50$ mV (inset) in control conditions (1) and during (2) the onset of the inward current activated by 50 μM muscarine. V_{rev} of the muscarine-activated current, obtained from the crossing of the regression curves of the two currents (dotted lines), is about -10 mV. External solution: standard Tyrode (Sol. A); electrode solution: high K-aspartate (Sol. C).

experiments were performed at a holding potential of −50 mV with Sol. A in the bath and Sol. C in the pipette. In 14 out of 44 experiments (32%), the activation of an inward current could be observed in response to 50 μM muscarine (Fig. 1C); in four cases it was preceded by an outward component. In five additional cells only the outward component was present. Similarly to the current clamp observations, only the inward current observed in voltage clamp experiments is described in this paper. The mean peak amplitude of the inward current was −20.8 ± 10.5 pA. During the inward responses, flickering events were often detected. Current–voltage relationships, obtained by applying a voltage ramp from −100 to +50 mV before and during agonist stimulation, show that the inward current activated by muscarine (an example of which is shown in Fig. 1D) has a V_{rev} of −14.5 ± 6.4 mV ($n=5$), suggesting that this component could be a nonselective cationic current.

In order to investigate if calcium permeation could be associated with the inward current (as already shown for the bFGF-activated current; [24]), a further set of experiments was performed substituting Na^+ and K^+ with TEA^+ ions in the external solution (Sol. B) and K^+ with Cs^+ and 85% Cl^- with aspartate in the electrode solution (Sol. D): in these conditions, E_{Cl} was about −50 mV so that an inward current, if present at $V_h=-50$ mV, could only be carried by Ca^{2+} ions. In 9 out of 22 neurons (43%), 50 μM muscarine elicited an inward current, which showed a latency ranging from a few seconds up to 60 s, with a mean peak inward amplitude of −28.4 ± 17.5 pA. A typical recording is shown in Fig. 2A. In all experiments the current decayed in the presence of the agonist, with a duration of the order of 1 min. Current–voltage (I–V) relationships obtained before and during agonist stimulation (Fig. 2B) gave for the inward current a V_{rev} of −9.8 ± 4.2 mV ($n=8$), consistent with the hypothesis that this current is carried through nonselective calcium-permeable cationic channels.

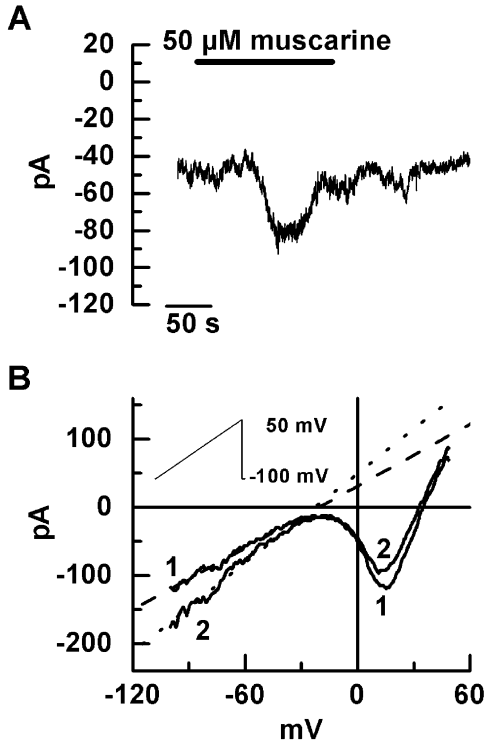


Fig. 2. The inward current elicited by 50 μM muscarine observed with Ca^{2+} (2 mM) as the only external permeant cation. (A) Muscarine-induced inward current recorded at $V_h=-50$ mV. Na^+ was replaced with TEA in the external solution (Sol. B); K^+ was replaced with Cs^+ and 85% Cl^- with aspartate in the electrode solution (Sol. D). Low-pass filter 1 kHz. (B) Current–voltage relationships obtained from another cell, with same solutions as in (A), before (1) and during (2) the muscarine-induced inward current. The regression curves (dotted lines) cross at about −30 mV.

3.3. Muscarinic receptor stimulation activates cationic channels

In an attempt to characterize at the single channel level the conductances involved in the activation of the inward currents described above, a first set of recordings was performed in the cell-attached configuration with the patch electrode filled with a standard Tyrode solution (Sol. A) to which 20 mM TEA, 4 mM 4AP and 1 μM TTX were added. External solution was high in KCl (Sol. F), in order to set V_m near 0 mV; the patch was clamped to −50 mV. TTX was added to the pipette in order to block voltage-dependent Na^+ channels; TEA and 4AP were added to block K^+ conducting channels, which could be expected to be activated by muscarinic stimulation, based on whole cell recordings.

In seven patches (out of 26) we observed the opening of inward conducting channels. Fig. 3A shows a typical response at $V_h=-50$ mV. In the presence of 50 μM muscarine in the bath, sustained channel openings and several current levels were observed. Channels were characterized by openings of long duration, of the order of seconds. Two current levels were more frequently observed, respectively, of −0.23 ± 0.06 and −0.35 ± 0.04 pA; these values were obtained by means of amplitude histograms (Figs. 3B and C). From I–V analysis, obtained by applying voltage ramps during channel openings (Fig. 3D), the single channel conductance corresponding to the higher current level (Fig. 3C), resulted of 6.1 ± 0.1 pS with a reversal potential of −4.7 ± 0.9 mV. The mean conductance for this current level, calculated by averaging the slope of the regression lines obtained from four different experiments, resulted to be 7.8 ± 1.0 pS with a $V_{rev}=10.9\pm7.9$ mV. Fig. 3E shows the time course of the patch activity NP_o: in this experiment the open probability returned to the basal level after washout of the agonist; in others the activity decayed even in the presence of muscarine.

3.4. The cationic channels are calcium-permeable and can be activated by $InsP_3$

As previously reported [21,23], muscarinic agonists are able to elicit calcium influx in CG neurons; moreover,

