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MEMBRANE CURRENT THROUGH ADENOSINE-TRIPHOSPHATE-REGULATED POTASSIUM CHANNELS IN GUINEA-PIG VENTRICULAR CELLS

BY AKINORI NOMA AND TOHRU SHIBASAKI
From the National Institute for Physiological Sciences, Myodaiji, Okazaki 444, Japan

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SUMMARY

1. The question whether activation of the ATP-regulated K channel is responsible for macroscopic anoxia-induced outward currents was examined in ventricular cells isolated enzymatically from guinea-pig heart. Gigaseal patch-clamp electrodes were used for a whole-cell voltage clamp. Membrane currents were compared in the same cell while the cell interior was dialysed by perfusing the electrode with different solutions.

2. When the cell was dialysed with various ATP-deficient ($\leqslant 2$ mM) internal solutions, the Ca current decreased in a dose-dependent manner to less than 10% of control at 0·5 mM-ATP. A slight (*ca.* 25%) decrease of the slope conductance for hyperpolarizing current was observed. When a delayed rectification on depolarization followed by a marked outward current tail on repolarization was present under control conditions, this time-dependent outward current was also depressed.

3. An increase in a time-independent outward current was observed accompanied by marked current fluctuations. The outward current showed a reversal potential near the K equilibrium potential, inward rectification, and no relaxation on voltage jumps.

4. The power density spectrum of the current fluctuations showed a pattern similar to the spectrum calculated from the single-channel currents of ATP-regulated K channels. The amplitude of the single-channel current, estimated from the fluctuations, was almost equal to that of the single-channel current. The total number of channels within one cell was estimated as 2000–3000.

5. It is concluded that the ATP-regulated K channels are responsible for the increase in the outward current and the shortening of the action potential duration under various anoxic conditions.

INTRODUCTION

The properties of a K channel, which is blocked by intracellular adenosine 5′-triphosphate (ATP), have been studied previously by the improved patch-clamp technique (Kakei & Noma, 1984; Trube & Hescheler, 1984). It was found that the K channel was activated when intracellular ATP was lowered below 2 mM (Kakei, Noma & Shibasaki, 1985). The experiments described in this paper were designed to

separate the outward-current component generated by the ATP-regulated K channels from the current changes induced in other current systems on depleting intracellular ATP, and to estimate the contribution of the ATP-regulated K channel to the whole-cell current.

Depletion of intracellular ATP may decrease the Ca current, as demonstrated by internal dialysis with ATP-deficient solutions (Irisawa & Kokubun, 1983). The electrogenic Na–K-pump activity may be depressed, which in turn results in a decrease of the pump-induced outward current (Isenberg & Trautwein, 1974; Glitsch, 1979; Isenberg, Vereecke, Van der Heyden & Carmeliet, 1983), and also in an alteration of intracellular ionic concentrations. Furthermore, it has been suggested that an increase in intracellular Ca may depress the Ca current (Kohlhardt & Kübler, 1975; Kohlhardt, Mnich & Maier, 1977), and increase the K conductance (Bassingthwaighte, Fry & McGuigan, 1976; Isenberg, 1977*a*, *b*) or activate non-selective cation channels (Colquhoun, Neher, Reuter & Stevens, 1981; Yellen, 1982; Matsuda, 1983). An increase in intracellular Na may activate a specific K channel (Kameyama, Kakei, Sato, Shibasaki, Matsuda & Irisawa, 1984).

In order to minimize the above complexities arising from the reduction of intracellular ATP, we dialysed single ventricular cells using the tight-seal suction electrode (Matsuda & Noma, 1984; Nakayama, Kurachi, Noma & Irisawa, 1984). If the cell is dialysed with a Na-free solution, the activity of the Na–K pump will be suppressed in control condition (D. C. Gadsby & A. Noma, unpublished observation) and thus, an alteration of the intracellular ionic composition on depleting intracellular ATP will not occur. Furthermore, we added 5 mM-EGTA in the pipette solution to increase the buffering capacity of intracellular free Ca level.

The outward current which is induced by depleting intracellular ATP showed characteristics similar to those of the ATP-regulated K channel. We suggest that the ATP-regulated K channel is the major current system which increases the outward current described under various anoxic conditions (for review, see Carmeliet, 1978).

METHODS

The preparation of single ventricular cells from the guinea-pig heart and the techniques of recording the whole-cell current and the single-channel current are essentially the same as in previous reports (Matsuda & Noma, 1984; Kakei & Noma, 1984).

Solutions. The composition of Tyrode solution was (in mM): NaCl, 136·1; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; $NaHPO_4$, 0·33; glucose, 5·5; and the pH was adjusted to 7·4 with 5 mM-HEPES–NaOH. The standard pipette solution (internal solution) contained (in mM): aspartate, 90; KCl, 20; $MgCl_2$, 1; ATP (dipotassium salt), 5; creatine phosphate (dipotassium salt), 10; EGTA, 5; HEPES, 5; and the pH was adjusted to 7·4 by adding KOH. When the concentration of ATP was varied, ATP was replaced with 4-fold higher molar aspartate to keep the K concentration constant. In some experiments creatine phosphate was replaced with 2-fold higher molar aspartate. In preliminary experiments, the effects of varying ATP concentration on the Ca current and the time-dependent K currents were found to be dependent on the Mg concentration. We found that the membrane current changed with intracellular ATP concentration in a dose-dependent manner when the ratio Mg/ATP was kept constant. Since the amplitude of the Ca current was larger when the ratio Mg/ATP was 0·2 compared with the Ca current obtained with a ratio of 0 or 1, we varied the Mg concentration in proportion to (0·2) the ATP concentration. In the CN-containing internal solution, ATP was omitted and 1 mM-KCN was added. In some experiments, 50 μM-2,4-dinitrophenol (DNP) or 0·5 mM-2-deoxy-D-glucose (2-DG) was also added in place of CN. All experiments were carried out at 35–36 °C.

Whole-cell clamp and internal dialysis. The single-pipette whole-cell clamp technique was used (Hamill, Marty, Neher, Sakmann & Sigworth, 1981; Hagiwara & Ohmori, 1982). To avoid the liquid junction potential and also to facilitate the formation of the gigaohm seal, the pipette (tip diameter of 4–5 μm) was first filled with Tyrode solution. After the formation of the gigaohm seal, the pipette solution was replaced with the internal solution containing 5 mM-ATP. After several minutes for equilibration of the pipette solution, a brief stronger suction was applied to the inside of the pipette to rupture the patch membrane. An abrupt decrease in the input resistance and an increase in the capacitive current indicated rupture of the membrane. When the rupture of the patch membrane was insufficient, as indicated by a relatively long-lasting capacitive current (>1 ms), the brief suction was repeated until the capacitive current decayed within 1 ms. In order to improve the time resolution of the clamp, a series resistance compensation circuit was involved in the single-pipette voltage-clamp amplifier (for details, see Matsuda & Noma, 1984). The holding potential was set at -35 or -40 mV to inactivate the Na current.

