

SINGLE-CHANNEL STUDY OF THE CYCLIC AMP-REGULATED CHLORIDE CURRENT IN GUINEA-PIG VENTRICULAR MYOCYTES

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SUMMARY

1. Properties of the cyclic AMP-regulated Cl^- channel were studied in guinea-pig ventricular myocytes with the patch clamp technique. Cell-attached patch recordings were performed, while the cell was dialysed with a cyclic AMP (0.2–0.5 mM)-containing internal solution through a second patch pipette. The latter pipette was also used to monitor the whole-cell Cl^- conductance.

2. The whole cell showed a large Cl^- conductance for 10–15 min after the beginning of cell dialysis. The activity of single Cl^- channels began to appear in some of the cell-attached patches during this time.

3. The channels showed a high open probability (0.69 ± 0.14 , mean \pm s.d., $n = 12$) at the time of their appearance, and the open probability did not appreciably increase thereafter, even when the whole-cell Cl^- conductance increased further with time.

4. An increase in the number of active channels was observed in some patches with progression of the cell dialysis. In such cases, the newly activated channels also showed a high open probability.

5. The above results are consistent with the hypothesis that the cyclic AMP system makes the 'latent' Cl^- channels available without influencing their own kinetic behaviour. The available channels may intrinsically exhibit a high open probability.

6. Chloride channel currents could also be recorded in the outside-out patches excised from the cyclic AMP-loaded cells. The I - V relation of these currents showed outward rectification under the condition of symmetrical Cl^- gradients, suggesting that the channel itself or a related structure has the property of rectifying current flow.

7. The channel seemed to have at least one open state and two closed states; the open-time histograms showed one exponential component with the values of time constant scattering around 1 s, while the closed-time histograms showed two exponential components with the values of time constant scattering around 0.2 and 1 s. These time constants showed no clear voltage dependence.

INTRODUCTION

In cardiac cells, β -adrenergic stimulation modulates voltage-dependent cation channels such as the Ca^{2+} channel (Reuter, 1983; Kameyama, Hofmann & Trautwein, 1985; Kameyama, Hescheler, Hofmann & Trautwein, 1986; Tsien, Bean, Hess, Lansman, Nilius & Nowycky, 1986; Hartzell & Fischmeister, 1987; Fischmeister & Shrier, 1989) and the delayed rectifier K^+ channel (Tsien, Giles & Greengard, 1972; Brown & Noble, 1974; Carmeliet & Mubagwa, 1986; Yazawa & Kameyama, 1990). Cardiac anion channels are also influenced by catecholamines; recently, β -adrenergic stimulation has been found to induce a novel membrane current in cardiac cells (Egan, Noble, Noble, Powell & Twist, 1987; Egan, Noble, Noble, Powell, Twist & Yamaoka, 1988), and this current was identified as a cyclic AMP-dependent Cl^- current (Bahinski, Nairn, Greengard & Gadsby, 1989; Harvey & Hume, 1989; Matsuoka, Ehara & Noma, 1990). Bahinski *et al.* (1989) obtained evidence that cyclic AMP-dependent protein kinase (PKA) is involved in the activation of this Cl^- current.

A class of Cl^- channels that is most likely to underlie the above Cl^- current has recently been identified on the single-channel level (Ehara & Ishihara, 1990). This channel is practically voltage independent, and the single-channel current shows outward rectification, in accordance with the properties of the macroscopic Cl^- current. In the present study, we attempted to further characterize the single Cl^- channel, with special reference to the regulatory role of cyclic AMP. To activate the Cl^- channel, the ventricular myocyte was loaded with cyclic AMP using a patch pipette under the whole-cell configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). We recorded the Cl^- channel activity with another patch pipette, while monitoring the development of macroscopic Cl^- current by the whole-cell clamp.

METHODS

Preparation of single cells

Single ventricular cells were obtained from guinea-pig hearts using an enzymatic dissociation technique similar to that described before (Powell, Terrar & Twist, 1980; Isenberg & Klöckner, 1982). Briefly, guinea-pigs (280–350 g) were killed by sodium pentobarbitone overdose (70–90 mg/kg, i.p.). The chest was opened and the heart was quickly excised. The heart was hung on a Langendorff-type perfusion system and was perfused first with Tyrode solution and subsequently with Ca^{2+} -free Tyrode solution. When the heart beat ceased, the perfusate was changed to Ca^{2+} -free Tyrode solution containing collagenase (0.06 mg/ml, Yakult, Tokyo, Japan). After 10–15 min of this enzyme treatment, the cells were dissociated in the high- K^+ , low- Cl^- solution ('KB medium', Isenberg & Klöckner, 1982) and stored in this medium before use.

Patch clamp and recording technique

Cell-attached single-channel recordings and whole-cell recordings were simultaneously performed using two patch pipettes, following the technique described by Hamill *et al.* (1981). The pipette for the cell-attached recording had a resistance of 3–5 $\text{M}\Omega$ when filled with the pipette solution. The pipette for the whole-cell recording, which contained the solution for cell dialysis, had a resistance of 2–3 $\text{M}\Omega$. The two pipettes were set on the cell surface with a distance of six to ten sarcomeres.

