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Single chloride-permeable channels of large conductance in cultured cardiac cells of new-born rats

A. Coulombe[1]*, H. Duclohier[1], E. Coraboeuf[1]***, and N. Touzet[2]**

[1] Laboratoire de Physiologie comparée et Laboratoire des Biomembranes et Ensembles neuronaux associé au CNRS, Université Paris XI, F-91405 Orsay Cedex, France
[2] Laboratoire d'Embryologie Moléculaire, Université Paris-XI, F-91405 Orsay Cedex, France

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Abstract. Large conductance channels were observed in the membrane of cultured cardiac cells of new-born rats studied with the patch-clamp technique in cell-attached and inside-out configurations. These channels were observed in $\simeq 4\%$ of the patches. In the cell-attached configuration they exhibited outward rectification and partial inactivation. In the inside-out configuration no rectification occurred but inactivation was present, mainly during hyperpolarizations. Two channels with large single unit conductances (400–450 pS) and one with a smaller conductance (200–250 pS) were frequently observed in the same patch. The two large channels generally had different kinetics. Under steady-state conditions the opening probability of the faster channel appeared to be voltage-independent. The slower channel was activated by depolarization. In asymmetrical solutions the permeability ratios P_{Na}/P_{Cl} were 0.03 and 0.24 for the larger and smaller channels, respectively; corresponding values for P_{Ba}/P_{Cl} were 0.04 and 0.09. It is proposed that in cardiac membranes the chloride permeability system is composed of widely dispersed microclusters forming grouped channels of different types and sizes.

Key words: Single channel recording, chloride channels, cultured rat cardiac cells, cardiac membranes, ionic permeabilities

Introduction

Cardiac ionic conductances for sodium, calcium and potassium ions have been recently investigated at the single channel level. Several novel types of channels, undetected at the macroscopic level, have also been discovered e.g. Ca_i-activated non selective channels, ATP-regulated K-channels and Na_i-activated K channels. In contrast, relatively little is known concerning the chloride conductance of cardiac tissues either at the macroscopic or microscopic level. Carmeliet (1961a, b) and Hutter and Noble (1961) first suggested that passive movements of chloride ions may play a role in generating the cardiac action potential because replacement of chloride by impermeant anions induced elevation and lengthening of the plateau and slowing of the initial repolarization. It has usually been assumed that in cardiac tissues the chloride conductance contributes 10%–30% of the total resting membrane conductance (Carmeliet 1961a, b; Hutter and Noble 1961; Deck and Trautwein 1964; Fozzard and Lee 1976; Goldman and Morad 1977; Lenfant and Goupil 1977; Nosek and Lieberman 1981). However this value may very well have been greatly overestimated because the replacement of external chloride by impermeant anions also reduces K permeability (Carmeliet and Verdonck 1977). Therefore, the Cl conductance may be very low (Piwnica-Worms et al. 1983; Vaughan-Jones 1979). The increase in amplitude and duration of the action potential plateau which occurs in low-Cl media cannot be taken as a reliable index of the Cl conductance since it has been shown that these media markedly enhance the cardiac slow response and contraction (Nosek 1979) as well as the slow inward current (Horackova and Vassort 1982). These uncertainties prompted us to use the patch-clamp technique to obtain further information about chloride channels in cardiac membranes.

In the past three years large conductance anionic channels (130–450 pS) have been described in several tissues including rat muscle cells (Blatz and Magleby 1983), Schwann cells (Gray et al. 1984),

* Present address: Department of Biophysics, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4
** Present address: Laboratoire de Neurobiologie Cellulaire, Gif-sur-Yvette, France
*** To whom offprint requests should be sent

Ascaris muscle (Martin and Thorn 1984), epithelial cells (Nelson et al. 1984; Kolb et al. 1985) and macrophages (Schwarze and Kolb 1984). In the present paper we show that large conductance chloride permeable channels also exist in membranes of cultured cells of new-born rat hearts. Some of the present results have already appeared in preliminary form (Coulombe and Duclohier 1984).

Materials and methods

Heart ventricles from 2–3 day old rats were used to prepare isolated culture cells according to the technique described by Robinson and Legato (1980). This technique was slightly modified by using four 15 min incubations instead of twelve and by adding 100 U/ml penicillin to the culture medium. Foetal calf serum was also substituted for horse serum. Four to six day-old cells were ordinarily used. Older cells were submitted to mild trypsin treatment in order to facilitate pipette adhesion. All experiments were performed at 24 °–26 °C, a temperature at which cardiac tissues exhibit large anion permeability (Macchia and Bankston 1983). The normal Tyrode solution had the following composition (m*M*): NaCl, 155; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 11. The composition of other solutions is given in the text. HEPES (5 m*M*) was always used to buffer the pH (7.4).

