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Mechanism by which metabolic inhibitors depolarize cultured cardiac cells

(dinitrophenol/automaticity/sodium ions/calcium ions)

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ABSTRACT **To elucidate the means by which metabolic inhibition depolarizes cardiac cells, spontaneously beating chicken embryonic myocardial cell aggregates were voltage clamped during superfusion with 2,4-dinitrophenol and iodoacetic acid. In aggregates continuously clamped in the pacemaker potential range, abrupt exposure to these metabolic inhibitors produced a slow transient inward current. This inward current was not due to an alteration of the pacemaker current, I_{K2}, because it could still be elicited after I_{K2} was abolished by Cs^+ ions. The inward current was increased by hyperpolarization and decreased by depolarization. It became larger and more sustained if intermittent action potentials were allowed during exposure or if the aggregates were pretreated with either 10 mM Ca^{2+} or 2.7 μM acetylstrophanthidin. The inward current was suppressed by removal of extracellular Na^+ or Ca^{2+}. These observations suggest that early depolarization of cultured cardiac cells by metabolic inhibitors involves some of the same mechanisms as the transient inward current of digitalis toxicity—specifically, an effect of intracellular Ca^{2+} ions on membrane permeability. Similar phenomena could occur during other forms of metabolic inhibition such as myocardial ischemia.**

A conspicuous effect of energy depletion in cardiac muscle is reduction of the transmembrane potential between beats. In spontaneously beating preparations, hypoxia or metabolic inhibitors produce an increase in beat frequency and reduction of the maximum diastolic potential, followed by arrest in a partially depolarized state (1). Although loss of intracellular K^+ must ultimately produce depolarization in the absence of energy, it is uncertain whether this occurs rapidly enough to explain the above effects. Another early effect of metabolic inhibition in cardiac muscle is an increase in intracellular free Ca^{2+} (1). Recordings obtained in sheep Purkinje fibers with Ca^{2+}-sensitive microelectrodes show that 2,4-dinitrophenol (DNP) produces a marked increase in intracellular Ca^{2+} activity within 2 min, which parallels the decline in the membrane potential (2). Measurements of Na^+-dependent $^{45}Ca^{2+}$ efflux from guinea pig myocardium indicate that cyanide has a similar effect (3). When "calcium overload" occurs in digitalis toxicity, phasic release and reuptake of sequestered intracellular Ca^{2+} lead to a transient inward current (TI) (4–6). This current may traverse a recently discovered cation channel, which has equal permeability to Na^+ and K^+ and is opened by the presence of Ca^{2+} at the inner surface of the cell membrane (7). Similar mechanisms might explain the early depolarizing effect of metabolic inhibition.

In the present study, changes in ionic current have been recorded during application of metabolic inhibitors to chicken embryonic myocardial cell aggregates. The combination of a glycolysis inhibitor (iodoacetic acid) and an electron transport uncoupler (DNP) has been used, based on reports that glycolysis alone can sustain metabolism in these cells (8). The suitability of myocardial cell aggregates for microelectrode voltage clamp is well-established (9–12). A principal advantage of this preparation is the relative absence of diffusion barriers, which allows the immediate effects of a test agent to be resolved (13, 14). Metabolic inhibitors are shown to induce an activity-dependent inward current whose sensitivity to voltage and extracellular ions is similar to that of the digitalis-induced TI. Several alternative explanations for the inward current can be ruled out.