The whole cell was usually voltage clamped at -40 mV, and, to monitor the membrane conductance, ramp pulses (triangle wave, -140 to $+60$ mV, $dV/dt = \pm 1$ V/s) were applied, the hyperpolarizing portion being used for measurement of the I - V relation. The membrane capacitance was measured by dividing the half-amplitude of the current jump at the peak of ramp by the slope of the ramp pulse. The liquid junction potential developing at the pipette tip after rupture of the

patch membrane was assumed to be -12 mV, according to the previous works (Imoto, Ehara & Matsuura, 1987; Matsuura, Ehara & Imoto, 1987). The cell membrane potentials described in the text are those corrected for the junction potential.

Single-channel currents were recorded with a patch clamp amplifier (EPC-7, List, Darmstadt, FRG), and the whole-cell currents, with a whole-cell clamp system (TM-1000, ACT ME, Tokyo, Japan). Data were stored on a digital audiotape using a PCM data recorder (RD101T, TEAC, Tokyo, Japan). Analysis of the single-channel data was made with a computer (PC98 RL, NEC, Tokyo, Japan) to which the data were fed from the digital audiotape via a low-pass filter (E-3201A, NF, Tokyo, Japan) with an appropriate cut-off frequency.

Solutions

The normal Tyrode solution contained (mM): NaCl, 140; KCl, 5.4; MgCl₂, 0.5; CaCl₂, 1.8; glucose, 10; Hepes, 5 (pH 7.4 with NaOH). The external solution used for recording the whole-cell conductance was a K⁺-free, Ca²⁺-free solution containing (mM): NaCl, 140; MgCl₂, 2; Hepes, 10 (pH 7.4 with NaOH). Ouabain (10 μ M), BaCl₂ (2 mM) and nifedipine-HCl (Sigma, 1 μ M) were added to suppress the Na⁺-K⁺ pump, K⁺ channels and Ca²⁺ channels, respectively. The low-Cl⁻ external solution was prepared by substituting 140 mM NaCl with equimolar sodium aspartate. The composition of the internal solution for whole-cell dialysis was (mM): CsOH, 90; aspartate, 90; CsCl, 30; MgCl₂, 2; TEA-Cl, 20; EGTA, 5; Tris-ATP, 5; glucose, 10; Hepes, 5 (pH 7.2 with CsOH). This solution also contained 0.2–0.5 mM cyclic AMP to activate Cl⁻ current. The pipette solution for single-channel recordings contained (mM): NaCl, 150; MgCl₂, 2; Hepes, 5 (pH 7.4 with NaOH). All experiments were performed at 35 ± 1 °C.

RESULTS

Activation of Cl⁻ channels by internal cyclic AMP

For the whole-cell recording, our experimental conditions with selected external and internal media are those suitable for recording the background currents of cardiac cells (Ehara, Matsuoka & Noma, 1989; Matsuoka *et al.* 1990). Since the internal solution contained cyclic AMP in the present study, it was expected that the cell would exhibit a substantial Cl⁻ conductance after the whole-cell configuration had been established. The experiment shown in Fig. 1 confirmed this. The membrane conductance of the cyclic-AMP-loaded cell was large, and when the external solution was changed from a Cl⁻-rich solution to a low-Cl⁻, aspartate-rich one, there was a large reduction of the outward current during the ramp pulses, as expected. Subsequent addition of anthracene-9-carboxylic acid (9AC, 2 mM), an inhibitor of Cl⁻ channels (Gögelein, 1988), to the bath further reduced the inward current (Fig. 1A and B).

The Cl⁻ conductance was almost maximal within 30 s to 1 min after the membrane rupture in whole-cell recording, and then changed little with time, in most cells. In some cells, a gradual, slight increase in the conductance occurred for a few minutes with progression of cell dialysis. At a later stage, however, the Cl⁻ conductance always decreased spontaneously (run-down, Fig. 1A and C). The current component that declined with time exhibits characteristic features of the Cl⁻ current (Fig. 1C, inset): it has a reversal potential of about -30 mV, and its I - V relation shows outward rectification (cf. Bahinski *et al.* 1989; Harvey & Hume, 1989; Matsuoka *et al.* 1990). The run-down usually began 10–15 min after cell dialysis. The nature of this phenomenon is unknown.

It was possible to record the activity of single Cl⁻ channels in cell-attached patches during dialysis of the whole cell with cyclic AMP. We observed such a channel activity in less than 5% of patch recordings. The low success rate is at least partly due to the low density of Cl⁻ channels on the membrane (Ehara & Ishihara, 1990).

