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Effects of chloride ion substitutes and chloride channel blockers on the transient outward current in rat ventricular myocytes

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Abstract

The Ca_i^{2+}-insensitive transient outward current, i_{to}, was studied at 20–24°C in rat ventricular myocytes with the whole cell recording patch-clamp technique. The current was recorded before and after replacement of chloride by methanesulfonate or aspartate or in the absence and the presence of chloride channel blockers, SITS or 9-anthracene carboxylic acid. In control conditions (in the presence of external divalent cations, Ca^{2+} and Cd^{2+}, Cd^{2+} being used to suppress Ca^{2+} current), i_{to} inactivation was composed of a fast and a slow component. When methanesulfonate was substituted for external Cl^-, the peak current decreased to a variable extent, but the inactivation of the remaining current was still composed of a fast and a slow component. In contrast, the inactivation of the difference current was well fitted by a single exponential. The time to peak of the difference current was shorter than that of the current recorded either in the absence or the presence of methanesulfonate. Both activation- and steady-state inactivation–voltage curves were either unchanged ($n = 4$) or shifted by a few mV (5.5 mV, $n = 14$) towards positive potentials when methanesulfonate was substituted for Cl^-. The current remaining in methanesulfonate reversed at potentials closed to E_K. The difference current was composed of a peak and a steady-state component. The peak was suppressed by 4-aminopyridine whereas the steady-state component was not. The peak was also suppressed when pipette solution contained Cs^+ instead of K^+ but was still present when the Hepes concentration in both external and pipette media was increased 5-fold (50 mM vs. 10 mM). When aspartate was substituted for Cl^- or when 2 mM SITS was added to the external solution (in the absence of Ca^{2+} and Cd^{2+} because aspartate is known to chelate Ca^{2+} ions and possibly other divalent cations), i_{to} was reduced to a similar extent in the two cases and the difference current was composed of a peak (inactivation fitted by a single exponential) and a steady-state component. The SITS-sensitive transient current reversed at a potential close to E_{Cl}. When 5 mM 9-anthracene carboxylic acid was added to external solution (in the presence of Ca^{2+} and Cd^{2+}), the peak of the difference current was similar to that observed when Cl^- was substituted by methanesulfonate. The difference current resulting from the substitution of methanesulfonate for chloride was not changed when the pipette solution contained either 50 mM EGTA (instead of 5 mM) or 10 mM EGTA and 10 mM BAPTA. The nature of Cs^+- and 4-aminopyridine-sensitive transient outward current suppressed by chloride ion substitutes or chloride channel blockers is discussed.

Keywords: Transient outward current; Chloride current; Methanesulfonate; Aspartate; Caffeine; Chloride ion substitute; (Rat ventricular myocytes)

1. Introduction

In rat ventricular cells, the existence of a large and long-lasting 4-aminopyridine-sensitive transient outward current [1–6] offers a logical explanation for the very short duration of the initial part of the action potential plateau of the rat ventricle and the low amplitude of its late component [7]. Since the discovery, in cardiac tissues, of the transient outward current [8,9], much attention has been paid to the ionic nature of the current carrier (for reference see [10]).

Because the transient outward current was reduced in chloride-free solution, it was initially suggested that the current was carried mainly by chloride ions [11–14]. However, Kenyon and Gibbons [15], suggested that this apparent Cl^- dependence was due to changes in external Ca^{2+} activity caused by chelation of free Ca^{2+} by the Cl^- substitutes. This suggestion was strengthened by the

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demonstration of the dependence of the transient outward current on external free Ca^{2+} [16,17], and by the fact that the current was much less sensitive to replacement of external Cl^- when free Ca^{2+} was maintained at a constant level [18,19]. In addition, the transient outward current was blocked by K^+ channel antagonists, such as tetraethylammonium and 4-aminopyridine, suggesting that, in fact, K^+ ions were the main charge carriers of the current. Subsequent studies demonstrated that the transient outward current can be divided into two components, i.e., a longer-lasting, 4-aminopyridine sensitive, Ca_i^{2+}-independent component referred to as i_{lo}, i_t, i_{to} or i_{to1} and considered as being carried by K^+ ions, and a brief Ca_i^{2+}-dependent component referred to as i_{bo}, $i_{K(Ca)}$ or i_{to2} [20–25]. In ferret ventricular myocytes, a small component of 4-aminopyridine and Cd^{2+}-insensitive transient outward current was described as being a chloride current [26] and, more recently the brief Ca_i^{2+}-dependent component of the transient outward current was shown to be carried by Cl^- ions in cardiac tissues of the rabbit as well as in canine ventricular myocytes and, as a result, was referred to as $i_{Cl(Ca)}$ [27–30]. Here, we report that in adult rat ventricular myocytes the Cs^+- and 4-aminopyridine-sensitive, Ca_i^{2+}-independent component of transient outward current, i_{lo}, was depressed by substitution of chloride ions by methanesulfonate or aspartate, or by addition of 2 mM SITS or 5 mM 9-anthracene carboxylic acid. In addition, the SITS-sensitive component of the transient current was shown to reverse at a potential close to E_{Cl}.

2. Materials and methods

2.1. Preparation of myocytes and solutions

Ventricular myocytes from adult rat hearts were enzymatically dissociated as previously described [5]. Cells were mechanically dispersed by gently shaking a small piece of tissue cut from either right or left ventricular myocardium in a plastic Petri dish containing the standard extracellular solution in which the myocytes were maintained. In order to record the calcium-insensitive transient outward current with minimal contamination from Ca^{2+}-sensitive currents, calcium current was suppressed by external Cd^{2+} and Ca^{2+} release from the sarcoplasmic reticulum was blocked both by external ryanodine and internal EGTA or EGTA and BAPTA. In addition, the sodium current was eliminated by substituting choline chloride for NaCl, the possible activation of muscarinic potassium currents by choline being prevented by addition of atropine. Therefore, the standard extracellular solution in which cells were maintained contained (in mM): 135 NaCl, 5.4 KCl, 1.1 $MgCl_2$, 0.18 $CaCl_2$, 0.5 $CdCl_2$, 10 Hepes, 10 glucose, 1 ribose, 0.001 ryanodine, 0.01 atropine sulfate; pH was adjusted to 7.4 with NaOH. The standard superfusion medium contained (mM): 135 choline chloride, 5.4 KCl, 1.1 $MgCl_2$, 1.8 $CaCl_2$, 0.5 $CdCl_2$, 10 Hepes, 1 ribose, 10 glucose, 0.001 ryanodine, 0.01 atropine sulfate; pH was adjusted to 7.4 with KOH. The intracellular pipette medium was a nominally calcium-free solution, containing (mM): 130 K-aspartate, 10 KCl, 4 MgATP, 3 $MgCl_2$, 5 phosphocreatine (di-Tris salt), 10 glucose, 5 EGTA, 10 Hepes; pH was adjusted to 7.2 with KOH. In some experiments the concentration of EGTA was increased either to 50 mM or to 10 mM in the simultaneous presence of 10 mM BAPTA. The external concentration of Cl^- was altered by replacing choline chloride with equimolar concentration of choline aspartate or choline methanesulfonate (choline-CH_3SO_3). To avoid large liquid junction potentials between pipette and bath solution, arising from the reduction of external Cl^- concentration, the altered solution contained a residual Cl^- concentration of either 12.2 mM, coming in part from the unchanged divalent salt concentration, or 5.4 mM coming from KCl in divalent-free solution. Otherwise, the junction potential problem was treated in the same way as Zygmunt and Gibbons [28], i.e., the Ag|AgCl electrode was connected to the Petri dish by a 3 M KCl agar bridge. In a few experiments the standard superfusion medium contained NaCl instead of choline chloride and sodium methanesulfonate was used instead of choline methanesulfonate. SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) (Serva, Heidelberg, Germany), 9-anthracene carboxylic acid (Sigma Chemical) were dissolved directly in the superfusate to achieve the desired concentration. SITS was prepared and used in a darkened room. All experiments were conducted at room temperature (20–24°C).