METHODS

Myocardial cell aggregates were prepared from trypsin-dispersed chicken embryonic ventricular cells by a modification of DeHaan's procedure, previously described (14, 15). After 4–12 days in culture, the aggregates were plated onto glass slides, immersed in physiological saline on the stage of an inverted microscope, and penetrated with microelectrodes. The composition of the saline was 137 mM NaCl/2.7 mM KCl/0.4 mM NaH_2PO_4/1.8 mM $CaCl_2$/1.0 mM $MgCl_2$/5.5 mM dextrose/6.0 mM Hepes, pH 7.4 at 37 ± 0.2°C. Recordings were obtained either in current or voltage clamp by using a Dagan 8100 single electrode voltage clamp at a sampling frequency of 500 Hz. Adequacy of membrane potential control was verified in several experiments by an independent microelectrode. Microelectrodes were filled with 3 M KCl and had tip resistances of 20–40 MΩ. The largest sustained currents were <20 nA. Current was normalized for membrane area, which was estimated as $(4/3)\, k\, \pi\, ab^2$, in which a and b are the major and minor hemiaxes of the aggregate (in μm) and k is 4.4×10^{-9} cm^2/μm^3, a constant derived from the histologic data of Nathan and DeHaan (9). Aggregates were superfused via a 270 μm (inside diameter) polyethelene catheter connected to a Buchler peristaltic pump. Recordings obtained with ion-sensitive electrodes showed that a flow rate of 60 μl/min produced nearly complete (>90%) exchange of solute over a region several times larger than an aggregate's diameter (60–130 μm) within 2–4 sec. DNP and iodoacetic acid were always applied together, each in a concentration of 0.1 mM. Data were recorded by an oscilloscope camera or by a Gould–Brush 2400 strip chart recorder. Grouped data were expressed as mean ± σ. Statistical significance (P value) was determined by Student's t test (two-tailed distribution).

RESULTS

Effects of Metabolic Inhibitors During Spontaneous Beating. A spontaneously beating myocardial cell aggregate was abruptly superfused with DNP and iodoacetic acid in Fig. 1.

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Abbreviations: DNP, 2,4-dinitrophenol; TI, transient inward current.

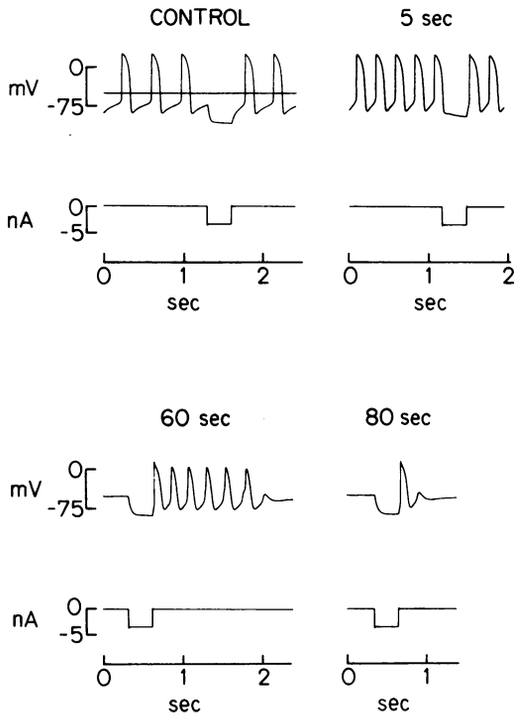

FIG. 1. Effects of metabolic inhibitors on a spontaneously beating chicken embryonic myocardial cell aggregate. Upper traces show intracellular potential and lower traces, applied current. After the control recording, the aggregate is abruptly superfused with 0.1 mM DNP and 0.1 mM iodoacetic acid. Beat frequency increases markedly within 5 sec, and the aggregate arrests in a partly depolarized state within 60 sec. The resting potential at 80 sec is indicated on the control record by a horizontal line.