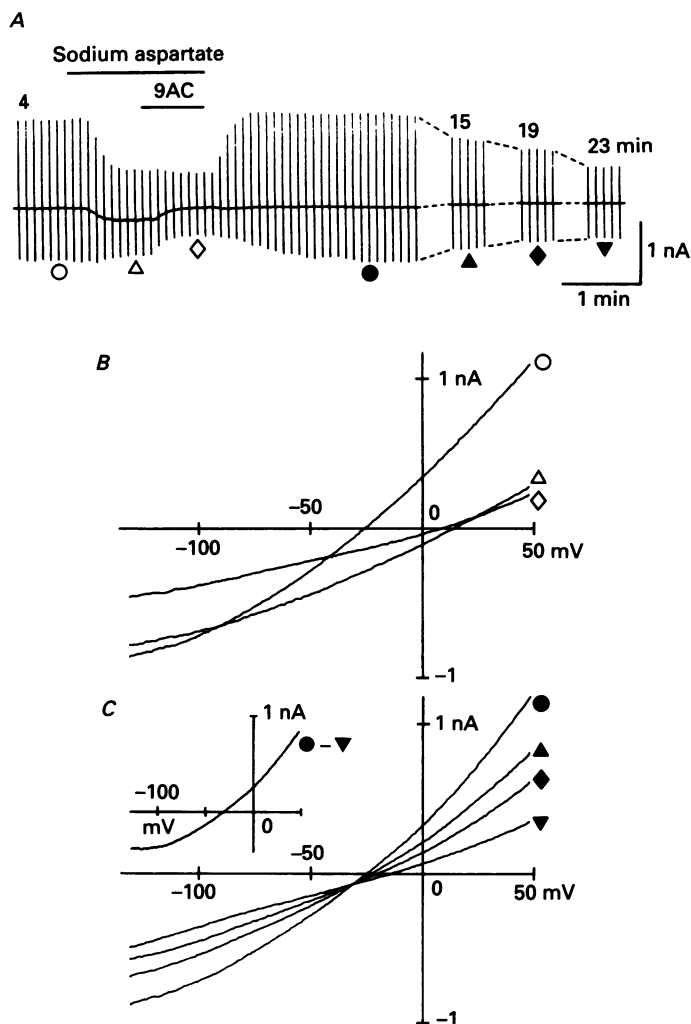


Fig. 1. Whole-cell Cl^- current recorded in a ventricular myocyte dialysed with an internal solution containing 0.5 mM cyclic AMP. *A*, chart record of membrane current. Holding potential was set at -40 mV and ramp pulses were applied every 6 s. The bath solution was changed from Cl^- -rich to low- Cl^- , aspartate-rich (sodium aspartate) solution for the time indicated by bar, and anthracene-9-carboxylic acid (9AC, 2 mM) was further added to the bath (bar). Numbers above the traces indicate the time (min) which elapsed after the beginning of cell dialysis. Current traces indicated by the symbols are the sources for $I-V$ relations shown in *B* and *C*. *B*, $I-V$ relations obtained in control solution (○) and in sodium aspartate solution before (△) and after (◇) addition of 9AC. *C*, run-down of Cl^- current. $I-V$ relations obtained 9 (●), 15 (▲), 19 (◆), and 23 min (▼) after the beginning of cell dialysis are shown. Inset shows difference current (● - ▼) obtained by subtracting late $I-V$ relation from early $I-V$ relation.

Another feature of the channel activity noted was that more than 80% of the active patches showed multichannel activity with two to four channels. These findings might indicate a non-homogeneous distribution of channels on the membrane. It should also be noted that channel activity was never observed in non-dialysed cells.

Like the macroscopic Cl^- current, the activity of single Cl^- channels also showed a run-down. The current trace shown in Fig. 2A was recorded from a cell-attached patch when the macroscopic Cl^- current was large (Fig. 2C, curve *a*). The record showed a three-step change in the current level, indicating the presence of at least

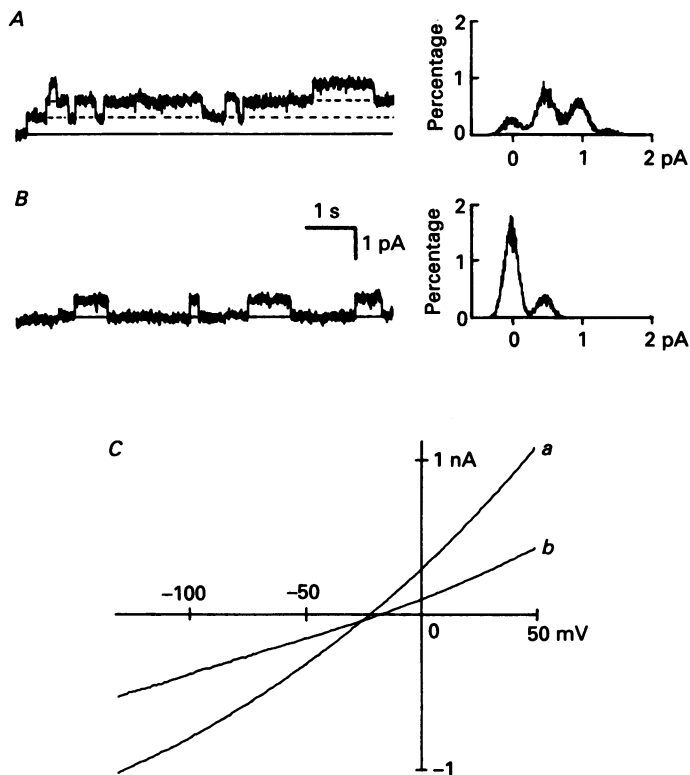


Fig. 2. Run-down of single Cl^- channel. *A* and *B*, trace of Cl^- channel currents and amplitude histogram obtained from the same patch at an early and a late stage (about 5 and 14 min after beginning of cell dialysis) of experiment, respectively. The patch membrane potential was held at +50 mV. The whole-cell pipette contained 0.5 mM cyclic AMP. Amplitude histograms were constructed from data sampled for 1 min. *C* shows I - V relations of whole-cell current observed at the times when each of the records in *A* and *B* was obtained (*a* and *b*).

three active channels (Fig. 2A). Nine minutes later, the current record in the same patch showed a depressed channel activity (Fig. 2B), associated with a reduction of macroscopic current (Fig. 2C, curve *b*). Thus, analysis of the single Cl^- channel was possible for 10–15 min after the beginning of cell dialysis.

