

# Properties of the Calcium-activated Chloride Current in Heart

ANDREW C. ZYGMUNT and W. R. GIBBONS

From the Department of Physiology and Biophysics, University of Vermont, Burlington, Vermont 05405

**ABSTRACT** We used the whole cell patch clamp technique to study transient outward currents of single rabbit atrial cells. A large transient current,  $I_A$ , was blocked by 4-aminopyridine (4AP) and/or by depolarized holding potentials. After block of  $I_A$ , a smaller transient current remained. It was completely blocked by nisoldipine, cadmium, ryanodine, or caffeine, which indicates that all of the 4AP-resistant current is activated by the calcium transient that causes contraction. Neither calcium-activated potassium current nor calcium-activated nonspecific cation current appeared to contribute to the 4AP-resistant transient current. The transient current disappeared when  $E_{Cl}$  was made equal to the pulse potential; it was present in potassium-free internal and external solutions. It was blocked by the anion transport blockers SITS and DIDS, and the reversal potential of instantaneous current-voltage relations varied with extracellular chloride as predicted for a chloride-selective conductance. We concluded that the 4AP-resistant transient outward current of atrial cells is produced by a calcium-activated chloride current like the current  $I_{Cl(Ca)}$  of ventricular cells (1991. *Circulation Research*. 68:424-437).  $I_{Cl(Ca)}$  in atrial cells demonstrated outward rectification, even when intracellular chloride concentration was higher than extracellular. When  $I_{Ca}$  was inactivated or allowed to recover from inactivation, amplitudes of  $I_{Cl(Ca)}$  and  $I_{Ca}$  were closely correlated. The results were consistent with the view that  $I_{Cl(Ca)}$  does not undergo independent inactivation. Tentatively, we propose that  $I_{Cl(Ca)}$  is transient because it is activated by an intracellular calcium transient. Lowering extracellular sodium increased the peak outward transient current. The current was insensitive to the choice of sodium substitute. Because a recently identified time-independent, adrenergically activated chloride current in heart is reduced in low sodium, these data suggest that the two chloride currents are produced by different populations of channels.

## INTRODUCTION

We recently identified chloride as the charge carrier of a calcium-activated current in rabbit ventricular myocytes (Zygmunt and Gibbons, 1991a). The current, which we called  $I_{Cl(Ca)}$ , appears as a transient outward current during voltage clamp depolarizations. Calcium-activated chloride currents have been identified in *Xenopus* oocytes

Address reprint requests to Dr. W. R. Gibbons, Dept. of Physiology and Biophysics, University of Vermont, Medical Research Facility, Colchester, VT 05446-2500.

(Miledi, 1982; Barish, 1983), neurons (Owen, Segal, and Barker, 1984), smooth muscle (Byrne and Large, 1987), and secretory cells (Marty, Tan, and Trautmann, 1984), but this was the first clear evidence that such a current is present in heart.

Transient outward currents in heart have a long and confusing history. We will consider only recent data from rabbit myocytes here, and defer more general review for the Discussion. When voltage-clamped rabbit atrial, atrioventricular node, or ventricular myocytes are depolarized to voltages positive to  $-20$  mV, transient outward currents dominate the total current (Nakayama and Irisawa, 1985; Giles and Imaizumi, 1988). A majority of the outward current is blocked by 4-aminopyridine (4AP). The current blocked by 4AP is voltage dependent and does not appear to be activated by internal calcium (Clark, Giles, and Imaizumi, 1988; Zygmunt and Gibbons, 1991a). We shall refer to the 4AP-sensitive current as  $I_A$  because it resembles the  $I_A$  current of neurons (Connor and Stevens, 1971).

In the presence of 4AP, to block  $I_A$ , Giles and Imaizumi (1988) detected a residual transient outward current superimposed on  $I_{Ca}$  in rabbit atrial and ventricular cells. This 4AP-resistant transient current was larger in atrial than in ventricular cells. Hiraoka and Kawano (1989) also demonstrated a 4AP-resistant transient outward current in rabbit ventricular myocytes. It was blocked by caffeine or ryanodine, and inhibited by replacing bath calcium by strontium, suggesting activation by internal calcium. Both groups assumed that the calcium-activated transient was carried by potassium.

We (Zygmunt and Gibbons, 1991a) confirmed the presence of a small 4AP-resistant transient outward current in rabbit ventricular myocytes, which was activated by the calcium transient that causes contraction. Under our experimental conditions,  $I_{Cl(Ca)}$  seemed to cause all of the 4AP-resistant current. However, calcium-activated potassium channels (Callewaert, Vereecke, and Carmeliet, 1986) and calcium-activated nonspecific cation channels (Kass, Lederer, Tsien, and Weingart, 1978; Colquhoun, Neher, Reuter, and Stevens, 1981; Ehara, Noma, and Ono, 1988) have been reported in heart, and we could not rule out contributions of potassium or nonspecific cation current to 4AP-resistant transient outward current in other cells or under other conditions.

Most of our ventricular myocyte experiments were performed in the presence of isoproterenol because  $I_{Cl(Ca)}$  was small in the ventricular cells; isoproterenol potentiated the current and made it easier to study (Zygmunt and Gibbons, 1991a). We believed that isoproterenol enhanced the current simply by increasing the size of the underlying calcium transient, and did not materially alter  $I_{Cl(Ca)}$  properties. That assumption could be challenged, however. A time-independent chloride current,  $I_{Cl(cAMP)}$ , is activated in cardiac cells by adrenergic agonists (Harvey and Hume, 1989; Bahinski, Nairn, Greengard, and Gadsby, 1989). As the abbreviation implies,  $I_{Cl(cAMP)}$  appears to be regulated by a cAMP-dependent pathway.  $I_{Cl(cAMP)}$  provides a clear precedent for actions of adrenergic agonists on chloride conductances.

The reported larger size of the 4AP-resistant transient current in atrial cells than in cells from the ventricle (Giles and Imaizumi, 1988) raises questions and offers opportunities. The total 4AP-resistant transient might be larger in atrial cells because a calcium-independent transient current is present, because calcium-activated potassium or calcium-activated nonspecific cation currents make important contributions

to the 4AP-resistant current in atrial cells, or because  $I_{Cl(Ca)}$  is present and larger than in the ventricle. If a calcium-independent, 4AP-resistant transient current were responsible, it would be a current that had not previously been described. If potassium or nonspecific cation channels contributed more current than in ventricular cells, we might be able to detect these currents in the atrium. Finally, if  $I_{Cl(Ca)}$  were present and larger than in the ventricle, we should be able to extend our initial work on  $I_{Cl(Ca)}$  without using isoproterenol to potentiate the current and without the attendant risk that the current properties are fundamentally altered by the drug.

Some of this work has been reported as an abstract (Zygmunt and Gibbons, 1991b).

## METHODS

### *Cell Preparation*

Male New Zealand White rabbits (1.9–2.3 kg) were given 400 IU/kg heparin (sodium salt) and deeply anesthetized with 50 mg/kg i.v. pentobarbital sodium. Hearts were quickly removed and Langendorff perfused at a constant pressure (76 cm of  $H_2O$ ) by: (a) Tyrode's solution containing 1 mM  $CaCl_2$  for 1 min; (b) nominally Ca-free Tyrode's containing 0.020 mM EGTA and 0.1% bovine albumin for 5 min; (c) nominally Ca-free Tyrode's containing 1 mg/ml type II collagenase (Worthington Biochemical Corp., Freehold, NJ), 0.15 mg/ml type XIV protease (Sigma Chemical Co., St. Louis, MO), and 0.1% bovine albumin for 15 min. Perfusion solutions were warmed to 37°C and saturated with 100% oxygen.

The atria were placed in enzyme-containing Tyrode's solution for an additional 20 min. Single atrial cells were obtained by gentle agitation of the tissue. Cells were centrifuged at 1,000 rpm for 1 min and resuspended in Tyrode's solution containing 0.1 mM  $CaCl_2$  and 0.5 mg/ml gentamicin sulfate to reduce bacterial growth. After ~20 min,  $CaCl_2$  was added to bring the final concentration of calcium to 1 mM. We used cells that were quiescent in this solution.

### *Solutions*

The composition of the Tyrode's solution used to isolate cells was (mM): 135 NaCl, 5.4 KCl, 1.0  $MgCl_2$ , 0, 0.1, or 1.0  $CaCl_2$ , 10 glucose, 0.33  $NaH_2PO_4$ , 5 HEPES, pH adjusted to 7.4 with NaOH.

Internal (pipette) solutions are listed in Table I. Cells dialyzed with solutions containing <1 mM EGTA contracted when depolarized positive to the calcium current threshold; cells dialyzed with 10 mM EGTA did not contract (Zygmunt and Gibbons, 1991a). All external solutions contained (mM): 10 HEPES, 3.6  $CaCl_2$ , 1  $MgCl_2$ . Other ingredients will be listed in the figure legends and text as needed. When we reduced extracellular chloride, methanesulfonic acid was chosen as the chloride substitute because it has little effect on calcium ion activity or intracellular pH (Kenyon and Gibbons, 1977). When necessary, chloride ion activity was measured with a macroscopic chloride-selective electrode (model 9417B; Orion Research Inc., Boston, MA) and ion analyzer (model EA 920; Orion Research Inc.).

Concentrated solutions of  $CdCl_2$ , ryanodine (Calbiochem Corp., La Jolla, CA), apamin (Sigma Chemical Co.), charybdotoxin (IBF Biotechnics, Columbia, MD), and tetrodotoxin (Calbiochem Corp.) in water were diluted into bathing solutions to the final concentrations indicated in the text. Diisothiocyanostilbene-2,2'-disulfonic acid (DIDS; Calbiochem Corp.), 4-acetamido-4'-isothiocyano-2,2'-disulfonic acid stilbene (SITS; Calbiochem Corp.), caffeine (Sigma Chemical Co.), and 4AP (Aldrich Chemical Co., Milwaukee, WI) were added directly to bath solutions. Fresh concentrated solution of nisoldipine (Miles Inc., West Haven, CT) was made in 95% ethanol and diluted 1,000-fold into bath solution. Nisoldipine, SITS, and DIDS were prepared and used in a darkened room.

