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Ca^{2+}-activated Cl^- Current in Gastric Antral Myocytes

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=ABSTRACT=

The whole-cell mode of the patch clamp technique was used to study Ca^{2+}-activated Cl^- current ($I_{Cl_{Ca}}$) in gastric antral myocytes. Extracellular application of caffeine evoked Ca^{2+}-activated currents. In order to isolate the chloride current from background current, all known systems were blocked with specific blockers. The current-voltage relationship of caffeine-induced current showed outward rectification and it reversed at around E_{Cl^-}. The shift of reversal potential upon the alteration of external and internal chloride concentrations was well fitted with results which were calculated by the Nernst equation. Extracellular addition of N-phenylanthranilic acid and niflumic acid which are known anion channel blockers abolished the caffeine induced current. Intracellular application of a high concentration of EGTA also abolished this current. Application of c-AMP, c-GMP, heparin, or AlF_4^- made no remarkable changes to this current. Sodium replacement with the impermeable cation N-methylglucamine or with Cd^{2+} rarely affected this current.

From the above results it is suggested that the caffeine induced current was a Cl^- current and it was activated by intracellular Ca^{2+}.

Key Words: Patch-clamp technique, Ca^{2+}-activated Cl^- current, Gastric antral myocyte

INTRODUCTION

Smooth muscle has many different characteristics to other excitable tissues. Many smooth muscles are electrically self-excitable and are able to generate an action potential spontaneously. Furthermore, these muscles can, without any remarkable change of the action potential, contract in response to external stimuli. The responses were mostly explained with the concept of receptor and receptor operated channels. Methods to isolate healthy single cells and record channel currents (Neher and Sakmann, 1976; Hamill et al, 1981) have been developed. However it is possible to study the charteristics of excitability more deeply. The calcium ion is a modulator of many electrophysiological events occuring at the cellular and molecular levels in excitable cells(Kass et al, 1978; Matsuda, 1983). Three classes of ion channels are known to be sensitive to free intracellular Ca^{2+}: 1) Ca^{2+}-activated K^+ channels; 2) Ca^{2+}-activated non-selective cation channels; 3) Ca^{2+}-activated Cl^- channels(Marty, 1989). From these channels classes 2) and 3) were known to be components of the background current, and their physiological role being less important, less attention was paid to them than to 1). As their voltage- and time-dependencies were dis-

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covered, their importance in the genesis of the action potential and resting potential were noticed (Pacaud et al, 1989; Loirand et al, 1991). It is now suggested that they could control the electrical excitability and membrane potential in many excitable cells, and contribute to the maintenance of cellular pH and volume (Hille, 1992).

It has been reported that caffeine penetrates the cell membrane of striated muscle(Bicanchi, 1962) and acts directly on the sarcoplasmic reticulum to induce release of calcium ions(Herz and Weber, 1965). Increased intracellular Ca^{2+} induced the opening of some ion channels (Pacaud et al, 1989; Baron et al, 1991). For example, the Ca^{2+}-activated K^+channel, non-selective cation channel and Cl^- channel were reported from various excitable cells (Singer and Walsh, 1984; Loirand et al, 1991; Vogalis and Sanders, 1991; Soejima and Kokunbun, 1988). We have also reported caffeine-induced Ca^{2+}-activated currents in the gastric antral myocytes (Chung et al, 1992).

We have tried to exclude the background current, and blocked known currents with various blockers as Ba^{2+}, Cs^+, Ni^{2+}, 4-aminopyridine, verapamil and ouabain. The present study has two goals. First, we aimed to clarify the existence of the background current, and to identify the ions engaged in its formation. The second goal is to confirm their physiological characteristics and importance in gastric antral myocytes.

