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CHLORIDE-SENSITIVE NATURE OF THE ADRENALINE-INDUCED CURRENT IN GUINEA-PIG CARDIAC MYOCYTES

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SUMMARY

1. Ionic selectivity of an adrenaline-induced current was investigated in single guinea-pig ventricular cells by recording whole-cell currents using the patch clamp technique combined with internal perfusion. Other ionic currents and exchange currents known in ventricular cells were suppressed by appropriate inhibitors and the adrenaline-induced current was defined as a difference between currents obtained in the presence and absence of adrenaline.

2. The adrenaline-induced current was time independent and its *I–V* relation showed saturation of the inward current in the negative voltage range.

3. The reversal potential was approximately −20 mV with 140 mM-NaCl external solution and Cs^+-rich internal solution containing 51 mM-Cl^-. Replacing Na^+ with various monovalent and divalent cations (Li^+, K^+, Rb^+, Cs^+, Ca^{2+}, Sr^{2+} and Ba^{2+}) produced no appreciable change in the reversal potential.

4. Varying the external Cl^- concentration ($[Cl^-]_o$) in exchange for aspartate or benzenesulphonate greatly changed the reversal potential. The relationship between the reversal potential and $\log[Cl^-]_o$ indicated a slope of 59·5 or 53·6 mV per tenfold change in $[Cl^-]_o$ in the presence of 51 or 102 mM-Cl^- in the internal solution, respectively.

5. Anion substitutions did not appreciably affect the *I–V* relation before application of adrenaline, suggesting that the cell membrane had a low Cl^- conductance in the control state.

6. 4,4′-Dinitrostilbene-2-2′-disulphonic acid (DNDS; 1–10 mM), a specific inhibitor of membrane chloride permeability, depressed the adrenaline-induced current without changing the reversal potential.

7. The results suggest strongly that the adrenaline-induced current is carried mainly by Cl^-. However, the development of this current appears to depend also on external cations, since the magnitude of the adrenaline response varied depending on the external cation species, with no response in Tris-HCl or TEA-Cl solution. The external cations may facilitate the adrenaline response with a sequence of efficacy of $Na^+ > K^+$, $Rb^+ > Cs^+$, Li^+, divalent cations.

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INTRODUCTION

It is well established that catecholamines can increase the amplitude of time- and voltage-dependent membrane currents in cardiac muscle (for review see Noble, 1979). Catecholamines can also increase background currents. Isoprenaline or noradrenaline increases a membrane K^+ conductance via β-adrenoceptor stimulation in quiescent cells of coronary sinus (Boyden, Cranefield & Gadsby, 1983) and canine Purkinje fibres (Gadsby, 1982). Recently, isoprenaline was found to induce an inward current in ventricular myocytes and this response was very probably mediated by intracellular cyclic adenosine monophosphate (cyclic AMP) which was increased by β-adrenoceptor stimulation (Egan, Noble, Noble, Powell & Twist, 1987).

The ionic nature of the above isoprenaline-induced current is still unclear. Egan, Noble, Noble, Powell, Twist & Yamaoka (1988) showed that this current was insensitive to K^+ channel blockers, Ca^{2+} channel blockers and Na^+–K^+ pump inhibitors. It was also unaffected by Cs^+ which blocks the hyperpolarization-activated, non-specific channel (I_f). Substitution of external Cl^- with large anion did not change the current amplitude recorded at a negative holding potential, but replacement of external Na^+ with tetramethylammonium (TMA) abolished the isoprenaline response. The reversal potential of the current was difficult to measure because of the presence of other catecholamine-sensitive currents, but was considered to be close to that for Na^+. Thus it was presumed that the isoprenaline-induced current was carried by Na^+ (Egan *et al.* 1988). Harvey & Hume (1989*a*) also studied the properties of the same isoprenaline-induced current, and reported that the current was apparently time independent, and that its reversal potential was near -40 mV. They also suggested that the Ca^{2+} channel, Na^+–K^+ pump and ATP-sensitive K^+ channel are not involved.

In this study, we isolated the catecholamine-induced current in an attempt to measure its current–voltage (I–V) relation and reversal potential. These parameters are prerequisite for determining the ionic nature of the current and for differentiating it from other catecholamine-sensitive currents. To isolate the current, other membrane currents and exchange currents known in ventricular cells were inhibited by appropriate inhibitors. It will be shown that the adrenaline-induced current is very likely a Cl^- current, and that it also depends on co-existing cations.

METHODS

Preparation of single cells

Single ventricular cells were obtained from guinea-pig hearts using an enzymatic dissociation technique, which was similar to that described previously (Powell, Terrar & Twist, 1980; Isenberg & Klockner, 1982). Briefly, guinea-pigs (300–400 g) were anaesthetized with sodium pentobarbitone. The chest was opened and the aorta was cannulated under artificial respiration. The heart was excised under continuous coronary perfusion with Tyrode solution, and was hung on the Langendorff-type perfusion system. The heart was first perfused with a nominally Ca^{2+}-free Tyrode solution until the heart beat ceased, and then with Ca^{2+}-free Tyrode solution containing collagenase (Sigma type I, 0·4 mg/ml) and trypsin inhibitor (Sigma, 0·4 mg/ml) for about 30 min at 37 °C. After washing out collagenase by perfusing with 'KB medium' (Isenberg & Klockner, 1982), the left ventricle was further dissected into small pieces in a dish filled with KB medium. The dispersed cells were kept in KB medium before use.

Voltage clamp and recording technique

Whole-cell clamp experiments were performed following the original technique developed by Hamill, Marty, Neher, Sakmann & Sigworth (1981). To avoid the liquid junction potential (see below) and also to facilitate formation of the 'gigaohm seal', the pipette was first filled with Ca^{2+}-containing Tyrode solution. After formation of the gigaohm seal, the pipette solution was replaced with the internal solution by using an 'intrapipette perfusion device' (Soejima & Noma, 1984). A brief strong suction was then applied to the pipette interior to rupture the patch membrane. After equilibration of the intracellular medium with the pipette solution, the external solution was changed from Tyrode solution to various test solutions under the voltage clamp conditions.

The initial voltage reference was performed when Tyrode solution was present both inside and outside the electrode (see above), a condition in which there must be no liquid junction potential at the electrode tip. The command DC potential at which the current through the electrode was zero was taken as the reference potential. This reference potential was considered to remain unchanged after changing the pipette solution from Tyrode to internal solution, assuming that the junction potential at a 3 M-KCl–agar electrode set in the intrapipette perfusion device was negligible in these solutions. The junction potential which might develop during cell dialysis was also considered to be negligible. The reference electrode in the bath was usually 3 M-KCl–agar with an integral Ag–AgCl wire. However, in the experiments in which external Cl^- concentration was varied, we used a flowing KCl electrode instead of the KCl–agar in order to reduce possible changes in the junction potential which is sensitive to Cl^-. This electrode was a glass pipette connected to a reservoir of 3 M-KCl solution. A continuous small leak of the 3 M-KCl solution through the pipette tip was maintained by hydrostatic pressure. The electrode was placed down-stream from the preparations to avoid contamination of the bath solution with KCl.