*Recording*

Dissociated cells were placed in a 0.5-ml chamber on the stage of an inverted microscope and superfused at 3 ml/min. Whole cell currents were measured at room temperature (20–24°C) with an Axopatch-1C amplifier (Axon Instruments, Inc., Foster City, CA). Pipette tip resistances were 1.5–3.0 M $\Omega$ . Seal resistances were 5–40 G $\Omega$ .

Series resistance, estimated from the decay of the capacitive transient after establishment of whole cell recording, was ~2.5 times the pipette resistance. Electronic compensation reduced the series resistance by 65–75%. Currents recorded during this study did not exceed 1 nA; the voltage drop across the series resistance was therefore <3 mV. After establishment of whole cell recording, application of a small positive pressure to the pipette prevented resealing of the membrane and maintained a low series resistance.

The junction potential between the pipette solution and Tyrode's solution was approximately –6 mV (pipette negative). Junction potentials were zeroed before formation of the membrane–pipette seal in 1 mM CaCl<sub>2</sub> Tyrode's solution. This zeroing created an offset equal to the junction potential, but of opposite sign, that remained after establishment of whole cell

TABLE 1  
*Internal Solutions*

	Standard	K-free	High Cl	Intermediate Cl	Low Cl
	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
K-ASP	110			65	
Cs-ASP		110			130
KCl	20			65	
CsCl		20	130		
HEPES	10	10	10	10	10
MgATP	5	5	5	5	5
MgCl <sub>2</sub>	1	1	1	1	1
EGTA	0.2–0.6	0.2–0.6	0.2–0.6	0.2–0.6	10
pH	7.0 with KOH	7.0 with CsOH	7.0 with CsOH	7.0 with KOH	7.0 with CsOH

ASP = aspartate; ATP = adenosine triphosphate.

recording. Voltages reported in the text were corrected for this offset. We sometimes changed extracellular chloride after the patch electrode was sealed onto the cell. To avoid the development of a potential between the bath solution and the Ag/AgCl ground, we placed the bath ground in a separate pool of 3 M KCl, which was connected to the recording chamber by a 3 M KCl-agar bridge.

Currents were filtered with a four-pole Bessel filter at 5 kHz, digitized at 5–20 kHz, and stored on an IBM-AT computer (pCLAMP software; Axon Instruments, Inc.). Currents that were selected for presentation were digitally filtered with a low pass RC filter ( $\tau = 0.5$  ms). Filtering did not attenuate currents.

When recording the calcium-activated conductance, the pipette solution contained 0.1–0.8 mM EGTA. Visual inspection of the cells confirmed that this concentration of EGTA permitted a depolarization-induced transient rise in intracellular free calcium that was sufficient to cause contraction. We assume that the calcium buffering capacity of the internal solution reduced, but did not prevent, the loading and release of calcium from the sarcoplasmic reticulum. To ensure a steady-state loading of the sarcoplasmic reticulum (SR), many voltage clamp protocols included two “loading pulses” that preceded each test potential. A typical sequence consisted of

two pulses to 0 mV to activate  $I_{Ca}$  and load the SR, a 15-s rest at the holding potential and then the test pulse.

## RESULTS

### *Voltage-activated $I_A$*

In Fig. 1, the upper trace in each panel shows the total current of an atrial myocyte during 150-ms depolarizations to the voltages indicated. At voltages positive to  $-20$  mV, the total current showed an outward peak early in the pulse, then decayed almost to a steady level over 150 ms. As the cell was depolarized to more and more positive potentials, the outward current became larger and peaked sooner; the rate of decay appeared to be roughly similar at each voltage.

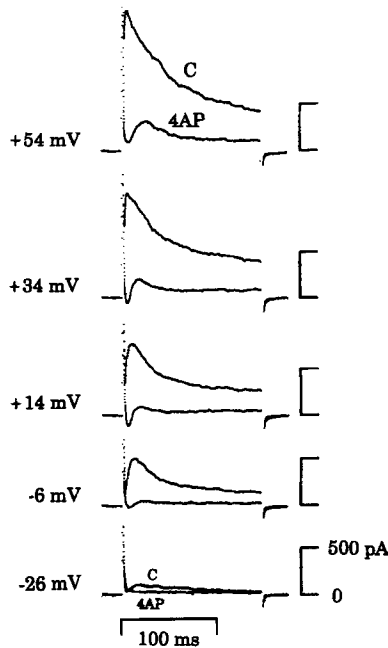


FIGURE 1. Block of  $I_A$  by 4AP. Currents obtained at various voltages are shown before (upper traces, C) and after (lower traces, 4AP) addition of 2 mM 4AP to the bath. Between pulses the cell was held at  $-86$  mV for 30 s. External solution included 150 mM *N*-methyl-D-glucamine (NMG)-Cl, 3 KCl, and 0.02 mM TTX; standard internal solution contained 0.3 mM EGTA.

The lower trace in each panel of Fig. 1 illustrates that outward current was greatly reduced in the presence of 2 mM 4AP. Positive to  $-20$  mV, a small transient outward current remained after 4AP treatment. In the records obtained at  $-6$ ,  $+14$ , and  $+34$  mV, the 4AP-resistant outward transient was preceded by a brief net inward current. We believe the biphasic trace represents one or more 4AP-insensitive transient outward currents superimposed on  $I_{Ca}$ , as in ventricular cells (Zygmunt and Gibbons, 1991a).

Shifting the holding potential to depolarized levels had much the same effect as 4AP (Fig. 2). In the absence of 4AP (Fig. 2A), the whole cell current during  $+44$ -mV depolarizations showed a large outward peak when the holding voltage was  $-86$  mV. In subsequent trials, the holding potential was depolarized in 10-mV steps. At a

holding voltage of  $-56$  mV, the majority of the outward current was gone, leaving a transient that resembled the currents recorded in 4AP in Fig. 1.

After addition of 2 mM 4AP to the bath, changing the holding potential over the range  $-86$  to  $-56$  mV had little effect on the total current (Fig. 2 *B*). In particular, the currents elicited from a  $-56$ -mV holding potential in the absence (Fig. 2 *A*) and in the presence of 4AP (Fig. 2 *B*) were very similar. Thus, a holding potential near  $-50$  mV inactivated the same current or currents that were blocked by 4AP. Similar results were obtained in 11 cells. The 4AP-sensitive current,  $I_A$ , presumably is carried by potassium (Clark et al., 1988; Giles and Imaizumi, 1988; Hiraoka and Kawano, 1989).

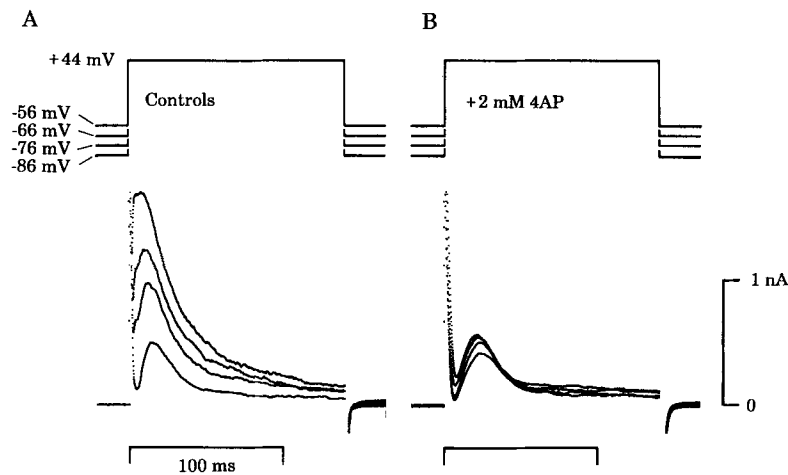


FIGURE 2. Depolarized holding potentials selectively inactivate  $I_A$ . Currents were obtained during 150-ms pulses to  $+44$  mV; pulses were separated by 30-s intervals. In *A*, the four traces were obtained from different holding potentials; the largest current was elicited when the holding potential was  $-86$  mV and the smallest when the holding potential was  $-56$  mV. The currents in *B* were obtained from the same cell after addition of 2 mM 4AP to the bath. Again, four traces are overlaid, obtained by depolarizations from the same four holding potentials. In both panels, external solution was the same as in Fig. 1. Standard internal solution containing 0.5 mM EGTA was used.

Our interest was in the transient current that remained when  $I_A$  was blocked. To ensure that the conductance for ions through  $I_A$  channels was negligible, subsequent experiments were performed in the presence of 2 mM 4AP, and holding potentials near  $-50$  mV were used. Fast sodium channels and T-type calcium channels (if these are present in rabbit atrial cells) should also have been inactivated by the depolarized holding potential.

#### *Tests for 4AP-resistant, Calcium-independent Transient Current*

Fig. 3 demonstrates experiments designed to see if the 4AP-resistant transient current included a calcium-independent transient outward current. In Fig. 3 *A*, the uppermost panel illustrates the voltage protocol. The holding potential was  $-50$  mV.

Brief (20 ms) presteps to  $-20$ ,  $+20$ , or  $+60$  mV were intended to elicit different peak  $I_{Ca}$ , which should in turn elicit intracellular calcium transients of different sizes. Each prestep was followed by a test step to  $+100$  mV. The extremely positive test step should increase the driving force on potassium and chloride, while essentially eliminating the driving force on calcium. The intracellular calcium transient initiated by the prestep could not change instantaneously when the second step was given. At the beginning of the second or test step, then, the current should consist of outward currents (calcium-activated current plus any calcium independent current) with little or no contamination by  $I_{Ca}$ .