METHOD

Isolation of single cells

New Zealand white rabbits of either sex, weighing 1~1.5 kg were used. The antral portion of the stomach was excised and dissected through the lesser curvature. The mucosa and longitudinal layer of the stomach were removed and the circular layer was retained. The bulk mass of smooth muscle was divided into small pieces of 2~8 mm^3 volume. Isolated small masses were stored in a medium (containing in mM: taurine 20, oxalate 14, glutamate 80, KH_2PO_4 10, KCl 25, glucose 11, EGTA 0.5, KOH 129 pH 7.3) for 30 minutes at 4°C. The digestion procedure of the gastric antral smooth muscle into single cells was performed in a Ca^{2+}- and Mg^{2+}-free physiological salt solution (containing in mM NaCl 140, KCl 5, NaH_2PO_4 0.25, HEPES 5, glucose 11, pH 7.4) containing collagenase(Wako) 1 mg/ml, bovine serum albumin 2 mg/ml, trypsin inhibitor 1 mg/ml, and dithioerythritol(DTE) 5 mM. Pieces of tissue were shaken in enzyme solution for 25 minutes at 35°C. After enzymatic digestion, gentle agitations with a glass dropper were performed for 5 minutes. Isolated single cells were stored for 30 to 120 minutes at 4°C before each patch recording experiment (Hamill et al, 1981).

Whole cell mode of patch clamp recording

Isolated single cells were dispersed in an experimental chamber of 300 μl volume, and allowed for 20 minutes to become attached the bottom of chamber. Perfusion with physiological salt solution for 5 minutes, cells which were relaxed and had a sharp margin were used. Patch electrodes were made with a vertical puller (model 700C, DKI, U.S.A.) and had 3 to 5 MΩ tip resistance when filled with internal solution (containing in mM CsCl 140, MgATP 5, $MgCl_2$ 5, NaH_2PO_4 1, EGTA 0.1, HEPES 5, pH 7.2). Patch-clamp studies were performed with a standard amplifier (model AXOPATCH 1D, Axon, U.S.A.), and data were recorded to a personal computer (IBM) through a four-pole Bessel filter of -3 dB frequency 1 kHz. Recordings were digitized with a labmaster non-DMA board and analyzed with pClamp software (version 5.5.1, Axon, U.S.A.). Most experiments were performed with ramp pulse protocols in the voltage clamp mode. We used an agar-KCl reference electrode to minimize the