Patch electrodes were fire-polished (resistance 2–10 MΩ when filled with isotonic salt solution) and their shanks coated with Sylgard. The patch-clamp method (Hamill et al. 1981), was used, either in cell-attached or inside-out configurations. The single current signals were recorded with an EPC 4 (Sigworth and Neher) or a Dagan 8900, filtered at 1 kHz (– 3 dB frequency) by a 6-pole Bessel low pass filter (Frequency Devices), stored on a FM tape recorder (EMI 7000) at 7½ inches/s and retrieved on a paper recorder (Gould 2400). Alternatively, records were digitized (rate 1 kHz–3 kHz) by a Minc 11/23 computer (DEC). Cumulative open or closed time histograms (number of events longer than *t*) were plotted against *t*. Distributions of open and closed times were fitted by a least squares stripping procedure. Ensemble averaging of single current traces was obtained using a Tektronix 7603 equipped with a programmable digitizer 7D20.

Results

I. Cell-attached configuration

Figure 1 shows four current traces obtained when the resting membrane (exhibiting no detectable currents) was submitted to long-lasting (≃ 1 s) depolarizing pulses (+ 40 mV) and two current traces when the membrane was hyperpolarized (– 40 and – 60 mV). During depolarizing pulses (Fig. 1 A) both small and large current fluctuations occurred. Small fluctuations are attributable to several of the previously described channels, e.g. 15 pS fast Na channel (Cachelin et al. 1983), 65 pS delayed K channels (Clapham and de Felice 1984), 20–25 pS and 80 pS background K channels (Bechem et al. 1983; Trube and Hescheler 1984). Large fluctuations (arrows) correspond to unusually large conductance channels of ≃ 425 pS as calculated from the current amplitudes measured at + 40 mV (Fig. 1 A) and + 60 mV (not shown). They occurred in only one out of 20 to 30 patches indicating a low density of the channels in the cardiac membrane. These channels were frequently open at the very beginning of the voltage pulse and then tended to close (Fig. 1 A). This suggests the existence of an inactivation process. However current fluctuations could persist for

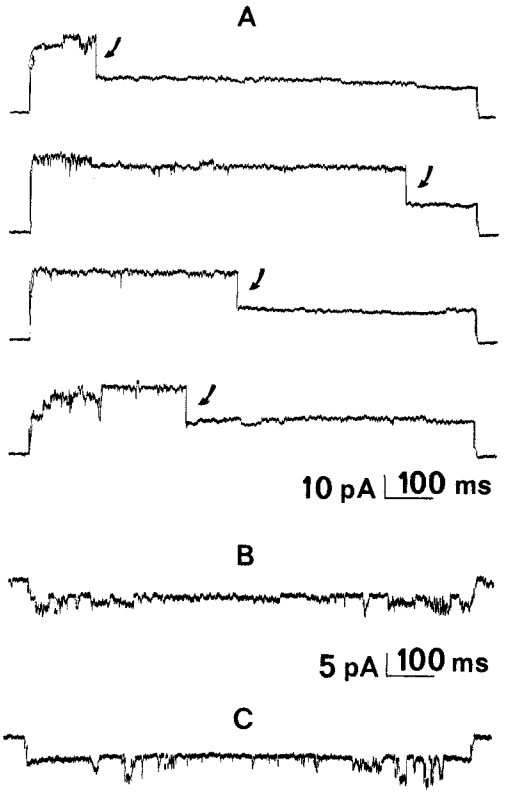

Fig. 1A–C. Single channel currents recorded from a cell-attached patch during depolarizing pulses of 40 mV (**A**) and hyperpolarizing pulses of 40 mV (**B**) and 60 mV (**C**) from the resting potential. Tyrode solution containing 2 m*M* Co was used both in the bath and the patch pipette. Pulse frequency 0.4 Hz. In this and the following figures upward- and downward-current deflections correspond to outward and inward currents respectively. Channel conductance in **A**: ≃ 425 pS, in **B** and **C** ≃ 90 pS. *Arrows* indicate large amplitude events

long periods of time and two levels of current frequently occurred. During hyperpolarizing pulses (Fig. 1B and C) similar large current fluctuations corresponded to a conductance of about 90 pS. Therefore the large currents observed in the intact cell (Fig. 1) undergo noticeable outward-going rectification (Fig. 2, open circles), which disappears after excision of the patch and exposure of the two sides of the membrane to Tyrode solution (Fig. 2, filled circles). The extent of this outward rectification varied in cell-attached patches (Fig. 2, open triangles and squares) possibly as a result of abnormal intracellular ionic concentrations and the loss of rectification was generally associated with the tissue having spent long periods (3–6 h) out of the incubator. Current-voltage relationships for these large fluctuations cross the voltage axis either close to the resting potential (Fig. 2A) or positive relative to this value (Fig. 2B).