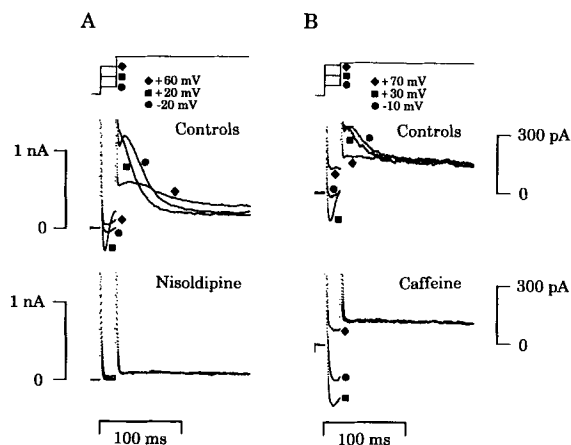


FIGURE 3. All 4AP-resistant transient outward current is activated by calcium released from the SR. *A* and *B* present data from two different cells. Both cells were bathed in external solution that included 150 mM NMG-Cl, 3 mM KCl, and 2 mM 4AP; both were dialyzed with standard internal solution containing 0.3 mM EGTA. In the experiment in *A*, the holding potential was  $-50$  mV. After "loading" pulses (see Methods) the cell was held at  $-50$  mV for 15 s. It was then given a

20-ms prestep to elicit  $I_{Ca}$  and to initiate an intracellular calcium transient. After the prestep, the test pulse was a 170-ms pulse to  $+100$  mV. At the top, the presteps and test pulse are illustrated. The center records are control currents recorded during and after  $-20$ -,  $+20$ -, and  $+60$ -mV presteps. The records at the bottom of *A* are currents recorded with the same voltage steps after addition of 500 nM nisoldipine to the external solution. For the experiment in *B*, loading steps were given followed by 15 s at the  $-50$ -mV holding potential. A 20-ms prestep was given to various voltages to elicit  $I_{Ca}$  and an intracellular calcium transient, followed by a 170-ms test step to  $+80$  mV. The records are arranged as in *A*. In the center are control currents. At the bottom of *B* are currents in the same cell after addition of 10 mM caffeine to the external solution.

In the currents marked "Controls" in Fig. 3 *A*, the calcium current during the prestep is most readily apparent in the trace recorded during a  $+20$ -mV prestep. The instantaneous size of the outward transient during the test step also was largest after the  $+20$ -mV prestep. Outward transients during the other test steps varied in proportion to the prestep voltage approximately as one would expect if the primary effect of varying the prestep was to vary  $I_{Ca}$  and the resulting intracellular calcium transient. 500 nM nisoldipine was then added to the bath solution, and the currents in the bottom panel were recorded. After nisoldipine, we could not detect inward  $I_{Ca}$  during any of the presteps, nor was there a measurable outward transient during any of the test steps (three cells). Equivalent results were obtained when 0.3 mM

cadmium was used to block  $I_{Ca}$  in six cells. Thus all of the 4AP-insensitive transient outward current was blocked when  $I_{Ca}$  was blocked.

The experiment in Fig. 3 *B*, performed on a different cell, used a voltage clamp protocol similar to that used in Fig. 3 *A*. The holding potential was again  $-50$  mV, the test voltage was  $+80$  mV, and the prestep voltages were  $-10$ ,  $+30$ , and  $+70$  mV. The control currents were similar to the control currents in Fig. 3 *A*. The currents in the bottom panel of Fig. 3 *B* were obtained after the addition of 10 mM caffeine to the bath. In the presence of caffeine, which interferes with SR calcium uptake and release (Blinks, Olson, Jewell, and Braveny, 1972; Fabiato and Fabiato, 1973), the outward transients during the test pulses were eliminated (nine cells). The calcium currents during the presteps, however, were increased. Ryanodine, a plant alkaloid that interferes with SR calcium release (Kenyon and Sutko, 1987), also eliminated the outward transients without blocking  $I_{Ca}$  (four cells; data not shown).

These data indicate that the 4AP-resistant transient outward current of rabbit atrial myocytes consists entirely of calcium-activated current or currents. The caffeine and ryanodine data indicate that the transients are activated by calcium released from the SR, rather than by calcium derived directly from  $I_{Ca}$ .

#### *Ionic Composition of 4AP-resistant Transient Outward Current*

The experiment in Fig. 4 was designed to tell us whether calcium-activated potassium or nonspecific cation current makes a significant contribution to the 4AP-resistant transient current. The voltage protocol is illustrated in *A*. From a  $-50$ -mV holding potential, a cell was stepped to  $+10$  mV for 20 ms to evoke a large  $I_{Ca}$  and to trigger a large intracellular calcium transient. Voltage was then stepped to  $+52$  mV to reduce  $I_{Ca}$ .

The current in Fig. 4 *B* was obtained when the voltage steps were performed in 162.2 mM extracellular chloride ( $E_{Cl} = -23$  mV). At  $+52$  mV there was a large driving force on potassium and chloride, and a large outward transient. The bath solution was then exchanged for one with 9.2 mM chloride, which made  $E_{Cl} = +52$  mV (i.e., equal to the membrane voltage during the final test step). In this solution there was no trace of a transient outward current (Fig. 4 *C*). The disappearance of the transient outward current in *C* occurred despite a very large driving force on potassium ions. If either calcium-activated potassium or calcium-activated nonspecific cation currents were significant, we would not have expected the 4AP-resistant transient outward current to disappear in Fig. 4 *C*. Similar results were obtained in four cells.

We also tested the sensitivity of 4AP-resistant transient outward current to agents that block calcium-activated potassium currents in other types of cells (see Blatz and Magleby, 1987, for review). The 4AP-resistant transient current was not detectably affected by tetraethylammonium (TEA) (see Fig. 9 *B*, which shows transient current recorded in external solution containing 138 mM TEA). The 4AP-resistant transient current also was not measurably affected by 100 nM charybdotoxin (three cells) or 100 nM apamin (tested in five cells). Although there was no indication that potassium current contributed to the 4AP-resistant transient current, other potassium currents might cause complications. We therefore omitted potassium from the external and internal solutions in subsequent experiments.



The disappearance of the 4AP-resistant transient outward current when  $E_{Cl}$  was made equal to the pulse voltage in Fig. 4 C suggested that the transient current was equivalent to  $I_{Cl(Ca)}$  in ventricle. Further evidence was provided by testing the sensitivity of the transient current to the anion transport blockers, 2 mM SITS (in 7 cells) or 0.1 mM DIDS (in 33 cells).

Fig. 5 shows the results of an experiment in which the voltage of test pulses was varied between  $-40$  and  $+80$  mV before and after addition of 0.1 mM DIDS to the bath. The current blocked by DIDS at a particular voltage was determined by subtracting the current recorded in DIDS from the control current at the same voltage. A DIDS-sensitive outward transient was first apparent at  $-20$  mV (Fig. 5 A). The peak current blocked by DIDS increased with voltage up to  $+60$  mV, then

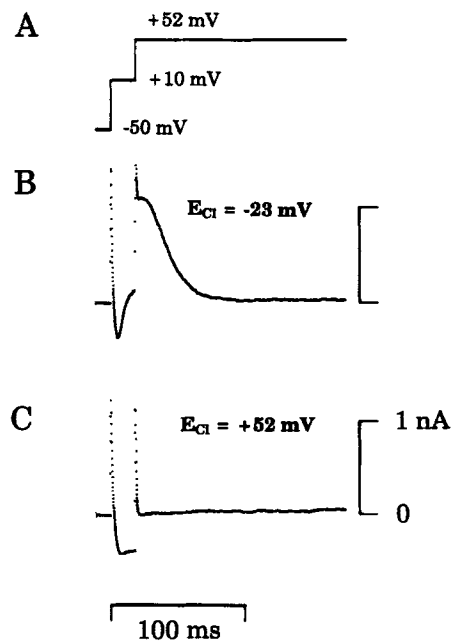


FIGURE 4. Neither calcium-activated potassium nor calcium-activated non-specific cation current makes a detectable contribution to the 4AP-resistant transient current. A illustrates the two-step voltage protocol used. Cells were given two loading pulses to 0 mV as described in Methods, held at the  $-50$ -mV holding potential for 15 s, then given a 20-ms prestep to  $+10$  mV to elicit  $I_{Ca}$  and to initiate an intracellular calcium transient. The test pulse that followed was a 170-ms depolarization to  $+52$  mV. The cell was dialyzed with intermediate chloride internal solution containing 0.3 mM EGTA. In A, the cell was bathed with external solution containing 150 mM NMG-Cl, 3 mM KCl, and 2 mM 4AP, giving a calculated  $E_{Cl}$  of  $-23$  mV. In B, the external bath solution included 150 mM NMG-methanesulfonate and 2 mM 4AP, which gave a calculated  $E_{Cl}$  of  $+52$  mV.

decreased markedly at  $+80$  mV. Time to peak current increased progressively with voltage to 32 ms at  $+60$  mV, and further to 74 ms at  $+80$  mV. 2 mM SITS gave results similar to 0.1 mM DIDS.

After the 4AP-resistant transient current was blocked by DIDS in the experiment illustrated in Fig. 5, the remaining ionic current should be primarily  $I_{Ca}$ . We have plotted peak  $I_{Ca}$  recorded in the presence of DIDS as a function of voltage in Fig. 5 B (open circles). On the same plot, we have shown the peak of the DIDS-sensitive currents from Fig. 5 A (filled circles). The voltage dependence of the peak inward current in the presence of DIDS was consistent with our assumption that the current is  $I_{Ca}$ . The peak DIDS-sensitive current had a bell-shaped dependence on voltage,

consistent with the expected behavior of a calcium-activated chloride current (Zygmunt and Gibbons, 1991a). Peak DIDS-sensitive current decreased markedly at +80 mV, but was not zero, whereas  $I_{Ca}$  appeared to have reversed. However, the voltage at which net calcium channel current reverses probably does not correspond to a reversal of calcium flux through the channel (Lee and Tsien, 1982). A bell-shaped relation between peak intracellular calcium and voltage, similar to the current-voltage relation of the DIDS-sensitive current in Fig. 5 B, has been reported in ventricular myocytes (Barceñas-Ruiz and Wier, 1987; Cannell, Berlin, and Lederer, 1987). Tentatively, we assumed that the 4AP-resistant transient outward current in atrial cells is composed of calcium-activated chloride current,  $I_{Cl(Ca)}$ .