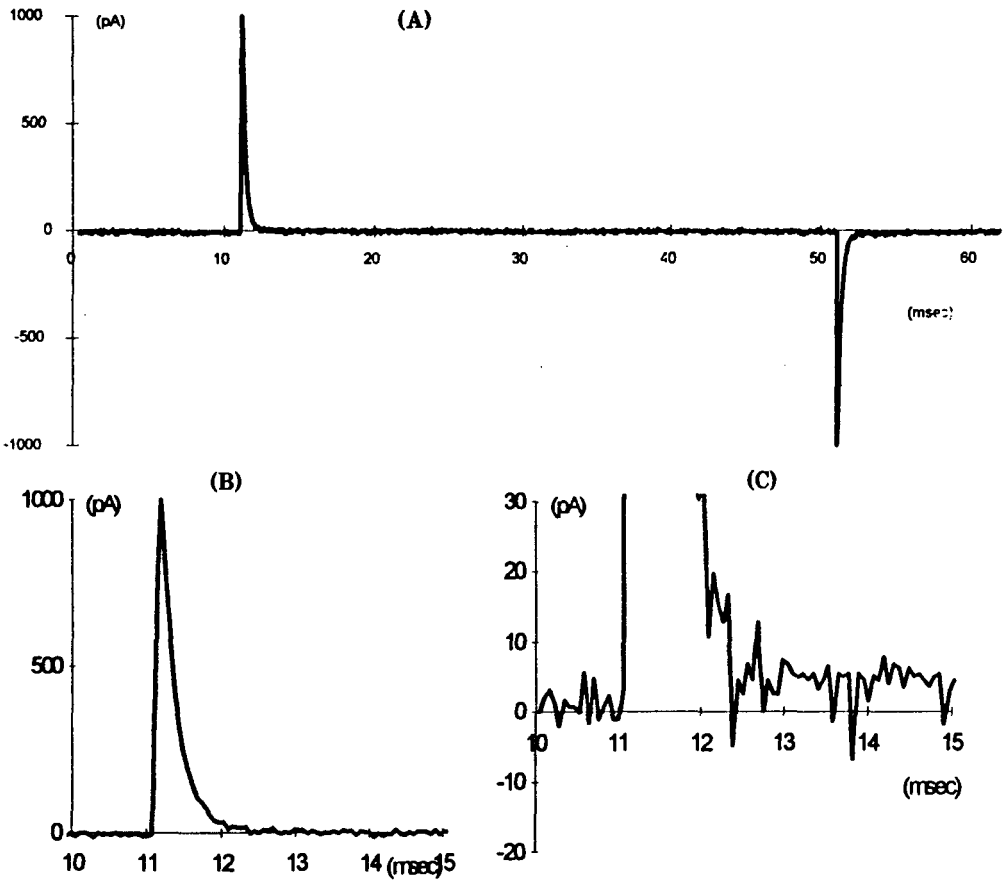

Fig. 1. Voltage clamp recording of whole-cell current from a gastric antral smooth muscle cell. Capacitive current transients were elicited by 10 depolarizing commands from − 60 mM (A). (B) indicates an enlarges view of a capacitive transients, a single exponential cureve decaying with time constant $\tau = 0.34$ msec. Rseries was estimated from the peak capacitive current to be ~ 10M Ω. Cell capacitance determined from integration of the current was ~ 32 pF. Steady-state current, induced by a command pulse (C), indicated that this cell had an input resistance of ~ 2M Ω.

shift of holding potential resulting from the changes of chloride ion concentration (lower than 10 mV). The command potential was changed at a rate of 800 mV per second starting at a negative holding potential. The current ampitude was then standardized to a membrane capacitance of 50 pF.

RESULTS

Calculation of the resistant component and cell capacitance with small depolarizing command pulses is illustrated in figure 1. Time constant and series resistance(R_{series}) were estimated from the peak capacitive current. Input resistance was calculated from the steady-state current and cell capacitance determined from the integration of current. We first examined the effect of caffeine and an anion channel blocker on the action potential in current-clamp mode (Fig. 2). Application of caffeine elongated action potential duration, subsequent addition of the anion channel blocker niflumic acid (10 μ

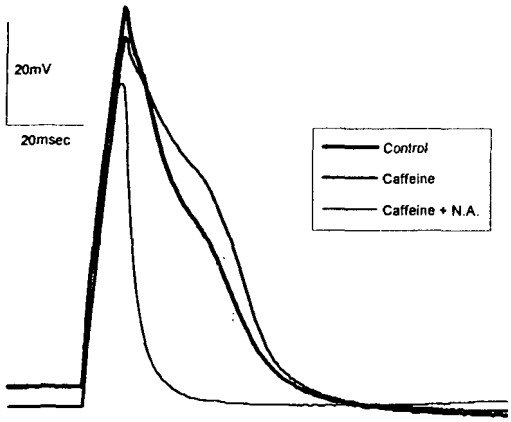

Fig. 2. Effect of the anion channel blocker niflumic acid (20 μM) on the action potential induced by constant current at reduced intracellular chloride concentration (25 mM). Application of caffeine prolonged the action potential and further addition of the anion channel blocker reduced the duration of action potential.

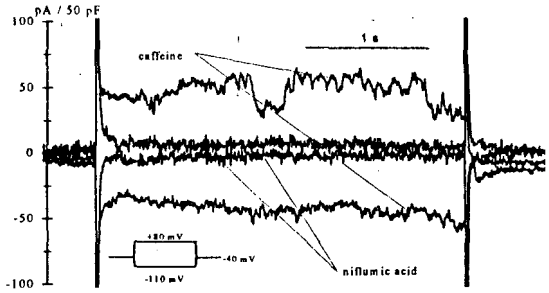

Fig. 3. Blockade of caffeine (10 mM) -induced square pulse-current responses by anion channel blocker (niflumic acid, 20 μM). The Main constituents of the outside bathing solution and intrapipette solution are shown as Ext. and Int. respectively. Expected equilibrium potential of Cl^- is denoted by E_{Cl^-}.
Ext.: NaCl, caffeine 10 mM with blockers
Int.: CsCl
E_{Cl^-}: ~ 0 mM