Ramp voltage clamp pulses (triangle wave; $dV/dt = \pm 0.9$ V/s) were employed to obtain the *I–V* relations, and the hyperpolarizing portion of the ramp pulses was used for evaluation of the *I–V* relation. To measure the membrane capacitance (C_m), the half-amplitude of current jump at the peak of ramp pulse was divided by the slope of the ramp pulse. The membrane conductance is expressed relative to the capacitive area of the membrane (pS/pF). The membrane current and voltage were stored on videotapes (video recorder, TOSHIBA, A-700HFD, Tokyo, Japan) using a PCM converter (SONY, PCM-501ES, Tokyo, Japan, modified for DC signal) for later computer analysis (NEC, PC98 XA, Tokyo, Japan).

Solutions

The normal Tyrode solution contained (in mM): NaCl, 140; KCl, 5·4; $MgCl_2$, 0·5; $CaCl_2$, 1·8; NaH_2PO_4, 0·33; glucose, 5·5; HEPES, 5·0 (pH = 7·4 with NaOH). The external solution used for recording the adrenaline-induced current was a K^+-free, Ca^{2+}-free solution containing (in mM): NaCl, 140; $MgCl_2$, 2·0; *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (HEPES), 5·0 (pH = 7·4 with NaOH). Ouabain (20 μM) and $BaCl_2$ (2·0 mM) were added to block Na^+–K^+ pump and K^+ channels, respectively. A Ca^{2+} channel blocker, nicardipine-HCl (Sigma, 1·0 μM; cf. Sanguinetti & Kass, 1984; Terada, Kitamura & Kuriyama, 1987), was also added.

When cation dependence of the adrenaline-induced current was examined, external 140 mM-NaCl was replaced with equimolar LiCl, KCl, RbCl or CsCl, or with 93 mM-$CaCl_2$, $SrCl_2$, or $BaCl_2$. Tris-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) or tetraethylammonium chloride (TEA-Cl) was also used as substitutes for NaCl. When 93 mM-divalent cations were used, the concentration of nicardipine was increased to 10 μM. When anion dependence was examined, external NaCl was totally or partially replaced with sodium aspartate or sodium benzenesulphonate on an equimolar basis.

The composition of the standard internal solution was essentially the same as that used in a previous study (Ehara, Matsuoka & Noma, 1989), and was (in mM): CsOH, 166; aspartate, 42; ethyleneglycol-bis(β-aminomethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA, Sigma), 42; Tris-ATP, 5·0; $MgCl_2$, 8·0; HEPES, 5·0 (pH = 7·4 with HCl). TEA-Cl (20 mM) was also added to block K^+ channels. The amount of HCl required for titration was measured in several experiments, and it was 15 mM on average. Therefore, the total Cl^- concentration of this solution was considered to be 51 mM. Chloride-rich internal solution was made by replacing 66 mM-CsOH in the above solution with 66 mM-CsCl (pH = 7·4 with CsOH). The Cl^- concentration of this solution was 102 mM. The free Ca^{2+} concentration of these high-EGTA internal solutions was estimated to be lower than 10^{-10} M (see Fabiato & Fabiato, 1979). Thus any activation of the non-selective cation channels

(Colquhoun, Neher, Reuter & Stevens, 1981; Ehara, Noma & Ono, 1988) and the Na^+–Ca^{2+} exchange current (Baker & McNaughton, 1976; Kimura, Noma & Irisawa, 1986) should be absent. To facilitate diffusion of the internal solution through the pipette tip, pipettes having a wide tip diameter (3–4 μm) were used. All experiments were performed at 37·0 ± 0·5 °C.

Egan *et al.* (1987, 1988) suggested that the isoprenaline-induced current is mediated by β_1-adrenoceptor. We used adrenaline (Dai-ichi), (±)isoprenaline-HCl (Sigma) and a selective β_1-agonist, denopamine (Tanabe). While 0·5–5 μM-adrenaline and 0·1–0·5 μM-isoprenaline induced substantial membrane responses, the concentration of denopamine required to produce appreciable amplitude of the response was higher than 5 μM. The magnitude of the current response produced by 10 μM-denopamine was approximately 15% of that induced by 0·5 μM-adrenaline. Therefore, adrenaline was used in most experiments. In some experiments, an inhibitor of membrane chloride permeability, 4,4′-dinitrostilbene-2-2′-disulphonic acid (DNDS, Tokyo Kasei), was added to external solution (Bretag, 1987; Bridges, Worrell, Frizzell & Benos, 1989).

RESULTS

Isolation of the adrenaline-induced current

In our experimental conditions with selected external and internal media (see Methods), most of the ionic currents and exchange currents known in cardiac cells were inhibited. The Ca^{2+} channel was blocked by nicardipine and by removing Ca^{2+} from the external solution. The K^+ channels were blocked by external Ba^{2+}, internal TEA and removal of K^+ from both the external and internal solutions. The Na^+–K^+ pump current was inhibited by ouabain and removal of external K^+ and internal K^+ and Na^+. The Na^+–Ca^{2+} exchange current was suppressed by removing external Ca^{2+} and internal Na^+. Furthermore, the intracellular free Ca^{2+} concentration was kept low by using high concentration of EGTA in the internal solution, to exclude contamination of any Ca^{2+}-mediated conductances and to inhibit the Na^+–Ca^{2+} exchange. The hyperpolarization-activated, non-specific current (I_f) is not known in guinea-pig ventricular cells, and our Cs^+-rich internal solution should block activation, if any, of this current. The transient outward current (I_{to}) is also not known in guinea-pig ventricular cells, and in the present study we found no evidence of I_{to}-like, time-dependent current in the presence and absence of catecholamine (see Fig. 1). Thus, we consider that the membrane currents identified so far in cardiac cells can hardly develop under our experimental conditions (except the Na^+ channel current which may develop only on rapid depolarizations from negative potentials), whether they are sensitive or insensitive to catecholamine, and that the remaining current reflects solely the background current.

Figure 1 shows the membrane currents in response to 200 ms square pulses from a holding potential of −40 mV. Under the control conditions (Fig. 1*A*), the current traces did not show any marked time-dependent changes during depolarizing pulses. During strong hyperpolarizations, a decay of the inward currents of unknown nature was observed within the initial 50 ms of hyperpolarizing pulses. Application of 0·5 μM-adrenaline resulted in an inward shift of the holding current accompanied by an increase in membrane conductance as shown in Fig. 1*B*. An increase of the miniature current fluctuations was also observed on the traces of the inward current during hyperpolarizing pulses.

Figure 1*C* shows isochronal current–voltage (*I–V*) relations measured near the end of the clamp pulses in the absence (○) and presence (●) of adrenaline. The control *I–V* relation was almost linear with a reversal potential of about 0 mV. The current

component induced by adrenaline was obtained at various voltages by subtracting the control current from the current obtained in the presence of adrenaline (Fig. 1*D*), and the *I–V* relation of the difference current is illustrated in Fig. 1*E*. This *I–V* relation shows a slight outward rectification and a reversal potential of about −20 mV. Because of the time-independent nature of the adrenaline-induced current (Fig. 1*D*), *I–V* relations were measured by using ramp pulses in the following experiments.