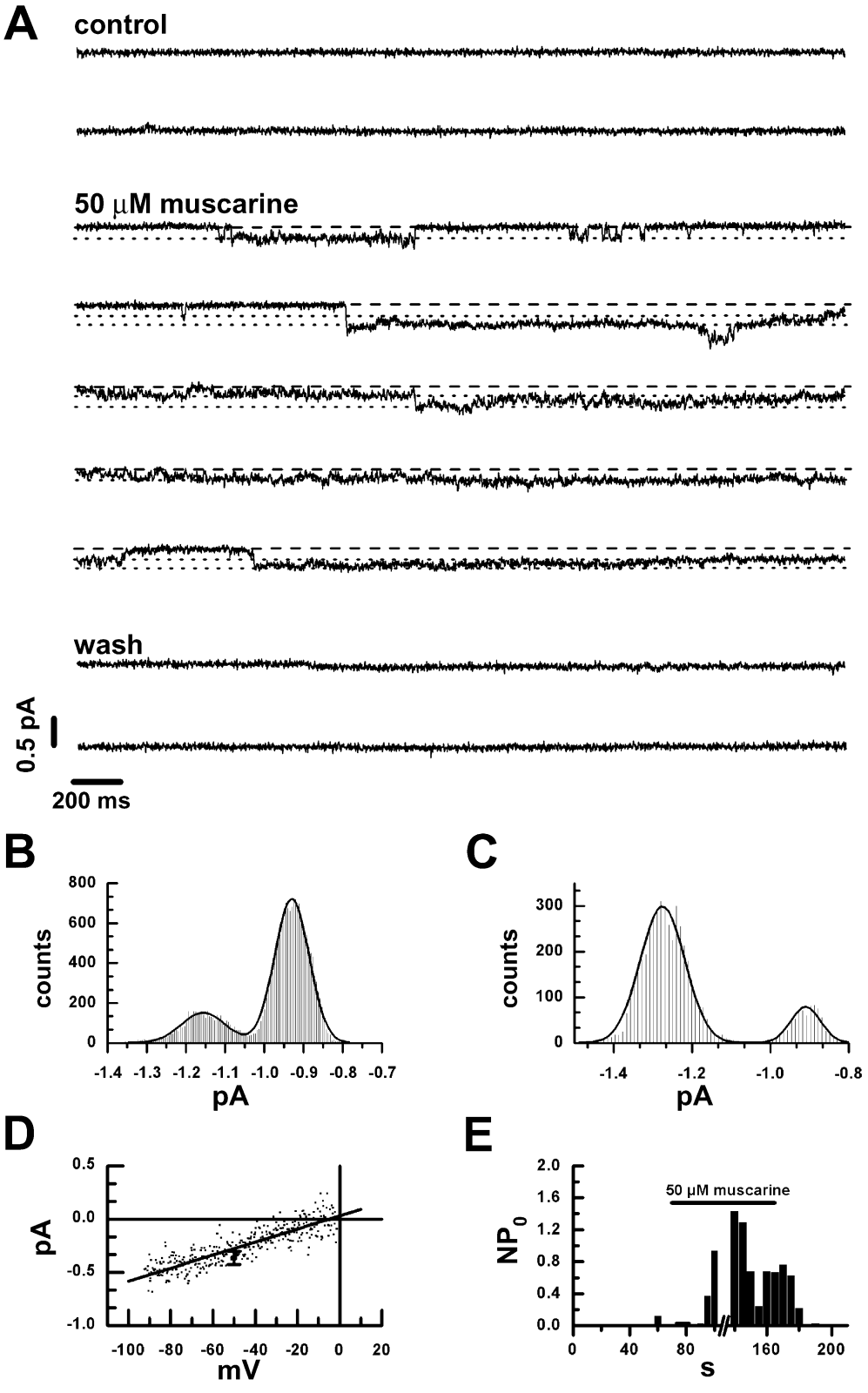


Fig. 3. Muscarine-activated cationic channels recorded in cell-attached configuration. All data refer to the same patch. (A) Stretches of recording prior to, after application of 50 μM muscarine to the bath, and after washing out the agonist. External solution was high-K^+ (Sol. F) and electrode solution was standard Tyrode (Sol. A). Dotted lines indicate current levels. Dashed line is baseline. $V_m = -50$ mV; low-pass filter 0.3 kHz. (B, C) Amplitude histograms identifying current levels, respectively, of $-0.23 \pm .06$ and -0.35 ± 0.04 pA. Peaks were fitted by Gaussian curves. (D) $I-V$ relationship obtained during a channel opening. The regression line gives a conductance of 6.1 pS and a reversal potential of -4.7 mV. Filled circle corresponds to the current level obtained from histogram in (C). (E) Patch activity NP_o recorded in the presence and in the absence of 50 μM muscarine in the bath.