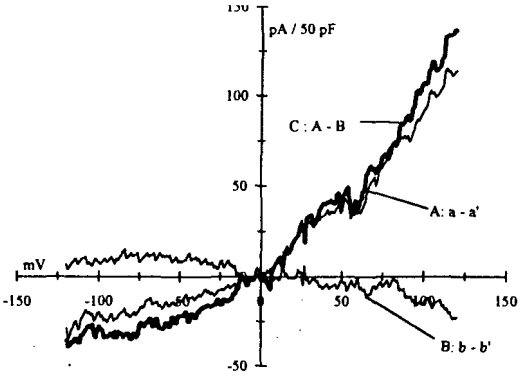

Fig. 4. Anion channel blocker (5 mM phenylanthranilic acid, PAA)-sensitive current responses to ramp pulses (0.8 V/s) in the caffeine (10 mM) -injected condition.
A: Control
B: Current response with PAA
Ext.: NaCl (140 mM) with blockers
Int.: CsCl (140 mM)
E_{Cl^-}: ~ 0 mV

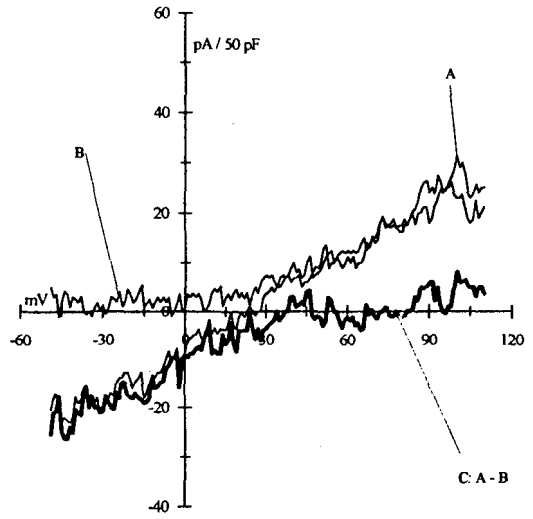

Fig. 5. Anion channel blocker (20 μM niflumic acid, NA)- sensitive diference currents in reduced Cl^-_o (10 mM) concentration.
Ext.: Na^+ Asp (140 mM) with blockers
Int.: CsCl (140 mM)
E_{Cl^-}: ~ +68 mV

M) reduced action potential duration more than in control experiments. Fig. 3 shows the effects of niflumic acid on the currents induced by caffeine under depolarized and hyperpolarized conditions. In each case niflumic acid blocked the current responses induced by caffeine. Many blockers were used to eliminate known currents. $BaCl_2$ (2 mM) and CsCl (2 mM) were used to block K^+ currents, $NiCl_2$ (2 mM) to block Ca^{2+} and Na^+/Ca^{2+} exchange currents, 4-amino-pyridine to block transient outward currents, verapamil (2 μM) to block Ca^{2+} current, and ouabain to block Na-K pump current (Giles and Ginneken, 1985; Kimura et al, 1987; Osterrieder et al, 1982). We held the membrane potential at -40 mV to inactivate Ca^{2+} current. To clarify the nature of these current responses we changed the concentration of intracellular and extracellular Cl^-. Ramp pulse was used to study the current-voltage relationships of these current (Fig. 4, 5, 6). Phenylanthranilic acid, another kind of anion channel blocker, was also used in these experiments. Anion channel blocker-sensitive currents were well fitted with the reversal potential as calculated from the Nernst equation. In order to exclude the possibility that these current responses contained a non-selective cation current we changed the major extracellular cation from Na^+ to the non-permeable cation n-methylglucamine(Fig. 7). The current responses induced by caffeine were rarely affected by Na^+ removal. We also used Cd^{2+} (2 mM) which is known to be a non-selective cation channel blocker (Inoue, 1991) to exclude the non-selective cation current more certainly (Fig. 8). For the further evaluation of these current responses, we used several second messangers such as, c-AMP (10 μM), c-GMP (10 μM), heparin (100 μg/ml), Al^{3+} (10 μM), and F^- (10 mM). Application of these agents had little affect on the current responses. Application of EGTA could chelate cations (Fabiato and Fabiato, 1979) such as Ca^{2+} and Mg^{2+}. We used a high concentration (10 mM) of