To examine the effect of varying intracellular ATP concentration on the membrane current, the interior of the suction pipette was perfused with different test solutions. The perfusion device for the pipette has been described elsewhere (Soejima & Noma, 1984). Briefly, a thin polyethylene tube having a tapered tip of about 70 μm diameter was inserted into the capillary and used as an inlet tubing. The perfusate was driven by a negative pressure of about 30 cmH_2O from a small reservoir (1 ml), through the inlet polyethylene tubing to near (*ca.* 150 μm) the tip of the electrode. Negative pressure was applied to a waste chamber connected to the outlet. The currents and potentials were recorded on magnetic tapes (TEAC R-210) for later analysis using a computer (HITAC E-600).

Variance and power spectrum. The output of the tape recorder was fed via an anti-aliasing filter (NF, FV-625A 48 dB/octave) into the computer. The record was digitized and broken into frames of 1024 samples each. The power spectrum was calculated using a fast fourier transform (one-sided power spectrum) for each frame. Both the variance and the power spectral density were averaged from six up to thirty-two frames and the spectrum was displayed in log–log coordinates.

RESULTS

Effects of varying intracellular ATP level on the membrane currents

When the pipette solution contained 5 mM-ATP, 10 mM-creatine phosphate, 1 mM-Mg and 5 mM-EGTA, the amplitude of the Ca current gradually increased by 5–20 % of its initial size and the holding current level shifted in the outward direction by 0·02–0·05 nA after rupture of the patch membrane enclosed within the tight-seal suction electrode. The current reached a new steady state within 5 min. Cells which showed a decrease of Ca current with time were discarded. After recording the control current–voltage relation, the pipette was perfused with an ATP-free internal solution containing 1 mM-CN in order to deplete intracellular ATP.

Within 3 min after starting the perfusion of the CN internal solution, the holding current shifted in the inward direction and stayed there for 3–7 min (Fig. 1*A*). Then, the current started to shift outward and, simultaneously, current fluctuations became evident. The increase of the outward current was nearly exponential in most experiments (upper trace), but a much slower increase was also observed in some experiments (lower trace). The former rapid increase of the outward current was accompanied by the development of rigor contracture. In general, the rate of increase was retarded when the concentration of creatine phosphate in the internal solution was increased between 0 and 10 mM or by adding a small amount of ATP (0·2–0·5 mM). The final effect of depleting intracellular ATP, however, was not affected by the presence or absence of creatine phosphate in the internal solution.

The amplitude of the Ca current, as monitored by repeating constant depolarizing pulses to $+5$ or $+15$ mV every 15–30 s, was gradually decreased by the perfusion of

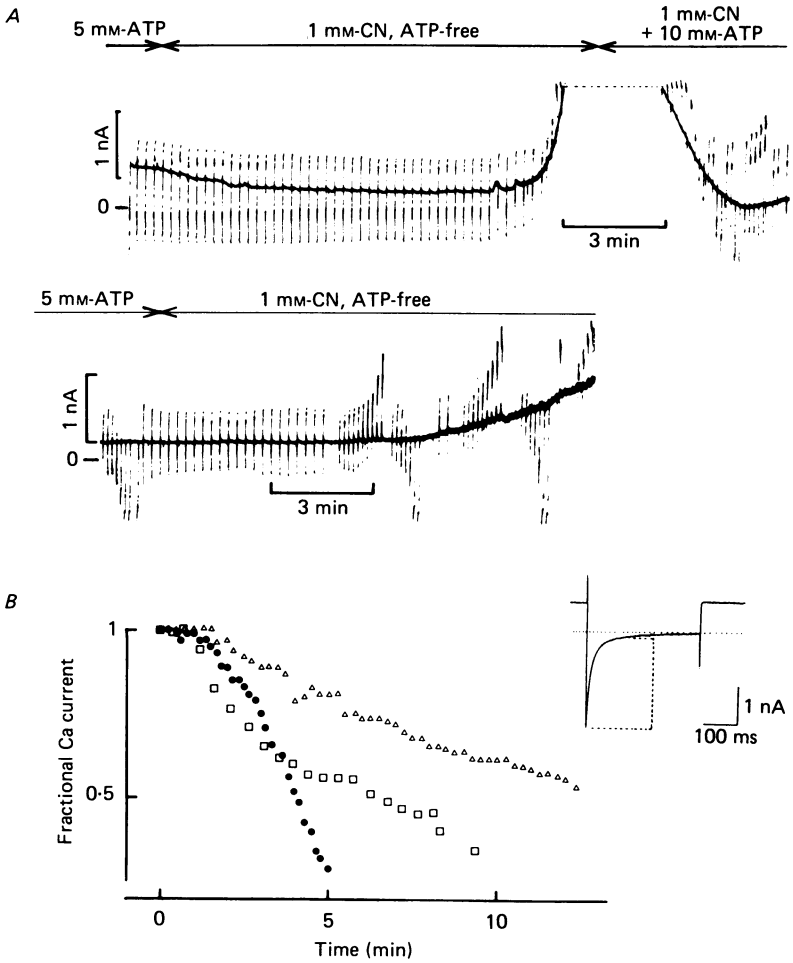

Fig. 1. *A*, time course of the change in the holding current at −35 mV during the perfusion of 1 mM-CN-containing ATP-free internal solution, recorded on a chart recorder. The spikes superposed on the current trace were induced by the clamp pulses. In the upper record near the end of the CN perfusion, the current was off the scale for about 3 min. The internal solutions contained no creatine phosphate. In the lower trace creatine phosphate was added to each perfusate. The thickening of the current trace reflects the increase of the current fluctuations. *B*, changes in the amplitude of the Ca current. The amplitude was displayed as a fraction of that obtained with the standard internal solution and the time on the horizontal scale indicates the duration of the dialysis with 1 mM-CN-containing, ATP-free internal solution. Different symbols indicate different experiments. The holding potential was −35 mV in every experiment.