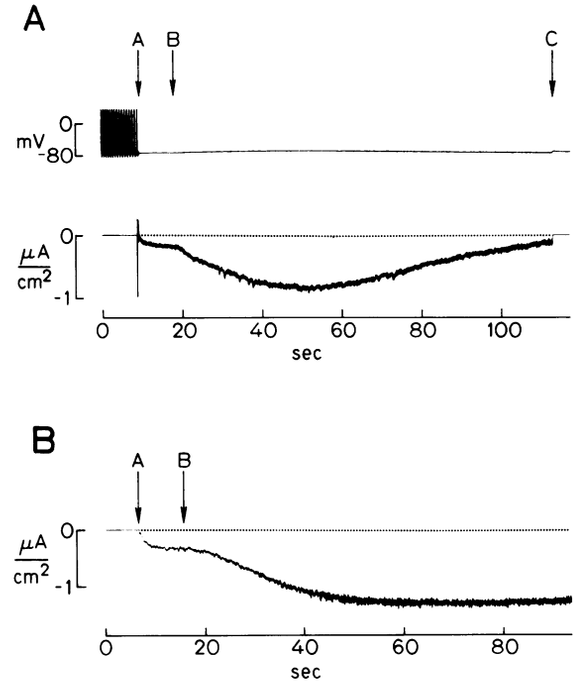

FIG. 2. (*A*) Ionic current induced by metabolic inhibitors. A spontaneously beating aggregate is voltage clamped at −71 mV between arrows A and C. Superfusion with DNP and iodoacetic acid, beginning at arrow B, causes a slow TI (lower trace). Current at arrow C is slightly outward compared to arrow B, and release of the clamp fails to reinitiate beating. (*B*) Ionic current induced by DNP and iodoacetic acid in high Ca^{2+}. The aggregate has been exposed to 10 mM Ca^{2+} for 10 min and then clamped at −65 mV at arrow A. Addition of DNP and iodoacetic acid at arrow B elicits a sustained inward current. Aggregate hemiaxes are 60 × 60 μm in *A* and 60 × 65 μm in *B*. Zero current during voltage clamp is indicated by dotted lines.

A marked increase in beat frequency occurred within 5 sec, along with a slight reduction of the maximum diastolic potential. Arrest of the aggregate occurred within 60 sec, but hyperpolarizing current pulses still initiated action potentials of decreased duration. A similar sequence was observed in 15 aggregates, of which 5 were periodically stimulated by hyperpolarizing pulses as in Fig. 1. In these 5 aggregates, the mean resting potential at 80 sec was −36.0 ± 11.7 mV. DNP and iodoacetic acid also promptly increased the beat frequency of aggregates that were not impaled with microelectrodes, but beating did not accelerate in aggregates superfused with saline alone. Except for their more rapid onset, the above effects were identical to those produced in rabbit sinoatrial node by 0.1 mM DNP (16).

Initiation of Inward Current by Metabolic Inhibitors. The depolarizing effect in Fig. 1 suggests that DNP and iodoacetic acid induce an inward shift in membrane current that could be measured under voltage clamp. This expectation was tested by using a single electrode voltage clamp, whose reported ability to control the membrane potential of myocardial cell aggregates in the pacemaker range (17) has been confirmed recently by dual impalements (figure 1*a* in ref. 12). In Fig. 2*A*, abrupt superfusion with DNP and iodoacetic acid (arrow B) caused a slow TI. Similar changes in membrane current occurred in eight other aggregates for which the average change in current has been plotted in Fig. 3*A*. A TI could be elicited several times in the same aggregate if the superfusion was discontinued for several minutes between trials.

The result obtained in Fig. 2*A* was not entirely consistent with the depolarizing effect of DNP and iodoacetic acid in nonclamped aggregates (Fig. 1) because the inward current was too short-lived. When the voltage clamp was released at arrow C in Fig. 2*A*, the aggregate did not depolarize but rested near the maximum diastolic potential. In eight aggregates that were clamped in the pacemaker potential range for at least the first 60 sec of superfusion, the mean resting potential measured at 80 sec was −67.8 ± 8.6 mV. This value was significantly larger ($P = 0.0001$) than the mean resting potential of the five aggregates that continued beating during an identical superfusion (above).

A possible explanation for the above discrepancy is that the metabolic inhibitor-induced current is augmented by electrical activity. This possibility was tested by allowing intermittent action potentials to occur during exposure. In Fig. 4, an aggregate was paced at a frequency of 150 beats/min by 200-msec hyperpolarizing current pulses and then clamped at −62 mV for ≈2 sec after every eighth beat. The current at the end of each clamp was constant during the control recording (time < 0) but became progressively inward during superfusion with DNP and iodoacetic acid (time > 0). The progressively increasing inward current observed between 20 and 45 sec of superfusion contrasts with the result obtained in continuously clamped aggregates (Fig. 2*B*), where current became progressively less inward during the same period. Mean currents obtained from seven experiments similar to Fig. 4 were plotted in Fig. 6*A*. The mean metabolic inhibitor-induced current was significantly more inward in these aggregates than in the continuously clamped aggregates (Fig. 3*A*) for all times after 30 sec ($P = 0.03$–0.008). Thus, both the amplitude and duration of the metabolic inhibitor-induced inward current were increased by intermittent electrical activity.