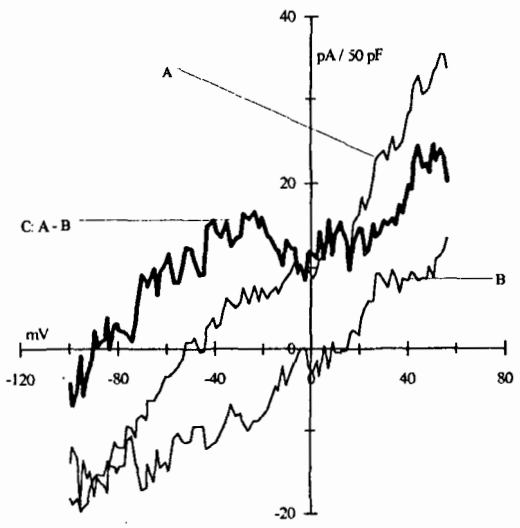

Fig. 6. Niflumic acid (NA) -sensitive difference currents in reduced Cl_i^- (10 mM) concentration.
Ext.: NaCl (140 mM) with blockers
Int.: Cs · Asp (140 mM)
E_{Cl^-}: ~ −68 mV

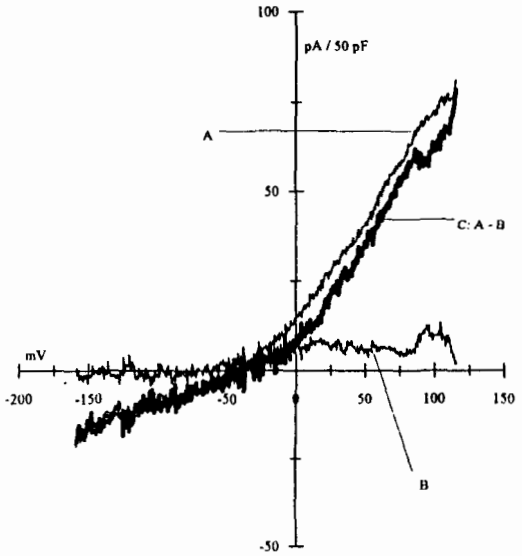

Fig. 7. NA-sensitive difference currents in Na_o^+-free conditions.
Ext.: NMG · Cl (140 mM) with blockers
Int.: CsCl (140 mM)
E_{Cl^-}: ~ 0 mV

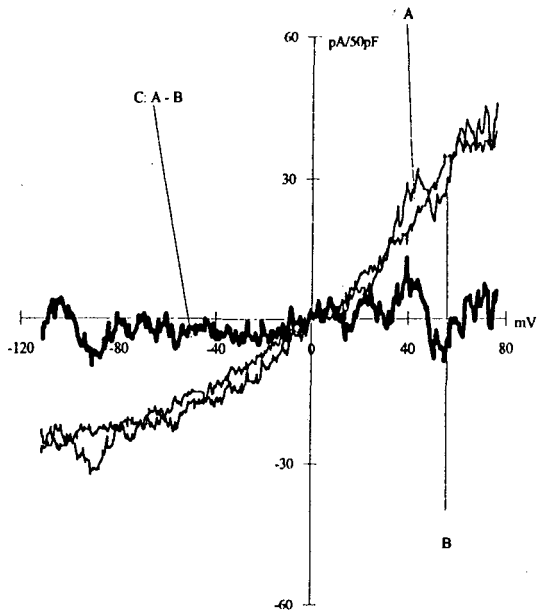

Fig. 8. Intrapippette c-AMP (10 μM) hardly affected the current responses to caffeine. Application of $(c\text{-}GMP)_i$, $(heparin)_i$, (AlF_4^-), and $(Cd_2^+)_o$ showed similar results.