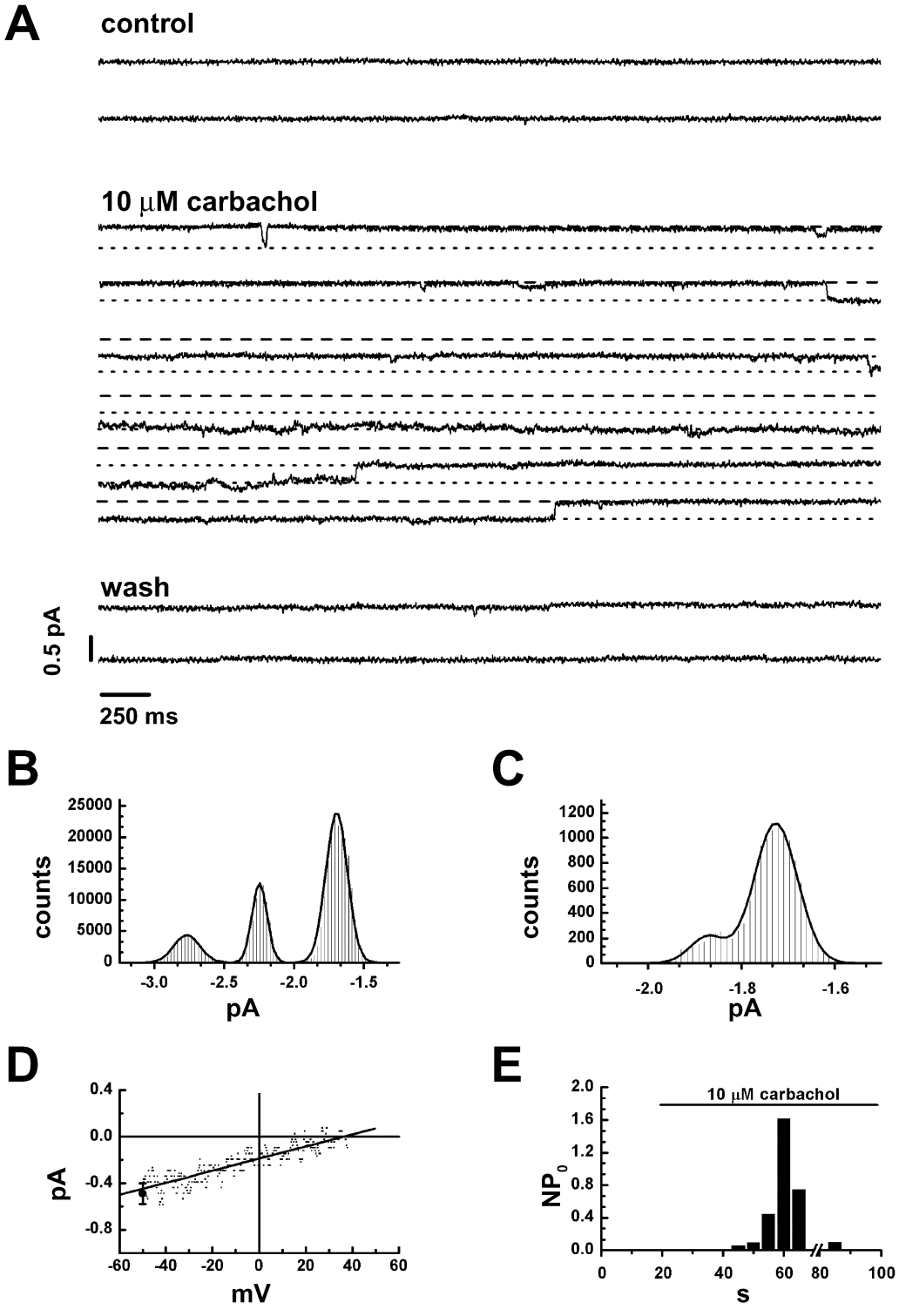


Fig. 4. The cationic channels activated by muscarinic agonists are calcium-permeable. All data refer to the same patch. (A) Stretch of recording showing several current levels activated by 10 μM carbachol. Dotted lines indicate the two most commonly observed levels. External solution: high-K^+ (Sol. F); electrode solution: 100 mM $CaCl_2$ (Sol. E) with 2 μM nifedipine. $V_m = -50$ mV; low-pass filter 0.3 kHz. (B) Amplitude histogram showing the two main current levels detected in (A), separated from the baseline, respectively, by -0.49 ± 0.09 and -1.10 ± 0.08 pA. (C) Amplitude histogram showing a lower amplitude level (-0.15 ± 0.05 pA). (D) $I-V$ relationship obtained during a channel opening. The regression line gives a conductance of 5.2 pS and a reversal potential of 36.5 mV. Filled circle corresponds to the current level (-0.49 ± 0.09 pA) obtained from histogram in (C). (E) Patch activity NP_o was markedly and transiently increased by addition of 10 μM carbachol to the bath.

whole cell data presented above point to a role for calcium ions in the inward current activated by muscarine. Therefore, 57 experiments were performed in the cell-attached configuration with the pipette filled with a solution containing 100 mM $CaCl_2$ (Sol. E) and with Sol. F in the bath, clamping the patch membrane to a holding potential of −50 mV.

In a first set of experiments, in response to the stimulation of MAChRs, by addition to the bathing solution of either carbachol (10–100 μM, $n=6$) or muscarine (50 μM, $n=3$), we observed marked and reversible channel activation. In six of such experiments 2 μM nifedipine was added to the pipette solution. An example is shown in Fig. 4A. In these conditions, too, channels were characterized by openings of long duration. Amplitudes of single channel current were evaluated with histograms (Fig. 4B and C). The