CN internal solution. The peak amplitude of the Ca current was measured from the current level 100 ms into the depolarizing clamp pulse, as a first approximation (visual estimate, Fig. 1*B* inset; see also Isenberg & Klöckner, 1982), and is plotted in Fig. 1*B*. The rate of decrease varied between different preparations, but in every experiment no obvious latency before the initiation of the current decay was detected. This finding is in strong contrast to the delay recorded before the development of the outward current (Fig. 1*A*). The depression of the Ca current was reversible when

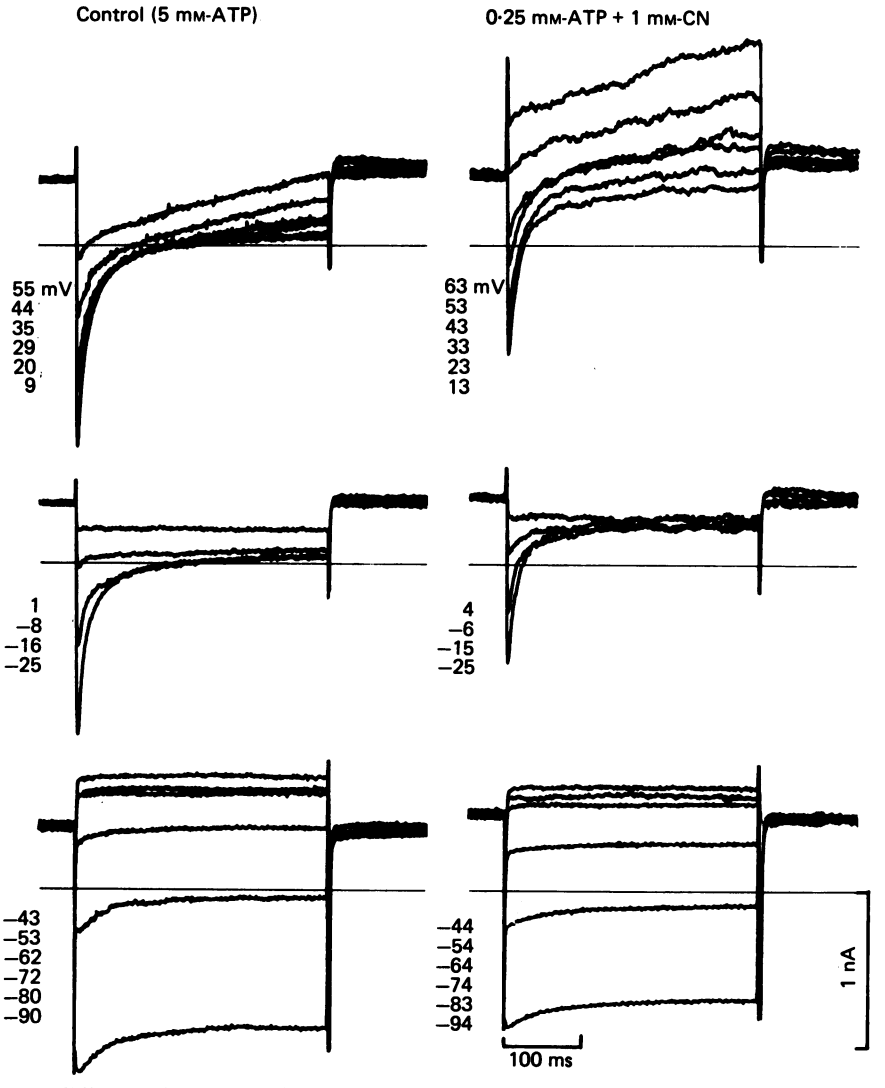

Fig. 2. Effects of internal dialysis with 1 mM-CN-containing, 0·25 mM-ATP internal solution on the membrane currents. The currents in the left column were obtained with the standard internal solution (creatine-phosphate-free). The potentials during the clamp pulses are indicated to the left of the superimposed current traces. The holding potential was −35 mV. The currents in the right column were obtained 6–8 min after the onset of the dialysis with the CN internal solution. Horizontal lines indicate the zero-current level. The current fluctuations in the right column are not due to an unstable clamp, but are due to intrinsic fluctuations of the membrane current. Note, the amplitude of the fluctuations decreased markedly as the amplitude of the hyperpolarizing clamp pulses increased. The transient inward deflexions at the offset of the hyperpolarizing pulses are due to the activation of the Na current.

the increase of the outward current was retarded by adding creatine phosphate in the pipette solution and the perfusate was switched back to the control before the outward current increased by more than 0·5 nA at +5 or +15 mV.

In order to observe the changes in the time-dependent current components without interference by the relatively large increase of the outward current (as shown in Fig. 1), the current records in Fig. 2 were obtained when the holding current was increased by only 0·06 nA, 6–8 min after perfusing with the CN internal solution. The Ca current on depolarization, as well as the current during hyperpolarizing pulses,

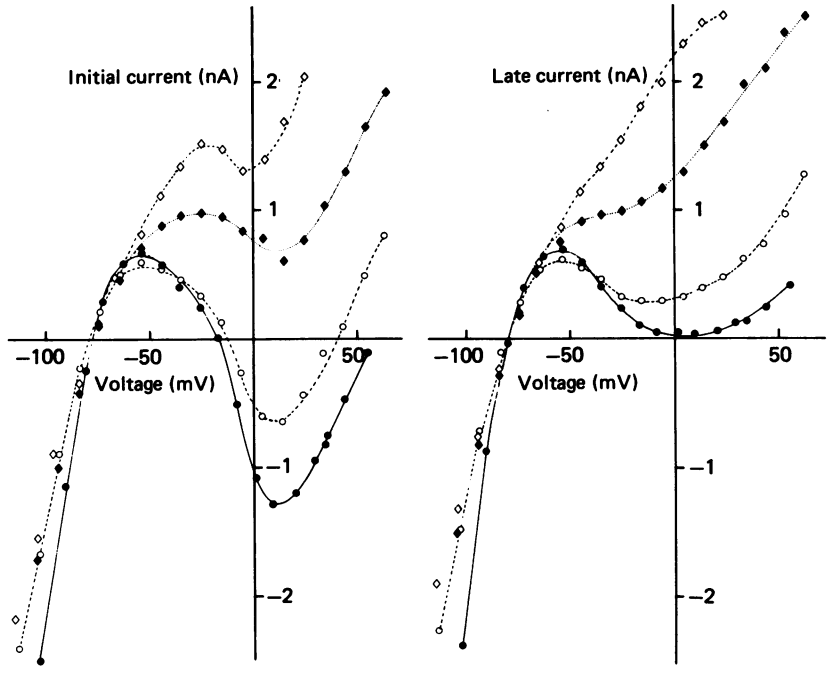

Fig. 3. Current–voltage relations measured in control (●), 6–8 min (○), 10–12 min (◆), and 15–17 min (◇) after the onset of the CN internal solution. The initial current was measured at the peak of the Ca current or 5–10 ms into the hyperpolarizing clamp pulse. The late current was measured near the end of the clamp pulse of 300 ms in duration. The data were obtained from the same experiment as shown in Fig. 2. Holding potential was −35 mV.