The Metabolic Inhibitor-Induced Current Is Distinct From I_{K2}. A possible explanation for the metabolic inhibitor-induced

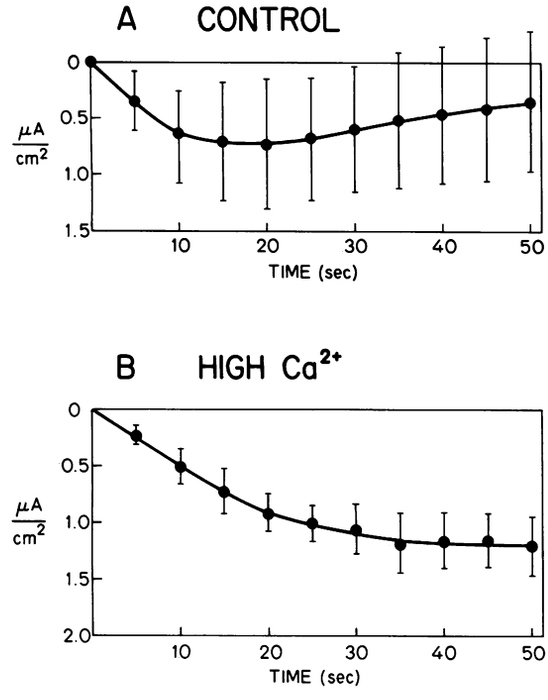

FIG. 3. Mean metabolic inhibitor-induced current in aggregates continuously clamped in the pacemaker potential range. Current measured at 5-sec intervals has been normalized for membrane area and expressed as the change in current since addition of DNP and iodoacetic acid. *A* is from eight aggregates studied in physiological saline (1.8 mM Ca^{2+}), whereas *B* is from five aggregates studied in 10 mM Ca^{2+}. The mean holding potential is -70.0 ± 7.2 mV in *A* and -69.0 ± 4.9 mV in *B*. Error bars show $\pm\sigma$.

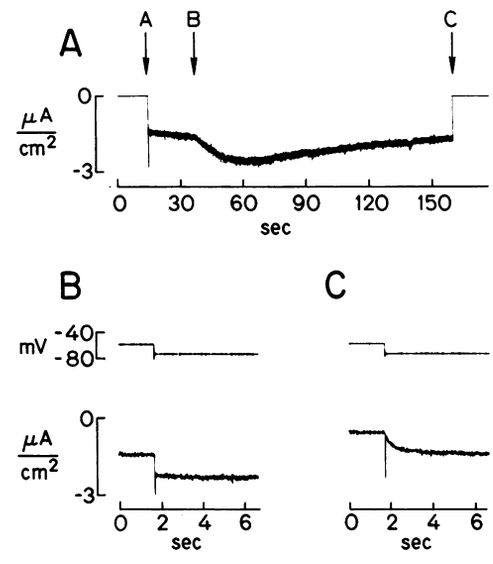

FIG. 5. Failure of Cs^+ ions to abolish the metabolic inhibitor-induced inward current. In *A*, the aggregate has been exposed to 3 mM Cs^+ for 10 min prior to impalement and then clamped at -57 mV between arrows A and C. Application of DNP and iodoacetic acid beginning at arrow B evokes the usual slow TI. In *C*, the same aggregate has been superfused for 5 min with physiological saline containing no DNP, iodoacetic acid, or Cs^+. A 16-mV hyperpolarizing voltage step evokes an inward current (the I_{K2}, pacemaker current), which increases exponentially with time. Return of the aggregate to 3 mM Cs^+ in *B* abolishes this time-dependent current within 2 min. Aggregate hemiaxes are 35×50 μm.