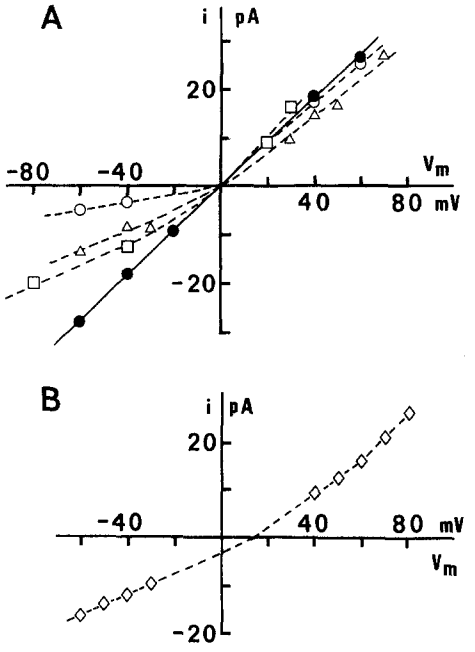

Fig. 2A and B. Current-voltage relationships for three different experiments in which the curves cross the voltage axis near the resting potential (**A**) and one experiment where the crossing point is positive to the resting potential (**B**). *Open symbols* (and *dotted lines*) correspond to cell-attached patches; *filled circles* (and *continuous line*) correspond to the same patch as that indicated in **A** by *open circles*, after passing to inside-out configuration. *Open* and *filled circles:* same solutions as in Fig. 1. *Other symbols:* normal Tyrode solution both in the bath and the pipette

II. Inside-out configuration

Both the pipette and the bath were filled either with Tyrode solution containing 2 m*M* Co (to block Ca channels) or with Ca-free Tyrode solution containing 5 m*M* Ba or Co, to prevent stimulation of Ca-activated K channels (Gorman and Hermann 1979). The experiment of Fig. 3 was performed in the first solution. It can be seen that large conductance channels (440 pS), similar to those recorded in Fig. 1A, were active during both depolarizing and hyperpolarizing pulses. It is also apparent that some inactivation occurs during hyperpolarization. Possible sub-levels of conductance were also detected at negative potentials (arrow). Inactivation is more prominent in Fig. 4 where 13 to 34 pulses have been averaged. Inactivation is very slow for depolarizing or small (− 20 mV) hyperpolarizing pulses. It becomes faster with large hyperpolarizations (τ inactivation $\simeq$ 120 ms for $\Delta V = -40$ mV and $\simeq$ 50 ms for $\Delta V = -60$ mV). It is clear from Fig. 3 that during hyperpolarizing pulses (− 40 mV) inactivation is not complete since reopenings can occur. The steady-state activity persisting under these conditions is typical for the fast channel described below.

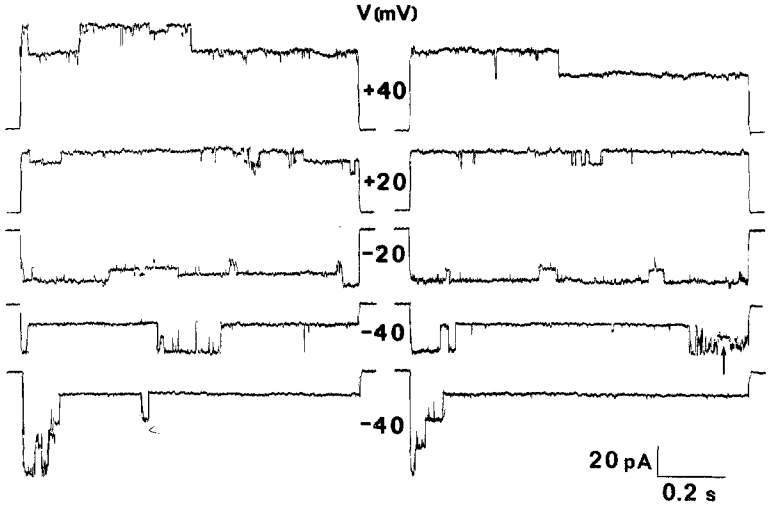

Fig. 3. Single channel currents recorded from an excised patch of membrane (inside-out patch) at different potentials. Normal Tyrode solution containing 2 m*M* Co both in bath and pipette. Pulse frequency 0.4 Hz. The *arrow* indicates possible subconductance state