which is probably due to the inward-rectifier K channel, was depressed after dialysis with the ATP-free solution (Fig. 2). In case the delayed rectification during depolarization and the outward current tail on repolarization were present (in some cells), these time-dependent outward currents were also depressed. In Fig. 3, the current–voltage relations obtained 6–8, 10–12, and 15–17 min after the perfusion of the CN internal solution were compared with the control. It is obvious that the effect developed progressively.

In order to get an average effect of intracellular ATP depletion, the outward current amplitude at +10 mV was measured; it increased from 0·14±0·17 to 0·39±0·29 nA, 4–8 min after starting CN internal solution (mean±S.D., $n = 6$). The amplitude of the Ca current (measured from the zero-current level at the peak in the

current–voltage relation) decreased to $60 \pm 12\%$ of control. The slope conductance near the resting membrane potential decreased to $75 \pm 15\%$ and the amplitude of the outward current at around -55 mV decreased to $82 \pm 5\%$ of control. Essentially the same results were obtained when 50 μM-DNP (in two experiments) or 0·5 mM-2-DG (seven experiments) was added to the ATP-free internal solution.

Dependence on intracellular ATP of the outward current and the Ca current

During the course of experiments, it was found that the above changes in the current components were induced even when the ATP concentration in the internal solution was simply decreased without adding metabolic inhibitors. In order to get more insight in the relation between intracellular ATP and the membrane currents,

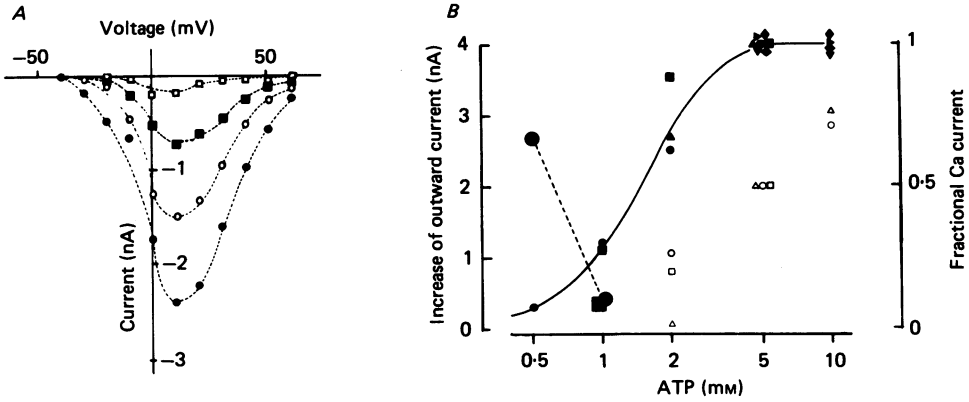

Fig. 4. *A*, the amplitude of the Ca current at different ATP concentration in the pipette. The maximum amplitudes at +10 mV are plotted in the right graph. The concentration of ATP was changed in a sequence of 5 (●), 2 (○), 1 (■) and 0·5 mM (□) in the same experiment. Note, the voltage dependence of the Ca current is not much altered by depleting intracellular ATP. Holding potential was −40 mV. *B*, effects on the outward current (dashed line) and on the Ca current (smooth curve) of dialysing the cell with internal solutions containing different ATP concentrations. The vertical scale on the left indicates the amplitude of the outward current at +10 mV induced by depleting intracellular ATP. The vertical scale on the right is for the Ca current. The amplitude of the Ca current was expressed as a fraction of that obtained with 5 mM-ATP in the pipette. In the absence of creatine phosphate in the pipette, the amplitude of the Ca current (open symbols) was smaller than in the presence of creatine phosphate and the amplitude of the Ca current at 5 mM-ATP was arbitrarily defined as 0·5. Different symbols indicate different experiments.

the cell was perfused with various ATP concentrations in the internal solutions. With 2 mM-ATP in the internal solution the increase in the outward current was detected in none of eight experiments, but below 1 mM-ATP it was observed in every experiment. In one experiment, the amplitude of outward current at +10 mV (in the late current) increased to steady levels of 0·39 and 2·69 nA at 1·0 and 0·5 mM-ATP, respectively (Fig. 4*B*). Below 0·5 mM-ATP (eight experiments), the development of rigor contracture interfered with the measurements of the steady amplitude of the increased outward current except in the experiment shown in Fig. 4*B*.

On the other hand, new steady peak amplitudes were reached for the Ca currents about 15 min after varying the ATP concentration in the range between 5 and 0·5 mM. When the concentration of ATP in the internal solution was decreased from 5 to 2 mM, the amplitude of the Ca current at the peak of the current–voltage relation (Fig. 4*B*) decreased to 74% of the control, while an increase of ATP to 10 or 20 mM did not induce appreciable change.

When creatine phosphate was omitted from the internal solution, the increase of the outward current was observed in each of three experiments when ATP was