histogram in Fig. 4B shows, in addition to the baseline peak, two other peaks separated from the baseline, respectively, by −0.5 and −1.1 pA (right to left), that could correspond to the opening of two identical channels. Moreover, a smaller current level of -0.15 ± 0.05 pA was also observed in this patch (Fig. 4C). From $I-V$ analysis of Fig. 4D, obtained by applying voltage ramps during channel openings, the single channel conductance corresponding to the first current level shown in Fig. 4B resulted to be of 5.2 ± 0.1 pS, with a reversal potential of 36.1 ± 0.7 mV. Fig. 4E shows the time course of the patch activity NP_o: the latency of the change in NP_o was of the same order of magnitude as the latency of the whole cell currents and the activation appears to be transient in nature: the channels closed in the presence of the agonist.

Fig. 5 shows samples from another experiment in which in addition to the level of about −0.50 pA (Fig. 5C and F), the lower amplitude level was better resolved (Fig. 5A and D). In Fig. 5B (the corresponding histogram is shown in Fig. 5E), simultaneous openings and transitions between the different levels, followed by a sharp return to the baseline level can be clearly detected: these features are in agreement with the hypothesis of the presence in the patch of one channel with two states of conductance.

Channels with similar behaviour were also recorded in 12 out of 60 experiments in the inside-out configuration after the addition of 5–20 μM $InsP_3$ to the bath. Bath solution in these experiments was Sol. G (high K-aspartate). Pipette solution was Sol. E. In these conditions, E_{Cl} was close to the holding potential (−50 mV). In three of the experiments in which channel activation was observed, 10 μM ω-conotoxin GVIA and 2 μM nifedipine were present in

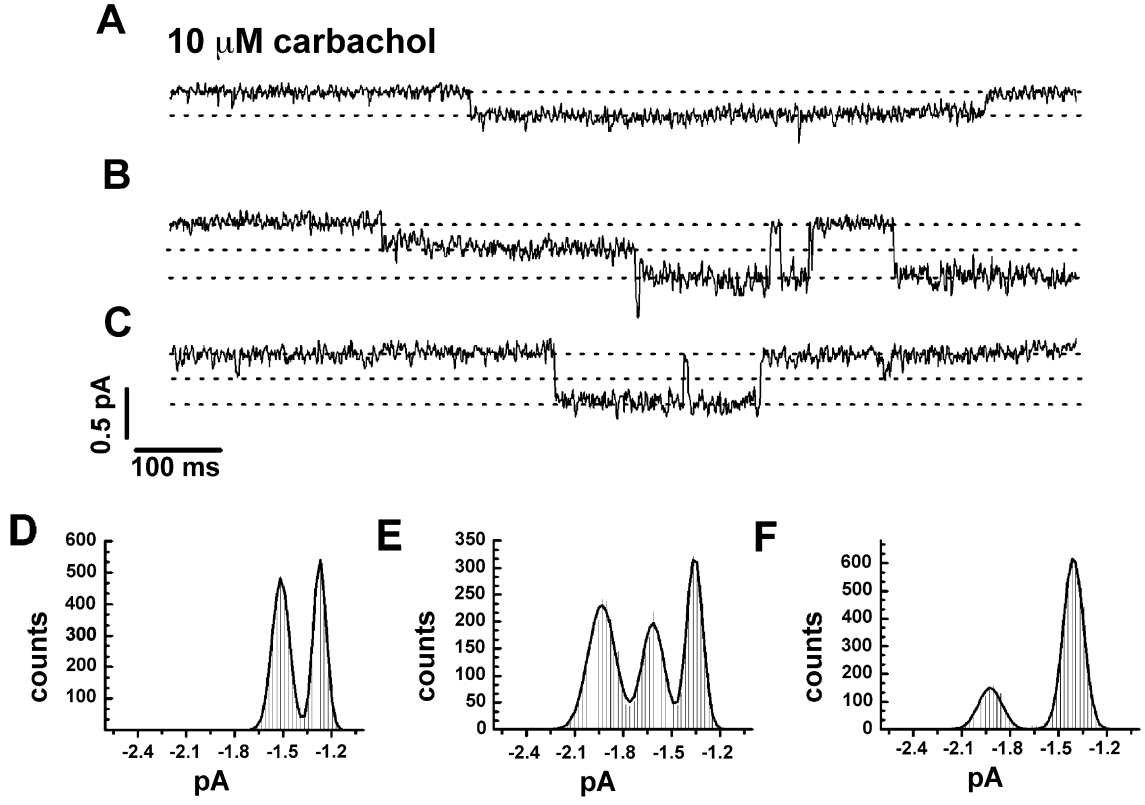
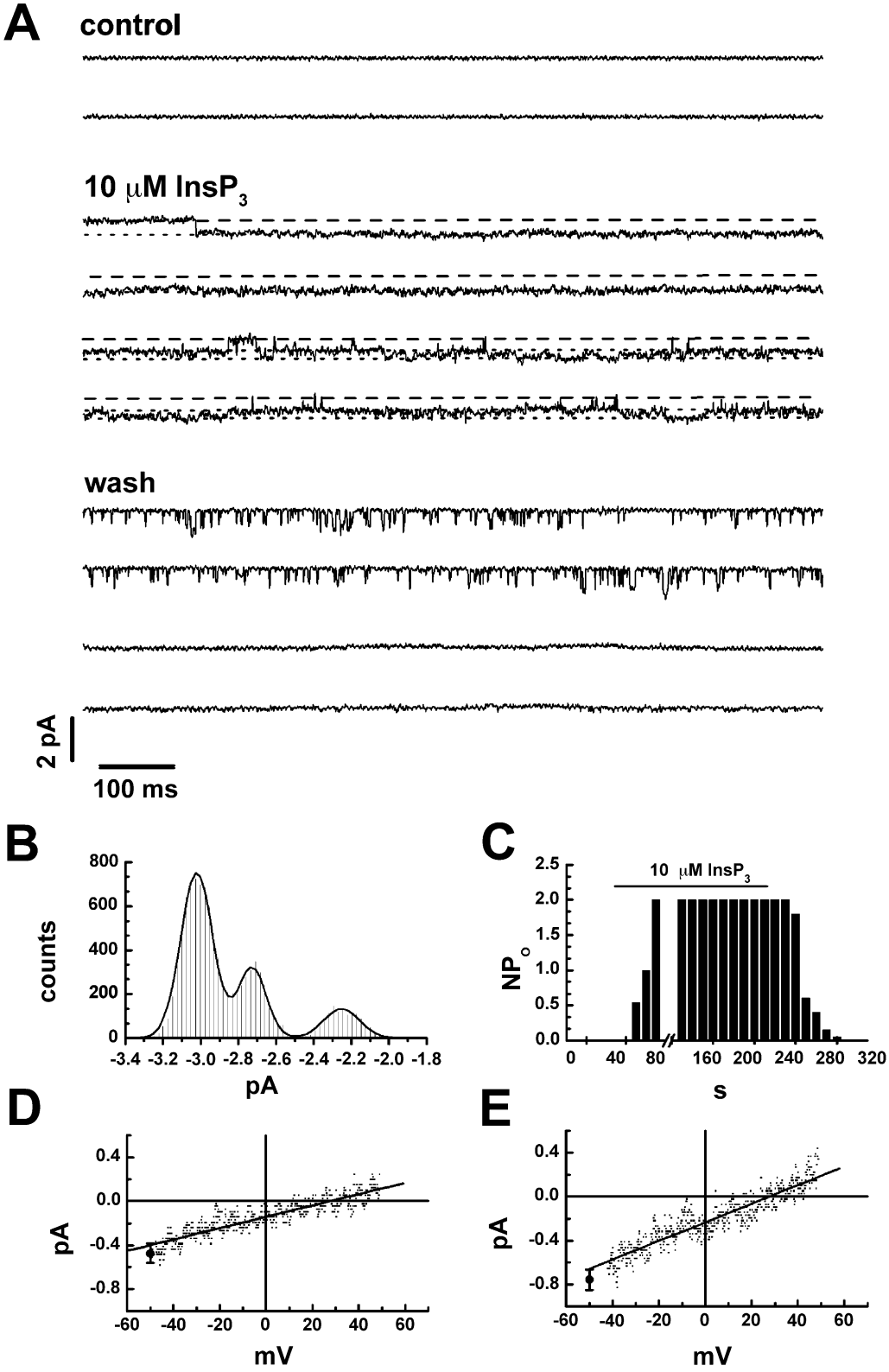