2.2. Current recordings and analysis

Macroscopic current recordings were obtained with the classical whole-cell voltage-clamp method using a patch-clamp amplifier with a 100 MΩ feedback resistor (Model 8900, Dagan Corp., Minneapolis, MN, USA). Patch pipettes (1–3 MΩ when filled with experimental solutions) were pulled from Pyrex capillaries (Corning code 7740, Corning Glass, Corning, NY) and were not fire-polished before use. The resistance in series with the cell membrane was compensated, whereas neither cell membrane capacitive current nor leakage current was compensated. A flow of solution from one of a series of five piped outlets continuously superfused the cell from which recording was being made. The flow rate of perfusion solutions was 50–100 μl/min. Currents were elicited by 700 ms or 1 s voltage steps in 10 mV increments from a holding potential of −80 mV. Under our experimental conditions, the total outward current was composed of a transient component identified as a calcium-independent transient current, i_{lo}, and a time-independent component, i_c. Voltage steps were applied at a frequency of 0.1 Hz, which allowed complete recovery of i_{lo} between pulses. Whole-cell currents were recorded without filtering (wide

band) and stored upon digital audio tape and/or digitized at 20 kHz and analyzed with a microcomputer (Tandon, MCS 486) using an S200 interface (Cambridge Research Systems, Cambridge, UK). They were retrieved on a HP Laserjet III printer (Hewlett-Packard, San Diego, CA). The cell capacitive current was exponentially fitted and then numerically subtracted from the recorded current trace. Peak current of the transient outward current was determined and the kinetic analysis of the current inactivation was performed according to Lefevre et al. [5]. The data for steady-state inactivation and activation were fitted to the theoretical Boltzmann function using the non-linear least-squares gradient-expansion algorithm of Marquardt. In the case of activation, the relative conductance was computed by determining the chord conductance as a function of membrane potential, assuming that the transient outward current was carried only by potassium ions, i.e., that it reversed at the K^+ equilibrium potential, E_K. As maximum experimental values did not reflect maximum chord conductances, we determined the latter using a computer-calculated fit to a Boltzmann relation, according to the equation:

$$G = G_{max}/\{1 + \exp[(V_m - V_{0.5})/k]\}$$

where G_{max} is the maximum chord conductance, G the chord conductance calculated at the membrane potential V_m, $V_{0.5}$ the potential at which the conductance is half-maximally activated, and k is the slope factor inversely proportional to the steepness of the activation curve. When possible, results are given as mean ± standard deviation (S.D.) of n determinations.

3. Results

3.1. External chloride ion substitution

3.1.1. Methanesulfonate

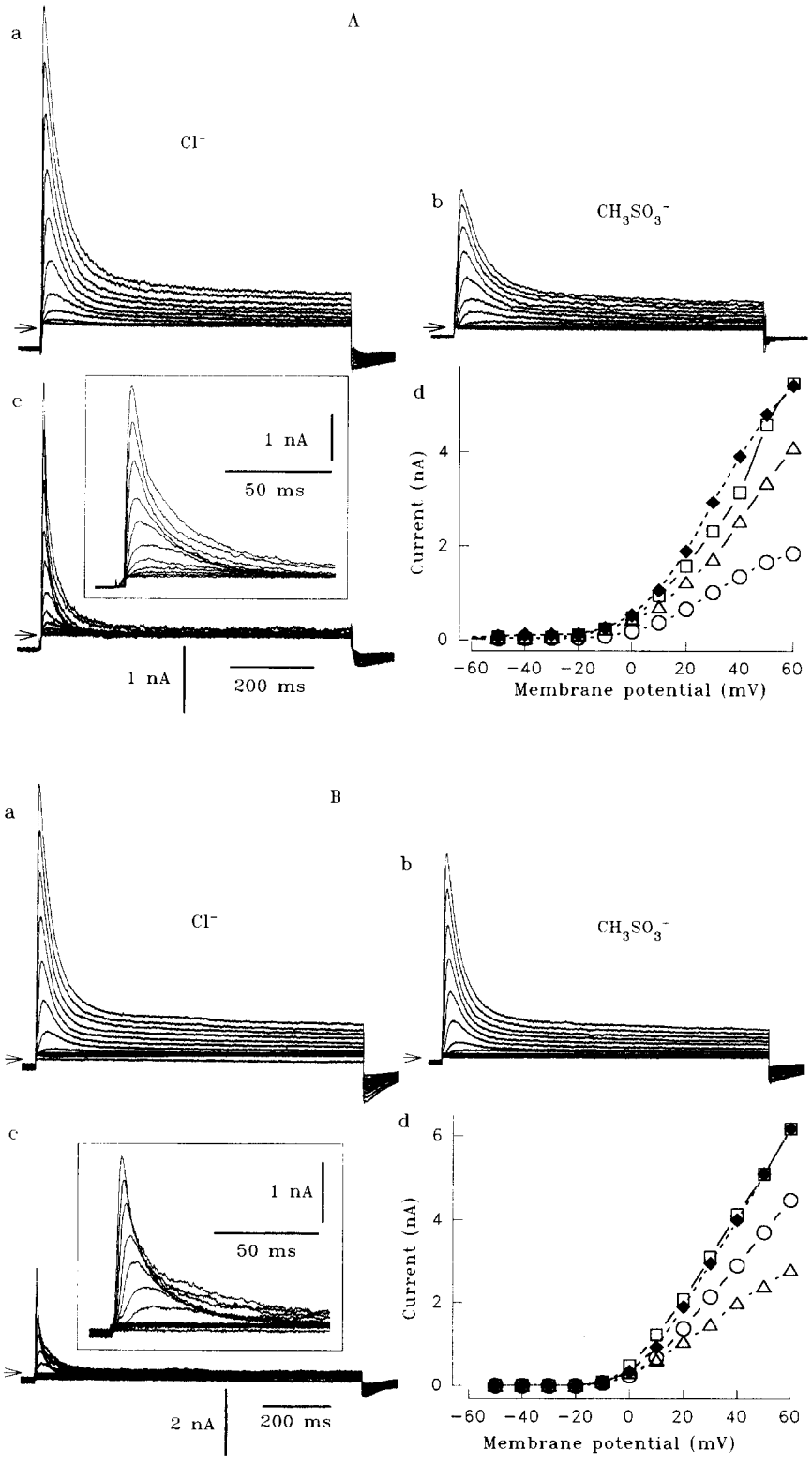
Fig. 1 shows the depressing effect of substitution of external Cl^- by methanesulfonate on transient outward current (i_{to}) in two different myocytes, one exhibiting a large effect (A) and the other a moderate and more usual effect (B) of chloride substitution. It is worth noting that the pipette solution was different in the two experiments. In A, it contained 5 mM EGTA whereas in B it contained 50 mM EGTA in order to maximally buffer cytosolic calcium. The reduction of peak current amplitude observed after suppression of external chloride was much smaller in B (26.6% at +60 mV) than in A (60.8%) but this effect cannot be attributed to the difference in EGTA concentration of the pipette solution because in other experiments we observed smaller current reductions with low EGTA concentrations and larger reductions with high EGTA concentrations. Fig. 1Aa and 1Ba show typical families of current traces obtained in control conditions, i.e., in the presence of 142 mM external Cl^- concentration. Depolarizations to membrane potentials more positive than −20 mV elicited large, rapidly activating transient outward currents, which increased in amplitude with increasing depolarizations. The current peak was reached within 15 ms for strong depolarizations (+60 mV). The current decayed rapidly over the first 100 ms, then more slowly, indicating the contribution of two kinetic components to the time-dependent fraction of outward current [5]. At the end of the voltage step (700 ms in A, 1000 ms in B) there remained a sustained component of outward current. In Fig. 1Ab and 1Bb the families of outward current traces were obtained when external Cl^- was substituted by methanesulfonate. i_{to} was sizeably decreased compared to control conditions whereas the sustained component although somewhat reduced was less affected by the substitution. This effect developed in less than 5 s, and was not always fully reversible, specially for long term exposures, possibly as a result of some detergent-like effect of high concentrations of methanesulfonate.