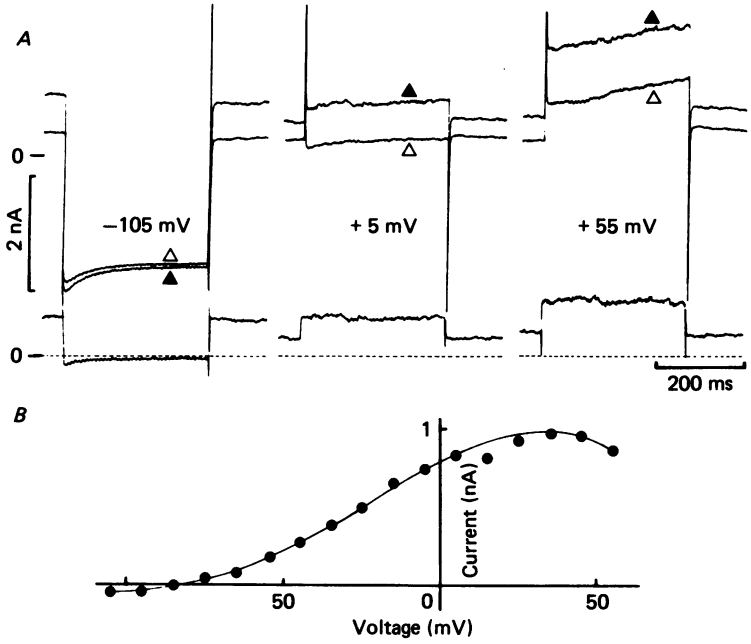

Fig. 5. The outward current induced by depleting intracellular ATP. The Ca current was blocked by replacing 0·9 mM-Ca with equimolar Co. The pipette solution was switched from the standard internal solution to the ATP-free one containing 1 mM-CN. The currents indicated by △ were obtained 5–7 min after starting the CN perfusion, and those indicated by ▲ were obtained 9–11 min after the perfusion. The differences were calculated between each pair of currents (lower trace). Holding potential was −35 mV.

decreased from 5 to 2 mM. The amplitude of the Ca current varied more extensively in the absence of creatine phosphate; it decreased to less than 32% of control (intracellular ATP, 5 mM) on decreasing intracellular ATP from 5 to 2 mM, and on increasing from 5 to 10 mM-ATP the amplitude increased to 150% of control. These results agree well with the view that creatine phosphate plays an important role in transferring high-energy phosphates in the reaction catalysed by creatine kinase (Seraydarian & Abbott, 1976; Ventura-Clapier & Vassort, 1980*a*). Thus, it was suggested that the intracellular ATP level can be varied by adding or removing ATP from the internal solution in the presence of creatine phosphate. Since both the Ca current and the outward current depend on intracellular ATP, analysis of the outward current induced by the ATP depletion requires blockade of the Ca current.

The current–voltage relation of the anoxia-induced outward current

The outward current induced by depleting intracellular ATP was measured as the difference between the control records and that obtained after depleting intracellular ATP. In order to block the Ca current, 0·9 mM-Ca was replaced with equimolar Co. Variation in the inward-rectifier K current and in the delayed outward current may cause an error in the estimation of the K current induced by the ATP depletion.

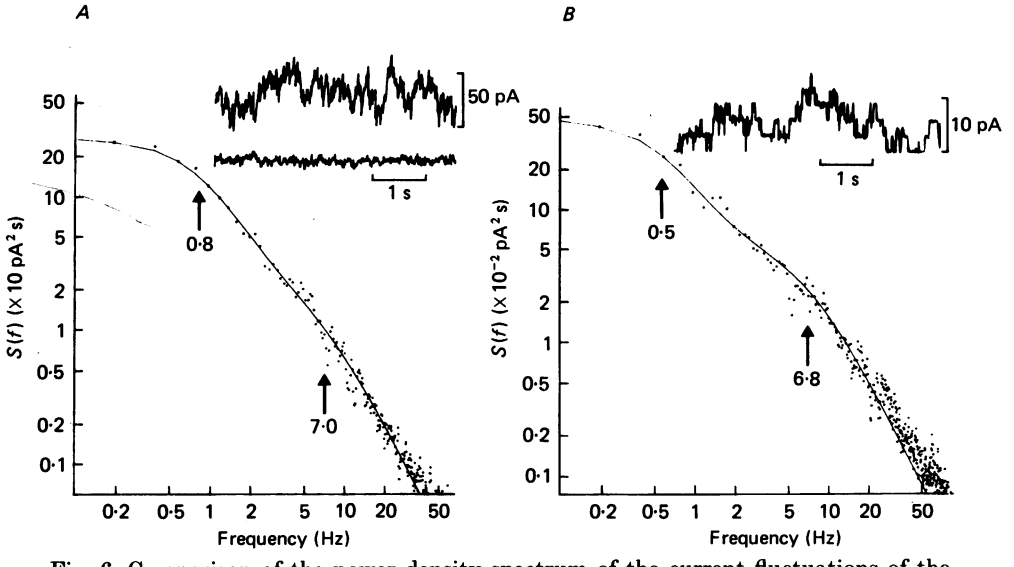

Fig. 6. Comparison of the power density spectrum of the current fluctuations of the outward current induced by depleting intracellular ATP (*A*), with the spectrum calculated from the single-channel recordings (*B*). The average spectra were calculated from the whole-cell current at −35 mV in a ventricular cell dialysed with the standard internal solution and with the ATP-deficient internal solution (1 mM-CN-containing, ATP-free solution) and the difference was calculated and illustrated in *A*. The continuous curves are the least-squares fit of the equation, where $S_1(0)$ and $S_2(0)$ are the zero-frequency amplitudes, and $f_{c,1}$ and $f_{c,2}$ are the corner frequencies of the two Lorentzian components:

$$S(f) = S_1(0)/(1+f/f_{c,1}) + S_2(0)/(1+f/f_{c,2}).$$

The parameters used were: $S_1(0) = 252{\cdot}7$ pA², $f_{c,1} = 0{\cdot}8$ Hz, $S_2(0) = 13{\cdot}3$ pA², and $f_{c,2} = 7{\cdot}0$ Hz. The current trace in the control (lower trace) and that after intracellular ATP depletion (upper trace) are shown. In *B*, the spectrum was calculated from the single-channel recordings using the patch-clamp electrode containing 5·4 mM-K. An example of a current trace is shown in the inset. The bathing solution for the open cell-attached patch was 140 mM-K-, 0·5 mM-ATP-containing solution. The background noise was relatively small compared to the channel noise, and was neglected. The spectrum was fitted with a sum of two Lorentzian components, with $S_1(0) = 0{\cdot}42$ pA², $f_{c,1} = 0{\cdot}5$ Hz, $S_2(0) = 0{\cdot}047$ pA², and $f_{c,2} = 6{\cdot}8$ Hz.

However, no selective blocker for these K currents which leaves the ATP-regulated K channel intact is available. Since the change in the inward-rectifier K current occurs within 1–3 min after switching the perfusate, the current traces obtained just before the rapid increase in the outward current were used as the control. The current traces obtained before (△) and after (▲) the increase of the holding current are shown in

Fig. 5*A*. The differences of these current traces are shown in the lower panel, where no clear time dependency is detected except at −105 mV.