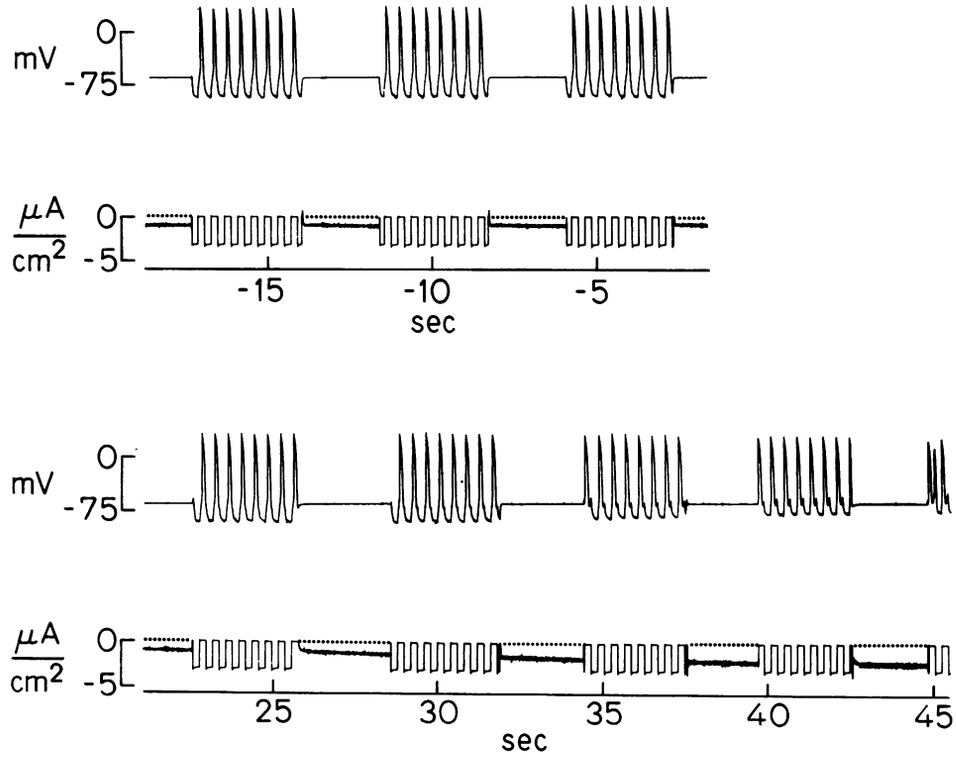

FIG. 4. Effects of DNP and iodoacetic acid on ionic current in an intermittently clamped aggregate. The aggregate is paced at 150 beats/min by hyperpolarizing current pulses and then clamped to -62 mV for ≈2 sec after every eighth beat. Current at the end of the clamps is constant during the control recording (upper traces) but shifts progressively inward during application of DNP and iodoacetic acid, which begins at time = 0. There is little time-dependent current during control clamps because the holding potential is near the positive end of the I_{K2} activation range. Aggregate hemiaxes are 45×45 μm.