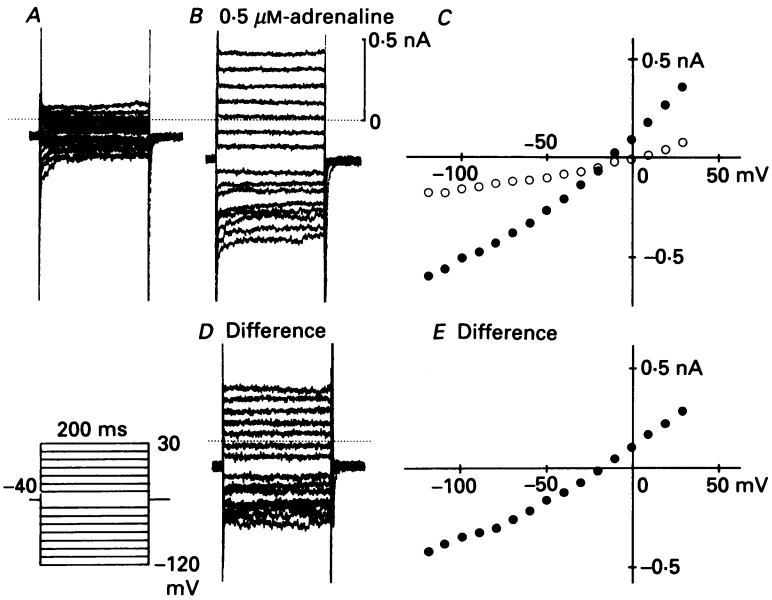

Fig. 1. Effects of adrenaline on the membrane current as examined by square voltage pulses. *A*, control current traces in response to various test potentials from holding potential of −40 mV. Pulse protocol is shown in lower left. The cell was then exposed to adrenaline and the membrane currents were recorded with the same pulse protocol (*B*). *C*, *I–V* relations of the current in the absence (○) and presence (●) of adrenaline. *D*, adrenaline-induced current measured by subtracting the control current (*A*) from the current obtained in the presence of the drug (*B*). *E*, *I–V* relation of the adrenaline-induced current. The current amplitudes were measured 200 ms after the onset of test potentials in all *I–V* relations.

Measurement of I–V relation with ramp pulses

Measurements of the *I–V* relation of the adrenaline-induced current performed by means of ramp pulses are shown in Fig. 2. The holding potential was set at −40 mV and the ramp pulses were applied every 6 s. Application of 0·5 μM-adrenaline induced an inward shift of the holding current accompanied by an increase in the amplitude of the current deflection in response to the ramp pulses (Fig. 2A). The observed *I–V* relations are shown in Fig. 2*B*, in which the *I–V* relations obtained from three consecutive ramps in the absence (○) and presence (●) of adrenaline are illustrated. These *I–V* relations showed essentially the same characteristics as those obtained by the square pulse protocol shown in Fig. 1.

In Fig. 2*B*, the three consecutive *I–V* relations were almost superimposable. This

indicates that spontaneous decay of the response during continuous presence of catecholamine reported earlier (Egan *et al.* 1987, 1988) was negligible during the short application used in the present study. After washing out adrenaline, the membrane conductance decreased to the control level within about 15 s. It should be

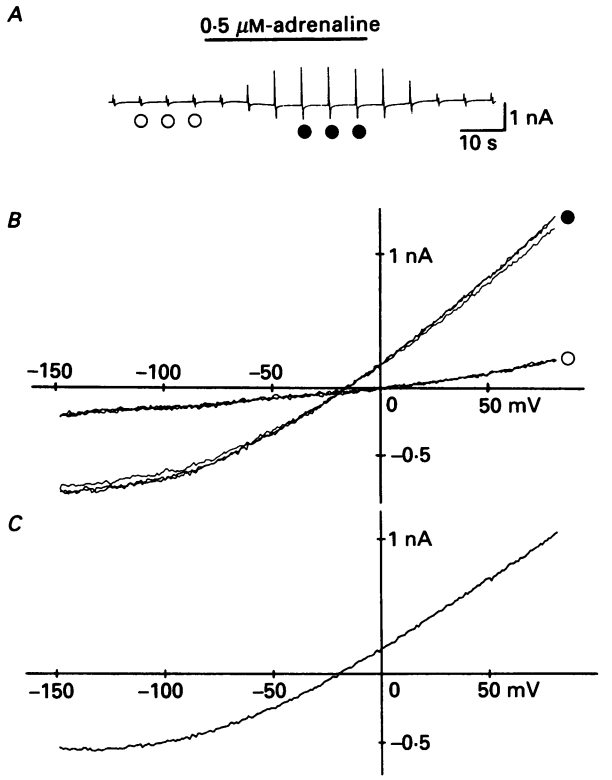

Fig. 2. Measurement of *I–V* relation of the adrenaline-induced current by ramp voltage pulses. *A*, chart record of membrane current. Holding potential was set at −40 mV and ramp pulses of triangular type (−150 to +100 mV, $dV/dt = \pm 0{\cdot}9$ V/s) were applied every 6 s. The cell was exposed to adrenaline for the time period indicated by bar. *B*, *I–V* relations obtained by ramp pulses in the absence (○) and presence (●) of adrenaline. Three consecutive current traces indicated by the same symbols in *A* are superimposed. *C*, *I–V* relation of the adrenaline-induced current obtained by subtracting average control *I–V* relation from average *I–V* relation obtained in the presence of the drug.

noted, however, that when adrenaline application was repeated, even with an interval of several minutes, the second response was usually smaller than the first one (sustained 'desensitization), in agreement with earlier observations (Egan *et al.* 1987, 1988).

The reversal potential of the control current was $9{\cdot}0 \pm 4{\cdot}5$ mV ($n = 17$) and the slope conductance, measured at the reversal potential, was 25 ± 28 pS/pF (see Table 1). The *I–V* relation of the adrenaline-induced current was determined as a difference between the averages of the three records obtained in the absence and presence of adrenaline, and is shown in Fig. 2*C*. The reversal potential of the difference current was $-19{\cdot}0 \pm 3{\cdot}1$ mV ($n = 10$). The outward current increased linearly with

depolarization, and the inward current saturated in amplitude in the potential range from −100 to −150 mV (Fig. 2*C*).

Application of 0·5 μM-isoprenaline also induced a current response similar to that produced by adrenaline (not shown). The *I–V* relation of the isoprenaline-induced current showed a reversal potential of −15·0±2·4 mV ($n = 4$), which was not significantly different from that of the adrenaline-induced current. These findings indicated that the currents induced by the two drugs are mediated by a common current system.

TABLE 1. Slope conductances and reversal potentials of control current and catecholamine-induced current

	Control current			Catecholamine-induced current		
External solution (mM)	Slope conductance (pS/pF)	E_r (mV)	(n)	Slope conductance (pS/pF)	E_r (mV)	(n)
140 NaCl	25±28	9±4	(17)	37±29	−19±3	(10)*
140 NaCl	—	—	—	61±12	−15±2	(2)**
140 LiCl	17±12	6±5	(10)	66±44	−14±3	(3)
140 KCl	160±32	31±4	(5)	21±14	−13±5	(3)*
140 RbCl	190±51	24±4	(9)	22±11	−10±5	(6)*
140 CsCl	42±25	10±2	(10)	10	−8	(1)
93 $CaCl_2$	19±11	13±3	(9)	64±36	−17±1	(5)
93 $SrCl_2$	18±6	13±5	(8)	40±27	−16±3	(6)
93 $BaCl_2$	23±13	14±4	(10)	36±44	−16±2	(4)

Slope conductance is expressed as relative to capacitive area of membrane (pS/pF). E_r, reversal potential. Values are presented as mean±S.D. Numbers in parentheses (n) are the number of experiments. Drug used was: *, 0·5 μM-adrenaline; **, 0·5 μM-isoprenaline; without asterisks, 5 μM-adrenaline.