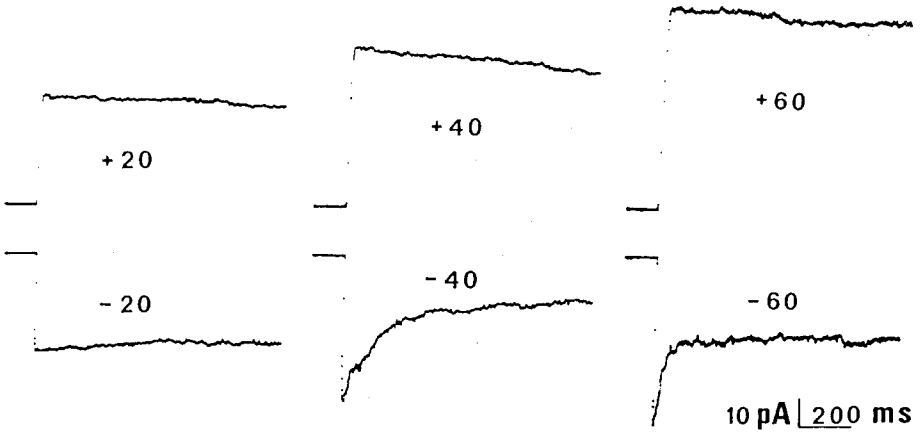

Fig. 4. Ensemble averages of single-channel currents at different potential steps. Numbers of single current traces averaged were: 34 at + 60 mV, 13 at + 40 mV, 13 at + 20 mV, 20 at − 20 mV, 27 at − 40 mV and 26 at − 60 mV. Same experiment as in Fig. 3

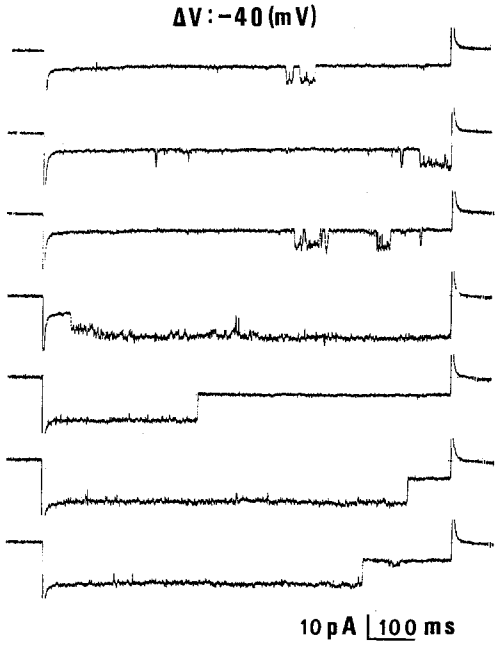

Fig. 5. Channel evolution during successive hyperpolarizing pulses applied from zero potential. Inside-out patch in symmetrical Ca-free Tyrode solution containing 5 m*M* Co. Capacity not compensated, channel conductance is ≃ 200 pS in the first three records and ≃ 300 pS in the last three. During the 4th record the channel conductance increases from the former to the latter value. Thereafter the channel inactivates during the pulses. Pulse frequency 0.5 Hz

Results similar to those described in Figs. 3 and 4 were obtained in three other experiments. However in other cases, inactivation was not detectable. The experiment reported in Fig. 5 suggests that for some unknown reason, the large conductance channels can demonstrate variable behaviour in the course of a given experiment. In Fig. 5 the membrane was bathed in Ca-free Tyrode solution containing Co. During the initial hyperpolarizing (− 40 mV) pulses a 200 pS channel showed short bursts of activity which occurred irregularly. During the fourth pulse its conductance increased to ≃ 300 pS, its opening became much longer and during subsequent pulses the channel repeatedly showed inactivation (τ inactivation ≃ 165 ms for 24 pulses at − 40 mV and ≃ 95 ms for 39 pulses at − 60 mV). Although we observed a similar phenomenon in two other experiments, we were unable to reproduce and analyse it adequately. Although such behavior cannot be attributed with certainty to the evolution of the same channel we observed in the three cases that the appearance of the large events were coincident with the disappearance of the small ones. In the remaining part of this paper we shall focus our attention on the steady-state behaviour and ionic selectivity of the large conductance channels. In many steady-state recordings two levels of current (L_1, L_2) were recorded. A typical result is illustrated in Fig. 6 in which large conductance channels (> 400 pS) were observed in Ca-free Tyrode solution containing 5 m*M* Co. The fact that jumps from a closed state (C) to L_2 or from L_2 to C were never observed (intermediate steps were always detectable at a faster recording speed) argues in favour of two separate channels rather than a two-state channel.