Role of cyclic AMP in activation of Cl^- channel

There is the question as to whether the cyclic AMP system regulates the open-close kinetics of Cl^- channels, or only activates the channels without influencing their own kinetic property. Therefore, we attempted to see how the channel activity appears and how it changes during diffusion of cyclic AMP into the cell. If the cyclic AMP system has a profound, concentration-dependent influence on the kinetics of the Cl^-

channel, it might be possible to record a channel activity that increases in a graded manner with progression of the cyclic AMP loading.

In the experiment shown in Fig. 3, we began the cell dialysis while monitoring the current through a cell-attached patch. The dialysing solution contained 0.5 mM cyclic

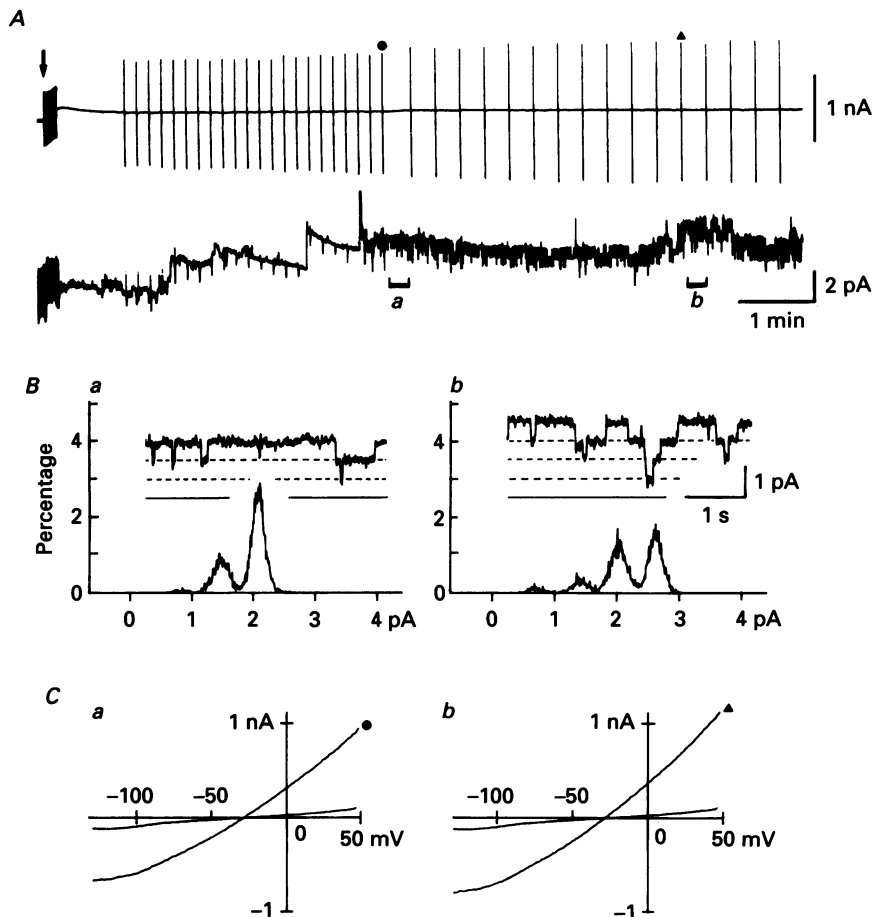


Fig. 3. *A*, simultaneous chart recordings of whole-cell current (upper trace) and current through a patch membrane (lower trace) obtained during dialysis of the cell with 0.5 mM cyclic AMP. The cell-attached patch membrane was clamped at +50 mV throughout. At arrow, cell dialysis was begun by rupturing the membrane beneath the whole-cell pipette. The thick vertical deflections of current trace seen at the beginning are artifacts produced by resistance-monitoring pulses. About 1 min after this moment, monitoring of the whole-cell conductance was begun by applying ramp pulses every 6 s (thin vertical deflections of whole-cell current). The pulse interval was increased to 20 s later. About 4.5 min after the onset of cell dialysis, activity of single Cl⁻ channels began to appear in the patch recording. Large fluctuations of the patch current seen before this instance are artifacts of unknown nature. *Ba* and *b*, amplitude histograms of channel currents obtained during the time periods indicated by *a* and *b* in *A*. Inset shows the original current traces. *Ca* and *b*, *I-V* relation of whole-cell current obtained from the current traces indicated by the symbols in *A* (● and ▲). *I-V* relation observed after run-down of the Cl⁻ current is also drawn in both graphs.

AMP. The whole-cell conductance (Fig. 3A, upper trace) gradually but slightly increased for the first 5 min after the beginning of cell dialysis in this case (see also Fig. 3C). This increase could be due to more adequate diffusion of cyclic AMP in the entire cell. In the patch recording (lower trace), activity of single Cl^- channels abruptly began to appear about 4.5 min after the beginning of cell dialysis. On the whole the magnitude of channel currents seemed to have three levels (see below). For a brief period (around the time indicated by *b* in Fig. 3A), the number of current levels increased.

In order to see the changes in open probability (P_o) of the channel during the course of the experiment, we constructed a series of amplitude histograms using the 15 s data of patch current sampled at 20 s intervals during the experiment. The lowest current level, which was clearly present in some segments of current record, was considered to be the zero current level. Examples of amplitude histograms are shown in Fig. 3B. In the histogram of Fig. 3Ba, the third current level shows the highest incidence, and there is no event at the fourth current level. If it is assumed that all channels had a similar P_o , this profile can be taken to indicate the number of active channels to be three, according to the theory of binomial distribution. In a similar way, the number of active channels was considered to be four in Fig. 3Bb. Then we calculated the mean patch current and mean P_o value from these amplitude histograms.