I–V relations in various cation solutions

The reversal potential of −15 to −20 mV in the foregoing experiments did not match the equilibrium potential of any major cations present outside and inside the cells. This finding raised the possibility that the catecholamine-induced current might be generated by one type of non-selective cation channels. Therefore, we first examined the effects of varying external cation species on the adrenaline response. The external 140 mM-NaCl was replaced with equimolar chloride salt of various monovalent cations (Li^+, K^+, Cs^+, Rb^+) or with 93 mM-chloride salt of divalent cations (Ca^{2+}, Ba^{2+} or Sr^{2+}). Representative *I–V* relations recorded before and after applying adrenaline in the various salt solutions are shown in Figs 3 and 4, and Table 1 summarizes the results.

In LiCl and CsCl solutions, the control *I–V* relation was quite similar to that observed in NaCl solution (see Fig. 2*B*). In KCl and RbCl solutions, however, the control *I–V* relation showed a large membrane conductance and a positive reversal potential. The large membrane conductance might be due to an increased conductance of the inward rectifier K^+ current, which was not completely blocked by external 2 mM-Ba^{2+} in the presence of high external K^+. The *I–V* curve at negative

potentials showed marked humps, which were probably caused by a time- and voltage-dependent action of Ba^{2+} on the inward rectifier (DiFrancesco, Ferroni & Visentin, 1984; Imoto, Ehara & Matsuura, 1987). The *I–V* curves of adrenaline-induced currents, obtained by subtracting the control from the current obtained in the presence of adrenaline, however, showed a smooth upward curvature in all types of the solutions containing monovalent cations, as in Fig. 2*C*. The humps in the original *I–V* curve observed in the KCl and RbCl solutions were absent in the difference current.

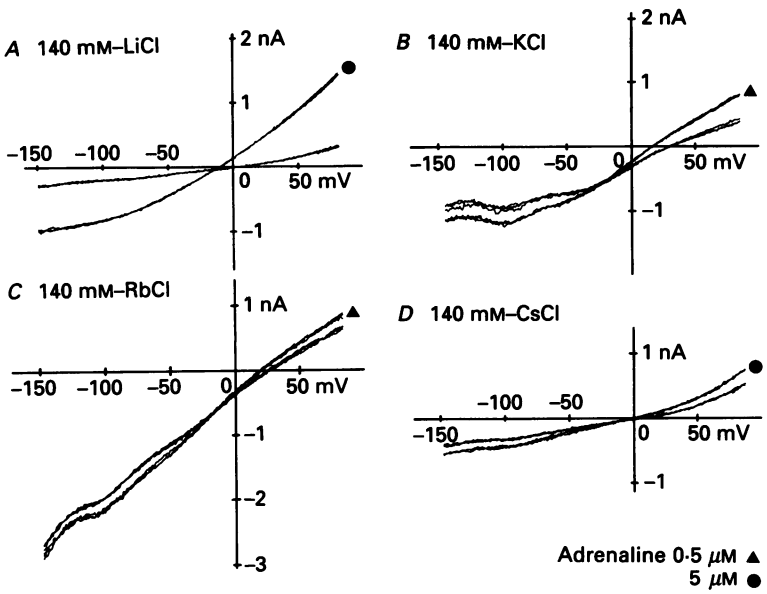

Fig. 3. The *I–V* relations observed in solution containing the chloride salt of various monovalent cations. NaCl (140 mM) in the external solution had been replaced with equimolar LiCl (*A*), KCl (*B*), RbCl (*C*) and CsCl (*D*). Three *I–V* relations observed consecutively in each solution in the absence and presence of adrenaline are superimposed. *I–V* relation after the drug is denoted by ▲ and ●.

Figure 4 shows representative results obtained with various divalent cations. In all solutions, the control *I–V* relation showed outward rectification at positive potentials and an inward rectification at negative potentials. There was no significant difference in both the slope conductance and the reversal potential of the control current recorded in these solutions.

Table 1 lists the slope conductance and reversal potential of both the control and adrenaline-induced current observed in various solutions. The slope conductance was calculated at the reversal potential of each current. Surprisingly, the reversal potential of the adrenaline-induced current depended little or only slightly on the species of cations present outside the cells. This might support the view that the current was generated by a class of non-selective cation channels. Alternatively, it is also possible that the current was not carried by any cations, but by Cl^-, which was present in every test solution examined.

On the other hand, the sensitivity of the cell to adrenaline was dependent on the

cation species. In KCl and RbCl solutions, the cell responded to 0·5 μM-adrenaline, but the amplitude of the response was smaller than that obtained in NaCl solution. In the solutions containing other monovalent cations or divalent cations, 0·5 μM-adrenaline failed to induce any appreciable response, but 10-fold higher concentrations produced a sizeable response at least in a fraction of the cells examined. The ineffectiveness of 0·5 μM-adrenaline in LiCl solution has already been noted by Matsuoka, Noma & Powell (1989). The adrenaline sensitivity of the cell appeared to be lowest in CsCl solution: only one out of four cells responded to 5 μM-adrenaline.

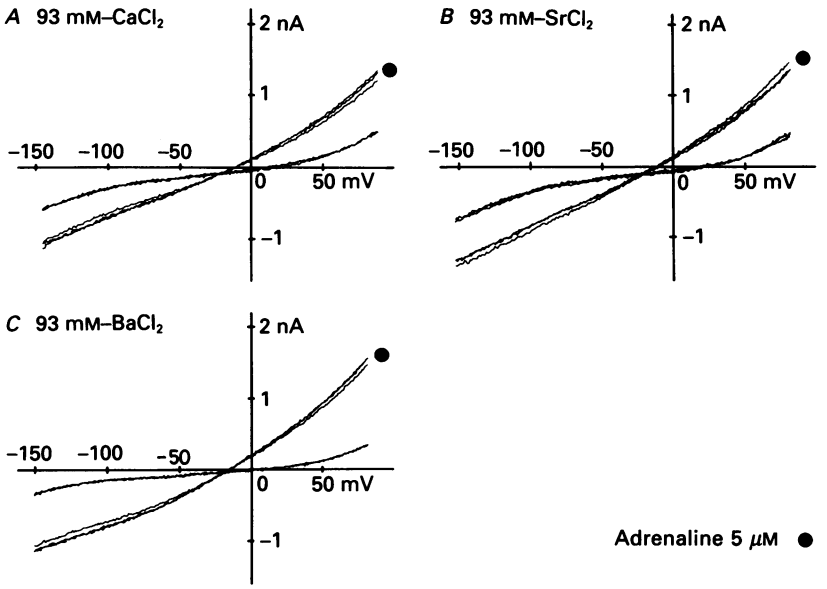

Fig. 4. The *I–V* relations observed in solutions containing chloride salt of various divalent cations. NaCl (140 mM) in the external solution had been replaced with 93 mM-$CaCl_2$ (*A*), $SrCl_2$ (*B*), and $BaCl_2$ (*C*). Details are the same as in Fig. 3.