In every experiment, the current obtained after ATP depletion showed increased current fluctuations, characterized by a relatively low frequency (spectrum shown in Fig. 6*A*). The holding current level varied between records, because the clamp pulses were applied when the outward current continued to increase. Therefore, the amplitude of the holding current (I) in each difference current was measured and the average value (I_{ave}) was calculated. To normalize the amplitude of the difference current, each current was multiplied by the ratio of I_{ave} to I, and the current–voltage relation was measured (Fig. 5*B*). The reversal potential of the difference current was −83 mV, a value close to the K equilibrium potential. In six ventricular cells it was -79 ± 4 mV. Inward rectification was observed positive to +25 mV. These characteristics of the outward current, i.e. time independency, reversal potential and rectification of the difference current, are very similar to those of the summation of the single-channel current of the ATP-regulated K channels (see Fig. 7 in the preceding paper, Kakei *et al.* 1985), and strongly suggest that the ATP-regulated K channels are responsible for the outward current induced by depleting intracellular ATP.

Estimation of the unit amplitude from the current noise

If the ATP-regulated K channel is responsible for the outward current induced by depleting intracellular ATP, the current fluctuations in the macroscopic current should be attributable to random variation of the number of open channels around its mean. This was tested by calculating the power density spectra of the fluctuations of the whole-cell currents (Fig. 6*A*). As shown by the high-gain a.c. records in the inset, the current fluctuations were markedly increased after depleting intracellular ATP when the mean current level shifted by 0·8 nA in the outward direction. The noise spectrum for the outward current was measured as the difference of the spectra obtained before and during the depletion of intracellular ATP. The power density distribution was fitted with a sum of two Lorentz components having corner frequencies of 0·8 and 7·0 Hz (Fig. 6*A*). As an average for six experiments (Table 1), the corner frequencies were $1{\cdot}2 \pm 0{\cdot}5$ and $9{\cdot}3 \pm 2{\cdot}6$ Hz for the slow and fast components, respectively. The ratio of the plateau level ($S(0)$) of the fast Lorentz component to that of the slow component was $0{\cdot}085 \pm 0{\cdot}048$. The finding that the noise spectrum could be separated into Lorentz components supports the view that the current fluctuations are due to first-order kinetics of the gating of ionic channels.

In the preceding paper (Kakei *et al.* 1985), the rate constants of the channel kinetics were measured from the open-time and the closed-time histograms (Fig. 9 in the preceding paper, Kakei *et al.* 1985). However, the time constant of the whole-cell current fluctuations cannot be directly compared with the rate constants obtained from the histograms, because the estimation of the time constant depends on the model, and it is difficult to establish a kinetic model for the gating of the ATP-regulated K channel. Therefore, we decided to compare directly the noise spectrum with that obtained from single-channel recordings (Fig. 6*B*), which showed superpositions of more than three channels at intracellular ATP concentrations of 0·5–0·7 mM. The single-channel current spectrum also showed two components and the corner frequencies were $0{\cdot}6 + 0{\cdot}2$ and $9{\cdot}0 + 4{\cdot}3$ Hz (Table 1). The ratio of the plateau level

TABLE 1. Whole-cell and single-channel currents

Whole-cell current noise

	Slow component			Fast component						Surface area	
No.	f_c (Hz)	Variance (pA²)	$S(0)$ (pA²)	f_c (Hz)	Variance (pA²)	$S(0)$ (pA²)	Ratio of $S(0)$	i (pA)	N	μm^2	$\mu m \times \mu m$
1	0·8	329·5	252·7	7·0	146·7	13·30	0·052	0·89	1750	3880	(70 × 20)
2	1·9	164·6	52·7	13·7	120·2	5·57	0·105	1·00	2290	4400	(80 × 20)
3	0·7	167·1	147·7	9·1	69·2	4·83	0·032	0·76	1724	3880	(70 × 20)
4	0·9	158·5	110·6	7·2	120·1	10·55	0·095	0·90	2167	3712	(80 × 16)
5	1·0	274·7	161·9	10·8	164·9	9·65	0·059	0·84	1988	4328	(94 × 16)
6	1·8	74·2	26·1	7·8	54·7	4·42	0·168	—	—	—	—
7	—	—	—	—	—	—	—	1·08	2657	5016	(100 × 18)
8	—	—	—	—	—	—	—	0·79	3329	5440	(100 × 20)
9	—	—	—	—	—	—	—	0·84	2762		
Mean ±S.D.		1·2 ±0·5 ($n = 6$)			9·3 ±2·6 ($n = 6$)		0·085 ±0·048 ($n = 6$)	0·88 ±0·10 ($n = 8$)	2333 ±552 ($n = 8$)	4379 ±642 ($n = 7$)	

Single-channel current recordings ($n = 9$)

Slow component: f_c(Hz)	Fast component: f_c(Hz)	Fast component: Ratio of $S(0)$
0·6 ±0·2	9·0 ±4·3	0·059 ±0·041

of the fast Lorentz component to that for the slow component was 0·059 ± 0·041. These findings strongly support the view that the current noise in the whole-cell current is due to a first-order relaxation process of the ATP-regulated K channel. The slight difference in the corner frequencies may be attributed to variations in the intracellular ATP in the ATP-depleted cells.

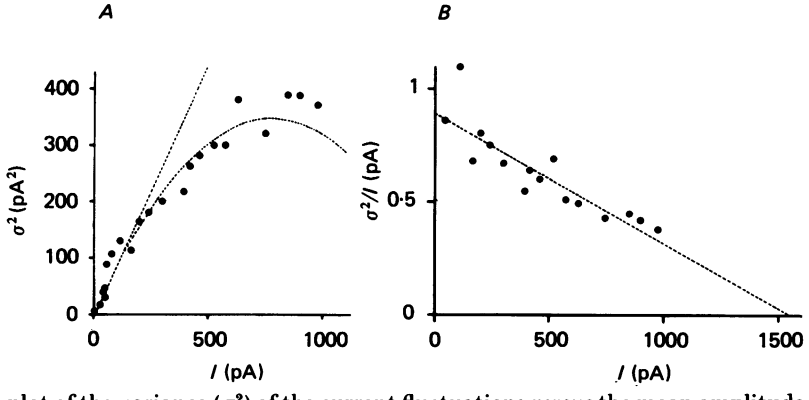

Fig. 7. In *A*, plot of the variance (σ^2) of the current fluctuations *versus* the mean amplitude (I) induced by depleting intracellular ATP. The slope of the relation gave a single-channel unit current of 0·89 pA. In *B*, σ^2/I *versus* I is plotted. The values of σ^2/I were averaged when I was relatively small. The line was drawn with single-channel unit current of 0·89 pA at $I = 0$ with the least-squares fit.