In three experiments, 10 mM EGTA + 10 mM BAPTA were used as intracellular Ca^{2+} buffer in order to eliminate the possibility that fast changes in internal Ca^{2+} concentrations were responsible for the effect of chloride substitution, since BAPTA is a faster buffer than EGTA. The reduction of peak amplitude observed in these experiments after suppression of external chloride was 31 ± 11%.

Fig. 1Ac and 1Bc show the families of current traces obtained by subtracting the current traces obtained in the presence of methanesulfonate from those recorded in control conditions. Difference currents appear as brief peaks of transient outward current followed by relatively small steady-state components. Current-voltage relationships (Fig. 1Ad and 1Bd) show that either in control or in chloride-free solution the current began to activate between −20 and 0 mV and increased with increasing depolarizations. After substitution of Cl^- by methanesulfonate, the peak amplitude measured at +60 mV was reduced to 72 ± 13% ($n = 43$). Normalization of peak current-voltage relationships obtained in methanesulfonate solution (filled diamonds) to those obtained in control solution (open square) indicates that chloride removal induced either a small negative shift (Fig. 1Aa) or almost no shift (Fig. 1Bd) in current-voltage relationships. In a different group of 11 cells (5 mM EGTA in the pipette solution) substitution of external chloride by methanesulfonate induced a mean positive shift of 5.5 mV of both activation-voltage and steady-state inactivation-voltage relationships (Fig. 2A).

The time to peak, for the three families of current traces of Fig. 1A, decreased with increasing membrane depolarization. Ranging between 38 and 9 ms in control conditions, the time to peak markedly increased in chloride-free solution, reaching values comprised between 92 and 20 ms. The time to peak of the difference current was always shorter at a given potential than those measured either in control or in chloride-free solutions. This fact (i.e., peaks

were not measured at the same time) explains why, at a given membrane potential, the current peak measured in Fig. 1Ab added to the difference current peak measured in Fig. 1Ac gives a total value higher than that of the control current peak measured in Fig. 1Aa. This is also visible in the current-voltage relationships shown in Fig. 1Ad.



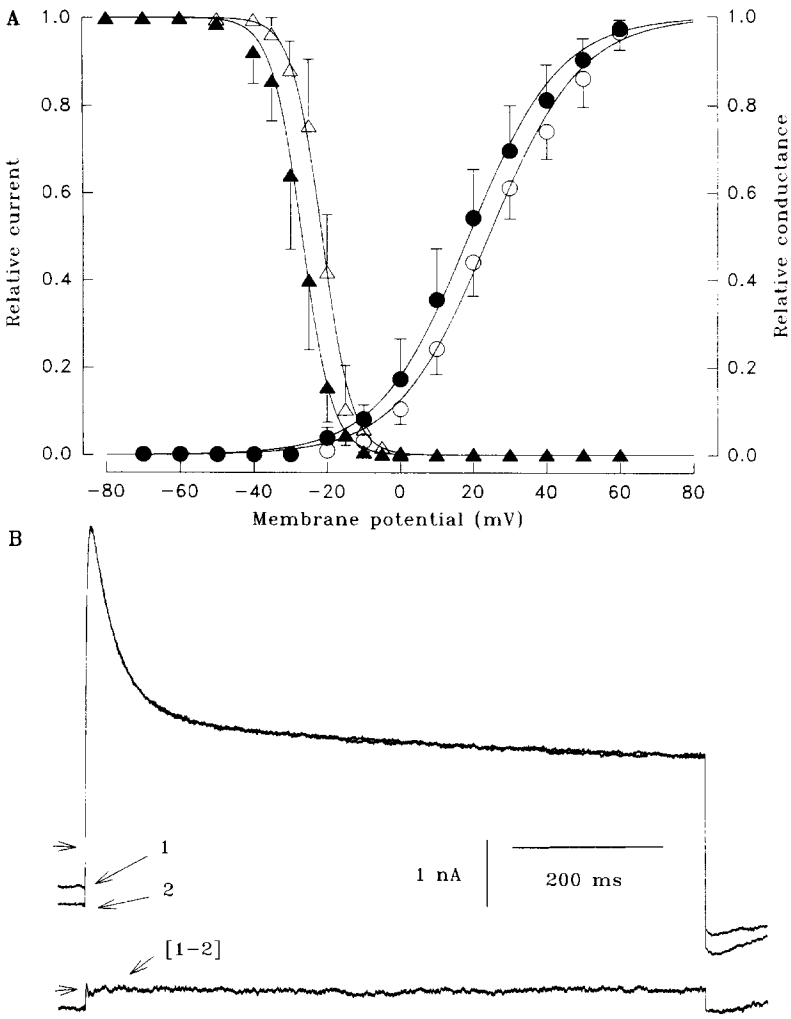


Fig. 2. (A) Effect of substitution of extracellular chloride by methanesulfonate on activation- and inactivation-voltage relationships of transient outward current. In the case of activation-voltage relationships, normalized chord conductance (relative conductance) obtained from 11 myocytes, was plotted versus membrane potential before (●, SD upward) and after (○, SD downward) chloride substitution by methanesulfonate. The curves were fitted to experimental data according to procedures described in the text. The values of $V_{0.5}$ and k (see Methods section) were 18.7 mV and 12.4 mV respectively for chloride medium versus 24.2 mV and 12.5 mV for methanesulfonate medium. In the case of steady-state inactivation-voltage relationships obtained from 9 myocytes before (▲, SD downward) and after (△, SD upward) chloride substitution by methanesulfonate, the curves were fitted to experimental data according to the following Boltzmann relation: $I/I_{max} = 1/\{1 + \exp[-(V_m - V_{0.5})/k]\}$, where $V_{0.5} = -26.8$ mV, $k = -4.1$ mV for chloride medium versus $V_{0.5} = -21.3$ mV and $k = -4.0$ mV for methanesulfonate medium. Holding potential was −80 mV. (B) Reduction of transient outward current resulting from chloride substitution by methanesulfonate is not due to a direct inhibitory effect of methanesulfonate. Currents were elicited by 1 s pulses to +60 mV from a holding potential of −80 mV. (A) Upper traces, currents in control choline chloride solution (1) and after addition of 100 mM methanesulfonate to the control solution (2). Lower trace, difference current [1,2].