I–V relations in Cl^- deficient solutions

Egan *et al.* (1988) observed that the isoprenaline-induced current was not appreciably affected by replacement of external Cl^- with isethionate ion. However, this observation was made only at one holding potential, and the *I–V* relation was not determined. We examined the effects of substituting external NaCl with sodium aspartate or sodium benzenesulphonate on the adrenaline-induced current. In the experiment shown in Fig. 5, 5 μM-adrenaline was applied to the cell in sodium aspartate solution. This produced a usual inward shift of the holding current accompanied by an increase in the current change during ramp pulses (inset of Fig. 5*A*). When sodium aspartate was replaced with NaCl in the presence of agonist, there was a slight outward shift of the holding current with a marked increase in the membrane conductance. These changes were reversible when the sequence of the solution change was reversed (not shown).

The *I–V* relations obtained under the above conditions are superimposed in Fig.

5*A*, and the *I–V* relations of the adrenaline-induced current obtained in the two external solutions are shown in Fig. 5*B*. A marked change in the reversal potential is evident in the adrenaline-induced currents: it changed from +42 mV in low-Cl^- (aspartate) solution to −22 mV in Cl^--rich solution (Fig. 2*B*). The slope conductance

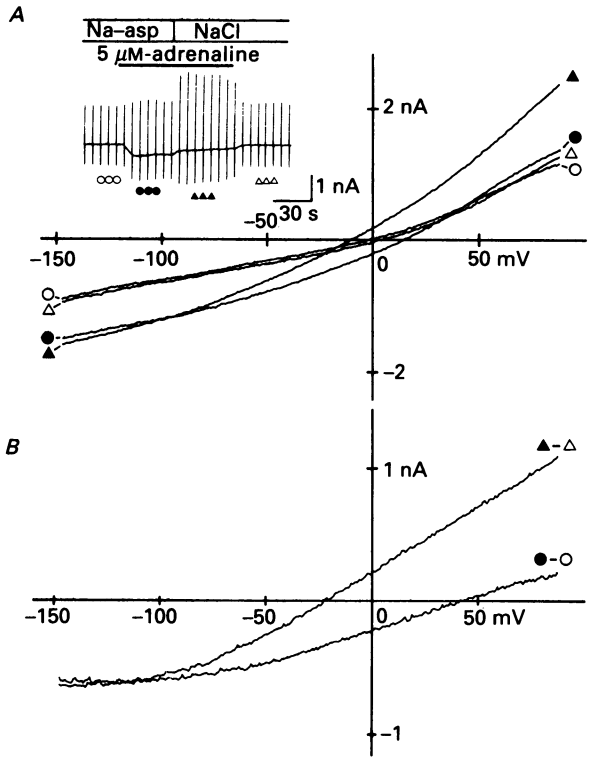

Fig. 5. *A*, *I–V* relations obtained in sodium aspartate (Na-asp) or NaCl solution, in the absence and presence of adrenaline. Inset shows the chart record of current obtained in this experiment. First, adrenaline (5 μM) was applied (bar in the inset) to the cell in aspartate solution, and then bathing solution was changed to Cl^- solution. Finally adrenaline was omitted. Averages of each three consecutive *I–V* relations obtained at the times indicated by the symbols in the inset are shown in the graph. ○, control in aspartate solution; ● and ▲, in the presence of adrenaline in aspartate and Cl^- solution, respectively; △, second control in Cl^- solution. *B*, *I–V* relation of the adrenaline-induced current (difference current) observed in aspartate solution (▲−△) and Cl^- solution (●−○).

was also affected by the anion substitution. Thus, the slope conductance at the reversal potential in low-Cl^- solution (45 pS/pF) was about a half of that in Cl^--rich solution (87 pS/pF). Similar results were obtained when sodium benzenesulphonate was used as another substitute for NaCl (not shown).

The above findings are compatible with the idea that the current was carried by Cl^- ions. Since the Cl^- concentration in Cl^--rich and low-Cl^- solution was 148 and 8 mM, respectively, and since the internal solution contained 51 mM-Cl^-, a negative reversal potential is expected for the Cl^- current in Cl^--rich solution and a positive one, in low-Cl^- solution. A decrease in the slope conductance is also expected for the

Cl^- current at reduced $[Cl^-]_o$. Quantitatively, however, the constant field equation (Hodgkin & Katz, 1949) predicts that the Cl^- conductance at the equilibrium potential decreases by 75% if $[Cl^-]_o$ is changed from 148 to 8 mM in the presence of 51 mM $[Cl^-]_i$, at a constant Cl^- permeability (P_{Cl}). The observed decrease was therefore smaller than the predicted value. The reason of this discrepancy is unknown but a desensitization of the response, which would lead to a decreased Cl^- current at later stage of adrenaline action (see inset of Fig. 5*A*), could be a factor.

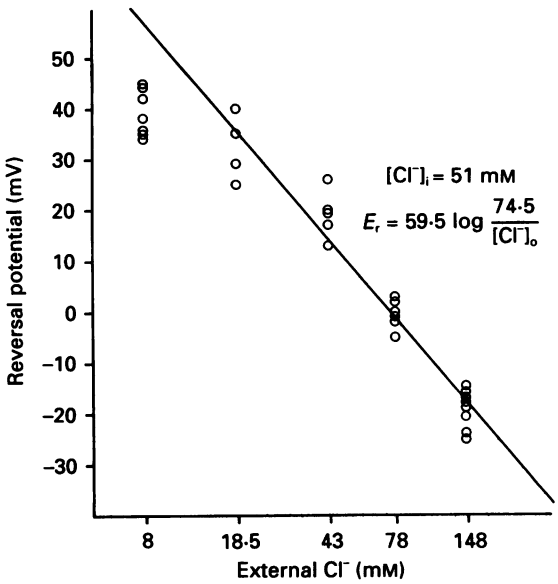

Fig. 6. Relationship between the reversal potential of adrenaline-induced current and external Cl^- concentration ($[Cl^-]_o$) obtained with internal solution containing 51 mM-Cl^-. The values of the reversal potential are plotted against $[Cl^-]_o$ in a logarithmic scale. Data from twenty-five cells are shown. The main cation was Na^+, and aspartate was the main substitute for Cl^-. The linear line was fitted to the data at $[Cl^-]_o \geqslant 18{\cdot}5$ mM according to regression analysis with the least-squares method, and had a slope of 59·5 mV per tenfold change in $[Cl^-]_o$ (59·5 mV/decade) with a theoretical $[Cl^-]_i$ of 74·5 mM.

With regard to the *I–V* characteristics, it is interesting to note that the magnitude of the adrenaline-induced current was almost the same at membrane potentials more negative than about −100 mV, whether the external solution was Cl^- rich or Cl^- deficient (Fig. 2*B*). On the other hand, the substitution of the external anions appeared to have little effect on the background membrane conductance (○ and △ in Fig. 5*A*), suggesting that the membrane Cl^- conductance was very small under the control condition.