Figure 4 shows the time course of the calculated P_o as well as that of the whole-cell conductance and mean patch current observed during the experiment shown in Fig. 3. It can be seen that the channel activity began to appear with a high P_o (0.63 in this case), and that the P_o value was roughly constant (around 0.8) for several minutes, though the whole-cell conductance showed a gradual increase.

A similar analysis was made in five other experiments in which we could record the channel activity for at least one minute after it appeared. The cells were dialysed with 0.2–0.5 mM cyclic AMP. In all cases, the P_o value, which was 0.4–0.9 at the beginning, did not show any appreciable increase during the experiment. In several other experiments, we could only briefly (< 30 s) record the channel activity after its appearance because of instability of the patch membrane. In these cases we determined only the initial P_o value using the short data segment (15–20 s). The initial P_o value, averaged from all of the available data, was 0.69 ± 0.14 (\pm S.D., $n = 12$). Channels with a low P_o (< 0.4) at the beginning of their appearance were not observed.

If it is assumed that the Cl^- channel is activated when the cyclic AMP concentration around the channel protein reaches a certain threshold concentration, the above observations appear to indicate that the channel, once activated by cyclic AMP, begins to function with a high P_o , and that P_o is not influenced further by cyclic AMP concentrations. It may be argued, however, that in our experiments with the dialysing solution containing high concentrations of cyclic AMP, its concentration beneath the patch membrane might have quickly risen to a level high enough to exert a maximal effect on the channel, so that we could not detect any graded activity of the channels that might depend on the degree of activation.

However, in some patches an increase in the number of active channels occurred with a progression of cell dialysis, as shown before (Fig. 3). This phenomenon could

be the result of an increase, within the submaximal range, of the cyclic AMP concentration beneath the patch. Therefore, we looked at the P_0 value around this event. In Fig. 4, the mean P_0 value, calculated on a basis of equal P_0 for each channel, did not decrease when the channel number increased (bar in Fig. 4), suggesting that

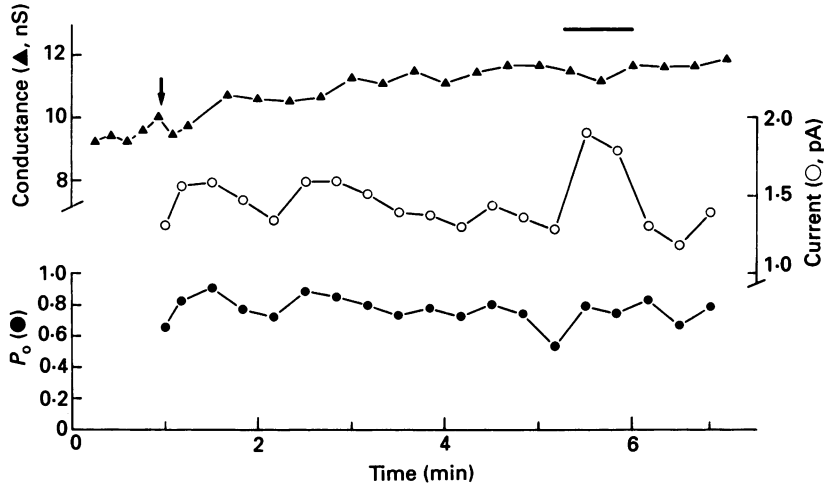


Fig. 4. Time course of whole-cell conductance (upper curve, ▲), mean patch current (middle curve, ○) and open probability of single Cl^- channel (P_0 , lower curve, ●) during the course of experiment shown in Fig. 3. Time 0 corresponds to the time point 3.5 min after the beginning of cell dialysis, and at arrow activity of single channels appeared in the patch. Number of active channels shortly increased from three to four during the period indicated by bar. Whole-cell conductance was calculated from the whole-cell $I-V$ relation at -30 mV, a voltage near the reversal potential of Cl^- current. Mean patch current and P_0 were calculated from the amplitude histograms which were obtained from the data periodically sampled for 15 s during the experiment. For calculation of P_0 it was assumed that every channel in the multichannel recordings had the same P_0 .

the 'new' channel also became active with a high initial P_0 . This point was further examined in more detail in the experiment shown in Fig. 5. In this experiment, the trace of channel currents initially showed a two-step change, but at the time indicated by the arrow in the figure it began to show a three-step change. To compare the P_0 values before and after this occasion, we constructed the amplitude histograms from the data sampled for 1 min immediately before and after the first appearance of the third current jump (Fig. 5B and C).

Assuming that the number of active channels increased from two to three, and that each channel had an equal P_0 , we made a binomial analysis to obtain a suitable P_0 value. The analysis showed that the P_0 value of 0.68 could roughly explain the relative heights of each current level in the histogram obtained before the increase in channel number (Fig. 5B). However, the histogram obtained after the increase in channel number did not match the relative heights of each current level expected from the binomial analysis based on the same P_0 value of 0.68 (Fig. 5C). Rather, the negative deviation of the experimental data from the theoretical value at the first current level and the positive ones at the second and third levels (Fig. 5C) suggest that the activity of the third channel began with a P_0 value greater than 0.68.

Thus, the Cl^- channel appears to exhibit a high P_o value in an all-or-nothing manner when it is activated by cyclic AMP. The channel does not seem to show any graded response that might depend on the degree of activation. The main effect of the cyclic AMP system on the Cl^- channels may be to regulate the number of active channels.