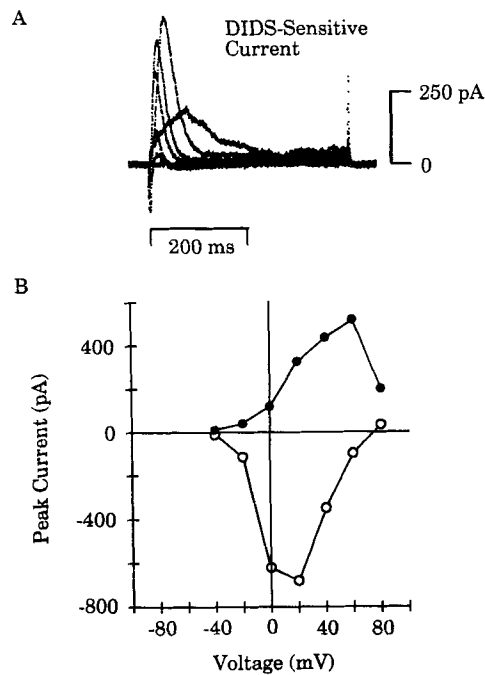


FIGURE 5. Current-voltage relation of DIDS-sensitive current. The cell was bathed in potassium-free external solution that included 150 mM NMG-Cl and 2 mM 4AP; it was dialyzed by potassium-free internal solution containing 0.6 mM EGTA. Two loading pulses were given as described in Methods. The cell was then rested at  $-50$  mV for 15 s and depolarized for 400 ms to a test voltage. Another pair of loading pulses and a 15-s rest preceded the next test. This process was performed for test voltages between  $-40$  and  $+80$  mV, then repeated in the presence of 0.1 mM DIDS. *A* shows the currents blocked by DIDS at various voltages; i.e., the current at each voltage before DIDS minus the current at the same voltage after DIDS. In *B* the peak currents in *A* are plotted as a function of the test voltage (filled circles). The open symbols on the plot are the peak inward current ( $I_{Ca}$ ) measured in the presence of DIDS.

It is only valid to use SITS or DIDS to separate  $I_{Cl(Ca)}$  from  $I_{Ca}$  if the drugs do not alter  $I_{Ca}$ . We evaluated this directly in cells repeatedly depolarized to  $+10$ - and  $+30$ -mV test voltages under conditions that isolate  $I_{Ca}$ . DIDS, tested at 0.5 mM (five times the concentration used to block  $I_{Cl(Ca)}$ ), had no effect on the amplitude or time course of  $I_{Ca}$ . SITS, tested at 6 mM (three times the concentration usually used to block  $I_{Cl(Ca)}$ ), had no detectable effect on amplitude or time course of  $I_{Ca}$  elicited at  $+30$  mV, but the peak current at  $+10$  mV was increased  $\sim 10\%$ . Because DIDS did not affect peak  $I_{Ca}$  or the time course of  $I_{Ca}$  at either of the tested voltages, we used it more often than we used SITS and we chose DIDS blockade for illustrations in this paper.

*Correlation of  $I_{Ca}$  and  $I_{Cl(Ca)}$* 

The experiment in Fig. 5 compared  $I_{Cl(Ca)}$  and  $I_{Ca}$  when we varied the activating pulse voltage. In such experiments, the conductance of  $I_{Ca}$  should vary with voltage, the driving force on each current changes as the voltage is changed, and the release of calcium from the SR to activate  $I_{Cl(Ca)}$  conductance may be a complex function of calcium entry via  $I_{Ca}$ . Thus, a complex relation can be expected between  $I_{Ca}$  and  $I_{Cl(Ca)}$ ; certainly it is not surprising that the current-voltage relations in Fig. 5 *B* were not mirror images. An alternative approach is to examine how  $I_{Cl(Ca)}$  depends on  $I_{Ca}$  when each is elicited at a fixed potential and the amplitude of  $I_{Ca}$  is varied by inactivating  $I_{Ca}$ .

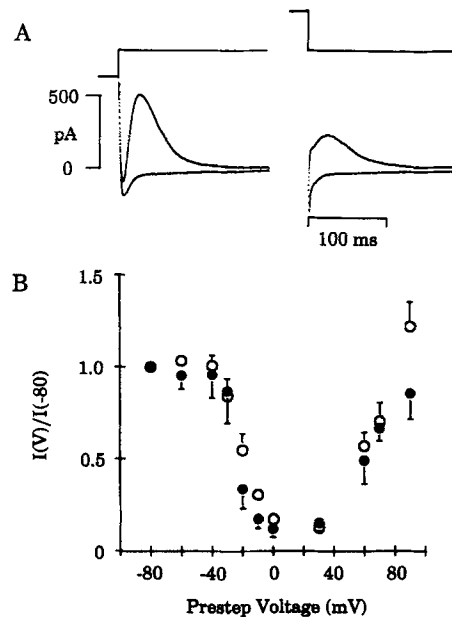


FIGURE 6. Relation between  $I_{Cl(Ca)}$  and  $I_{Ca}$  at a fixed test voltage. Cells were bathed in potassium-free external solution containing 150 mM NMG-Cl and 2 mM 4AP; they were dialyzed by potassium-free internal solution containing 0.2 mM EGTA. One loading pulse was given, then a 15-s rest at the  $-50$ -mV holding potential. Cells were then clamped to a conditioning prepulse voltage for 100 ms and stepped to  $+14$  mV for 200 ms. *A* illustrates currents obtained at  $+14$  mV before and after 0.1 mM DIDS. On the left, the prepulse voltage was  $-30$  mV; the upper current trace was the control and the lower trace was obtained in DIDS. On the right in *A*, the prepulse voltage was  $+70$  mV; the upper current trace was obtained during the control step from  $+70$  to  $+14$  mV and the lower current

trace was obtained during an identical step in the presence of DIDS. In *B*, the normalized amplitude of DIDS-sensitive  $I_{Cl(Ca)}$  versus prepulse voltage is plotted as filled circles (mean  $\pm$  SEM for five cells). The open circles are the inactivation of  $I_{Ca}$  as a function of prepulse voltage (mean  $\pm$  SEM for five cells). See text for details of current measurement and normalization.

$I_{Ca}$  inactivates via a combination of calcium-induced inactivation and voltage-dependent inactivation (Lee, Marban, and Tsien, 1985). We used a two-pulse protocol with rather brief conditioning pulses (see legend of Fig. 6), to emphasize calcium-dependent inactivation of  $I_{Ca}$ .

Fig. 6 *A* shows currents recorded at the  $+14$ -mV test potential in a representative cell, following two different presteps. The voltage steps are illustrated by drawings at the top of each panel. Below each voltage protocol are actual current records. On the left, the prestep was to  $-30$  mV. The test pulse to  $+14$  mV elicited a large  $I_{Cl(Ca)}$ ,

which is the upper current trace. After addition of 0.1 mM DIDS, the lower current trace, consisting primarily of  $I_{Ca}$ , was recorded during identical voltage steps. On the right, the conditioning voltage was +70 mV. When the voltage was stepped to the +14-mV test voltage, a transient outward  $I_{Cl(Ca)}$  was recorded in control conditions (upper current trace on the right). After DIDS, the lower current was recorded during an identical step from +70 to +14 mV.

When the prestep voltage was negative to the +14-mV test potential, the peak outward DIDS-sensitive currents (peak differences between currents before and after DIDS) during the test pulses were measured to give  $I_{Cl(Ca)}$ . The peak inward current in the presence of DIDS was measured to estimate peak  $I_{Ca}$ . When the prestep voltage was positive to the test potential, as it was in the records on the right in Fig. 6A, we continued to measure the peak DIDS-sensitive current to obtain  $I_{Cl(Ca)}$ .  $I_{Ca}$ , however, consisted of a declining inward tail current under these conditions (see lower current trace on the right in Fig. 6A). To estimate peak  $I_{Ca}$ , we measured the current in the presence of DIDS 5 ms after the step to the test potential to allow ample time for decay of the capacity transient.

The estimates of  $I_{Cl(Ca)}$  and  $I_{Ca}$  obtained in this way were normalized by dividing by the chloride and calcium currents obtained when the conditioning prestep was to -80 mV. These measurements were done on five cells; the data are plotted as a function of prestep potential in Fig. 6B. The open circles represent  $I_{Ca}$  inactivation; the filled circles show how  $I_{Cl(Ca)}$  varied as inactivation diminished  $I_{Ca}$ . Both currents changed little when the prestep voltage was between -80 and -40 mV. Between -40 and 0 mV, both currents decreased together, and both were nearly eliminated by prepulses between 0 and +20 mV. The inactivation curve for  $I_{Ca}$  was biphasic, with little or no inactivation of  $I_{Ca}$  at very positive voltages where  $I_{Ca}$  and intracellular calcium release are small.  $I_{Cl(Ca)}$  amplitude behaved in the same way, correlating well with the amplitude of  $I_{Ca}$  up to at least +70 mV.

#### *Does $I_{Cl(Ca)}$ Inactivate?*

An important question is whether  $I_{Cl(Ca)}$  is transient only because the intracellular calcium that activates it is transient, because  $I_{Cl(Ca)}$  undergoes voltage-dependent inactivation, or both. A definitive answer to this question would require direct manipulation of calcium, for example, using an excised patch, but the behaviors of the whole cell currents offer significant clues.

Fig. 6 illustrated a very close correspondence between the amplitudes of  $I_{Cl(Ca)}$  and  $I_{Ca}$  when the driving forces on the currents did not vary and the amplitude of  $I_{Ca}$  was made to change by inducing inactivation. The close relation between  $I_{Cl(Ca)}$  and  $I_{Ca}$  in Fig. 6, especially the close correlation at positive voltages, is consistent with the hypothesis that both currents varied primarily because of inactivation of  $I_{Ca}$ ; i.e., that  $I_{Cl(Ca)}$  did not undergo significant independent inactivation.