The two channels observed in Fig. 6 and similar experiments appear to possess different kinetics because one channel frequently underwent relatively rapid and sustained fluctuations while the other remained either open or closed for long periods of time. For this reason we refer to the rapidly fluctuating channel as the fast channel and the separate slowly fluctuating one as the slow channel. The two channels have similar but, generally, not identical conductances. From a total of 10 different patches the largest difference was 31% (the smaller value being taken as 100%) whereas the smallest was less than 1% (mean 11% ± 8% SD). In 7 cases the fast channel had the larger conductance whereas in 3 cases the reverse situation occurred. The elementary

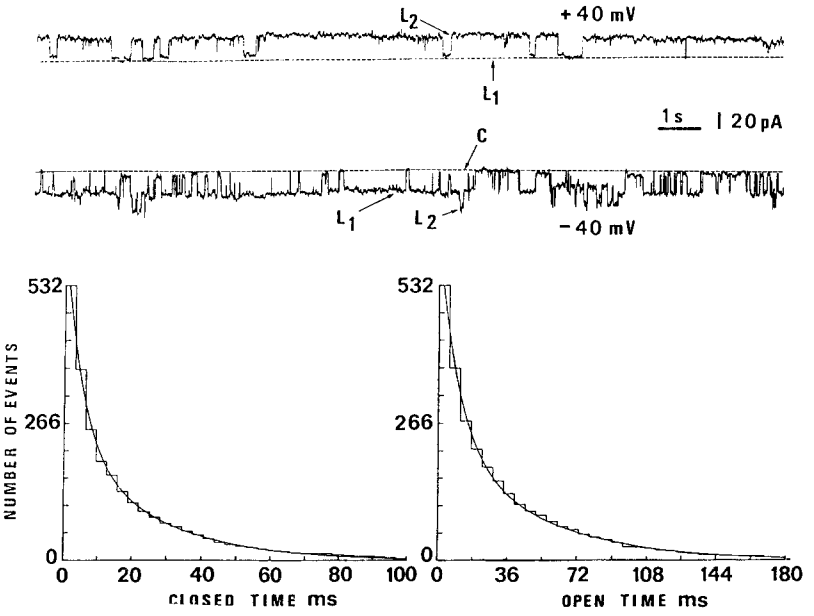

Fig. 6. *Upper part:* single channel currents recorded from inside-out patch (symmetrical Ca-free Tyrode solution containing 5 m*M* Co) during steady-state depolarization (upper record, + 40 mV) and hyperpolarization (lower record, − 40 mV) from zero potential. L_1 and L_2 indicate the two current levels both corresponding to ≃ 400 pS conductances. *C*: closed state. *Lower part:* histograms of the cumulative distributions of open and closed times calculated for a period of 24 s during which only one current level was observed (membrane potential − 40 mV, same patch as in the upper part). Double exponential curves were fitted to the histograms (see text)

channel conductance (pooled values for fast and slow channels) was 435 ± 27 pS (SD). The distribution of open times of the fast channel in one patch (Fig. 6) was calculated for a period of 24 s during which the slow channel did not open. The cumulative open time histogramm at − 40 mV shows a double exponential decay with time constant $\tau_{o1} = 9.5$ ms and $\tau_{o2} = 44.5$ ms. The histogramm of closed times also exhibits a fast and a slow component with $\tau_{c1} = 5.3$ ms and $\tau_{c2} = 24.5$ ms (see Table 1). The amplitude ratio of the exponentials (i.e. the relative area of the components of the probability density function) is 390/240 in both histograms.

We wished to test the possibility that the large conductance channels described above are chloride channels. We therefore exposed isolated membrane patches to $BaCl_2$ solutions where the only possible permeant ions are Cl and Ba and to choline chloride solutions where only Cl ions can permeate the channel. In the case of $BaCl_2$ solutions, Ba was chosen as the cation because of the well-known observation that seals form much more easily in the presence of high divalent ion concentrations (Corey and Stevens 1983). The observation that large conductance channels as well as smaller channels (456 ± 23 pS and 253 ± 30 pS, respectively, mean ± SD, 4 experiments) regularly occurred in symmetrical choline chloride solution (155 m*M* choline; 5 m*M* Co) suggests that these channels are chloride permeable.