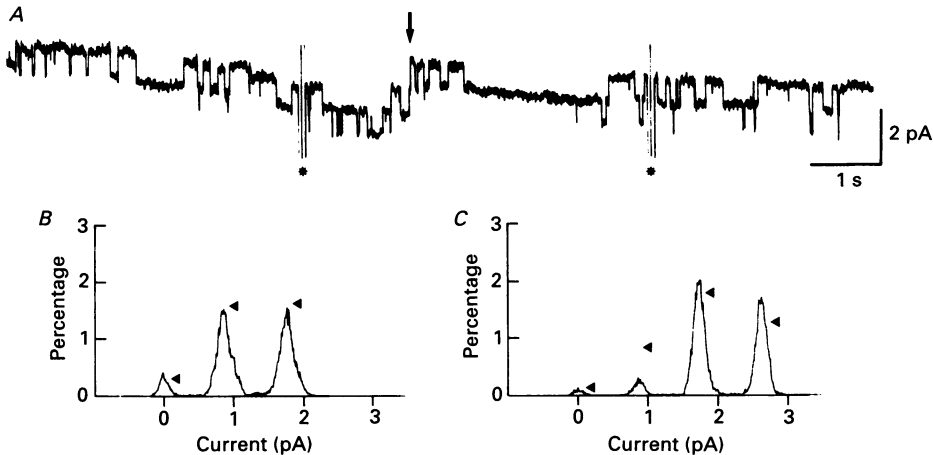


Fig. 5. Patch recording showing an increase in the number of active channels. *A*, original record of channel currents at a membrane potential of +50 mV. The current trace initially showed a two-step change, and at arrow it began to show a three-step change. Before this instance the two-step change had been recorded for about 2 min. The cell was dialysed with a cyclic AMP (0.25 mM)-containing solution. The vertical deflections denoted by * are artifacts produced by ramp pulses imposed to the whole cell. Current trace shows a slow, negative drift. *B* and *C*, amplitude histograms obtained from the data sampled for 1 min, immediately before and after the increase in channel number (arrow in *A*), respectively. Arrowheads indicate the relative heights that each of the three (*B*) and four (*C*) current levels should show, according to a binomial analysis with an assumption that each channel had P_o of 0.68. Note that in histogram *B* the relative height of each peak, instead of the area underlying the curve, was used for calculations.

Recording of Cl^- channel in outside-out patches

The patch membrane under the cell-attached pipette sometimes broke spontaneously while the cell dialysis with the other pipette was maintained. In such cases, it was possible to make outside-out patches (Hamill *et al.* 1981) in the former pipette after the bath solution was changed to normal Tyrode solution. Activity of Cl^- channels was found in some of these patches. This activity was considered to be initiated by cyclic AMP, since with the same pipette solution we could never detect a Cl^- channel activity in the outside-out patches derived from non-dialysed cells (15 experiments).

Figure 6 shows an example of the channel current and its $I-V$ relation observed in an outside-out patch. Since the Cl^- concentration gradient across the membrane was almost symmetrical, the reversal potential of the channel currents was near 0 mV, but the $I-V$ relation showed outward rectification (Fig. 6*B*). Similar observations were made in two other patches. These findings suggest that outward rectification of the Cl^- current is attributable at least in part to a property of the channel protein

or related structure. Similar outward rectification has also been noted for the current through agonist-activated, non-synaptic Cl^- channels in epithelial cells (Welsh, 1986; Halm, Reckemmer, Schoumacher & Frizzell, 1988) and lymphocytes (Chen, Schulman & Gardner, 1989) with symmetrical Cl^- gradients. The unit conductance

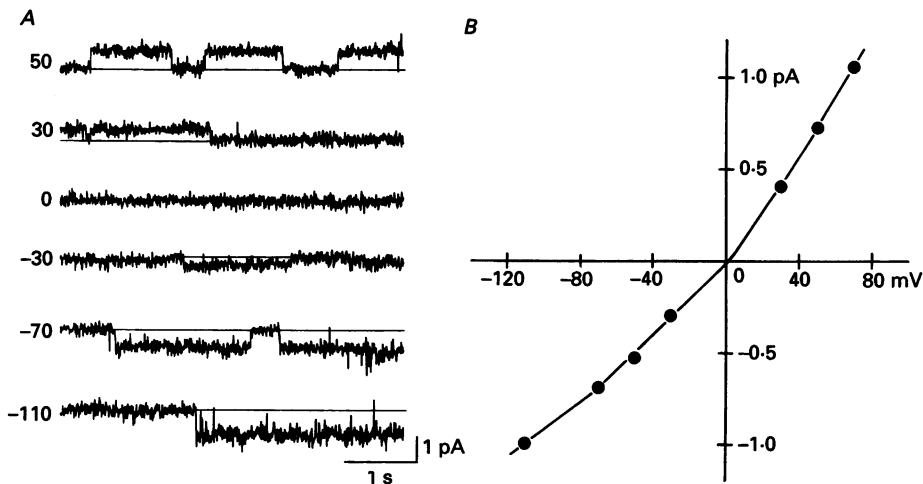


Fig. 6. Chloride channel current observed in an outside-out patch. The patch was excised from a cyclic AMP-loaded cell in normal Tyrode solution. The cell had been dialysed with a solution containing 0.25 mM cyclic AMP. Pipette solution was the usual one mainly composed of 150 mM NaCl, thus providing an almost symmetrical distribution of Cl^- ions across the membrane. *A*, original current traces filtered at 0.5 kHz. Numbers at the left of each trace are the patch membrane potential (mV). *B*, I - V relation of the channel current shown in *A*. Note a slight outward rectification. Linear part of the outward current has a slope of 16 pS.

of the cardiac Cl^- channel measured under these conditions was, on average, 15.3 pS ($n = 3$) for outward currents, a value slightly larger than that observed in cell-attached patches (Ehara & Ishihara, 1990).