To obtain further information on whether  $I_{Cl(Ca)}$  undergoes independent inactivation, we examined recovery of  $I_{Ca}$  from inactivation using a protocol that allowed us to simultaneously measure recovery of  $I_{Ca}$  and  $I_{Cl(Ca)}$ . Experiments were done on four cells. Internal and external solutions contained the same chloride concentrations, to give calculated  $E_{Cl} = 0$  (see the legend of Fig. 7 for the compositions of the solutions). A 20-ms prestep to 0 mV therefore elicited  $I_{Ca}$  alone and initiated an intracellular

calcium transient. This prestep was then followed by a second 180-ms step to +80 mV, near  $E_{Ca}$ . The second step therefore elicited essentially pure  $I_{Cl(Ca)}$ .

After 15 s at the -50-mV holding potential, the first double pulse was given (pulse 0 in the inset of Fig. 7A). At different intervals after the first pulse, an identical second pulse was given (pulse 1 in the inset of Fig. 7A). During the variable interval between the pulses, the voltage was -50 mV.  $I_{Ca}$  was measured during the presteps and  $I_{Cl(Ca)}$  was measured during the second steps. The ratio of each current during pulse 1 to the corresponding current during pulse 0 was plotted as a function of the interval between the pulses in Fig. 7A. Note the parallel recovery of  $I_{Ca}$  and of  $I_{Cl(Ca)}$ . The early course of recovery of the two currents is expanded in Fig. 7B. Fig. 7C

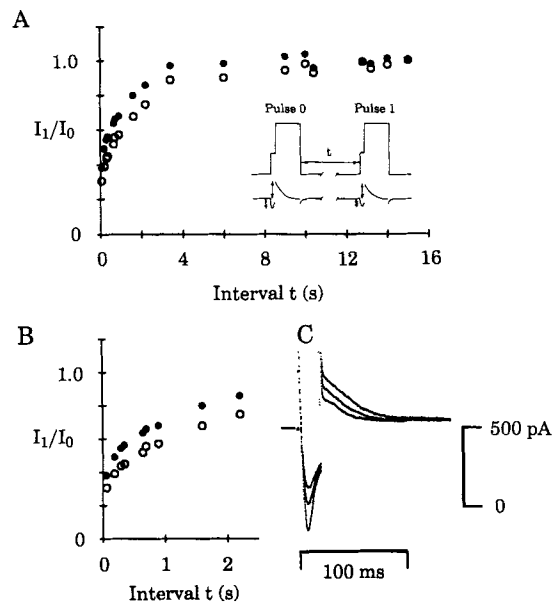


FIGURE 7. Recovery of  $I_{Cl(Ca)}$  and  $I_{Ca}$ . In this example, the cell was bathed in an intermediate chloride external solution containing 122 mM NMG-Cl, 30 mM NMG-methanesulfonate, and 2 mM 4AP. It was dialyzed by high chloride internal solution containing 0.3 mM EGTA. Thus internal and external chloride concentrations were equal. The inset in A illustrates the voltage protocol. Pulse 0 and pulse 1 were identical; each consisted of a 20-ms prestep to 0 mV followed by a 180-ms step to +80 mV. Pulse 0 followed a 15-s rest at the -50-mV holding potential, while the interval between pulse 0 and pulse 1 was varied

between 0 and 15 s.  $I_{Ca}$  and  $I_{Cl(Ca)}$  were measured and normalized as described in the text, and plotted as a function of the interval between pulses 0 and 1 in A. Filled circles are normalized  $I_{Cl(Ca)}$  and open circles are normalized  $I_{Ca}$  for a representative cell. B shows the early portion of the recovery plot on an expanded time scale. C shows actual current records for recovery intervals of 60, 350, and 1,600 ms.

shows three of the pulse 1 currents, recorded at pulse intervals of 60, 350, and 1,600 ms, illustrating the simultaneous recovery of  $I_{Ca}$  and  $I_{Cl(Ca)}$ .

#### Reversal Potential of $I_{Cl(Ca)}$

Quasi-instantaneous current-voltage relations for  $I_{Cl(Ca)}$  were determined by a double-step protocol similar to the one illustrated in Fig. 4A. Cells were given two loading pulses (see Methods), held at the -50-mV holding potential for 15 s, then stepped to

a conditioning prepulse voltage of 0 mV for 20 ms to elicit an intracellular calcium transient. After the prepulse, the voltage was stepped to a test potential for 170 ms. The process was repeated for test potentials between  $-70$  and  $+90$  mV, in the presence and absence of 0.1 mM DIDS. At each test voltage, the current blocked by DIDS was determined 5 ms (to allow settling of capacity current) after the beginning of the test pulse. This difference was taken to be our best estimate of the instantaneous  $I_{Cl(Ca)}$ .

Fig. 8 *A* illustrates the quasi-instantaneous current–voltage relations for four atrial cells when inside and outside chloride concentrations were equal. The inside and outside solutions were assayed with a chloride-sensitive macroscopic electrode;  $E_{Cl}$  based on these measurements was calculated to be  $+0.8$  mV. For the four cells, the mean reversal potential of the current was  $-8.2 \pm 2.7$  mV (mean  $\pm$  SEM,  $n = 4$ ).

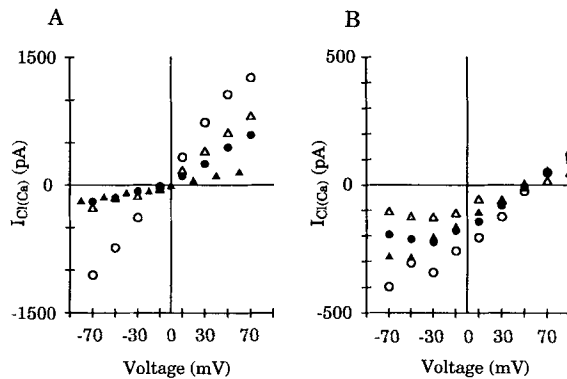


FIGURE 8. Reversal potentials of quasi-instantaneous current–voltage relations of  $I_{Cl(Ca)}$ . *A* shows current–voltage data, measured as described in the text, for four cells. They were bathed by external solution containing 140 mM NMG-methanesulfonate, 11 mM NMG-Cl, and 2 mM 4AP, and dialyzed by high chloride internal solution containing 0.3 mM EGTA.  $E_{Cl}$  was determined to be  $+0.8$  mV (see text). *B* shows

current–voltage data for four additional cells. These were bathed by external solution containing 122 mM NMG-Cl, 30 mM NMG-methanesulfonate, and 2 mM 4AP, and dialyzed by high chloride internal solution containing 0.3 mM EGTA.  $E_{Cl}$  was determined to be  $+51.9$  mV (see text).

Cell-to-cell variation in the currents probably was a result of cell-to-cell variation in the calcium loading of the cells. It cannot be attributed simply to different cell sizes because normalizing the current with respect to total membrane capacity did little to change the variability. Although the currents varied, the reversal potentials varied little; the range was  $-0.5$  to  $-12.5$  mV.

Fig. 8 *B* shows data from four additional cells, in which extracellular chloride was reduced to make calculated  $E_{Cl} = +51.9$  mV (solution concentrations of chloride again determined with an ion-selective electrode). The mean reversal potential of the current was  $55.8 \pm 2.5$  mV (mean  $\pm$  SEM,  $n = 4$ ), range 49–60 mV. Thus the reversal potential shifted approximately as predicted for a chloride current.

A high degree of outward rectification appeared in ventricular cells with near physiological chloride concentrations (Zygmunt and Gibbons, 1991a). With symmetrical chloride, or with inside chloride higher than outside, less rectification was apparent and current clearly reversed in the atrial cell data in Fig. 8. However,

outward rectification still was apparent in some cells, even when chloride was higher inside the cell than outside.

*Effects of Sodium and Sodium Substitutes on  $I_{Cl(Ca)}$*

Although cations do not appear to carry much of the time-independent, adrenergically activated  $I_{Cl(cAMP)}$ , the current is nevertheless very sensitive to extracellular cations. To see if  $I_{Cl(Ca)}$  is similar in this regard, we tested whether substituting different cations for external sodium affects  $I_{Cl(Ca)}$ .

Our experiments to date on  $I_{Cl(Ca)}$  have been done using zero sodium internal and external solutions, to eliminate  $I_{Na}$  and sodium-calcium exchange current. Clearly zero sodium external solution does not prevent  $I_{Cl(Ca)}$ , but it might have reduced it substantially, or the choice of cation to replace sodium might have affected results. Fig. 9 shows that this is unlikely. Fig. 9A illustrates  $I_{Cl(Ca)}$  in a cell in which we began

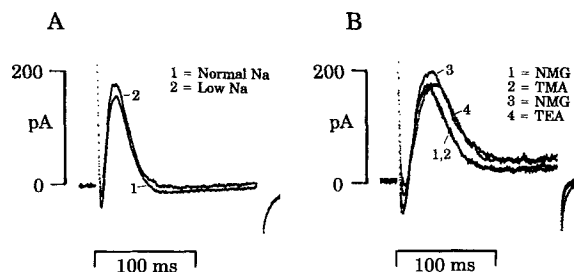


FIGURE 9. Effect of low sodium and of different sodium substitutes on  $I_{Cl(Ca)}$ . In A, trace 1 shows membrane current recorded during a 160-ms pulse to +60 mV. Test pulses were separated by two loading pulses to 0 mV and a 15-s rest at the -50-mV holding potential. External solution contained 150

mM NaCl and 2 mM 4AP, in addition to the usual HEPES,  $CaCl_2$ , and  $MgCl_2$ . Internal solution was potassium-free solution containing 0.3 mM EGTA (Table I). Trace 2 shows current recorded during an identical pulse in the same cell, after the external solution was changed to one containing 100 mM TMA chloride, 50 mM NaCl, and 2 mM 4AP. B shows currents recorded during 160-ms pulses to +60 mV in a different cell. Internal solution was potassium-free solution containing 0.5 mM EGTA (Table I). External solutions were: 150 mM NMG chloride for traces 1 and 3, 138 mM TMA chloride for trace 2, and 138 mM TEA chloride for trace 4. All external solutions contained 2 mM 4AP and the usual HEPES,  $CaCl_2$ , and  $MgCl_2$ .

recording in 150 mM sodium external solution, and then recorded current after 100 mM of the sodium in the external solution was replaced by tetramethylammonium (TMA).