Unitary amplitude and total number of ATP-regulated K channels estimated from the whole-cell current fluctuations

If all K channels are identical, non-interacting, and have only one conductance state, the variance (σ^2) of the fluctuations is given by the following equation,

$$\sigma^2 = i(1-p)\,I, \tag{1}$$

where i is the unit amplitude of the single-channel current, p is the probability of the open state of the channel and I is the mean amplitude of the total current. The increase of outward current on depleting intracellular ATP was continuously recorded into consecutive frames of 2 or 5 s duration each. During this frame length the mean current was assumed to increase linearly and measured by fitting a line using the least-squares method. The noise record was obtained by subtracting the fitted line from the original record. Since the variance varied between frames, the averages were obtained from six to twenty consecutive records showing a similar mean current amplitude and are plotted in Fig. 7*A* against the mean current. Close to the zero current ($p \sim 0$ in eqn. 1), the slope of the relation gives the unit amplitude i, which was 0·89 pA. This value agrees well with that obtained in the single-channel current recording with 5·4 mM-K on the outer surface of the membrane (1·17 ± 0·11 pA at −40 mV, Fig. 3 in the preceding paper, Kakei *et al.* 1985). In order to get the total number of channels (N) within the cell, eqn. (1) was modified to give,

$$\sigma^2/I = i - I/N, \tag{2}$$

and the data were replotted in Fig. 7*B*. From the slope of the fitted line, the value of N obtained was 1750. In Table 1, the results of eight experiments are summarized. The average of i was $0{\cdot}88 \pm 0{\cdot}10$ pA and of N was 2333 ± 552.

DISCUSSION

When single ventricular cells were dialysed with ATP-free internal solutions, not only the Ca current but also the delayed outward current and the inward-rectifier K current were progressively depressed. After a delay of several minutes, the depletion of intracellular ATP induced a time-independent outward current accompanied by marked current fluctuations. When the concentration of ATP in the dialysing internal solution was varied in a stepwise manner, the amplitude of the Ca current decreased in a dose-dependent manner over the concentration range of 0–5 mM-ATP. The increase of the time-independent outward current was observed below 1 mM-ATP. Since Na–K-pump activity was most probably depressed even in control dialysis with Na-free internal solution (D. C. Gadsby & A. Noma, unpublished observation), alteration of the intracellular ionic composition due to a change in pump activity is unlikely to occur on depleting intracellular ATP.

In summary, the following five findings strongly support the hypothesis that the increase of the outward current in the whole cell on depleting intracellular ATP is due to removal of the block of the ATP-regulated K channel. (1) The outward current is carried by K ions (Fig. 5*B*). (2) Both the whole-cell current (Fig. 5*A*) and the single-channel current (Fig. 7*B* in the preceding paper, Kakei *et al.* 1985) did not show relaxation on voltage jumps. (3) The current–voltage curve of the whole-cell current (Fig. 5*B*) shows inward rectification quite similar to that of the summated single-channel current (Fig. 7*C* in the preceding paper, Kakei *et al.* 1985). (4) The noise spectrum of the whole-cell current revealed two Lorentzian components, which are also observed in the power density spectrum of the single-channel current (Fig. 6). (5) The unit amplitude estimated from the fluctuations of the whole-cell current (Table 1) agrees well with the amplitude of the single-channel current.

Contribution of the ATP-regulated K channel in non-dialysed cells

Taniguchi, Noma & Irisawa (1983) demonstrated that the injection of ATP through an intracellular micro-electrode depressed the outward current induced by treating single ventricular cells with CN. Recently, Bechem & Pott (1984) also observed an increase of the outward current in atrial cardioballs after rupture of the patch membrane, when the gigaseal patch electrode contained an ATP-free internal solution. Furthermore, ATP-regulated K-channel currents were recorded with cell-attached patch recording from ventricular or atrial cells superfused with CN or DNP (Noma, 1983; Kakei & Noma, 1984; Trube & Hescheler, 1984; Noma, 1985). The outward current induced by hypoxia in papillary muscle (Vleugels, Vereecke & Carmeliet, 1980) and that induced by DNP in single ventricular cells (Isenberg *et al.* 1983) are also time independent, and show inward rectification. These findings strongly suggest that the ATP-regulated K channel is the major system generating the anoxia-induced outward current also in the non-dialysed cardiac cells.

Total number of channels per cell

In order to quantitate the contribution of the ATP-regulated K channels, it is necessary to know the total number of channels and also the dependence on intracellular ATP of the open-state probability of the K channel. From the relation between the mean amplitude and variance of the whole-cell current fluctuations, the total number of channels was estimated and ranged between 1724 and 3329 (Table 1). This result can be compared to the data for single-channel recordings (Kakei *et al.* 1985). We did not measure the density of the ATP-regulated K channels directly, but showed that the density of the ATP-regulated K channel is almost equal to that of the inward-rectifier K channel. The density of the inward-rectifier K channel over the surface membrane was estimated as 1 channel/1·8 μm^2 (Sakmann & Trube, 1984). A similar value of channel density was also suggested for rabbit ventricular cells (Noma, Nakayama, Kurachi & Irisawa, 1984). Assuming the area of the surface membrane of the ventricular cell to average 4379 μm^2 (thickness = 6 μm), a value of 2432 channels per cell was obtained. If the channels are distributed on the membrane of T tubules or caveolae, a larger number of channels may be estimated for the whole cells. Thus, the numbers of channels estimated by whole-cell and by single-channel current recordings agree well. It may be concluded that a single ventricular cell has approximately 2000–3000 ATP-regulated K channels.

Intracellular ATP dependence of open-state probability

We measured this dependence using three different methods. In the whole-cell clamp method, the threshold concentration of ATP in the internal solution was between 1 and 2 mM for the appearance of the outward current. This finding agrees well with the data obtained with the open cell-attached patches (Fig. 10 in the preceding paper, Kakei *et al.* 1985). The dose–response curve measured from the excised inside-out patches, however, was shifted to a lower concentration range by a factor of $\frac{1}{4}$.