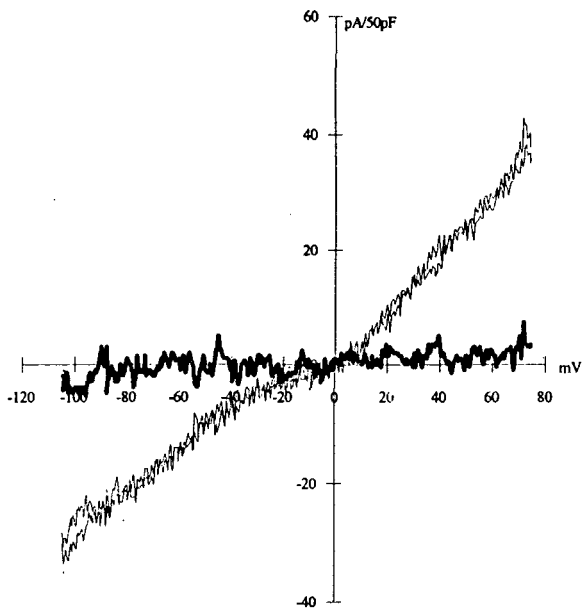

Fig. 9. Difference currents were almost completely abolished by increased intracellular EGTA concentration (10 mM). External bathing solution was the normal physiological salt solution which contained various agents to block all known current system.

intracellular EGTA to reduce intracellular Ca^{2+} concentration and furthermore to inactivate Ca^{2+}-activated currents (Fig. 9). The current responses evoked by caffeine addition were almost abolished by a high dose of intracellular EGTA.

DISCUSSION

The ionic currents induced by an increase of intracellular Ca^{2+} concentration are Ca^{2+}-activated K^+ cuurent, Ca^{2+}-activated non selective cation current, and Ca^{2+}-activated Cl^- current. They are found in various smooth muscle cells (Benham et al, 1986; Byrne and Large, 1987; Pacaud et al, 1989). According to a recent study non-selective cation current and Cl^- current are involved in the generation of the action potential, and K^+ and Cl^- currents affect action potential duration(Pacaud et al, 1989).

Caffeine acts on intracellular organelles to release stored Ca^{2+} into the cytosol and this phenomenum can evoke Ca^{2+}-activated currents (Pacaud et al, 1989; Baron et al, 1991). Therefore we focused on background current which has received little attenttion because its role and nature was unknown and its existence was controversial. Recent studies have shown that the background current contains anionic and non-selective cation currents which are involved in formation of the resting potential and the action potential (Pacaud et al, 1989; Loirand et al, 1991). Caffeine induced elongation of the action potential and application of an anion channel blocker reduced the action potential duration even more than control. From these results we could expect that anion movement may be involved in the regulation of the action poten-

tial in the excited state of gastric antral myocytes.

We examined whether Ca^{2+}-activated currents other than K^+ current exist in the gastric antral smooth muscle. We used many blockers to eliminate known currents and to extract the background current component of whole cell current. Caffeine induced current responses showed outward rectification and reversed at 0 mV in the same intracellular and extracellular Cl^- concentration. It was difficult to distnguish Cl^- current from non-selective cation current because latter can also be induced by the application of caffeine and its reversal potential was almost same at that in the control condition in our experiment. We therefore used the non-permeable cation Cd^{2+} (Inoue, 1991) which is known to be a non-selective cation channel blocker. It rarely affected caffeine induced current responses. The application of anion channel blockers, however, almost ablolished these currents. The shift of reversal potential according to changes of intra- and extracellular Cl^- concentrations were well fitted using the Nernst equation. From these results we thought that the current responses activated by caffeine were Cl^- currents.

We studied the characteristics of these current responses with second messengers which could modulate them. We tested c-AMP, a known activator of intracellular phosphorylation of myosin chain (Adelstein et al, 1978); c-GMP, an intracellular secondary activator that is reported to have various effects on smooth muscle cells (Gerzer et al, 1981; Kukovertz et al, 1979; Pfitzer et al, 1984; Schultz et al, 1977); heparin, known to be an inhibitor of IP_3 induced Ca^{2+} release (Kobayashi et al, 1988); and $AlF4^-$, reported to be a G protein activator (Cockcroft, 1987). We applied these modulators intracellularly but they rarely affected caffeine induced current responses.

According to our results we can suggest that the currents induced by caffeine were mostly Ca^{2+}-activated Cl^- currents. The currents seemed to be involved in determining action potential duration and were mainly regulated by intracellular Ca^{2+} concentration. Their exact roles in the resting and excited states, and characteristics, including single channel conductance and modulation remain to be studied.

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