Quantitative relationship between reversal potential and $[Cl^-]_o$

The Cl^- dependence of the adrenaline-induced current was systematically studied by determining the reversal potential at various $[Cl^-]_o$, where $[Cl^-]_o$ was varied by replacing NaCl mainly with sodium aspartate. Figure 6 summarizes the results obtained with the standard internal solution ($[Cl^-]_i$ = 51 mM), where the measured reversal potentials are plotted against $[Cl^-]_o$ in a logarithmic scale. The reversal

potential appeared to be a linear function of $\log[Cl^-]_o$, although the data at 8 mM $[Cl^-]_o$ substantially deviated from this linearity. When the regression line was calculated for the linear portion ($[Cl^-]_o \geqslant 18{\cdot}5$ mM), it had a slope of 59·5 mV per tenfold change in $[Cl^-]_o$ (59·5 mV/decade) (Fig. 6). This value was very near to the value expected for a Cl^- electrode from the Nernst equation.

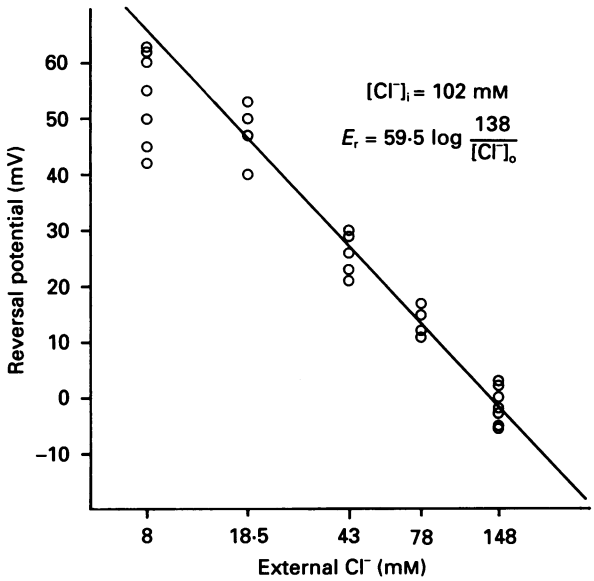

Fig. 7. Relationship between the reversal potential of adrenaline-induced current and $[Cl^-]_o$ obtained with internal solution containing 102 mM-Cl^-. Data from twenty cells are shown. Details are the same as in Fig. 6. The regression line, obtained for the data at $[Cl^-]_o \geqslant 18{\cdot}5$ mM, had a slope of 53·5 mV/decade with a theoretical $[Cl^-]_i$ of 138 mM.

The reversal potential also changed when $[Cl^-]_i$ was varied. The results in Fig. 7 were obtained by using an internal solution containing 102 mM $[Cl^-]_i$. The reversal potential was shifted by about 20 mV in the positive direction compared with those obtained with 51 mM $[Cl^-]_i$ shown in Fig. 6. The shift of 20 mV is very near to the value (18 mV) expected from the Nernst equation. The regression line obtained for $[Cl^-]_o \geqslant 18{\cdot}5$ mM had a slope of 53·5 mV/decade in this case (Fig. 7). These results strongly support the view that the major charge carriers for the adrenaline-induced current are Cl^- ions. The deviation of the relationship from linearity at the lowest $[Cl^-]_o$ may be explained by a contamination of other currents in our 'adrenaline-induced current', in which the contamination would become relatively large at low $[Cl^-]_o$ and hence at reduced Cl^- current. On the other hand, the theoretical $[Cl^-]_i$ giving best regression was 74·5 and 138 mM for the pipette solution containing 51 and 102 mM-Cl^-, respectively. This result remains to be explained (see Discussion).

Effects of inhibitor of chloride permeability

DNDS is a potent inhibitor of the membrane Cl^- permeability in various biological membranes (Bretag, 1987; Bridges *et al.* 1989). Therefore, we examined the effects of this agent on the adrenaline-induced current. Figure 8 shows the result of such an

experiment. DNDS (1 mM) substantially, but not completely. suppressed the adrenaline response without changing the reversal potential, and this effect was at least partially reversible. Similar results were obtained in other experiments with 1 mM (one cell) and 10 mM (two cell)-DNDS. These results support the view that the adrenaline-induced current is carried by Cl^- ions.

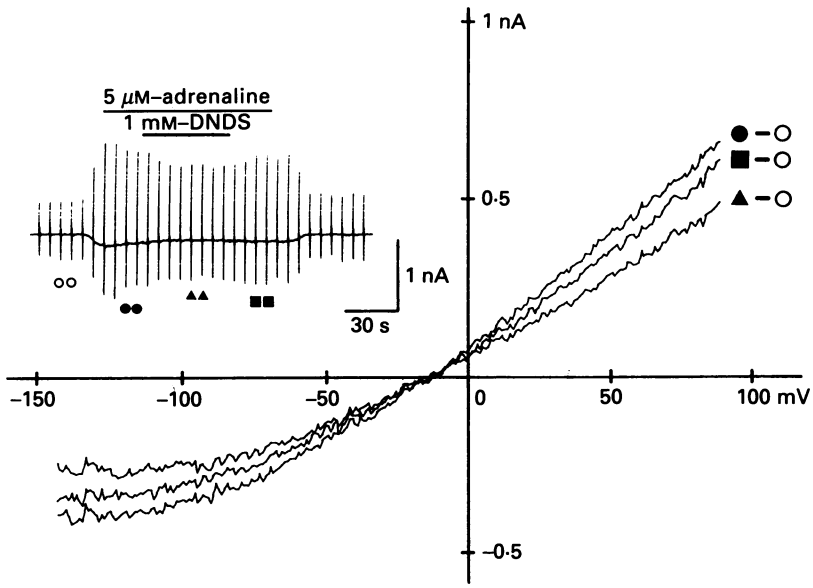

Fig. 8. Effects of DNDS on the adrenaline-induced current. DNDS (1 mM) was added to bath in the presence of 5 μM-adrenaline (see inset). The graph shows average *I–V* relation of the adrenaline-induced current observed in the presence or absence of DNDS. The average adrenaline-induced current was obtained as usual, using sets of two consecutive *I–V* relations obtained at the times indicated by symbols in the inset. ● – ○, control adrenaline-induced current; ■ – ●, adrenaline-induced current reduced by DNDS; ▲ – ○, second control.

Dependence of adrenaline response on cations

It has been shown that isoprenaline fails to induce the inward current in ventricular cells when external NaCl is replaced with TMA-Cl (Egan *et al.* 1988). This finding provided a basis for the proposal that isoprenaline increases the membrane Na^+ permeability. We have confirmed this finding. In the experiment shown in Fig. 9, adrenaline was first applied to the cell in NaCl solution. After recording the *I–V* relation of the usual response (●), we replaced NaCl with Tris-HCl. This Na^+ removal resulted in a marked decrease in the membrane conductance in spite of the continuous presence of Cl^- ions (▲). The subsequent removal of adrenaline did not further affect the *I–V* relation (△). The results were the same if Tris-HCl was replaced with NaCl in the presence of adrenaline (Fig. 9*B*). Similar results were obtained in four other experiments with Tris-HCl. Furthermore, when TEA-Cl was used as another substitute for NaCl, application of adrenaline again failed to induce the inward current (four experiments). The disappearance of adrenaline response in TEA or Tris solutions does not relate to any muscarinic action (cf. Egan *et al.* 1988;

Harvey & Hume, 1989*a*) of these compounds, since the results were the same if the TEA and Tris solutions contained 1–10 μM-atropine (not shown).