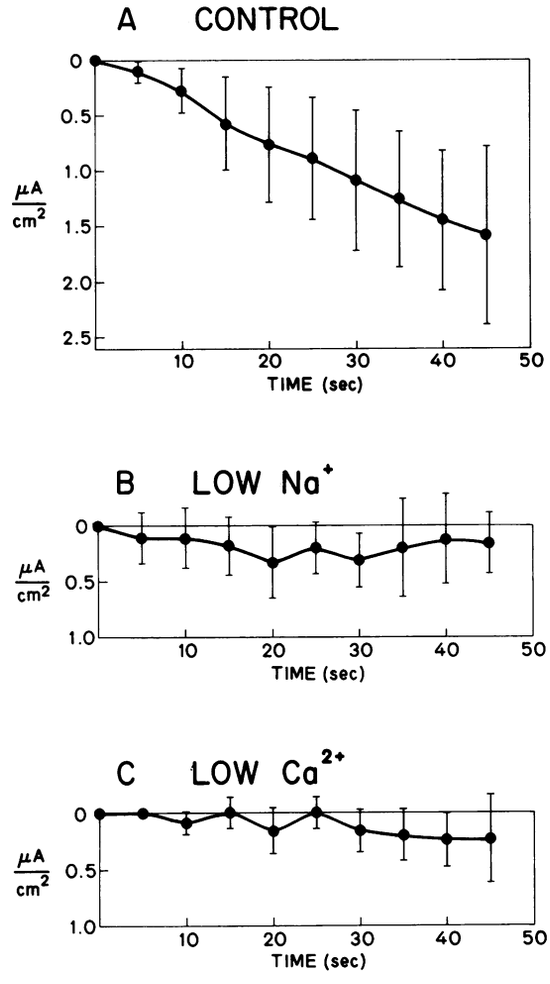

FIG. 6. Mean metabolic inhibitor-induced current in aggregates intermittently clamped according to the protocol in Fig. 4. Current is measured at the end of nine consecutive clamps to a holding potential in the pacemaker range and expressed as in Fig. 3. *A* is from seven aggregates studied in physiological saline. *B* is from six aggregates exposed to Na^+-free (Tris) saline during and for >60 sec prior to superfusion with DNP and iodoacetic acid. *C* is from five aggregates exposed to 0.1 mM Ca^{2+} during and for >45 sec prior to application of DNP and iodoacetic acid. The mean holding potential is -70.6 ± 8.1 mV in *A*, -72.0 ± 7.3 mV in *B*, and -67.2 ± 2.0 mV in *C*.

current is that it results from altered behavior of the normal pacemaker mechanism. Myocardial cell aggregates have a time- and voltage-dependent pacemaker current that is similar to the I_{K2} current of cardiac Purkinje fibers (11, 18). In Purkinje fibers, I_{K2} is abolished by low concentrations of Cs^+ ions (19, 20). Therefore, Cs^+ was used to investigate possible involvement of I_{K2} in the metabolic inhibitor-induced current of myocardial cell aggregates. Application of 3 mM Cs^+ (Fig. 5*B*) abolished I_{K2} within 2 min. In Fig. 5*A*, the same aggregate was superfused with DNP and iodoacetic acid after 10 min in 3 mM Cs^+. The resulting inward current in 3 Cs^+-treated aggregates was comparable to that observed in the absence of Cs^+. Thus, the metabolic inhibitor-induced current was not due to altered behavior of I_{K2}.

Suppression of the Metabolic Inhibitor-Induced Current by Na^+ and Ca^{2+} Removal. Another possible explanation for the metabolic inhibitor-induced current is that it results from increased permeability of the membrane to specific extracellular cations. To test this possibility, the experiment in Fig. 4 was repeated after reduction of extracellular Na^+ or Ca^{2+}. In six aggregates, extracellular Na^+ was removed by superfusion with Na^+-free (Tris) saline (Fig. 6*B*). Na^+ removal alone decreased the amplitude and upstroke velocity of the action potential. The inward holding current during voltage clamp decreased briefly in low Na^+ but then increased, reaching a stable value within 60 sec. Addition of DNP and iodoacetic acid produced little further change in holding current. The inward shift in Fig. 6*B* was significantly smaller than in normal Na^+ (Fig. 6*A*) for all times after 20 sec ($P = 0.02$–0.001).

Reduction of extracellular Ca^{2+} also suppressed the metabolic inhibitor-induced current. Fig. 6*C* shows the effect of DNP and iodoacetic acid in five aggregates pretreated with 0.1 mM Ca^{2+}. Low Ca^{2+} profoundly depressed contraction and decreased the amplitude of the action potential plateau. Low Ca^{2+} also caused an inward shift in the holding current in the pacemaker range, which reached a steady state within 45 sec. Addition of DNP and iodoacetic acid produced little further change in membrane current. The mean change in current was significantly less than in control saline (Fig. 6*A*) at all times except 10 sec ($P = 0.03$–0.002). The foregoing results are consistent with a metabolic inhibitor-induced increase in membrane permeability to extracellular cations. However, suppression of the inward current by *both* Na^+ and Ca^{2+} removal would be difficult to explain unless one of these ions affected the underlying permeability process.