Peak outward current in the low sodium solution was 115% of peak outward current in normal sodium. In five cells in which 100 mM of external sodium was replaced by either TMA or TEA, the peak current in the low sodium solution averaged  $128 \pm 12\%$  (mean  $\pm$  SEM) of the current in 150 mM external sodium. This measure was of total current, not specifically of  $I_{Cl(Ca)}$ . However, some cells lasted long enough for addition of DIDS and repetition of the low and normal sodium recording; these showed that the DIDS-sensitive current was increased in low sodium bathing solution. Thus, lowering external sodium increased  $I_{Cl(Ca)}$ .

Different sodium substitutes may reduce  $I_{Cl(cAMP)}$  by different amounts (Bahinski et al., 1989; Matsuoka, Ehara, and Noma, 1990; Harvey, Clark, and Hume, 1990). In

six cells we examined whether the choice of sodium substitute had a marked effect on  $I_{Cl(Ca)}$ . Fig. 9 *B* illustrates four currents recorded from one cell. The voltage protocol used was identical to the one used for the cell in Fig. 9 *A*. Trace 1 was obtained with *N*-methyl-*D*-glucamine (NMG) replacing sodium, 2 after changing to a bath solution in which TMA replaced sodium, 3 after return to NMG solution, and 4 after changing to a solution in which TEA replaced sodium. To ensure that chloride ion activity in the external solution did not change when solutions were changed, the concentrations of TEA chloride and TMA chloride were adjusted to give equal chloride ion activity in all solutions. The bath solution changed within 15 s; 2 min or more were allowed between recordings to ensure a complete change of solution. The peak current in the various solutions changed little; the differences observed seemed to be within the range of current variation expected over more than 6 min of recording.

## DISCUSSION

### *Two Separable Transient Current Components Are Present in Atrial Cells*

During voltage clamp depolarizations positive to  $-20$  mV we have confirmed that a large  $I_A$  is activated in rabbit atrial myocytes. After  $I_A$  has been blocked by 4AP and/or by a depolarized holding potential, a second transient outward current remains. The 4AP-resistant transient current is smaller than  $I_A$ , but it is still a very significant current (Giles and Imaizumi, 1988). The substantial  $I_A$  and 4AP-resistant transient outward currents probably are largely responsible for the triangular shape of the atrial action potential.

The intracellular calcium transient that mediates the twitch is responsible for activating the 4AP-resistant transient outward current in atrial cells, as is true in ventricular cells (Zygmunt and Gibbons, 1991*a*). All of the 4AP-resistant outward transient seems to be calcium activated. Atrial cells do not, therefore, include a 4AP-resistant, calcium-independent transient outward current.

### *Calcium-activated Potassium and Nonspecific Cation Currents Were Not Observed*

We could not detect any contribution of current through calcium-activated potassium channels to the 4AP-resistant transient outward current (Fig. 4). Most noncardiac cells have calcium-activated potassium channels (Blatz and Magelby, 1987), and calcium-activated potassium channels have been reported in cardiac Purkinje cells (Callewaert et al., 1986). Because of this, several authors have assumed that calcium-activated transient outward current in cardiac cells is caused by calcium-activated potassium channels (Giles and Imaizumi, 1988; Hiraoka and Kawano, 1989; Tseng and Hoffman, 1989). It is therefore somewhat surprising that in rabbit ventricle (Zygmunt and Gibbons, 1991*a*), and now in rabbit atrium, we have been unable to detect any contribution of calcium-activated potassium channels to outward transients. At this time, we do not know of any unequivocal demonstration of a macroscopic current produced by calcium-activated potassium channels in heart (see also Eisner and Vaughan-Jones, 1983).

Experiments like the one illustrated in Fig. 4 should also demonstrate current through calcium-activated nonspecific cation channels if this current is significant (although current might be reduced somewhat by the zero sodium solutions used).



No current attributable to calcium-activated nonspecific cation channels could be measured. This also is surprising, because calcium-activated nonspecific cation channels have been detected in cultured heart cells (Colquhoun et al., 1981) and in freshly isolated adult heart cells (Ehara et al., 1988), and the macroscopic transient inward current  $I_{Ti}$  seen in calcium-overloaded cardiac cells has been attributed to calcium-activated nonspecific cation current (Kass, Tsien, and Weingart, 1978; Cannell and Lederer, 1986).

The apparent absence of significant calcium-activated potassium or nonspecific cation current raises several questions. Are calcium-activated potassium channels present only in Purkinje fibers, and absent or very sparse in atrium or ventricle? Or, alternatively, are rabbit atrial and ventricular cells exceptional because they have few or no calcium-activated potassium channels? Is it possible to provoke  $I_{Ti}$  in rabbit cells and if so, what produces the current? These questions can only be answered by further experiments.

#### *Comparison of Atrial and Ventricular $I_{Cl(Ca)}$ Results*

The disappearance of the 4AP-resistant transient current when  $E_{Cl}$  was made equal to the pulse potential (Fig. 4), the block of the current by SITS (2 mM) and DIDS (0.1 mM), the bell-shaped current voltage relation (Fig. 5 B), and the time course of the DIDS-sensitive current at different voltages all are similar to results in rabbit ventricle (Zygmunt and Gibbons, 1991a). These points of similarity are sufficient to demonstrate that the 4AP-resistant transient current in rabbit atrial cells is equivalent to the calcium-activated chloride current,  $I_{Cl(Ca)}$ , in ventricle (Zygmunt and Gibbons, 1991a). The very similar behavior of  $I_{Cl(Ca)}$  in atrial cells, where isoproterenol was not used to augment the current, to the current in ventricular cells (Zygmunt and Gibbons, 1991a), where isoproterenol was present in most experiments, supports our assumption that isoproterenol did not fundamentally alter the  $I_{Cl(Ca)}$  we recorded in ventricular cells.

In our experiments on ventricular cells, we were unable to demonstrate reversal of  $I_{Cl(Ca)}$  in solutions that gave an approximately normal (i.e., low) ratio of inside to outside chloride. The small size of the current and its apparent outward rectification made it impossible to be sure where the current reversed. We have had more success with atrial cells, using symmetrical chloride or a high inside to outside chloride ratio. Both the actual reversal potentials and the magnitude of the shift were near the predictions for a chloride current.

When cells were bathed in low external chloride (Fig. 8 B), outward rectification was still apparent. This is in the opposite direction to rectification predicted by constant field theory. At this point, we cannot say if the rectification we observed in the whole cell current is a property of the open  $I_{Cl(Ca)}$  channel, a manifestation of voltage-dependent gating of the channel, a result of block by other ions, or a reflection of unexpected voltage dependence or kinetics of the calcium transient that regulates the current.

#### *Correlations of $I_{Ca}$ and $I_{Cl(Ca)}$*

When 100-ms depolarizing presteps were used to inactivate  $I_{Ca}$ , the amplitude of  $I_{Cl(Ca)}$  corresponded closely to that of  $I_{Ca}$  over the voltage range  $-80$  to  $+70$  mV, despite

the strongly biphasic nature of  $I_{Ca}$  inactivation when short inactivating pulses are used (Fig. 6). This result is consistent with the view that inactivation of  $I_{Ca}$  leads to reduced calcium release from the SR and ultimately to diminution of the calcium transient that activates  $I_{Cl(Ca)}$ . In other words, the result is consistent with the hypothesis that changes of  $I_{Cl(Ca)}$  in Fig. 6 were not caused by voltage-dependent inactivation of  $I_{Cl(Ca)}$ .

Similarly, parallel recovery of the two currents after brief (200 ms in Fig. 7) pulses is consistent with the assumption that  $I_{Ca}$  recovery mediates recovery of the calcium transient, which in turn causes apparent recovery of  $I_{Cl(Ca)}$ . Inherent voltage-dependent inactivation of  $I_{Cl(Ca)}$ , if it happens at all, presumably requires longer than 100–200 ms. During depolarizing pulses,  $I_{Cl(Ca)}$  decays over  $\sim 150$  ms. We suggest that this decay is caused primarily by decay of the underlying calcium transient, rather than by voltage-dependent inactivation of  $I_{Cl(Ca)}$ .

*Proposed Relations among  $I_{Ca}$ , Calcium, and  $I_{Cl(Ca)}$*

As we presented and discussed our data, we had to consider mechanisms piecemeal. We would now like to attempt a more coherent overview of how  $I_{Ca}$ , intracellular calcium, and  $I_{Cl(Ca)}$  may be related. This overview is based on our observations of  $I_{Cl(Ca)}$  and on present concepts of excitation–contraction (E–C) coupling in heart. See reviews by Gibbons (1986) and Gibbons and Zygmunt (1991) for details of the relevant E–C coupling background.

In heart,  $I_{Ca}$  appears to cause an initial increase of free calcium in cells, which then triggers release of additional calcium from stores in the SR. The amount of calcium released from the SR is believed to be proportional to  $I_{Ca}$  and to the contents of the SR. We have tried to keep calcium loading of the SR approximately constant in individual experiments, so to a first approximation we should be able to assume that calcium released was proportional to  $I_{Ca}$ .

The release of calcium from the SR causes an intracellular free calcium transient that activates contraction. Our data indicate that the same intracellular calcium transient activates the conductance for  $I_{Cl(Ca)}$ . In regard to E–C coupling, a frequent question is whether the calcium that activates a contraction comes almost entirely from the SR, almost entirely from calcium influx from the interstitial space (i.e., via  $I_{Ca}$ ), or from a mixture of the two sources. In our experiments, ryanodine prevented  $I_{Cl(Ca)}$  but did not block  $I_{Ca}$ . Caffeine, which interferes with calcium uptake and release by the SR (Blinks et al., 1972; Fabiato and Fabiato, 1973), increased  $I_{Ca}$  but eliminated  $I_{Cl(Ca)}$ .