To explain the above findings, we suggest that the adrenaline-induced Cl^- current requires the presence of Na^+ when it is activated. Since the adrenaline-induced

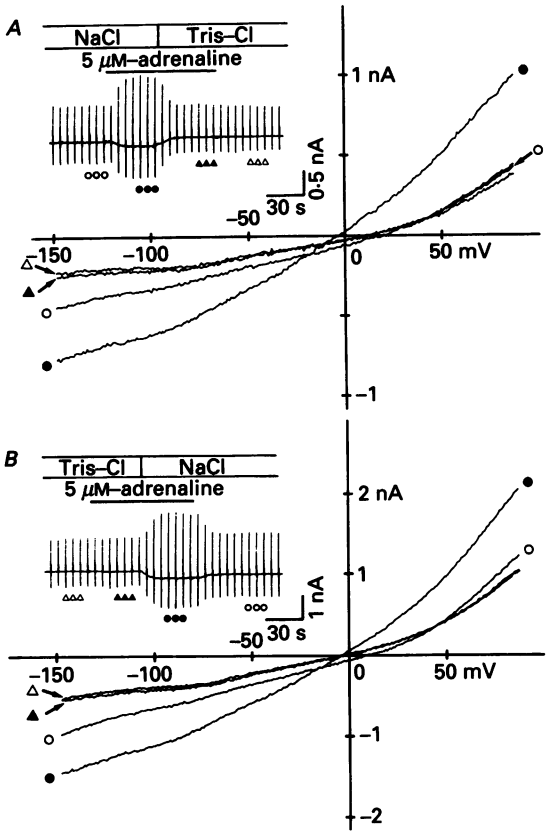

Fig. 9. Effects of replacement of external NaCl with Tris-HCl on the adrenaline-induced current. *A*, adrenaline (5 μM) was applied to the cell in NaCl solution, and then the bathing solution was changed to Tris-HCl solution (see inset). Finally, adrenaline was omitted from the latter solution. Averages of each three consecutive *I–V* relations obtained at the times indicated by symbols in the inset are shown in the graph. ○, control; ● and ▲, in the presence of adrenaline in NaCl and Tris-HCl solution, respectively; △, second control in Tris-HCl solution. *B* shows the result of an experiment similar to that in *A*, but the sequence of solution change was reverse in this case. *A* and *B* show the data from different cells.

current also developed in the external solutions containing various cations other than Na^+ (Figs 3 and 4), development of the adrenaline response may generally require the presence of cations, irrespective of the cation species. On the other hand, when in Fig. 9 the *I–V* relation in Tris-HCl solution (▲ or △) was compared with the control *I–V* relation (○), the slope conductance was smaller in the former than in the latter, and both *I–V* relations crossed at the membrane potential of a very positive value (about +45 mV). These findings indicate the presence of a background Na^+ conductance in the membrane.

Relationship to background conductance

There were marked differences among different cells with respect to amplitudes of both the control conductance and the membrane responses to adrenaline. During the course of this study, we noticed that cells having smaller control conductance

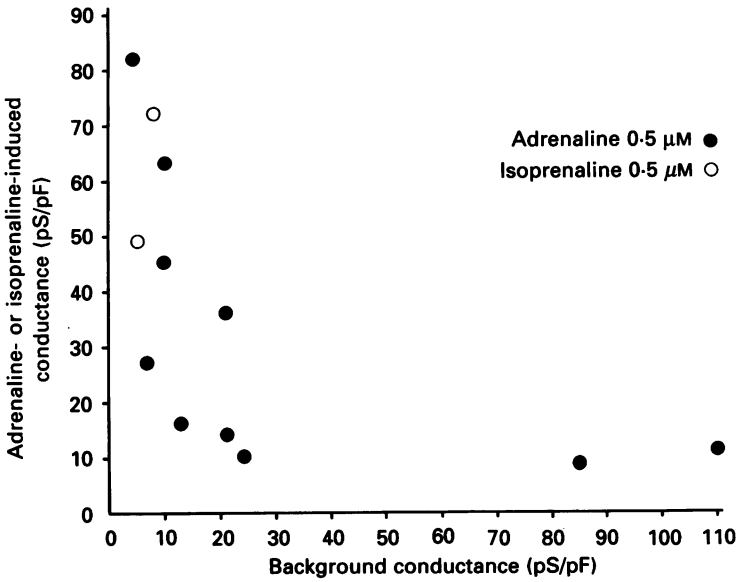

Fig. 10. The relationship between slope conductance of the adrenaline- or isoprenaline-induced current and the background conductance. Background conductances were calculated from the slope of I–V relation in the absence of the drug, at the reversal potential of the drug-induced current. The drug-induced conductances were obtained by measuring slope of the drug-induced current at the reversal potential. The calculated values were normalized in reference to capacitive area of the membrane. Each point represents data obtained from one cell at its first exposure to catecholamine.

generated larger current responses to adrenaline. This is demonstrated in Fig. 10, where the slope conductance of the adrenaline- or isoprenaline-induced current observed in individual cell is plotted against the control conductance of the same cell. The slope conductances were measured at the reversal potential of the catecholamine-induced current. A negative correlation between these two parameters is evident. If the control Cl^- conductance is very small relative to the total conductance (Fig. 5*A*), it follows that catecholamines create a larger Cl^- conductance in the cells in which the non-Cl^- background conductance is smaller, although the underlying mechanism remains to be explained.

DISCUSSION

Properties of the adrenaline-induced current

Catecholamines affect a large variety of current systems in cardiac cells (for review see Noble, 1979). Therefore, blockade of these current systems was a prerequisite for isolation of the adrenaline-induced current. In the present study, the membrane ionic

currents and exchange currents known in ventricular cells except the Na^+ channel current were suppressed by appropriate inhibitors and ion substitutions. The *I–V* relation was evaluated using the ramp pulses, where the Na^+ channel was inactivated by depolarization. We consider that the adrenaline-induced current measured as a difference current mostly represents a current system whose ionic nature has not been identified so far.

Egan *et al.* (1988) suggested that the isoprenaline-induced response in guinea-pig ventricular cells is mediated by β_1-adrenoceptor, adenylate cyclase and intracellular cyclic AMP (see also Harvey & Hume, 1989*a*). Our preliminary experiments confirmed that internal application of cyclic AMP induced essentially the same current as the adrenaline-induced current (S. Matsuoka, T. Ehara & A. Noma, unpublished data). Other lines of evidence also suggest that the adrenaline-induced current in the present study is generated by the same current system as that for the isoprenaline- and forskolin-induced current reported earlier (Egan *et al.* 1987, 1988; Harvey & Hume, 1989*a*). Firstly, the adrenaline-induced current was recorded in the presence of the K^+ channel blockers, Ca^{2+} antagonists, EGTA in the pipette solution, and Cs^{2+}, all of which were shown to be inert for the isoprenaline-induced current in the previous study. Secondly, the adrenaline-induced current and their isoprenaline-induced current were commonly absent when external NaCl was replaced with chloride salt of large inorganic molecules (TEA, Tris and TMA).