Inactivation of i_{lo} can be generally well fitted by two exponentials with a fast (30 ms ≤ τ ≤ 60 ms) and a slow (200 ms ≤ τ ≤ 600 ms) time constant, respectively [5,31]. The inactivation of the current recorded in Cl^--free media can also be fitted by two exponentials. Slow time constant values were not appreciably modified by methanesulfonate

Fig. 1. Effect of substitution of extracellular chloride by methanesulfonate on transient outward current recorded in rat ventricular myocytes. (A) and (B) correspond to two different myocytes; a and b, current traces recorded in normal chloride solution (Cl^-, a) and in methanesulfonate solution ($CH_3SO_3^-$, b). c, difference currents (expanded traces are shown in the inset). Currents were elicited by 700 ms (A) and 1000 ms (B); depolarizing voltage steps applied at 0.1 Hz, from a holding potential of −80 mV in 10 mV increments, between −50 and +60 mV. d, peak current-voltage relationships in chloride solution (□) and in methanesulfonate solution (○); difference current (△); normalization of current-voltage relationship obtained in methanesulfonate solution (◆) respectively to that obtained in chloride. Peak current amplitudes were measured with respect to the time-independent component of outward current determined at each potential (by kinetic analysis when necessary). In these and other current traces, arrows indicate zero current level. The pipette solution contained 5 mM EGTA in A and 50 mM EGTA in B.

substitution at negative potentials but were increased by 15–25% at positive potentials. Fast time constants were also increased in Cl^--free media. In contrast, the inactivation of the difference current can always be adequately fitted by a single exponential (and a steady-state component). In the potential range between +10 and +40 mV the time constant of inactivation of the difference current was sizeably smaller than that of the fast component of the current remaining in the absence of external Cl^-. As a result, although fairly large at the time of its peak, the difference current carry a relatively smaller quantity of charges than the current persisting after chloride removal. The current remaining in the presence of methanesulfonate is a K^+ current. This was shown by the fact that in

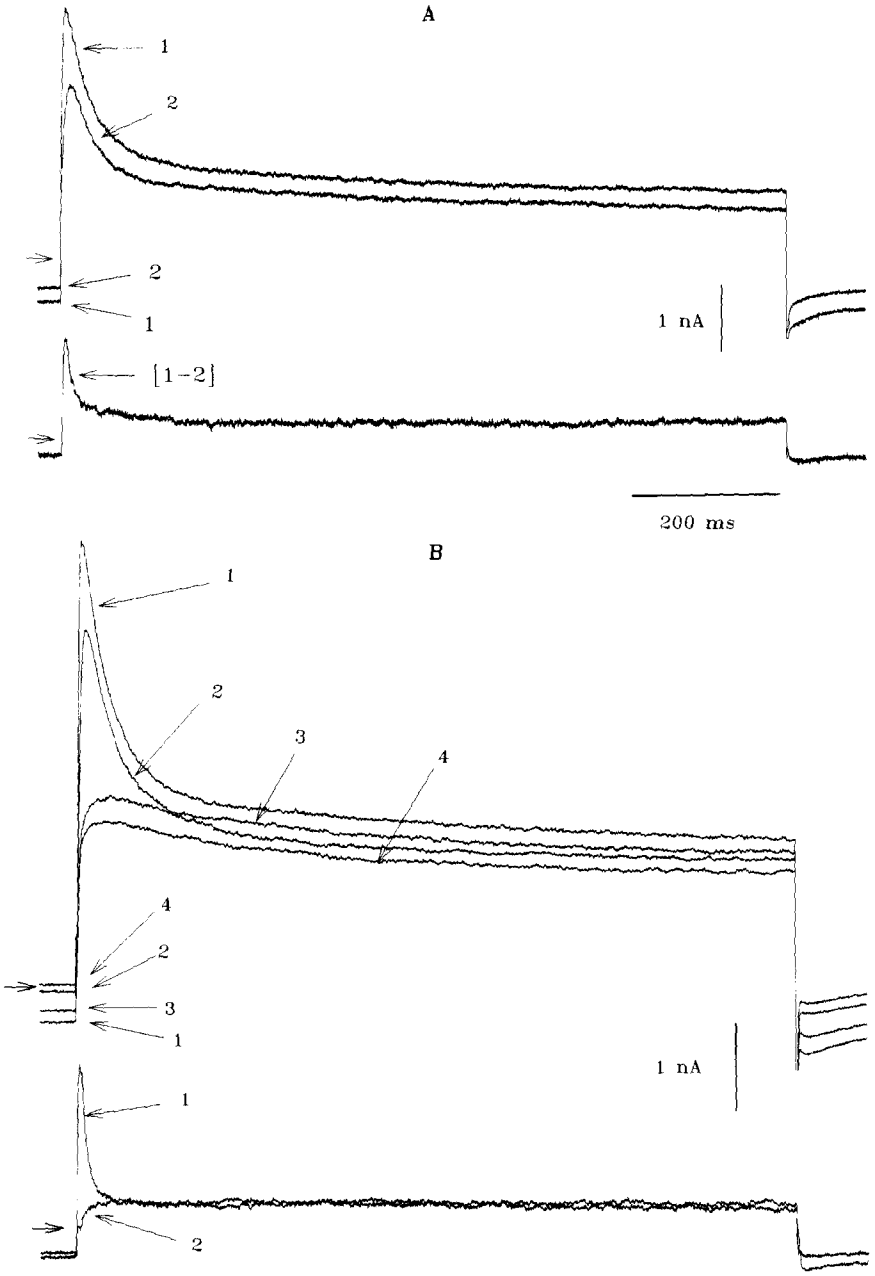


Fig. 3. (A) Reduction of transient outward current resulting from chloride substitution by methanesulfonate is not due to a change in internal pH. Both external and pipette media contained 50 mM Hepes. Upper traces, currents in control choline Cl solution (1) and after substitution of the external chloride by methanesulfonate (2). Lower trace, difference current [1,2]. (B) Inhibitory effect of 3 mM 4-aminopyridine on transient outward currents. Upper traces, currents recorded in control condition (1), after substitution of methanesulfonate for chloride (2), after addition of 4-aminopyridine in control condition (3) and after addition of 4-aminopyridine in methanesulfonate (4). Lower traces, difference current (control minus methanesulfonate) in the absence (1) and the presence of 4-aminopyridine (2). Currents were elicited by 1 s pulses to +60 mV from a holding potential of −80 mV.

Cl^--free medium tail currents following 20 ms pulses at +40 mV reverse at -77.0 ± 2.2 mV ($n = 5$), i.e., very close to the estimated E_K (−81 mV) (not illustrated).

We checked that methanesulfonate did not alter by itself the transient outward current. The current traces (Fig. 2B) obtained in control medium with and without addition of 100 mM methanesulfonate are strictly superimposed during depolarizing steps to +60 mV from a holding potential of −80 mV, although the background current was somewhat reduced in the presence of methanesulfonate. Because replacement of extracellular chloride by either aspartate or methanesulfonate could give rise to significant intracellular pH changes (the extracellular pH being systematically adjusted at the normal value), we measured the effect of substituting methanesulfonate to chloride after having increased 5-fold (50 mM instead of 10 mM) the concentration of Hepes in the pipette solution and external media tested. The result of a typical experiment is shown in Fig. 3A. It can be seen that chloride substitution resulted in a sizable reduction in peak current amplitude by 30.2% ($26.2 \pm 9.1\%$, $n = 5$). In the experiment shown in Fig. 3A, the shift towards positive potentials of the activation- and inactivation-voltage curves resulting from replacement of chloride by methanesulfonate was only of 1.2 mV. In a few experiments, external chloride removal was performed in the presence of normal external sodium by replacing