These data indicate that most of the calcium that causes  $I_{Cl(Ca)}$  is derived from the SR; the calcium supplied by  $I_{Ca}$ , even by caffeine-augmented  $I_{Ca}$ , is insufficient to provoke  $I_{Cl(Ca)}$ . Because we believe the same calcium transient evokes contraction and  $I_{Cl(Ca)}$ , this argument can be extended: the principal source of calcium to activate contraction under the conditions of our experiments was the SR. We must caution, however, that the conditions of our experiments were highly controlled and artificial; this conclusion may or may not apply to intact heart stimulated at a physiological rate.

When an intracellular calcium transient activates the conductance for  $I_{Cl(Ca)}$ , the current that results should depend both on the size of the intracellular calcium transient and on the driving force for chloride ions. Thus, we would not expect the

current-voltage relations of  $I_{Cl(Ca)}$  and  $I_{Ca}$  (Fig. 5) to be mirror images of each other. Even if one assumed a linear relation between peak  $I_{Ca}$  and the peak of the intracellular calcium transient, and another linear relation between the peak of the intracellular calcium transient and the peak conductance for  $I_{Cl(Ca)}$ , the shape of the current-voltage relation for  $I_{Cl(Ca)}$  would not be an inverted version of the  $I_{Ca}$  current-voltage relation because the chloride driving force would be low at negative voltages and very high at very positive voltages.

Our data may allow a very tentative inference about the relation between  $I_{Ca}$  and calcium and that between calcium and  $I_{Cl(Ca)}$ . During the experiments in which  $I_{Ca}$  was inactivated (Fig. 6), we were surprised by the close correspondence between peak  $I_{Ca}$  and peak  $I_{Cl(Ca)}$ . Experiments like these allow comparison of  $I_{Ca}$  and  $I_{Cl(Ca)}$  as both currents range from nearly zero to nearly maximum, and the comparisons are always made at the same voltage (+14 mV in the experiments summarized in Fig. 6). Because the chloride driving force was constant, the changes of  $I_{Cl(Ca)}$  should reflect changes in its conductance. The close correspondence between  $I_{Cl(Ca)}$  conductance and  $I_{Ca}$  could be accidental, but it is more likely that there is a reasonably linear relation between peak  $I_{Ca}$  and peak conductance for  $I_{Cl(Ca)}$  when loading of the SR is kept constant by careful experimental design.

#### *Relation of $I_{Cl(Ca)}$ to $I_{Cl(cAMP)}$*

Two chloride currents have recently been identified in heart.  $I_{Cl(cAMP)}$  is a time-independent current that is activated by adrenergic stimulation; it is negligible in the absence of adrenergic agonists and does not require intracellular calcium (Bahinski et al., 1989; Harvey and Hume, 1989).  $I_{Cl(Ca)}$  is time dependent, is present in the absence of adrenergic stimulation, and is activated by intracellular calcium. These are striking differences, but we have pointed out that the disparate results might be explained by a single type of chloride channel if adrenergic stimulation shifted the relation between intracellular calcium and channel open state probability (Zygmunt and Gibbons, 1991a). We believe it is important to establish whether  $I_{Cl(cAMP)}$  and  $I_{Cl(Ca)}$  are caused by the same or by different channels.

In a recent abstract, Harvey and Hume (1991) reported that 0.1–1.0 mM SITS increased isoproterenol-activated  $I_{Cl(cAMP)}$ ; in some cells, SITS activated  $I_{Cl(cAMP)}$  even in the absence of isoproterenol. SITS blocked  $I_{Cl(Ca)}$  in our experiments. If the drug activated  $I_{Cl(cAMP)}$  in our cells, that would suggest that the two chloride currents are through different channels. We did not, however, see any indication that SITS activated  $I_{Cl(cAMP)}$ .

Lowering extracellular sodium reduces  $I_{Cl(cAMP)}$  by an unknown mechanism. The ion that replaces sodium also makes a difference; some substitutes appear to virtually eliminate  $I_{Cl(cAMP)}$  (Bahinski et al., 1989; Matsuoka et al., 1990; Harvey et al., 1990). Lowering extracellular sodium to one-third normal did not decrease  $I_{Cl(Ca)}$  in our experiments; the current actually increased slightly, as we would expect if lowering extracellular sodium caused increased intracellular calcium accumulation in the weakly calcium-buffered interiors of the cells. When extracellular sodium was zero, changing from NMG to TMA or TEA had no significant effect on the magnitude or time course of  $I_{Cl(Ca)}$ . These sodium substitutes are ones reported to depress  $I_{Cl(cAMP)}$  by

differing amounts. The sodium results provide further reason for believing that  $I_{Cl(Ca)}$  and  $I_{Cl(cAMP)}$  are produced by different channels. In addition, the sodium substitution data illustrate that our experiments on  $I_{Cl(Ca)}$  are unlikely to be compromised by the choice of sodium substitute.

#### *Reports of Single Chloride Channels in Heart*

Unitary chloride currents through a 55-pS channel have been recorded from calf sarcolemmal membranes incorporated into lipid bilayers (Coronado and Latorre, 1982). These channels were open in 10 nM calcium. Low density (present in 4% of patches) chloride channels with conductances between 60 and 450 pS and extremely complex gating have been reported in cultured neonatal rat ventricle (Coulombe, Duclouhier, Coraboeuf, and Touzet, 1987). These channels functioned in inside-out patches excised into zero calcium solution. Because these channels functioned in zero or very low internal calcium, they are unlikely to be responsible for  $I_{Cl(Ca)}$ .

Ehara and Ishihara (1990) recorded a low conductance (13 pS) chloride channel in on-cell patches of guinea-pig ventricular myocytes. Activity of the channels was induced by adrenaline or dibutyryl cAMP. These channels are very likely to be responsible for  $I_{Cl(cAMP)}$ . To date, we know of no recordings of single channels in heart that are candidates to produce  $I_{Cl(Ca)}$ . Single calcium-activated chloride channels have been reported, however, in a variety of cell types other than heart, including *Xenopus* oocytes (Takahashi, Neher, and Sakmann, 1987), cultured pig intermediate lobe endocrine cells (Taleb, Feltz, Bossu, and Feltz, 1988), and distal nephron cells (Marunaka and Eaton, 1990). At present, we cannot say if the channels that produce  $I_{Cl(Ca)}$  in heart are similar to or very different from calcium-activated chloride channels in other types of cells.

#### *Relation of $I_{Cl(Ca)}$ to Transient Currents in Other Heart Cells*

Two operationally defined components of transient outward current were first identified in Purkinje fibers. The larger component of transient outward current in Purkinje fibers is blocked by millimolar concentrations of 4AP, and is believed to be carried by potassium ions (Kenyon and Gibbons, 1979*a, b*). In the presence of 4AP, a smaller transient current remains that is reduced in low chloride (Kenyon and Gibbons, 1979*b*). Kenyon and Gibbons (1979*b*) suggested that the 4AP-insensitive transient outward current might be a chloride current. Further efforts to test this suggestion gave equivocal results (Kenyon and Sutko, 1987), and for lack of evidence to the contrary potassium has often been assumed to be responsible for the 4AP-insensitive current as well as for the 4AP-sensitive current.

Siegelbaum and co-workers reported that the 4AP-insensitive transient outward current of Purkinje fibers is closely correlated with contraction, indicating that the current is calcium activated (Siegelbaum and Tsien, 1980). The consensus, therefore, has been that a voltage-activated transient outward current and a calcium-activated transient outward current coexist in Purkinje fibers (Coraboeuf and Carmeliet, 1982; Lipsius and Gibbons, 1982). The voltage-activated current is believed to be carried by potassium. The charge carrier of the 4AP-insensitive, calcium-activated transient current has been less certain.

Until recently, it seemed that transient outward currents might be unique to Purkinje fibers. However, as whole cell patch clamp methods have been applied to isolated cardiac cells, homologous currents have been identified in other parts of the heart. A 4AP-sensitive transient outward current has been reported in isolated myocytes of rat ventricle (Josephson, Sanchez-Chapula, and Brown, 1984), rabbit crista terminalis (Giles and van Ginneken, 1985), rabbit atrioventricular node (Nakayama and Irisawa, 1985), rabbit atrium (Clark et al., 1988; Giles and Imaizumi, 1988), rabbit ventricle (Giles and Imaizumi, 1988; Hiraoka and Kawano, 1989), human atrium (Escande, Coulombe, Faivre, Deroubaix, and Coraboeuf, 1987), mouse ventricle (Benndorf and Nilius, 1988), and dog ventricle (Tseng and Hoffman, 1989). The 4AP-sensitive transient current has been called  $I_{qr}$ ,  $I_v$ ,  $I_{to}$ ,  $I_{to1}$ ,  $I_A$ ,  $I_{lo}$ , and  $I_{eo}$ . The current we have called  $I_A$  is certainly equivalent to the 4AP-sensitive transient outward current in other heart cells.

A calcium-related current like the 4AP-insensitive transient current of Purkinje fibers has been seen in seal atrium (Maylie and Morad, 1984), rabbit atrium (Clark et al., 1988; Giles and Imaizumi, 1988), rabbit ventricle (Giles and Imaizumi, 1988; Hiraoka and Kawano, 1989), human atrium (Escande et al., 1987), and dog ventricle (Tseng and Hoffman, 1989).  $I_{Cl(Ca)}$  appears to be quite similar to the currents these groups recorded, and it is tempting to postulate that the calcium-activated current in other species is composed of  $I_{Cl(Ca)}$ . However, we cannot presently rule out the possibility that, in other species or under other experimental conditions, calcium-activated transient current may include measurable calcium-activated potassium current or nonspecific cation current in addition to or instead of  $I_{Cl(Ca)}$ .

We thank Dr. Joseph Patlak and Dr. Mark Nelson for helpful discussions and suggestions. Miles Inc., West Haven, CT, graciously supplied nisoldipine.

This work was supported by USPHS grant HL-14614. Dr. Zygmunt is a fellow of the Vermont Affiliate of the American Heart Association.