Asymmetrical $BaCl_2$ solutions contained 96 m*M* $BaCl_2$ in the pipette and 24 m*M*, made isotonic with sucrose, in the bath. Under such conditions, the reversal potential should be − 35.6 mV for channels exclusively permeable to Cl ions and + 17.8 mV for Ba selective channels. The current-voltage relationships shown in Fig. 7A indicate that the channels are predominantly chloride permeable, the P_{Ba}/P_{Cl} ratios were 0.04 for the larger channel and 0.09 for the smaller (with no correction for activity coefficient). When asymmetrical NaCl solutions were used (150 m*M* in the bath and 600 m*M* in the pipette without compensation for different osmolarities) the current voltage relationships crossed the voltage axis at − 30.4 (for the 598 pS channels) and − 18.5 mV (for the 296 pS channels) (Fig. 7B). The

Table 1. Mean open (τ_{o1}, τ_{o2}) and closed times (τ_{c1}, τ_{c2}) and opening probability (p_f) of the fast fluctuating large channel. Inside-out patch configuration

E_m	τ_{o1} [s]	τ_{o2} [s]	τ_{c1} [s]	τ_{c2} [s]	p_f*	*N*
Symmetrical Tyrode Ca^{++}-free solutions + 5 m*M* Co^{++}						
− 60	0.008	0.150	0.01	0.11	0.57	285
− 40	0.0095	0.044	0.0053	0.0245	0.64	1,170
− 20	0.0025	0.0096	0.0018	0.0096	0.50	1,925
+ 20	0.021	0.180	0.0068	0.11	0.62	1,450
+ 40	0.0058	0.124	0.0055	0.092	0.56	1,050
				mean =	0.57	
Symmetrical $BaCl_2$ (96 m*M*) solutions						
− 70	0.030	0.60	0.020	0.53	0.53	251
− 60	0.040	0.23	0.011	0.26	0.47	977
− 40	0.018	0.89	0.017	0.63	0.58	690
− 30	0.030	0.90	0.011	1.1	0.45	232
− 20	0.009	0.22	0.0095	0.15	0.59	374
				mean =	0.52	

* $p_f = \tau_{o1}/(\tau_{o2} + \tau_{c2})$
N = number of events (1 opening + 1 closure = 1 event)

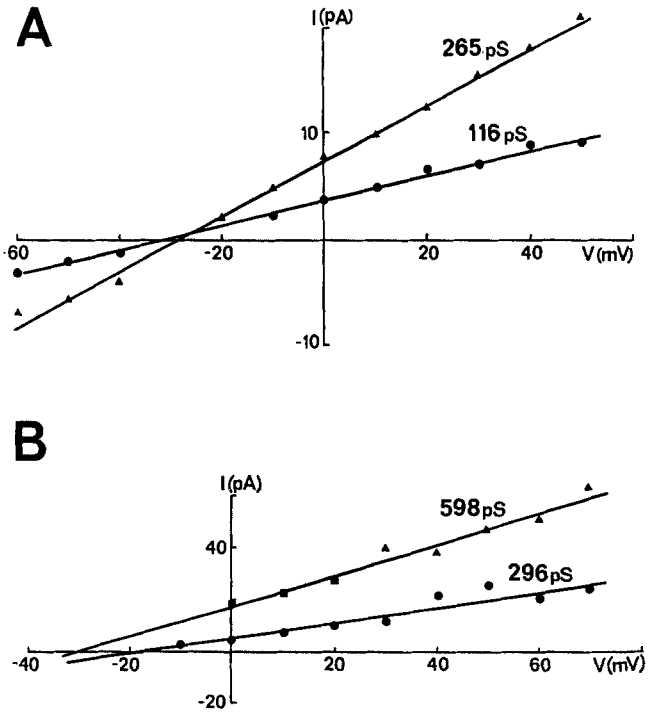

Fig. 7A and B. Current-voltage relationships obtained from inside-out patches in asymmetrical isotonic solution. **A**: $BaCl_2$ 96 m*M* in the pipette, 24 m*M* in the bath. **B**: NaCl 600 m*M* + 5 m*M* Co in the pipette; 150 m*M* + 5 m*M* Co in the bath. Channel conductances are indicated on the curves. The difference of conductance values between **A** and **B** is due to the difference of chloride concentration in the corresponding media. *Triangles:* slow and fast large conductance channels (pooled values); *circles:* smaller conductance channel. *Triangles* and *circles* are from the same cells, *squares* are from a different experiment

corresponding P_{Na}/P_{Cl} ratios were 0.06 and 0.27 assuming an activity coefficient of 1. When the real values of the activity coefficients for NaCl were taken into account (i.e., at 25 °C, $\alpha = 0.75$ at 0.15 *M* and 0.67 at 0.6 *M*) the corresponding values for P_{Na}/P_{Cl} ratios fell to 0.03 and 0.24 respectively.