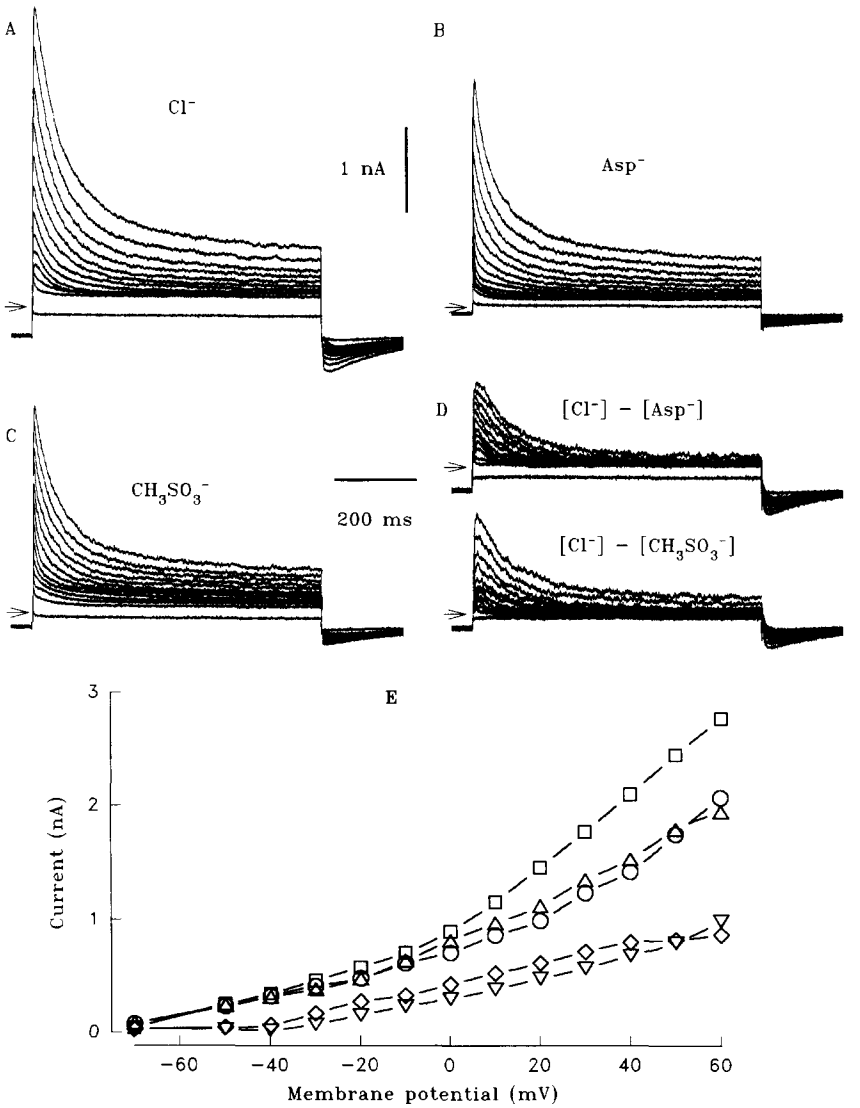


Fig. 4. Effect of substitution of extracellular chloride by aspartate or methanesulfonate on the transient outward current of the same myocyte in the absence of extracellular calcium and cadmium. (A) Chloride solution, Cl^-. (B) Chloride replaced by aspartate, Asp^-. (C) Chloride replaced by methanesulfonate, $CH_3SO_3^-$. (D) Difference currents (as indicated above each current trace family). Same voltage step protocol as in Fig. 1, except that depolarizing pulses were to −70 mV, then between −50 and +60 mV. (E) Effects of substitution of external chloride by aspartate or methanesulfonate on peak amplitude of transient outward current (expressed as a function of membrane potential) in the absence of extracellular calcium and cadmium. Symbols are: Cl^- (□), Asp^- (○), $CH_3SO_3^-$ (△), difference currents, Cl^- minus Asp^- (◇), and Cl^- minus $CH_3SO_3^-$ (▽).

130 mM NaCl by 130 mM sodium methanesulfonate. Except for the presence of large and brief initial peaks of inward Na current (which disappeared frequently with time as a result of the well-known $h_\infty - E_m$ shift towards negative potentials), the characteristics of the difference current (NaCl minus sodium methanesulfonate) were not different from those recorded in the absence of external Na^+.

3.1.2. Effect of extracellular 4-aminopyridine or intracellular Cs^+

Although the above described results suggest that the current suppressed by substitution of methanesulfonate for chloride ions is a chloride current, we tested the effects of two K^+ channel blockers, i.e., 4-aminopyridine and Cs^+ ions. The upper traces of Fig. 3B show the effect of 3 mM 4-aminopyridine on the transient outward currents recorded either in control conditions or in the presence of methanesulfonate, whereas the lower traces of Fig. 3B show the effect of 4-aminopyridine on the difference current. It can be seen that the brief peak of the difference current was entirely suppressed by 4-aminopyridine, leaving unaffected a more slowly activating sustained component of outward current. When potassium aspartate (130 mM) normally present in the pipette solution was replaced by 130 mM caesium aspartate, the transient outward current recorded just after membrane disruption progressively decreased in amplitude and after ≈ 3 min was almost entirely suppressed (not illustrated). When extracellular choline chloride was further replaced by choline methanesulfonate, the residual sustained current flowing during the pulse was somewhat reduced (as was also the holding current), but the difference current was entirely flat, demonstrating that no chloride-sensitive transient current subsisted in the presence of intracellular Cs^+. Four different experiments gave the same result.

3.1.3. Aspartate

Because aspartate is known to chelate calcium ions and therefore alter extracellular calcium activity [15] and possibly that of other divalent ions when used as a substitute for Cl^-, experiments using this anion were performed in Ca^{2+}-free, Cd^{2+}-free solution. In these conditions, calcium current was suppressed as a result of calcium removal and the possibility that sodium current might flow through calcium channels was excluded because experiments were performed in Na^+-free media. Fig. 4 shows current traces recorded in the same cell before (A) and after substitution of aspartate (B) or methanesulfonate (C) for chloride. The difference currents are shown in Fig. 4D. It can be seen that i_{lo} was reduced to almost the same extent by the two Cl^- substitutes. In 10 different experiments, the amplitude of i_{lo} measured at +60 mV was reduced to $68 \pm 11\%$ of its control value in the presence of methanesulfonate and to $71 \pm 14\%$ of its control value in the presence of aspartate.

In the experiment shown in Fig. 4E both the threshold of control current and those of currents measured in Cl^--free media are markedly shifted to more negative potentials (by ≈ 50 mV) as compared with those measured in Fig. 1. This results from the well-known dependence of threshold and current-voltage relations on external divalent cation concentration (in the case of rat transient outward current, see [32]). Interestingly, the shift appears to be less for the difference current than for that recorded in Cl^--free conditions, since comparison of Fig. 4A and 1Ad and 1Bd shows that thresholds were shifted from ≈ -20 to ≈ -70