Fig. 5. (A–C) Samples from another experiment (same solutions as in Fig. 4) in which two current levels could be detected. Simultaneous openings and transitions between the different levels, followed by a sharp return to the baseline level, could be observed. (D) Amplitude histogram obtained from the trace shown in (A) showing the presence of a level of -0.24 ± 0.05 pA; (E) histogram from trace (B) showing the presence of levels of -0.26 ± 0.05 and -0.56 ± 0.08 pA; (F) histogram from trace (C) showing the presence a level of -0.52 ± 0.07 pA.

the pipette. An example, obtained in the presence of these voltage-dependent calcium channel blockers in the electrode, is shown in Fig. 6. $InsP_3$ activated two inward current levels of − 0.47 and − 0.76 pA (Fig. 6A and B). The latter may be due to the simultaneous opening of the two levels (of about − 0.2 and − 0.5 pA) observed in cell-attached experiments in response to muscarinic receptor stimulation and previously described in Figs. 4 and 5. Actually, in other



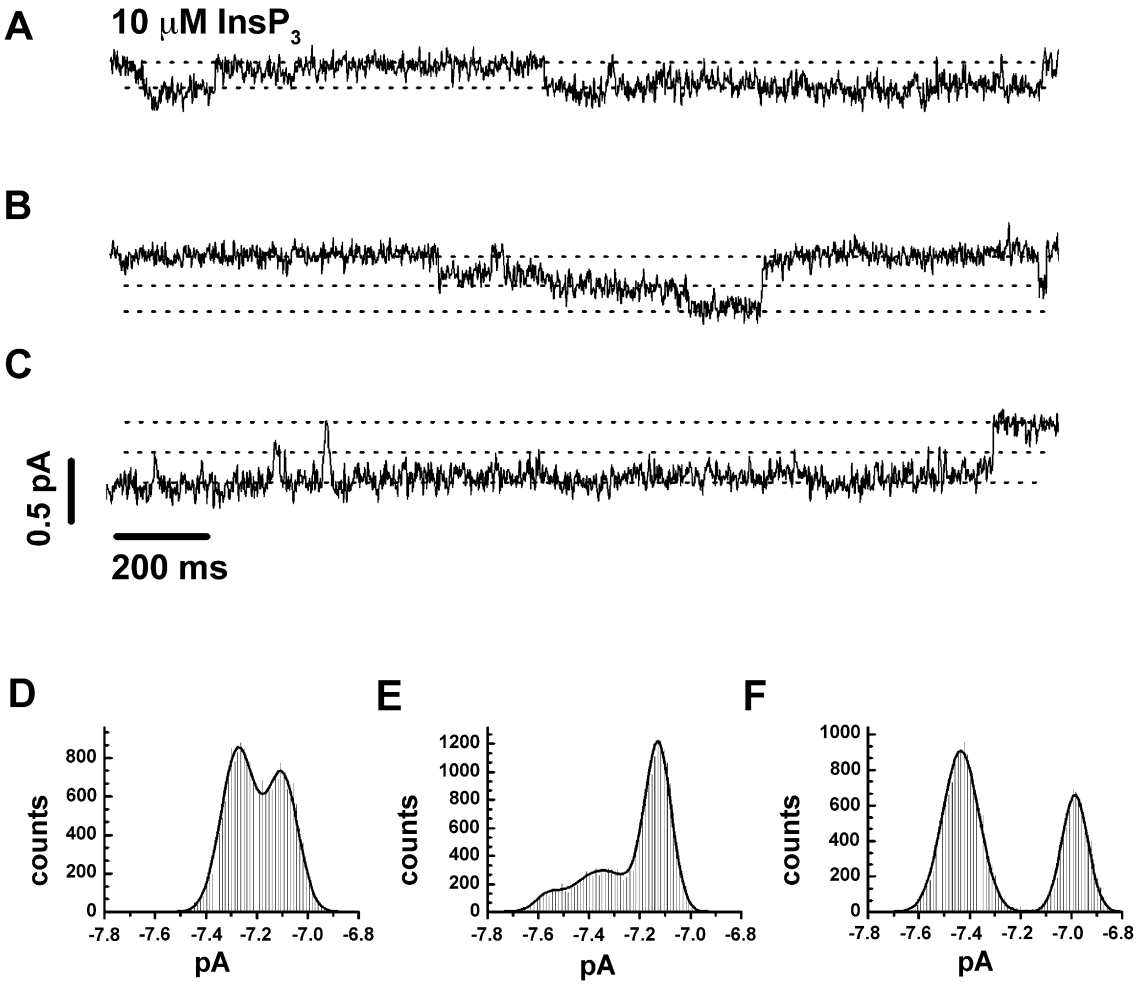


Fig. 7. (A–C) Samples from another inside-out experiment in which 10 μM $InsP_3$ activated two current levels. In (A) only the lower level is present; in (B) and (C) the two levels can be detected, with simultaneous transitions to the baseline level. (D) Amplitude histogram obtained from the trace shown in (A) showing the presence of a level of -0.18 ± 0.07 pA; (E) histogram from trace (B) showing the presence of a level of -0.22 ± 0.07 and -0.42 ± 0.08 pA; (F) histogram from trace (C) showing the presence of a level of -0.45 ± 0.07 pA.

experiments (Fig. 7), two current levels of about -0.20 and -0.45 pA could be resolved, showing simultaneous openings and transitions between different levels, followed by a sharp return to the baseline level (a behaviour comparable to that shown in Fig. 5 for cell-attached experiments). The conductances for the two current levels of Fig. 6A resulted to be 5.6 ± 0.1 and 8.5 ± 0.1 pS with a reversal potential, respectively, of 24.1 ± 0.6 and 27.7 ± 0.4 mV. As shown in Fig. 6C, the addition of the intracellular messenger induced a sustained activation of the channel; NP_o returned to basal levels only after $InsP_3$ washout (see also Fig. 6A): at the beginning of the wash the open time durations were strongly reduced and a flickering behaviour was evident.

In six other experiments, an example of which is shown in Fig. 8, it was possible to compare on the same patch the channel openings induced by carbachol in the cell-attached mode with those observed after exposure of the cytoplasmic side of the excised patch to $InsP_3$. The experiment of Fig. 8 was performed with 2 μM nifedipine in the pipette. Again, at a holding potential of -50 mV, two current levels could

Fig. 6. Calcium-permeable cationic channels activated by $InsP_3$. (A) Channel openings with amplitudes comparable to those described in Figs. 4 and 5 could be observed in excised patches after addition of 10 μM $InsP_3$ to the bath. External solution was high-K-aspartate (Sol. G); electrode solution was E, with 2 μM nifedipine and 10 μM ω-conotoxin. $V_m = -50$ mV; low-pass filter 0.3 kHz. (B) Amplitude histogram showing two current levels separated from the baseline, respectively, by -0.47 ± 0.09 and -0.76 ± 0.08 pA. (C) NP_o was markedly and reversibly increased by $InsP_3$. (D, E) $I-V$ relationships obtained by applying a voltage ramp during openings of channels corresponding to the lower (D) and higher (E) levels shown respectively in (A) and (B). The regression lines give conductances of 5.7 and 8.5 pS and reversal potentials of 24.1 and 27.7 mV, respectively. Filled circles correspond to the current levels obtained from histograms in (B).

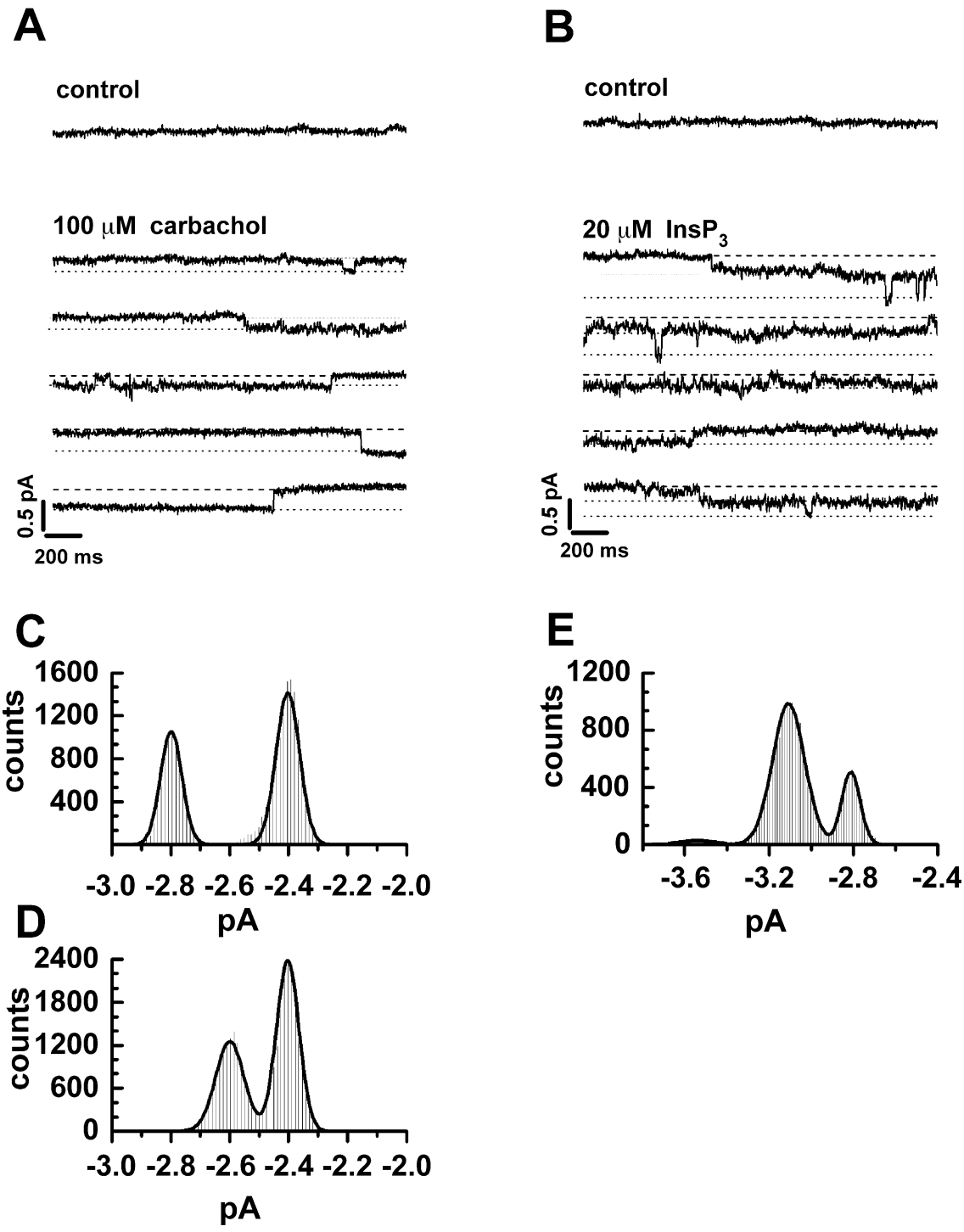


Fig. 8. Muscarinic agonists and $InsP_3$ open similar calcium-permeable channels in the same patch. (A) Channel openings recorded in cell-attached mode in response to 50 μM carbachol. (B) After patch excision, 20 μM $InsP_3$ caused the activation of a channel of similar amplitude. External solutions were F in (A), G in (B); electrode solution was E with 2 μM nifedipine. $V_m = -50$ mV; low-pass filter 0.3 kHz. (C) Amplitude histogram corresponding to the last two traces in (A). Single channel current level was of -0.40 ± 0.04 pA. (D) Amplitude histogram corresponding to first three traces in (A). Single channel current level was of -0.20 ± 0.05 pA. (E) Amplitude histograms corresponding to the first two traces in (B). Single channel current levels were of -0.43 ± 0.08 and -0.29 ± 0.08 pA.