Our measurements of the reversal potential of the adrenaline-induced current (Figs 3–7) under various experimental conditions strongly suggest that the adrenaline-induced current is carried mainly by Cl^- ions. The decrease in the slope conductance at reduced $[Cl^-]_o$ (Fig. 5*B*) and the inhibition of the response by DNDS (Fig. 8) also support this view. The conclusion of the adrenaline-induced Cl^- conductance is different from that of the previous study (Egan *et al.* 1988).

The *I–V* relation of the adrenaline-induced current showed an upward curvature and the inward current tended to saturate in amplitude at negative potentials. Although the mechanism of these characteristics is unknown, it is interesting to note that an *I–V* relation of similar shape was obtained for the isoprenaline-induced current by measuring the amplitude of current responses at various holding potentials without using extensive internal perfusion or channel blockers, in the previous study (Fig. 11 in Egan *et al.* 1988). Thus, the amplitude of the inward Cl^- current is almost equal at potentials negative to -100 mV, whether the external solution was Cl^- rich or Cl^- deficient (Fig. 5), in agreement with the previous observation that substitution of Cl^- with isethionate did not affect development of the isoprenaline-induced current at a large negative holding potential (Egan *et al.* 1988).

To fit the relationship between the reversal potential and $[Cl^-]_o$ using the Nernst equation, a value of $[Cl^-]_i$ higher than that in the pipette solution was assumed. If Cl^- ions follow a passive distribution across the membrane depending on the membrane potential (Carmeliet, 1961; Hutter & Noble, 1961), and if this was not compensated by the diffusion of the pipette solution, $[Cl^-]_i$ might vary according to the holding potential. The holding potential of -40 mV, however, is more negative than the equilibrium potential for Cl^- given from the pipette and the bath Cl^- concentrations. Therefore, it is not necessarily correct to assume that a net influx and intracellular accumulation of Cl^- ions develop at the holding potential. Alternatively,

the recorded membrane potential might include an unknown junction potential, leading to an estimation of erroneously higher $[Cl^-]_i$. However, when we examined the Ca^{2+} current using nicardipine-free, Ca^{2+}-containing external solution, its threshold voltage and peak *I–V* relation were normal (S. Matsuoka, T. Ehara & A. Noma, unpublished observation), suggesting that there was no significant artificial voltage shift in our measurements. Thus, we have no definite conclusions at present on these results.

Cation dependence of adrenaline response

Adrenaline failed to increase the membrane conductance in Tris-HCl or TEA-Cl solution (Fig. 9). This finding is consistent with the observation that isoprenaline response was absent when external NaCl was replaced with TMA-Cl (Egan *et al.* 1988). However, we do not consider that Na^+ is the major charge carrier responsible for the isoprenaline- or adrenaline-induced inward current. Since the adrenaline response persisted in other solutions containing chloride salts of various monovalent or divalent cations (Figs 4 and 5), we consider that development of this response requires the presence of not only Cl^- but also cations.

It is important to note that, although the concentration range of adrenaline used was rather narrow (0·5–5 μM), the adrenaline sensitivity of the cell varied significantly if different cation species were used in the external solution. In relation to this, the increase of the Ca^{2+} current by adrenaline is also partially inhibited by replacing external Na^+ with Li^+ (Matsuoka *et al.* 1989). All these findings seem to indicate existence of a cation-species-dependent modulation or potentiation of the adrenaline response. If this were the case, our observation suggests that the sequence of efficacy of cations to facilitate the response is roughly: $Na^+ > K^+$, $Rb^+ > Cs^+$, Li^+, divalent cations.

The adrenaline response may develop via a series of events occurring on or within the membrane and in the cytoplasm, and these events may include complicated intermediate steps between agonist–receptor binding and the final conductance change in the membrane. If cations act as a modulator of the response, it is important to clarify which of the above events is sensitive to concentration and species of cations. On the other hand, the Cl^- conductance reported here does not seem to require intracellular Ca^{2+}, since pipette solutions contained high concentrations (42 mM) of EGTA. This contrasts with the Cl^- channels in airway epithelial cells (Frizzell, Rechkemmer & Shoemaker, 1986; Welsh, 1986). More precise studies like single-channel analysis in the excised patch membrane, as well as biochemical measurements should be done to elucidate the mode of action of cations in the adrenaline response.

Cl^- conductance in cardiac muscle

In the absence of adrenaline, replacement of Cl^- with impermeant anions affected the *I–V* relation of the background current only very little, suggesting that the resting Cl^- conductance was very small or absent in isolated ventricular cells (Fig. 5). This result is in striking contrast with the previous studies on the multicellular preparations (Carmeliet, 1961; Hutter & Noble, 1961; Ehara, 1971), which indicated presence of the Cl^- conductance in cardiac membrane. In ventricular muscle, about 30% of the total membrane conductance was attributed to Cl^- permeability (Hutter

& Noble, 1961; Ehara, 1971). In studies on cardiac sarcolemmal vesicles incorporated into lipid bilayer membrane, a distinct single-channel activity representing Cl^- currents has been recorded (Coronado & Latorre, 1982), although, to our knowledge, such single-channel Cl^- currents have not been reported in patch clamp studies on isolated cardiac cells.

The reason for the above discrepancy is unclear. However, one possible explanation would be to assume that in the multicellular preparations the cells are continuously exposed to a spontaneous release of catecholamine from intramural nerve endings. In such cases, the background conductance of the cell would be substantially contaminated with the catecholamine-induced Cl^- conductance. Alternatively, the membrane, by unknown mechanisms, might have lost its Cl^- permeability during the procedure of cell isolation, the internal perfusion or other experimental manoeuvres. In this case, the induction of Cl^- conductance by adrenaline can be one type of unmasking phenomenon.

Role of the adrenaline-induced current

The possible pathophysiological roles of the catecholamine-induced current as a depolarizing current in working cardiac muscle have been discussed extensively by Egan *et al.* (1988). The catecholamine response like that reported in this paper is not a unique event for isolated, internally perfused single ventricular cells. In multicellular preparations (guinea-pig papillary muscles: Nakaya, Tohse, Hattori & Kanno, 1987; J. Hasegawa. & T. Ehara, unpublished observation), adrenaline or isoprenaline produces also a membrane depolarization under more physiological conditions. However, these results do not show *a priori* that similar responses would also develop in heart muscle *in vivo*, since the living heart muscle is always exposed to circulating catecholamines even though their concentrations may be low, and since the above catecholamine responses exhibit a strong 'desensitization'-like phenomenon (Egan *et al.* 1987, 1988; Nakaya *et al.* 1987; present study). Thus, evaluation of the physiological role of the adrenaline-induced current must await further investigations.

Since this paper was submitted, two papers showing that isoprenaline-induced increase in the membrane conductance in ventricular cells is due to cyclic AMP-mediated activation of a Cl^- current have appeared (Harvey & Hume, 1989*b*; Bahinski, Nairn, Greengard & Gadsby, 1989).

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