III. Analysis of fast and slow channels under steady-state conditions

Twelve experiments in which currents were recorded under steady-state conditions showed the coexistence of a fast and a slow channel. From the values reported in Table 1 it appears that the opening probability of the fast channel calculated as $\tau_{o2}/(\tau_{o2}+\tau_{c2})$, i.e. neglecting the shortest events, does not significantly change with membrane potential. The analysis of amplitude histograms shows that the total open time of the two channels increases when the membrane is less hyperpolarized or is depolarized. This fact taken together with the weak voltage-dependence of the fast channel indicates that the slow channel opens with depolarization. Because the amplitude histogram did not fit a binomial distribution (Miller 1982), we used a more generalized approach to analyze the normalized time-averaged probability distributions (multinomial distribution). Assuming that each channel is governed by a probability parameter p_f (fast channel) and p_s (slow channel), the equation for the probability distribution is

$$(p_f + (1-p_f)) \cdot (p_s + (1-p_s)) = 1\,,$$

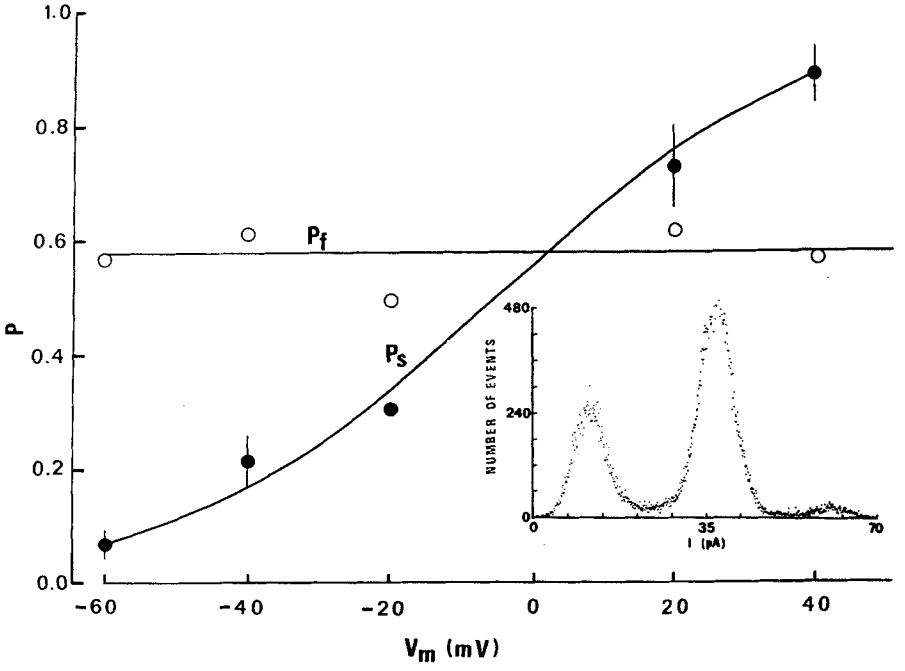

Fig. 8. Probabilities of opening of rapidly (*open circles*) and slowly fluctuating large conductance channels (*filled circles*) as a function of membrane potential. Same experiment as in Fig. 6. Inside-out patch in symmetrical Ca-free Tyrode solution containing 5 m*M* Co. *Inset:* example of current amplitude histogram (membrane potential: −40 mV; recording duration: 1 min). Further details in text

where $1-p_f$ (or $1-p_s$) represents the probability for a fast (or a slow) channel being closed. The preceding equation gives

$$p_f \cdot p_s + p_f + p_s - 2p_f p_s + 1 - (p_f + p_s) + p_f \cdot p_s = 1 . \quad (1)$$