be observed: channel amplitudes in both configurations were in good agreement. From the best fit of the amplitude distributions we obtained two single channel current levels of -0.40 ± 0.04 (Fig. 8C) and -0.20 ± 0.05 pA in the cell-attached mode (Fig. 8D), and of -0.43 ± 0.08 and -0.29 ± 0.08 pA in the inside-out configuration (Fig. 8E).

Finally, as shown in Fig. 9, channel activity induced by $InsP_3$ was reversibly blocked in all patches tested ($n = 3$) by addition of 1 μg/ml heparin to the bathing solution. Moreover, in whole cell experiments, in the presence of heparin in the pipette (Sol. D) and substituting Na^+ and K^+ with TEA^+ ions in the external solution (Sol. B), muscarine failed to induce an inward current in all cells tested ($n = 11$; see Fig. 9, inset).

In conclusion, we observed channel activation in response to muscarinic stimulation in 15 out of 57 cell-attached patches: the mean conductance for the higher level, as calculated by averaging the slope of the regression lines obtained for a total of five recordings from different experiments, was of 5.6 ± 0.6 pS with a $V_{rev} = 31.4 \pm 9.9$ mV; for the lower level, averaging of data from ramps in four experiments gave values of 2.3 ± 0.6 pS, with a reversal

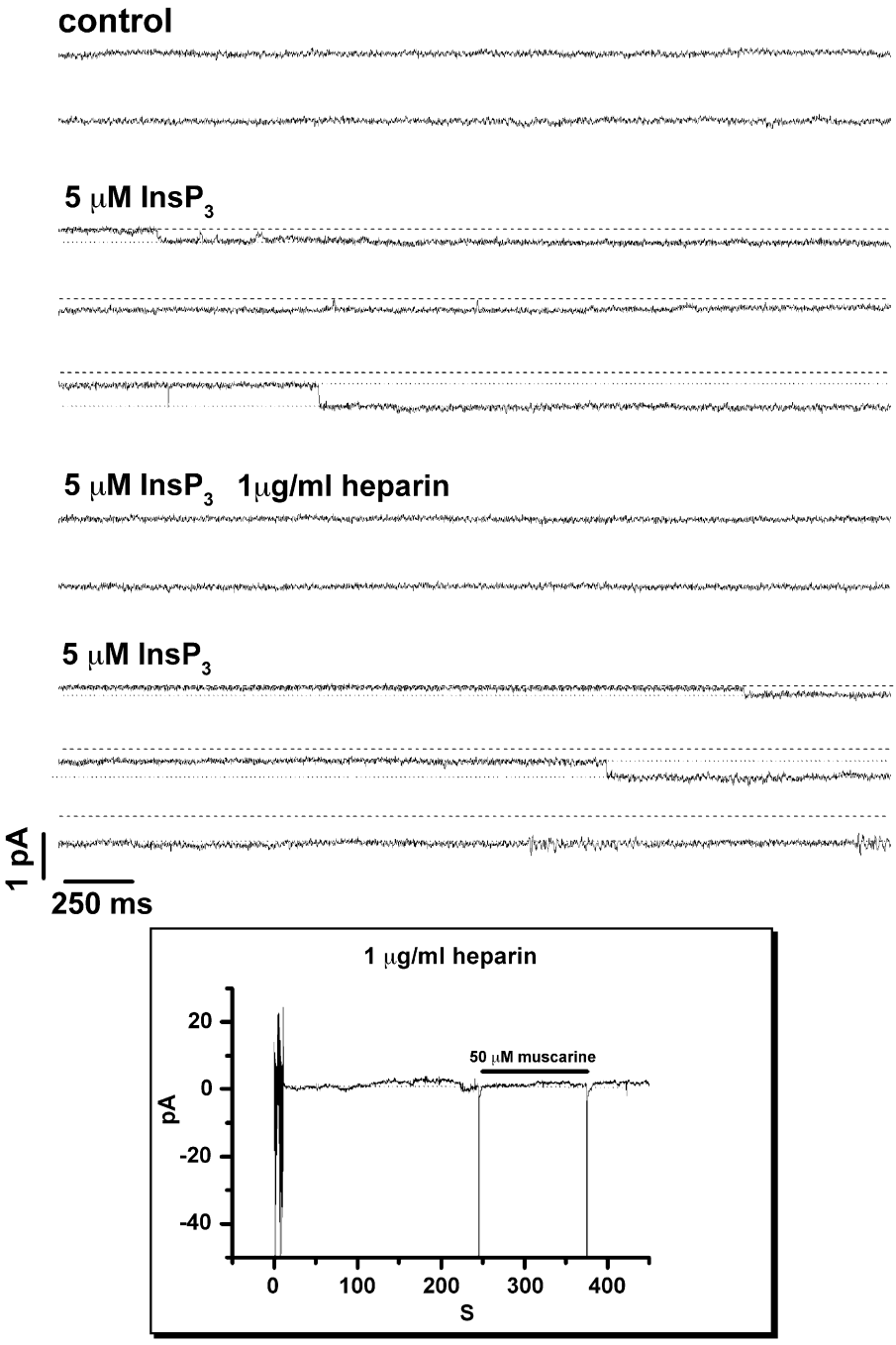


Fig. 9. Heparin reversibly blocks the $InsP_3$-activated channels. $InsP_3$, 5 μM, activated a channel with two conductance levels; addition of 1 μg/ml heparin to the bath reversibly blocked it. Solutions, V_m and filter as in Fig. 8. Inset: A whole cell experiment (representative of other 10) in the presence of 1 μg/ml heparin in the pipette: muscarine could not elicit any inward current. Solutions as in Fig. 2; $V_h = -50$ mV.

potential of 40.3 ± 8.7 mV. In 12 out of 60 inside-out patches, addition of $InsP_3$ in the bath activated a channel with similar properties: from three inside-out recordings, a mean conductance of 5.3 ± 0.7 pS, with $V_{rev} = 29.4 \pm 7.0$ mV, was obtained for the higher level.

4. Discussion

In this paper we have shown that, in chick embryo CG neurons at a rather early developmental stage, muscarinic stimulation induces a complex electrical response, which involves the activation of a voltage independent calcium-permeable channel. Furthermore, we provide evidence for a role of the second messenger $InsP_3$ in the activation of this channel. Since we have shown previously that the neurotrophic factor bFGF opens $InsP_3$-sensitive calcium-permeable channels [24], the data presented here give a first suggestion of a convergence of a neurotransmitter and a neurotrophic factor in the activation of a common target and in the induction of calcium influx.