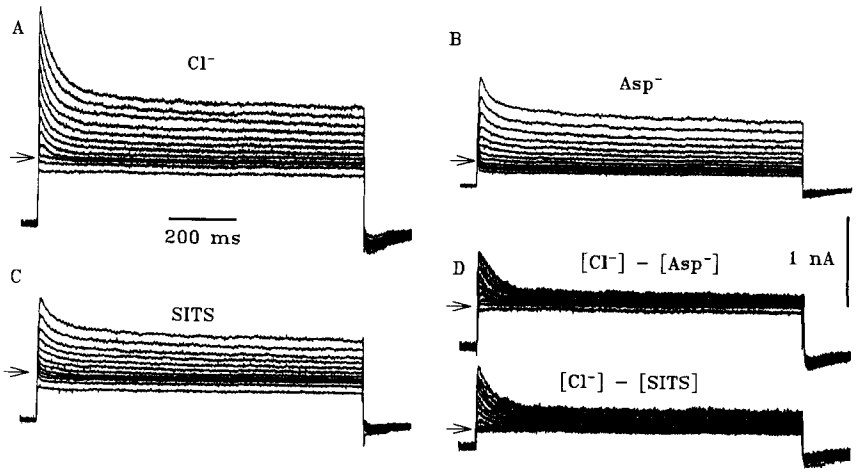


Fig. 5. Effects of SITS and of substitution of extracellular chloride by aspartate on the transient outward current of the same myocyte in the absence of extracellular calcium and cadmium. (A) Chloride solution (Cl^-). (B) Chloride replaced by aspartate (Asp^-). (C) 2 mM SITS in chloride solution. (D) Difference currents as indicated above each current trace family. Currents were elicited by 1 s depolarizing voltage steps applied at 0.1 Hz, from a holding potential of −80 mV in 10 mV increments, between −60 and +60 mV.

mV for the latter and only from ≈ −20 to ≈ −40 mV for the former.

3.2. Chloride channel blockers

3.2.1. SITS

The next step was to study the effects of Cl^- channel blockers on i_{lo} and compare these effects to those observed when external Cl^- was replaced by impermeant anions. Because both aspartate and methanesulfonate have been comparatively studied in the absence of divalent ions (Fig. 4), we also recorded comparatively the effects of aspartate and SITS in the absence of both Ca^{2+} and Cd^{2+}. Fig. 5 shows the effects of aspartate substituted for chloride and those of 2 mM SITS in the same cell. Both actions resulted in a similar decrease in i_{lo} (Fig. 5B and C), thus unmasking similar chloride-sensitive and SITS-sensitive transient outward current components. The major difference between the two components shown in Fig. 5D results from a larger steady-state outward current component suppressed by SITS compared with that suppressed by Cl^- removal. The difference currents shown in Fig. 5D have time courses which are not markedly different from those shown in Fig. 4D. Results similar to those reported in Fig. 5 were obtained in eight other myocytes.

In order to test whether the reversal potential of the SITS-sensitive transient current behaves as a chloride electrode we increased the pipette chloride concentration to 140 mM and adjusted the extracellular chloride concentration (42.5 mM) so that the equilibrium potential for Cl^- ion, E_{Cl}, reached a value of +30.5 mV. Fig. 6A shows the result of one of these experiments. The currents shown in the figure are difference currents resulting from the subtraction of currents recorded in the absence and the presence of 2 mM SITS when the membrane was submitted to varying depolarizing pulses from a holding potential of −80 mV. It can be seen that a peak of transient inward current occurred at potentials ranging from −10 mV to +20 mV. This current was suppressed at +30, +40 mV, then became outward at more positive potentials. We performed the same protocol using another extracellular chloride concentration, i.e., 12.9 mM. In Fig. 6B the reversal potential was plotted as a function of the $\log_{10}([140]/[Cl^-]_o)$ and a linear regression gave a slope of −59.0 mV per 10-fold change in $[Cl^-]_o$.

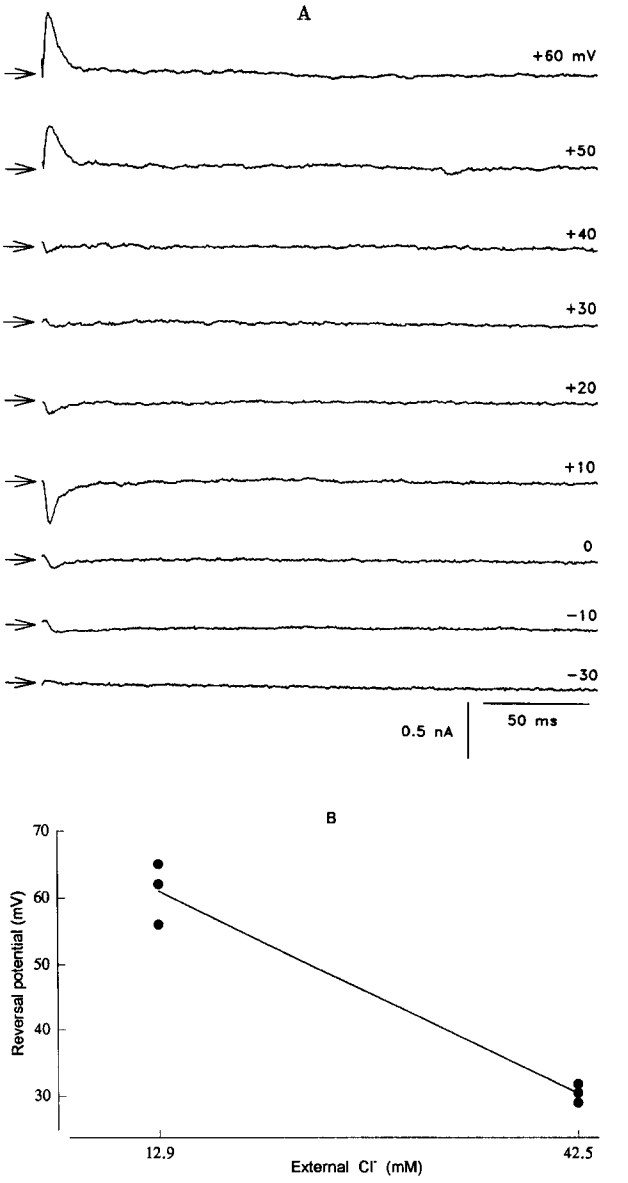


Fig. 6. Dependence of reversal potential of SITS-sensitive component of transient outward current on Cl^- gradient. (A) Current traces showing the voltage dependence of the SITS-sensitive component of the transient outward current. In this experiment the calculated E_{Cl} was +30.5 mV (bathing solution 42.5 mM Cl^-; pipette solution 140 mM Cl^-). Currents were elicited at a frequency of 0.1 Hz by 1 s voltage steps to the indicated potentials, from a holding potential of −80 mV. (B) Relationship between reversal potential of the SITS-sensitive component of transient outward current and $\log_{10}([Cl^-]_i/[Cl^-]_o)$ obtained with $[Cl^-]_i = 140$ mM. The least-square fit of the data yielded a slope of −59.0 mV per 10-fold change of $[Cl^-]_o$.

3.2.2. 9-Anthracene carboxylic acid

The action of 9-anthracene carboxylic acid was studied in the presence of divalent cations (Ca^{2+} and Cd^{2+}). Therefore, the effects of this chloride channel blocker can be more adequately compared with the results shown in Fig. 1 than with those shown in Figs. 4 and 5. Fig. 7 shows current traces obtained in control chloride medium (A) and after application of 5 mM 9-anthracene carboxylic acid (B), whereas the difference current traces are shown in C and corresponding current-voltage relationships in D. The effect of 9-anthracene carboxylic acid developed in less than 20 s and was fully reversible. Comparison of Fig. 1Ac and 1Bc and 7C shows that the 9-anthracene carboxylic acid sensitive current resembles that suppressed by methanesulfonate, although it inactivates somewhat more slowly and has a larger steady-state component.