*Original version received 8 July 1991 and accepted version received 23 November 1991.*

#### REFERENCES

- Bahinski, A., A. C. Nairn, P. Greengard, and D. C. Gadsby. 1989. Chloride conductance regulated by cyclic AMP-dependent protein kinase in cardiac myocytes. *Nature*. 340:718–721.
- Barceñas-Ruiz, L., and W. G. Wier. 1987. Voltage dependence of intracellular  $[Ca^{2+}]_i$  transients in guinea pig ventricular myocytes. *Circulation Research*. 61:148–154.
- Barish, M. E. 1983. A transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *Journal of Physiology*. 342:309–325.
- Benndorf, K., and B. Nilius. 1988. Properties of an early outward current in single cells of the mouse ventricle. *General Physiology and Biophysics*. 7:449–466.
- Blatz, A. L., and K. L. Magleby. 1987. Calcium-activated potassium channels. *Trends in Neuroscience*. 10:463–467.
- Blinks, J. R., C. B. Olson, B. R. Jewell, and P. Braveny. 1972. Influence of caffeine and other methylxanthines on mechanical properties of isolated mammalian heart muscle. *Circulation Research*. 30:367–392.
- Byrne, N. G., and W. A. Large. 1987. Action of noradrenaline on single smooth muscle cells freshly dispersed from the rat anococcygeus muscle. *Journal of Physiology*. 389:513–525.

- Callewaert, G., J. Vereecke, and E. Carmeliet. 1986. Existence of a calcium-dependent potassium channel in the membrane of cow cardiac Purkinje cells. *Pflügers Archiv*. 406:424–426.
- Cannell, M. B., J. R. Berlin, and W. J. Lederer. 1987. Effect of membrane potential changes on the calcium transient in single rat cardiac muscle cells. *Science*. 238:1419–1423.
- Cannell, M. B., and W. J. Lederer. 1986. The arrhythmogenic current  $I_{Ti}$  in the absence of electrogenic sodium-calcium exchange in sheep cardiac Purkinje fibres. *Journal of Physiology*. 374:201–219.
- Clark, R. B., W. R. Giles, and Y. Imaizumi. 1988. Properties of the transient outward current in rabbit atrial cells. *Journal of Physiology*. 405:147–168.
- Colquhoun, D., E. Neher, H. Reuter, and C. F. Stevens. 1981. Inward current channels activated by intracellular Ca in cultured cardiac cells. *Nature*. 294:752–754.
- Connor, J. A., and C. F. Stevens. 1971. Voltage clamp studies of a transient outward membrane current in gastropod neural somata. *Journal of Physiology*. 213:21–30.
- Coraboeuf, E., and E. Carmeliet. 1982. Existence of two transient outward currents in sheep cardiac Purkinje fibers. *Pflügers Archiv*. 392:352–359.
- Coronado, R., and R. Latorre. 1982. Detection of K and Cl channels from calf cardiac sarcolemma in planar lipid bilayer membranes. *Nature*. 298:849–852.
- Coulombe, A., H. Ducholier, E. Coraboeuf, and N. Touzet. 1987. Single chloride-permeable channels of large conductance in cultured cardiac cells of new-born rats. *European Biophysical Journal*. 14:155–162.
- Ehara, T., and K. Ishihara. 1990. Anion channels activated by adrenaline in cardiac myocytes. *Nature*. 347:284–286.
- Ehara, T., A. Noma, and K. Ono. 1988. Calcium-activated non-selective cation channel in ventricular cells isolated from adult guinea-pig hearts. *Journal of Physiology*. 403:117–133.
- Eisner, D. A., and R. D. Vaughan-Jones. 1983. Do calcium-activated potassium channels exist in the heart? *Cell Calcium*. 4:371–386.
- Escande, D., A. Coulombe, J.-F. Faivre, E. Deroubaix, and E. Coraboeuf. 1987. Two types of transient outward currents in adult human atrial cells. *American Journal of Physiology*. 252 (*Heart and Circulatory Physiology* 21):H142–H148.
- Fabiato, A., and F. Fabiato. 1973. Activation of skinned cardiac cells. *European Journal of Cardiology*. 1/2:143–155.
- Gibbons, W. R. 1986. Cellular control of cardiac contraction. In *The Heart and Cardiovascular System*. H. A. Fozzard, E. Haber, R. B. Jennings, A. M. Katz, and H. E. Morgan, editors. Raven Press, New York. 747–778.
- Gibbons, W. R., and A. C. Zygmunt. 1991. Excitation-contraction coupling in heart. In *The Heart and Cardiovascular System*. 2nd ed. H. A. Fozzard, E. Haber, R. B. Jennings, A. M. Katz, and H. E. Morgan, editors. Raven Press, New York. 1249–1279.
- Giles, W. R., and Y. Imaizumi. 1988. Comparison of potassium currents in rabbit atrial and ventricular cells. *Journal of Physiology*. 405:123–145.
- Giles, W. R., and A. C. G. Van Ginneken. 1985. A transient outward current in isolated cells from the crista terminalis of rabbit heart. *Journal of Physiology*. 368:243–264.
- Harvey, R. D., C. D. Clark, and J. R. Hume. 1990. Chloride current in mammalian cardiac myocytes. *Journal of General Physiology*. 95:1077–1102.
- Harvey, R. D., and J. R. Hume. 1989. Autonomic regulation of a chloride current in heart. *Science*. 244:983–985.
- Harvey, R. D., and J. R. Hume. 1991. Paradoxical agonist effects of the disulfonic stilbene derivative SITS on the cardiac chloride current. *Biophysical Journal*. 59:90a. (Abstr.)

- Hiraoka, M., and S. Kawano. 1989. Calcium-sensitive and insensitive transient outward current in rabbit ventricular myocytes. *Journal of Physiology*. 410:187–212.
- Josephson, I. R., J. Sanchez-Chapula, and A. M. Brown. 1984. Early outward current in rat single ventricular cells. *Circulation Research*. 54:157–162.
- Kass, R. S., W. J. Lederer, R. W. Tsien, and R. Weingart. 1978. Role of calcium ions in transient inward currents and aftercontractions induced by strophanthidin in cardiac Purkinje fibres. *Journal of Physiology*. 281:187–208.
- Kass, R. S., R. W. Tsien, and R. Weingart. 1978. Ionic basis of transient inward current induced by strophanthidin in cardiac Purkinje fibres. *Journal of Physiology*. 281:209–226.
- Kenyon, J. L., and W. R. Gibbons. 1977. Effects of low-chloride solutions on action potentials of sheep cardiac Purkinje fibers. *Journal of General Physiology*. 70:635–660.
- Kenyon, J. L., and W. R. Gibbons. 1979a. Influence of chloride, potassium, and tetraethylammonium on the early outward current of sheep cardiac Purkinje fibers. *Journal of General Physiology*. 73:117–138.
- Kenyon, J. L., and W. R. Gibbons. 1979b. 4-Aminopyridine and the early outward current of sheep cardiac Purkinje fibers. *Journal of General Physiology*. 73:139–157.
- Kenyon, J. L., and J. L. Sutko. 1987. Calcium- and voltage-activated plateau currents of cardiac Purkinje fibers. *Journal of General Physiology*. 89:921–958.
- Lee, K. S., E. Marban, and R. W. Tsien. 1985. Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *Journal of Physiology*. 364:395–411.
- Lee, K. S., and R. W. Tsien. 1982. Reversal of current through calcium channels in dialysed single heart cells. *Nature*. 297:498–501.
- Lipsius, S. L., and W. R. Gibbons. 1982. Membrane currents, contractions, and aftercontractions in cardiac Purkinje fibers. *American Journal of Physiology*. 243 (Heart and Circulatory Physiology 12):H77–H86.
- Marty, A., Y. P. Tan, and A. Trautmann. 1984. Three types of calcium-dependent channel in rat lacrimal glands. *Journal of Physiology*. 357:293–325.
- Marunaka, Y., and D. C. Eaton. 1990. Effects of insulin and phosphatase on a Ca-dependent Cl channel in a distal nephron cell line (A6). *Journal of General Physiology*. 95:773–789.
- Matsuoka, S., T. Ehara, and A. Noma. 1990. Chloride-sensitive nature of the adrenaline-induced current in guinea-pig cardiac myocytes. *Journal of Physiology*. 425:579–598.
- Maylie, J., and M. Morad. 1984. A transient outward current related to calcium release and development of tension in elephant seal atrial fibres. *Journal of Physiology*. 357:267–292.
- Miledi, R. 1982. A calcium-dependent transient outward current in *Xenopus laevis* oocytes. *Proceedings of the Royal Society London B*. 215:491–497.
- Nakayama, T., and H. Irisawa. 1985. Transient outward current carried by potassium and sodium in quiescent atrioventricular node cells of rabbits. *Circulation Research*. 57:65–73.
- Owen, D. G., M. Segal, and J. L. Barker. 1984. A Ca-dependent Cl conductance in cultured mouse spinal neurones. *Nature*. 311:567–570.
- Siegelbaum, S. A., and R. W. Tsien. 1980. Calcium-activated transient outward current in calf cardiac Purkinje fibres. *Journal of Physiology*. 299:485–506.
- Takahashi, T., E. Neher, and B. Sakmann. 1987. Rat brain serotonin receptors in *Xenopus* oocytes are coupled by intracellular calcium to endogenous channels. *Proceedings of the National Academy of Science, USA*. 84:5063–5067.

- Taleb, O., P. Feltz, J.-L. Bossu, and A. Feltz. 1988. Small-conductance chloride channels activated by calcium on cultured endocrine cells from mammalian pars intermedia. *Pflügers Archiv.* 412:641–646.
- Tseng, G.-N., and B. F. Hoffman. 1989. Two components of transient outward current in canine ventricular myocytes. *Circulation Research.* 64:633–647.
- Zygmunt, A. C., and W. R. Gibbons. 1991a. Calcium-activated chloride current in rabbit ventricular myocytes. *Circulation Research.* 68:424–437.
- Zygmunt, A. C., and W. R. Gibbons. 1991b. A calcium-activated chloride current in rabbit ventricular and atrial myocytes. *Biophysical Journal.* 59:545a. (Abstr.)