Another feature of the channel current noted here was that the inward current showed little of the bursting activity (Fig. 6*A*) that is evident in current records obtained from cell-attached patches (Ehara & Ishihara, 1990). This indicates that the high-frequency closures during the burst seen in the latter case do not necessarily reflect the kinetic property of the channel itself. It is possible that, in intact on-cell channels, some intracellular substances, which may be washed out in outside-out patches, cause fast-blocking phenomena.

It is also interesting to note that the channels in these patches were often active with a high P_o for more than 15 min after patch excision, despite the virtual absence of cyclic AMP in the pipette solution bathing the cytoplasmic side of the membrane. If it is assumed that cyclic AMP, which was originally present near the membrane, was quickly diluted or washed off by the pipette solution after formation of the outside-out patches, the above finding indicates that the channel, once activated by the cyclic AMP system, can be deactivated little or only slowly under the condition of excised patches, even if cyclic AMP is no longer present.

Analysis of the channel kinetics

Figure 7 shows a result of the kinetic analysis of the channel. In this case the inward currents at -80 mV observed in an outside-out patch were examined. The open-time histogram could be fitted to a single exponential, and showed a time

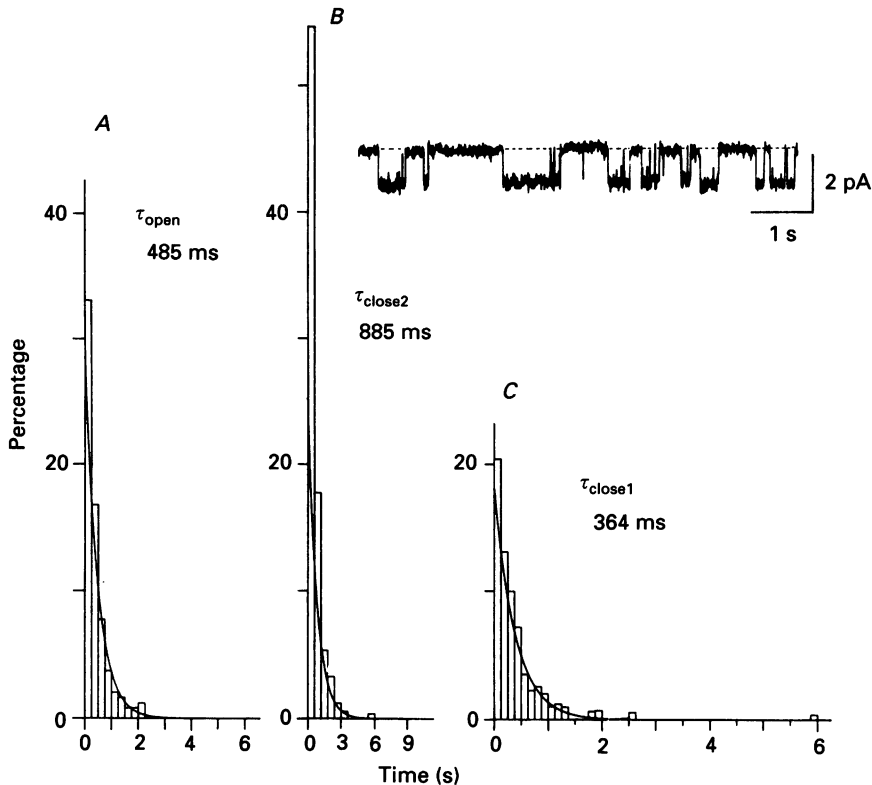


Fig. 7. Kinetic analysis of Cl^- channel. Data were obtained from an outside-out patch which was clamped at -80 mV, and 256 events were analysed. The experimental conditions were the same as in Fig. 6. *A*, histogram of open time. Time bins are 250 ms. The histogram was fitted to a single exponential with a time constant (τ_{open}) of 485 ms. *B* and *C*, histograms of closed time. Time bins are 600 ms for *B* and 125 ms for *C*. τ_{close1} and τ_{close2} are the time constants for the fast and slow components of closed time. Inset shows original current trace filtered at 0.2 kHz.

constant for open time (τ_{open}) of 485 ms. The closed-time histogram could not be fitted to a single exponential, and appeared to have at least two components, with faster (τ_{close1}) and slower (τ_{close2}) time constants of 364 and 885 ms, respectively.

Figure 8 summarizes the kinetic data obtained at various membrane potentials in a number of patches. These patches include both cell-attached and outside-out varieties. Although scattering of the data was considerably large, each of the three time constants did not appear to show any consistent variation with membrane potential. It may be concluded that both τ_{open} and τ_{close2} of the channel take values

around 1 s with a τ_{close1} value of around 0.2 s, and that the channel is practically voltage independent.