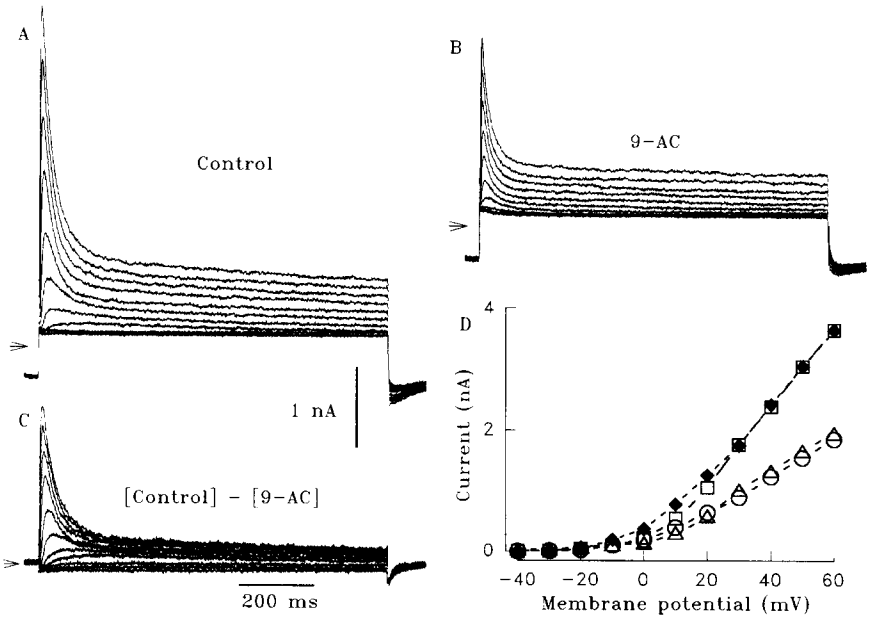


Fig. 7. Effect of 9-anthracene carboxylic acid on the transient outward current in the presence of external calcium and cadmium. (A) Control solution. (B) After addition of 5 mM 9-anthracene carboxylic acid. (C) Difference current. (D) Current-voltage relationships in control conditions (□), in the presence of 9-anthracene carboxylic acid (○) in the case of the difference current (△) and normalization of current-voltage relationship obtained in in the presence of 9-anthracene carboxylic acid (◆), respectively, to that obtained in control conditions. Currents were elicited by 1 s depolarizing voltage steps applied at 0.1 Hz, from a holding potential of −80 mV in 10 mV increments, between −60 and +60 mV.

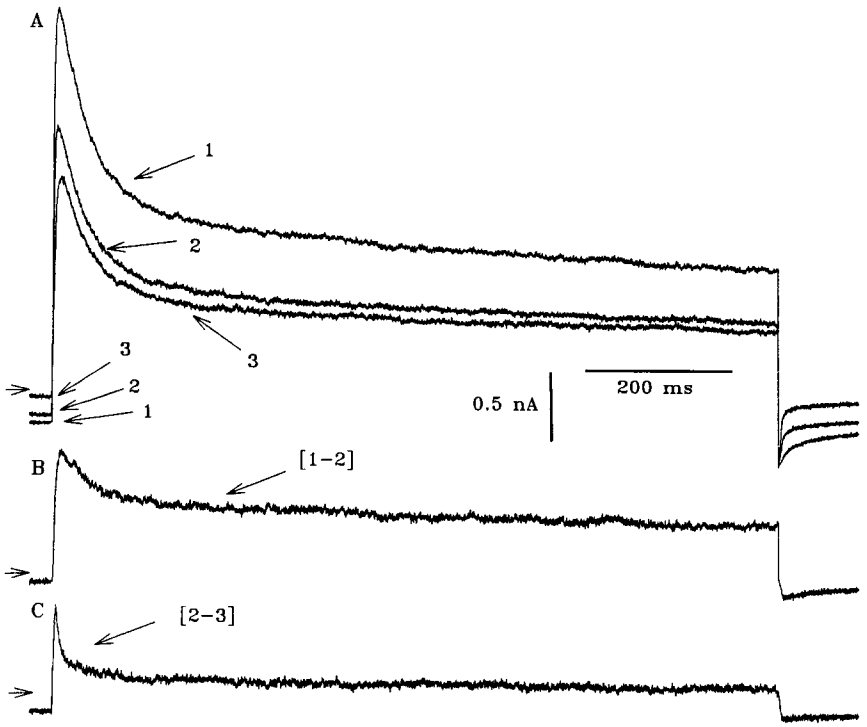


Fig. 8. Inhibitory effect of 10 mM caffeine on transient outward current and persistence of the current decrease induced by substitution of methanesulfonate for chloride in the presence of caffeine. Currents were elicited by 1 s pulses to +60 mV from a holding potential of −80 mV. (A), current in control choline Cl solution (1), after addition of 10 mM caffeine to the control solution (2) and after substitution of external chloride by methanesulfonate in presence of 10 mM caffeine (3). (B) and (C) Difference currents, respectively 1 − 2 and 2 − 3.

3.3. Effect of caffeine

Some of our previous observations indicated that, in the absence of calcium current (suppressed by 0.5 mM Cd^{2+} in the external solution) and therefore in the absence of Ca^{2+}-induced Ca^{2+} release and Ca^{2+}-induced outward current (ryanodine being in addition present in the superfusion medium), the transient outward current was reduced by caffeine in rat ventricular myocytes. Therefore, we examined the possibility that the current component suppressed by caffeine was the same as that suppressed by removal of external chloride. As shown in Fig. 8A (traces 1 and 2) the addition of 10 mM caffeine to the standard superfusion medium produced an inhibition of outward current. The caffeine-sensitive component of outward current (Fig. 8B) was composed of an initial slowly decaying component followed by a sustained component. When external chloride was replaced by methanesulfonate in the continuous presence of caffeine, the residual outward current was further reduced. The difference current (Fig. 8C) was quite similar to that obtained when methanesulfonate was substituted for chloride in the absence of caffeine (see, for example, Fig. 3B). It can therefore be concluded that the current suppressed by removal of external chloride is different from that suppressed by caffeine.

4. Discussion

The main result reported in the present paper is that the 4-aminopyridine-sensitive transient outward current, i_{to} (or i_{to1}), recorded in rat ventricular myocytes was consistently reduced (i) by replacement of external chloride with impermeant or poorly permeant anions such as methanesulfonate or aspartate and (ii) by addition of substances generally recognized as being chloride channel blockers such as 2 mM SITS or 5 mM 9-anthracene carboxylic acid. In addition, the transient current suppressed by SITS was found to reverse at potentials close to E_{Cl}, whereas the current remaining in methanesulfonate reversed at potential close to E_K. These observations suggest that, in rat ventricular myocytes, i_{to} is not a current carried exclusively by K^+ ions, as it is generally assumed to be in this type of cell [1] and in other types of cardiac cell from different species [20,22,33–36], but might be composed of two components, one of them being carried by Cl^- ions. However, the observation that the component of transient outward current suppressed by methanesulfonate was also suppressed by either 3 mM 4-aminopyridine or substitution of Cs^+ for K^+ in the pipette solution is very surprising because both substances are classical K^+ channel blockers. Most chloride currents are usually studied in the presence of internal Cs^+ in order to eliminate K^+ currents, thus demonstrating that the corresponding permeable channels are not blocked by Cs^+ ions [28,29,37–40]. It is therefore necessary to examine the different possibilities of artifacts capable of accounting for our experimental results.