We have studied CG neurons obtained from E7–E8 embryos for the following reasons: first of all, we were interested in comparing the responses to muscarinic stimulation with those induced by bFGF on the same cell population and at the same stage; moreover, this stage represents the starting point of a crucial phase in embryonic development, involving stabilization of synaptic connections, maturation of specific neuronal phenotypes and definition of cell populations through the mechanism of neuronal death. This choice limits the possibility of directly comparing our data with those obtained at later stages; however, our results provide the first evidence that the muscarinic pathway is already functional at an early stage and are suggestive of a role of calcium influx in this phase of embryonic development.

We have observed that in some cells muscarinic stimulation elicited, in addition to a depolarizing inward current, an outward one (and the corresponding hyperpolarizing response in current clamp mode) that could be observed alone or superimposed on the inward one. A brief account of the latter component has been given in a preliminary report [25]; here we have analyzed in detail only the inward component, and the channels that underlie it. While we have analysed only the depolarizing responses, we cannot exclude that in some cases their amplitude could be reduced by the presence of a hyperpolarizing component. It is worth noting that our percentages of cells giving inward currents (or depolarizing responses) are only slightly lower than those reported by Rathouz et al. [23], which refer to calcium signals elicited by muscarinic stimulation in neurons obtained from chick CGs at a later stage.

It is important to point out that in many preparations the muscarinic pathway is involved in the modulation of a class of K^+ channels (M channels) [26]. In developing CG neurons, Furukawa et al. [20] have described the activation of a slow inward current that they ascribed to the inhibition of a M-current. The current could be seen in about 50% of the cells tested, at holding potentials more positive (-20 mV) than those used in our voltage-clamp protocols, and in neurons taken at a later developmental stage (E14). We cannot exclude in principle that, at least in some neurons, this component may be present and contribute to the muscarine-activated inward current. Anyway, from $I–V$ analysis and ionic substitution protocols in the whole cell mode, it can be concluded that the inward current we have observed at $V_h = -50$ mV is not due to a reduction of a resting conductance, but rather to the activation of a cationic, non-selective pathway permeable to calcium ions.

The cationic current has a rather slow time course, inactivates, at least partially, in the presence of the agonist, and can be observed, with similar amplitude and frequency of occurrence, both with a standard physiological solution and with calcium as the only extracellular cation. Therefore, its role in the complex pattern of ionic currents elicited by cholinergic stimulation in CG neurons is likely to be connected with calcium influx. Other authors [21–23], by means of calcium cytofluorimetrical measurements, have provided evidence of muscarine-activated calcium influx in this preparation, even if at a later stage, and have proposed different pathways for its activation. Rathouz et al. [23] have suggested that calcium influx could be due either to activation of voltage-dependent calcium channels or to a mechanism dependent on depletion of calcium from internal stores (I_{CRAC}) [27] induced by the activation of M3 receptors coupled to the phospholipase C–$InsP_3$ pathway. Sorimachi et al. [22], at the same developmental stage, have reported that blockers of L- and N-type voltage-dependent calcium channels markedly reduced the calcium signals observed in response to muscarinic stimulation; these authors concluded that the inward current described by Furukawa et al. [20] could have a depolarizing effect, which in turn could activate voltage-dependent calcium channels and generate calcium influx.

The I_{CRAC} hypothesis will be discussed in more detail below; as for the possibility of an involvement of voltage-dependent channels, while we cannot completely exclude the presence of a voltage-dependent component in the depolarizing responses observed in the current clamp mode, our voltage-clamp whole cell experiments were performed at a holding potential of -50 mV, at which marked activation of L- and N-type channels seems unlikely [28]. Even if ramp protocols can give only qualitative information on the behaviour of voltage-activated currents, in no case have we observed, following muscarinic stimulation, an increase in the peak of the voltage-dependent calcium current or a shift toward more negative values (see Fig. 2B). Additional evidence in favour of the presence of a voltage-independent pathway is provided by single channel experiments: we observed activation of calcium-permeable channels even in the presence of voltage-dependent calcium channel blockers.

In single channel experiments in the cell-attached mode, we have most commonly observed two current levels, either

in physiological ionic conditions or with Ca^{2+} as the only extracellular permeant cation. We have characterized these levels in terms of conductances and reversal potentials; the fact that the reversal potentials for the two levels are nearly identical and the occurrence of simultaneous openings and transitions between the different levels, followed by a sharp return to the baseline level, are suggestive of a single channel with two substates. Further characterization based on kinetic analysis by means of open and closed time histograms has been prevented by the fact that the channels usually showed very long open times. While we have not investigated the mechanism responsible for these long-lasting openings, it must be remarked that this behaviour can be observed even in inside-out experiments with $InsP_3$ in the bath, suggesting that the relevant step is the interaction between the second messenger and the channel.

We have previously reported [24] that bFGF, which exerts a trophic role on these cells [29], can induce, at the same developmental stage, calcium influx and long-lasting changes in $[Ca^{2+}]_i$ by activating voltage-independent calcium-permeable channels. Interestingly, one of the conductance values (2.3 ± 0.6 pS) calculated for the channel that activates in response to muscarinic stimulation is nearly identical to the one obtained for the channel that is activated by bFGF in the same cells (3.2 ± 0.2 pS) [24]. This growth factor, too, activated several current levels. While these levels were not described in detail, a level of 5.5 ± 1.0 pS with a $V_{rev} = 36.3 \pm 12.5$ mV was present, as obtained by I–V analysis from six experiments (Distasi, C., unpublished data). This provides the first experimental evidence of a possible convergence of the pathways activated by a neurotrophic factor and a neurotransmitter on the same target in a parasympathetic preparation, and suggests that cooperative mechanisms may be present in the action of these two agonists in vivo during embryonic development. In any case, by comparing electrophysiological measurements, it appears that the time course of activation of calcium-permeable channels (and of the related inward current) is different, being of longer duration (several minutes) with bFGF: this finding could be related to a different trophic role of the two molecules. Further investigation will help to clarify this issue.

The other major finding is that, in analogy with bFGF-activated channels, a channel showing properties similar to those of the muscarine-activated one can be opened by perfusion of excised patches with $InsP_3$, and that the two modes of activation can be observed on the same patch. This finding raises the problem of the nature of these channels, and of the role of the second messenger in their activation. Electrophysiological evidence for the existence in the plasma membrane of $InsP_3$-activated calcium-permeable channels has been provided for several cell types, from olfactory neurons [30–33] to neonatal and adult mammalian cardiocytes [34,35] and endothelial cells [36].