Under steady-state conditions the histograms representing these events must exhibit three peaks whose areas correspond (as required in the case of this particular multinomial distribution) to *(i)* the proportion of time spent in double opening ($p_f \cdot p_s$), (ii) the proportion of time spent in single openings ($p_f + p_s - 2p_f \cdot p_s$) and *(iii)* the proportion of time spent in double closures ($1-(p_f+p_s)+p_f \cdot p_s$). The histograms computed from experimental records indeed showed three peaks (inset of Fig. 8) whose areas were in agreement with the functioning of two independent channels with unequal opening probabilities. From data given in Table 1 (Co-containing Tyrode solution), p_f was calculated as $\tau_{o2}/(\tau_{o2}+\tau_{c2})$ for each potential (Fig. 8, open circles). The linear regression line for the voltage dependence is not significantly different from a horizontal line ($P < 0.001$) suggesting that p_f is potential-independent ($p_f = 0.56$). Using this value, Eq. (1) gives three methods of calculating p_s. Mean values of the three estimations reported in Fig. 8 (filled circles) indicate that, in contrast to p_f, p_s is potential-dependent and is fitted reasonably well by a sigmoïd, with a half saturation potential of -5.7 mV and an effective gating charge of 1.08. The values of the different time constants vary greatly and do not exhibit simple relationships as a function of voltage (suggesting that they depend on some other as yet unrecognized parameters).

Discussion

Large conductance channels predominantly permeable to Cl ions exist in cardiac plasma membranes of the new-born rat. Their density appears to be very low since they were recorded only in one patch out of each 20–30 patches. Large conductance anion-selective (LCA) channels with similar low apparent density (1 patch out of 10–20) have also been described in myotubes (Blatz and Magleby 1983; Schwarze and Kolb 1984) and macrophages (Schwarze and Kolb 1984). The channel conductance measured in our experimental conditions is very close to those reported for other LCA channels observed in the rat myotube (Blatz and Magleby 1983), in Schwann cells (Gray et al. 1984) and in epithelial cells (Nelson et al. 1984; Kolb et al. 1985). The lower value obtained after a fourfold reduction of [Cl] on one side of the membrane is also in agreement with other observations (Gray et al. 1984; Martin and Thorn 1984; Nelson et al. 1984; Schwarze and Kolb 1984).

Several non-cardiac LCA channels inactivate, usually within a few seconds, when submitted to pulses of either polarity (Blatz and Magleby 1983; Gray et al. 1984; Nelson et al. 1984; Schwarze and Kolb 1984; Kolb et al. 1985). We also observed a voltage-dependent inactivation process, but in inside-out patches this process was negligible for small positive or negative pulses from the resting potential and developped much faster for large hyperpolarizing than depolarizing pulses. Because, under our experimental conditions as in other reports (Gray et al. 1984), the rate of inactivation varied between patches, it is difficult to decide whether or not significant differences exist, from this point of view, between the chloride channels described here in cardiac cells and those studied in other tissues. Our inside-out membrane patch experiments show that *(i)* Inactivation was only partial since reopenings frequently developed in steady-state conditions. Such behaviour has also been observed occasionally by others (Gray et al. 1984). *(ii)* In three experiments a transition was observed between currents without inactivation (small bursts of activity occurring after long latency periods) and with inactivation. Although such transitions can be of artifactual origin, the fact that they were associated with a progressive change in channel conductance (fourth trace in Fig. 5) suggests that in our experimental conditions some plasticity of the large conductance chloride channels can exist. *(iii)* Although steady-state activation was the standard behaviour of the large conductance channels, it is clear that these channels do not constitute a uniform population. Very often, under steady-state conditions, two large chloride permeable channels (400–450 pS) with different characteristics (a fast one with little or no potential-dependence and a slow one activating with depolarization) coexisted in the same patch. In addition, a smaller chloride channel with a conductance about one-half that of the large ones (200–250 pS) was frequently present and, occasionally, a still smaller channel (50–60 pS). Moreover, flickering of the large channels to subconductance states was occasionally visible (see also Schwarze and Kolb 1984; Nelson et al. 1984; Gray et al. 1984). These preliminary observations illustrate the complexity of the cardiac chloride conductance system. We may speculate that chloride permeable macromolecules tend to gather in microclusters forming channels of different sizes and types (and, possibly, of somewhat unstable conformation) widely dispersed in the membrane. The diversity of chloride channels is not limited to

cardiac membranes. In cultured rat skeletal muscle Blatz and Magleby (1983) described first a chloride selective channel of large conductance (440 pS) then (Blatz and Magleby 1985), two additional chloride channels with relatively small conductances (45 and 61 pS), one with fast kinetics and the other with slow.

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