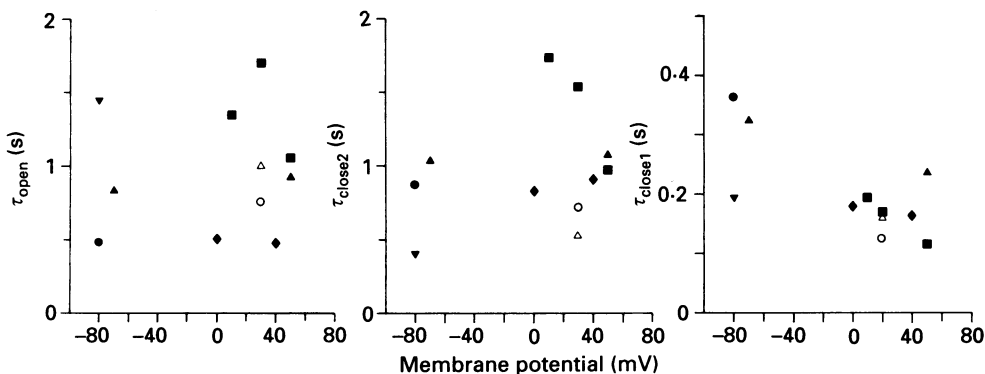


Fig. 8. Time constants of open-close kinetics as a function of membrane potential. τ_{open} , time constant of open time. τ_{close1} and τ_{close2} , time constants for the fast and slow components of closed time, respectively. Data from five outside-out patches (filled symbols) and two cell-attached patches (open symbols) are plotted.

DISCUSSION

Regulatory role of cyclic AMP in the activation of the Cl^- channel

Our main findings were that the Cl^- channel showed a high P_o immediately after it was activated by cyclic AMP, and that the P_o value remained almost unchanged during dialysis of the cell with cyclic AMP. In some patches, openings of an additional channel with a high P_o were observed during progression of the cell dialysis. These observations seem to be consistent with the hypothesis that the cyclic AMP system changes the channel situation from an unavailable state to an available one, without influencing the kinetic behaviour of the channel. The final step leading to the channel activation is phosphorylation of the channel protein or a related structure by cyclic AMP-dependent protein kinase (PKA) (Bahinski *et al.* 1989). Our results may also be interpreted to indicate that phosphorylation makes the 'latent' channels available.

On the other hand, phosphorylation also depends on the activity of phosphatases that counteract the action of PKA (Ingebritsen & Cohen, 1983). Therefore, the channel would show an unstable, low P_o due to alternation between phosphorylated and dephosphorylated states, if PKA is only weakly activated. In the present study, such an unstable behaviour of the channel was not observed, even at the beginning of channel activity. This could be due to massive activation of PKA by our dialysing solution (see below). It cannot be ruled out, however, that phosphatases were diluted or washed off by the cell dialysis, or inactivated by some components of the dialysing solution, so that a dephosphorylating reaction could hardly take place. The persistence of channel activity without cyclic AMP observed in outside-out patches (Fig. 6) could be related to such a mechanism.

Although it seems convincing that the cyclic AMP system exerts a switch-like action by which the channel becomes available, the relationship between the channel kinetics and cyclic AMP may still have ambiguous aspects. In particular, the

concentration of cyclic AMP or PKA beneath the patch membrane was uncertain in our study, and the possibility remains that their concentration was high enough to maximally act on the channel kinetics which otherwise should vary depending on the degree of phosphorylation. The recruitment of channel activity observed during cell dialysis (Fig. 5) was taken to render this possibility unlikely, but may not be direct evidence against it.

Furthermore, since many patches showed multichannel activity, and since we could obtain a stable long record only from such patches, we had to deduce the behaviour of the single channel from the multichannel activity. This must have introduced some complications into our analysis. Obviously, further studies, particularly studies on excised patches with controlled cyclic AMP concentrations beneath the membrane, are required to reach the final conclusion.

In cardiac cells, Ca^{2+} channels are modulated by phosphorylation via the cyclic AMP-PKA system (Kameyama *et al.* 1985; Kameyama *et al.* 1986; Tsien *et al.* 1986; Hartzell & Fischmeister, 1987; Fischmeister & Shrier, 1989). On the single-channel level, the current flow through the cardiac Ca^{2+} channel is thought to depend on a product of two variables, P_f and P_o , where P_f is the probability that the channel is available, and P_o the probability that the available channel will be open (Brum, Osterrieder & Trautwein, 1984; Tsien *et al.* 1986; Reuter, Kokubun & Prod'Hom, 1986). The non-phosphorylated channel has a low (but not zero) P_f , and phosphorylation is thought to increase P_o as well as P_f (reviewed by Hescheler & Trautwein, 1989). In addition, β -adrenergic stimulation never induces channel activity in cell-attached patches when no activity is detectable under the control condition (Cachelin, De Peyer, Kokubun & Reuter, 1983; Brum *et al.* 1984).

If our hypothesis on the Cl^- channel is correct, and if the P_f and P_o theory is also applicable to the Cl^- channel, phosphorylation exerts different actions on cardiac Cl^- and Ca^{2+} channels. In the case of Cl^- channels, P_f of the non-phosphorylated channel may be zero, and phosphorylation will convert it to 1, but P_o may not be influenced by phosphorylation. Whatever the mechanisms underlying the difference, the most characteristic feature of the Cl^- channels as regulated by the cyclic AMP system is that their activity can be induced in 'silent' patches by stimulation (present study; Ehara & Ishihara, 1990). The cardiac Cl^- might be denoted as a class of cyclic AMP- or PKA-gated channels.

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