4.1. Junction potentials and / or shifts in current-voltage relationships

That the effect of chloride substitutes results from the occurrence of junction potentials seems a priori unlikely for two reasons: (i) 3 mM KCl agar bridges were used between the Ag|AgCl electrode and the bath; (ii) chloride removal was never complete, since 7.2–12.2 mM Cl^- ions remained in the external solution. Of course, if in spite of these conditions, some junction potential still occurred as a result of external chloride reduction, the current assumed to be triggered by a depolarizing pulse to +60 mV might in fact be triggered by a less positive depolarizing pulse (for example +40 or +50 mV). In this case the current recorded during chloride substitution should indeed be smaller than that recorded in control solution even if no chloride current was suppressed by this intervention. If this was the case in our experiments, the potential corresponding to the current threshold and in fact the complete activation–voltage relationship should be appreciably shifted towards positive potentials. Although a small positive shift (+5.5 mV) indeed occurred in Fig. 2A, such a shift was absent in Fig. 1B and occurred in the negative rather the positive direction in Fig. 1A. It is worth noting that, because in our experiments currents were always triggered from a holding potential of −80 mV, the shift of the steady-state inactivation–voltage relationship shown in Fig. 2A was unable to influence our recordings. Moreover, the corresponding shift of the activation–voltage relationship could result in an underestimation of the current of only 2.5% at +60 mV. It is therefore unlikely that junction potentials are at the origin of the current depression induced by chloride ion substitutes.

The observation that chloride ion substitution and addition of a chloride channel blockers produces comparable effects either in the same myocytes (aspartate and SITS in absence of divalent ions, Fig. 5) or in different myocytes (methanesulfonate and 9-anthracene carboxylic acid, in presence of divalent ions Figs. 1 and 7) is of interest. In line with the above-mentioned possibility of artifact, such an effect of SITS and 9-anthracene carboxylic acid might result from a sizeable positive shift of the activation–voltage relationship. However, in the case of Fig. 7 such a shift is almost nil as shown by normalisation of activation–voltage relationships in absence and presence of methanesulfonate. Moreover, if one assumes that currents measured in control and methanesulfonate conditions are both pure K^+ current, i.e., reversed at E_K, the potentials of half activation were 26.7 mV and 25.6 mV, respectively, in the experiment of Fig. 7. It is therefore unlikely that the inhibitory effect of chloride channel blockers results from a shift in activation–voltage relationships.

4.2. Changes in intracellular pH or calcium level

It might be possible that replacing extracellular chloride by either aspartate or methanesulfonate gives rise to significant intracellular pH changes. It is indeed well known that a decrease in external chloride concentration increases the intracellular pH [41,42] as well as contraction and calcium current [43], an effect postulated to result from a reduced competition of chloride with extracellular bicarbonate on the HCO_3^-/Cl^- exchanger, thus inducing an increased entry of bicarbonate into the cell [44]. Such an effect cannot occur in our experiments, since HCO_3^- ions are absent from both extracellular and intracellular (pipette) solutions. In addition, we have shown that increasing massively the buffering capacity of the intracellular solution and of external media tested (50 mM Hepes, Fig. 3B) did not suppress the decrease in transient outward current induced by replacement of chloride with methanesulfonate, even when a long period of time (15–30 min.) was allowed for adequate cell dialysis before chloride substitution. Another possibility would be that the transient component of current suppressed by chloride ion substitutes or chloride channel blockers is not a Ca_i^{2+}-independent current but a Ca_i^{2+}-dependent chloride current similar to that described in rabbit and dog cardiac tissues [27–30]. This is, however, unlikely for three reasons: (i) in our experiments calcium current was suppressed either by adding 0.5 mM Cd^{2+} or by removal external Ca^{2+}; (ii) sarcoplasmic reticulum was emptied by either 1 μM ryanodine or 1 μM ryanodine + 10 mM caffeine; (iii) the difference current resulting from substitution of methansulfonate for chloride was not changed when pipette solution contained either 50 mM EGTA instead of 5 mM, or 10 mM EGTA and 10 mM BAPTA. For these reasons it seems unlikely that the difference current results from intracellular changes in either protons or Ca^{2+} ions.

4.3. Direct effect of chloride ion substitutes or chloride channel blockers

The fact that transient outward current was unaffected by addition of 100 mM methanesulfonate to control solution (Fig. 3B) shows that the reduction of i_{to} induced by substitution of methanesulfonate for chloride ions cannot result from a direct inhibitory effect of methanesulfonate. In a few experiments not described in the Results section, we tested the possibility that impermeant anions or chloride channel blockers might exert the effects described in the present paper by directly affecting the K^+ component of the transient outward current. We examined the effect of 2 mM SITS after the transient outward current had been previously reduced by substitution of methanesulfonate for chloride. We observed that in the presence of methanesulfonate, SITS did not change the residual transient outward current. The same absence of effect was observed when methanesulfonate was substituted for chloride in the continuous presence of SITS. It seems therefore unlikely that SITS and methanesulfonate exert their effect via some direct action on the channel responsible for the K^+ component of the transient outward current. Our results show that some differences exist in the characteristics of the currents suppressed by the different substitutes and blockers. Although the reasons for such minor differences cannot be presently clearly established, they might result from some lack of specificity of the blockers used. It is known that such blockers and specially the stilbene disulfonates appear to exert a wide variety of effects since, for example, SITS and DIDS, although blocking the Ca_i^{2+}-activated Cl^- current in rabbit ventricular cells [27], are not effective antagonists of the cAMP-activated chloride current and can even enhance this current in guinea pig ventricular myocytes via an effect on the β-adrenergic receptor [45]. They also block ATP-sensitive K^+ channels in the same type of cell [46]. It has been reported [28] and we have observed in several experiments (not illustrated) that 2–6 mM SITS exerts a limited effect on the calcium current i_{CaL}, an effect which, however, cannot interfere with our results, since the calcium current was suppressed in all our experiments as mentioned above.

Taken as a whole, the different arguments presented above tend to exclude the possibility that the transient outward current suppressed by chloride ion substitutes or chloride channel blockers is of artifactual origin and tend, a contrario, to favour the hypothesis that it is carried by chloride ions. The surprising fact remains that such a chloride current was suppressed by K^+ channel blockers such as 4-aminopyridine and Cs^+ ions. It is worth noting, however, that there is no information in the literature showing that all known chloride channels are Cs^+-insensitive. On the contrary, we have observed that the large conductance chloride channels previously described in ventricular cells from new-born rats [47] which are involved in cell volume regulation [48] are blocked by Cs^+, 5 mM internal Cs^+ inducing rapid channel flicker (Coulombe et al., unpublished results) similar to that induced by 20–50 μM external Cd^{2+} in single Ca^{2+} channels [49]. In a similar way, it may also be noted that fast chloride channels from cortical neurons and different other anion-selective ionic channels are blocked by internal tetraethylammonium, a typical K^+ channel blocker [50,51]. Whether the current described here results from the opening of a novel type of chloride channel or from chloride ions crossing another type of membrane structure is beyond the scope of the present paper. Another attractive possibility is that part of our observations result from the combination of the outward K^+ movement resulting from the transient outward current and an inward coupled movement of KCl via a cotransporter localized in the vicinity of K^+ channels. K^+–Cl^- and Na^+–K^+–$2Cl^-$ cotransports have been described in cardiac myocytes [52–55]. If K^+ ions crossing the membrane during the peak of transient outward current are coming out from, or/and entering into

some microcompartment in which diffusion is delayed (internal restricted space or external unstirred layer) this process can reduce the driving force for K^+ ions and therefore the peak current amplitude by reducing internal $[K^+]$ or/and increasing external $[K^+]$ (accumulation-depletion phenomena). The cotransporter might then allow recycling of the potassium and reduce or prevent accumulation-depletion, thus preventing the peak current depression. Suppression of external Cl^- or addition of cotransport blockers could therefore reduce the current and make the difference current appear to be carried by chloride ions. Further work is required to test this hypothesis.

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