It might be assumed that the excised patch membrane gives the real ATP dependence of the channel activity and that the concentration of intracellular ATP near the open cell-attached patch membrane is lower than the concentration in the bathing solution. A steady, lower intracellular ATP concentration than in the bath solution can only be possible when ATP is consumed continuously within the saponin-treated cell. However, the cell is not consuming ATP for contraction, because it remains completely relaxed by the addition of EGTA to the internal solution. The other major consumer of ATP, the Na–K pump, is not working because Na ions are absent from the system. Furthermore, the dose–response curve was not appreciably changed even when the distance between the recording electrode and the disrupted membrane was varied. Thus, it is difficult to assume a concentration gradient along the axis of the saponin-treated cell. We consider therefore that the channel protein was modified by excising the patch membrane and that the sensitivity of the channel to ATP was increased. The dose–response relation obtained in the open cell-attached patch may be relevant for the response of the whole-cell current.

The above view is supported by applying the relation between the total current

(I) and the open-state probability (p) to the whole-cell current, i.e. $I = pNi$. According to the dose–response curve in the open cell-attached patch (Fig. 10 in the preceding paper, Kakei *et al.* 1985) the open-state probability at 1 mM-intracellular ATP is 0·05–0·09. If the single-channel unit current is 2·5 pA at 0 mV, the total current produced by 2333 channels amounts to 0·29–0·52 nA at 1 mM-intracellular ATP. This value agrees well with the recorded current amplitude of 0·36–0·39 nA (Fig. 4*B*) when dialysed with 1 mM-ATP solution. When the cell was dialysed with 0·5 mM-ATP a steady outward current of 2·7 nA was recorded. This value may give an open-state probability of 0·54, which again agrees well with the dose–response curve of the open cell-attached patch.

Contribution of the ATP-regulated K channel

The intracellular ATP concentration under anoxic conditions may vary depending on how long and extensively the muscle is treated (Grinwald, Hearse & Segal, 1980; Ventura-Clapier & Vassort, 1980*a*). McDonald & MacLeod (1973*a*, *b*) measured the ATP concentration to be 0·5–1·5 μmol/g wet weight, when the plateau of the action potential was depressed by superfusing the cell with DNP in papillary muscle. During hypoxia, the cell content of creatine phosphate is greatly reduced before any decrease of intracellular ATP (Gudbjarnason, Mathes & Ravens, 1970). Thus, the potency of the creatine phosphate/creatine kinase/creatine system to buffer intracellular ATP may be severely impaired (for review, see Seraydarian & Abbott, 1976). Thus, the consumption of about 10 % of the available energy for each cardiac beat (Gibbs, 1978; Ventura-Clapier & Vassort, 1980*b*) cannot be compensated. This might cause a dynamic and local decrease of intracellular ATP below 2 mM in synchrony with the cardiac cycle in non-dialysed anoxic cells. Phasic activation of the ATP-regulated K channel, therefore, is likely to occur when the mean intracellular ATP concentration decreases below 2–3 mM. Furthermore, if the effect of metabolic inhibitors is assumed to be non-homogeneous (Baumgarten, Cohen & McDonald, 1981), it is quite possible that some of the cells within the central core of the papillary muscle are more extensively depleted of ATP, resulting in activation of the ATP-regulated K channels.

Isenberg *et al.* (1983) reported that the outward current induced by treating single ventricular cells with DNP is time independent. They suggested that this time-independent outward current is generated by the Ca-activated K conductance. However, we failed to record K-channel current even when the Ca concentration on the inner side of the membrane was raised to 100 μM (Kakei & Noma, 1984), although the ATP-regulated K channel was depressed. In the non-dialysed cells, the intracellular Na concentration may be raised, which in turn can activate the Na-activated K channel (Kameyama *et al.* 1984).

It has been postulated that the decrease of the Ca current is also responsible for the shortening of the ventricular action potential (Schneider & Sperelakis, 1974; Kohlhardt & Kübler, 1975; Nargeot, 1976; Nargeot, Challice, Tan & Garnier, 1978; Payet, Schanne, Ruiz-Ceretti & Demers, 1978). A new result of our study is that the Ca current is depressed in a dose-dependent manner when intracellular ATP is lowered below 5 mM. The ATP threshold of this effect is higher than that for the development of the outward current passing through ATP-regulated K channels. In

the non-dialysed cell, however, it is unlikely that depression of the Ca current occurs without the development of the outward current under mild hypoxic conditions. This is because local, phasic decreases of intracellular ATP may occur when intracellular ATP is lowered (see the previous section). Actually, the outward current markedly increases before a significant decrease in the Ca current is detected (Vleugels *et al.* 1980; Isenberg *et al.* 1983).

The Ca current inactivates within the initial 100 ms on depolarization and thus only its non-inactivated component can contribute to the plateau 100 ms after the rising phase of the action potential. The amplitude of this non-inactivating component, when measured as the difference between the control and that obtained after the application of the Ca blockers (Matsuda & Noma, 1984), is less than 50 pA in the single ventricular cell. If this component is depressed by the depletion of intracellular ATP to 2 mM as much as the peak amplitude (−30%), the current may change by only 15 pA. This amount of current corresponds to the current carried by six ATP-regulated K channels ($i = 2{\cdot}5$ pA). We suggest, therefore, that the shortening of the plateau during anoxic conditions is mainly due to activation of ATP-regulated K channels.

Depression of other current components

In the present study it was found that the holding current (−35 or −40 mV) decreased within 1–3 min after starting the perfusion with the ATP-deficient internal solution. This finding agrees well with the small decrease in the outward current observed by Isenberg *et al.* (1983) during the initial phase of superfusing the cell with 0·2 mM-DNP. When Irisawa & Kokubun (1983) dialysed ventricular cells with 3 and 13 mM-ATP internal solutions, they found a change in the outward current in proportion to the ATP concentration. We found that the decrease in the outward current is always accompanied by a decrease of the slope conductance over the hyperpolarizing voltage range. If this slope conductance is mediated by the inward-rectifier K channel (Sakmann & Trube, 1984), the decrease in the outward current is attributable to a depression of the inward-rectifier K channel. This view is supported by the fact that the inward-rectifier K channel is depressed by depleting intracellular ATP in the open cell-attached patch (in the preceding paper, Kakei *et al.* 1985).

During experiments using single ventricular cells, we observed a large variability of cell integrity within different cells. One rather direct indication of the integrity of membrane excitability is the leak conductance of the cell. When the cell becomes damaged during the isolation procedure, at the moment of applying the suction electrode, or following addition of metabolic inhibitors etc., the slope conductance of the cell rapidly increases. The ATP-regulated K channel, Na-activated K channel and Ca-activated cation channel may be responsible for these increased leak conductances.

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