On the other hand, it has been proposed that activation of calcium-permeable channels, seen in response to stimulation with agonists that bind to G protein-coupled receptors and that act through the phospholipase C–$InsP_3$ pathway, is not directly gated by this second messenger, but through a protein–protein coupling between $InsP_3$ receptors of the endoplasmic reticulum (ER) and membrane channels, identified with channels of the *Trp* gene family and others [37,38]. The model suggests that conformational changes in the $InsP_3$ receptor can transfer information to the channel and open it. In the case of TRP3 channels, it has also been shown that the two proteins are actually coupled, and that the N-terminal domain of the former is involved in the gating of the channel [39]. In addition, evidences have been provided that this mechanism is dependent not only on activation of $InsP_3$ receptors by their agonist, but also on the depletion of intracellular stores. In this context, it must be recalled that one of the hypotheses advanced by Rathouz et al. [23] to explain the activation of calcium influx by muscarinic stimulation in neurons from E14 CGs involved a store-dependent mechanism.

We have not tried in this paper to obtain information about the molecular nature of the calcium-permeable channels activated by muscarinic stimulation in our preparation, so we cannot exclude that TRP channels may be involved. Moreover, our data do not allow to assess if $InsP_3$ is able to induce channel openings by direct interaction with the channel or by an indirect mechanism, involving other associated proteins. Similarly, we cannot exclude the involvement of a mechanism dependent on store depletion, as proposed by Kiselyov et al. [37,38]. Actually, in CG neurons at this developmental stage, muscarine elicits a small $[Ca]_i$ transient, due to efflux from intracellular stores, only in a minority of the responsive cells (Lovisolo, D., Distasi, C. and Gilardino, A., unpublished data) similar to what has been observed for bFGF [24]; therefore, a mechanism of store-dependent influx, if present, cannot explain our results as a whole. Moreover, the conductance values, reversal potentials and kinetics of the channels described in the papers cited above are remarkably different from those reported in this paper; therefore, the issue of the actual mechanism by which $InsP_3$ mediates muscarinic activation of calcium influx in CG neurons is open to further investigation.

Acknowledgements

We thank D. Meandri and S. Bianco for technical assistance, and Dr. P. Zamburlin for help with the cultures. This work was financed by the Ministry of University and Scientific and Technological Research.

References

[1] S.A. Lipton, S.B. Kater, Trends Neurosci. 12 (1989) 265–270.
[2] J. Schwartz, Int. Rev. Neurobiol. 34 (1992) 1–23.
[3] J.M. Lauder, Trends Neurosci. 16 (1993) 233–240.

[4] S. Choo, J.H. Son, D.H. Park, C. Aoki, X. Song, G.P. Smith, T.H. Joh, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 2862–2866.
[5] N.J. Messenger, S.J. Rowe, A.E. Warner, Dev. Biol. 205 (1999) 224–232.
[6] W. Ma, D. Maric, B.S. Li, Q. Hu, J.D. Andreadis, G.M. Grant, Q.Y. Liu, K.M. Shaffer, Y.H. Chang, L. Zhang, J.J. Pancrazio, H.C. Pant, D.A. Stenger, J.L. Barker, Eur. J. Neurosci. 12 (2000) 1227–1240.
[7] B.S. Li, W. Ma, L. Zhang, J.L. Barker, D.A. Stenger, H.C. Pant, J. Neurosci. 21 (2001) 1569–1579.
[8] M.O. Poulter, J.L. Barker, A.M. O'Carroll, S.J. Lolait, L.C. Mahan, J. Neurosci. 12 (1992) 2888–2900.
[9] D.S. McGehee, L.W. Role, Annu. Rev. Physiol. 57 (1995) 512–546.
[10] L. Kaczmarek, M. Kossut, J. Skangiel-Kramska, Physiol. Rev. 77 (1997) 217–255.
[11] L.A. McKinnon, E.C. Gunther, N.M. Nathanson, J. Neurosci. 18 (1998) 59–69.
[12] R.W. Oppenheim, Annu. Rev. Neurosci. 14 (1991) 453–501.
[13] B. Pettmann, C.E. Henderson, Neuron 20 (1998) 633–647.
[14] S.E. Dryer, Prog. Neurobiol. 43 (1994) 281–322.
[15] S. Furber, R.W. Oppenheim, D. Prevette, J. Neurosci. 7 (1987) 1816–1832.
[16] S.D. Meriney, G. Pilar, M. Ogawa, R. Nuñez, J. Neurosci. 7 (1987) 3840–3849.
[17] J. Maderut, R.W. Oppenheim, D. Prevette, Brain Res. 444 (1988) 189–194.
[18] Z. Zhang, J.S. Coggan, D.K. Berg, Neuron 17 (1996) 1231–1240.
[19] C.W. Bowers, C.J. Schmidt, Dev. Biol. 154 (1992) 410–418.
[20] K. Furukawa, Y. Abe, M. Sorimachi, N. Akaike, Brain Res. 657 (1994) 185–190.
[21] M. Sorimachi, Brain Res. 627 (1993) 34–40.
[22] M. Sorimachi, K. Furukawa, Y. Abe, N. Akaike, Brain Res. 696 (1995) 67–75.
[23] M.M. Rathouz, S. Vijayaraghavan, D.K. Berg, J. Biol. Chem. 270 (1995) 14366–14375.
[24] C. Distasi, M. Torre, S. Antoniotti, L. Munaron, D. Lovisolo, Eur. J. Neurosci. 10 (1998) 2276–2286.
[25] C. Distasi, F. Di Gregorio, A. Gilardino, D. Lovisolo, in: M. Morad, P. Kostyuk (Eds.), Calcium Signaling, IOS Press, Amsterdam 2001, pp. 269–273, ISBN 1-58603-157-0.
[26] N.V. Marrion, Annu. Rev. Physiol. 59 (1997) 483–504.
[27] A.B. Parek, R. Penner, Physiol. Rev. 77 (1997) 901–930.
[28] H. Yawo, A. Momiyama, J. Physiol. 460 (1993) 153–172.
[29] K. Unsicker, H. Reichert-Preibsch, R. Schmidt, B. Pettmann, G. Labourdette, M. Sensenbrenner, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 5459–5463.
[30] D.A. Fadool, B.W. Ache, Neuron 9 (1992) 907–918.
[31] H. Hatt, B.W. Ache, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 6264–6268.
[32] D. Restrepo, J.H. Teeter, E. Honda, A.G. Boyle, J.F. Marecek, G.D. Prestwich, D.L. Kalinoski, Am. J. Physiol. 263 (1992) C667–C673.
[33] F.W. Lischka, M.M. Zviman, J.H. Teeter, D. Restrepo, Biophys. J. 76 (1999) 1410–1422.
[34] P.L. Merle, J.J. Feige, J. Verdetti, J. Biol. Chem. 270 (1995) 17361–17367.
[35] P.L. Merle, Y. Usson, M. Robert-Nicaud, J. Verdetti, J. Mol. Cell. Cardiol. 29 (1997) 2687–2698.
[36] L. Vaca, D.L. Kunze, Am. J. Physiol. 269 (1995) C733–C738.
[37] K. Kiselyov, X. Xu, G. Mozhayeva, T. Kuo, I. Pessah, G. Mignery, X. Zhu, L. Birnbaumer, S. Muallem, Nature 396 (1998) 478–482.
[38] K. Kiselyov, S.B. Semyonova, A.G. Mamin, G.N. Mozhayeva, Pflugers Arch. 437 (1999) 305–314.
[39] K. Kiselyov, G.A. Mignery, M.X. Zhu, S. Muallem, Mol. Cell 4 (1999) 423–429.