The role of Ca^{2+} was further investigated in aggregates exposed to 10 mM Ca^{2+} for 10–20 min prior to impalement. Ca^{2+}-rich saline presumably increased intracellular Ca^{2+} because the amplitude of contraction increased. The inward current induced by DNP and iodoacetic acid in Ca^{2+}-rich saline (Fig. 2*B*) differed from that observed in normal Ca^{2+} (Fig. 2*A*) because it was sustained. A similar result was obtained in five aggregates for which mean values were plotted in Fig. 3*B*. The inward current in 10 mM Ca^{2+} was significantly increased over control (Fig. 3*A*) at all times after 30 sec ($P = 0.04$–0.01). Therefore, the effect of 10 mM Ca^{2+} was similar to that of electrical activity, which also presumably increases intracellular Ca^{2+}.

Effect of Acetylstrophanthidin. The depolarizing effect of metabolic inhibitors is commonly ascribed to cessation of Na^+ pump activity. If the metabolic inhibitor-induced current were due to Na^+ pump depression alone, then it would not occur if the pump had been previously inhibited by digitalis. Gadsby and Cranefield (21) found that the Na^+ pump current induced in Purkinje fibers by transient K^+ withdrawal was abolished by a 2-min pretreatment with 2 μM acetylstrophanthidin. Accordingly, five myocardial cell aggregates were continuously exposed to acetylstrophanthidin (2.7 μM) for 10–30 min prior to addition of DNP and iodoacetic acid. Digitalis toxicity was manifested by a reduction in action potential duration. However, the metabolic inhibitor-induced current was not abolished but became larger and more sustained. The mean inward current shift after 30 sec in DNP and iodoacetic acid was 1.40 ± 0.50 μA/cm^2 in the acetylstrophanthidin-treated aggregates, which is significantly larger ($P = 0.02$) than the control value of 0.60 ± 0.56 μA/cm^2 (Fig. 3*A*). Thus, the effect of acetylstrophanthidin was similar to that of high Ca^{2+}.

Effect of Membrane Potential. If the metabolic inhibitor-induced current were due to a conductance change, then its amplitude would vary with membrane potential. To test this possibility, voltage-clamped aggregates were alternated between different holding potentials during exposure to DNP and iodoacetic acid. Hyperpolarization consistently increased the amplitude of the inward current, whereas depolarization decreased it. Therefore, DNP and iodoacetic acid induced a depolarizing conductance. The equilibrium potential of this conductance was not directly measured because the total current required to produce sufficient depolarization generally ex-

ceeded the capability of the single electrode voltage clamp. However, because the digitalis-induced TI (6) and the Ca^{2+}-activated inward current channels (7) both have linear current–voltage relations, the equilibrium potential of the metabolic inhibitor-induced current might be obtainable by linear extrapolation. The equilibrium potential thus estimated in five aggregates exposed to DNP and iodoacetic acid was -7.2 ± 13.4 mV. This is comparable to values reported for the digitalis-induced TI (4, 6) and is compatible with mediation by a channel having nearly equal permeability to Na^+ and K^+ (7).

DISCUSSION

This study shows that metabolic inhibitors depolarize chicken embryonic cardiac cells by inducing an inward current, which develops within seconds and becomes sustained if electrical activity is allowed to continue. These observations probably explain the depolarizing effect of metabolic inhibition to other spontaneously active cardiac preparations (1). Similar phenomena may occur in nonautomatic tissues, such as myocardium, but could easily be overlooked if low stimulation rates were employed.

The metabolic inhibitor-induced depolarization is not purely a consequence of Na^+ pump inhibition. Na^+ pump inhibition should produce some depolarization owing to the electrogenicity of the pump (21). Furthermore, if diffusion delays and resting K^+ efflux were large, then pump inhibition could cause early reduction of the K^+ equilibrium potential. However, these effects should be blocked or greatly diminished by prior digitalis intoxication. The paradoxical effect of acetylstrophanthidin, the voltage sensitivity of the metabolic inhibitor-induced current, and the spontaneous decay of this current in Fig. 2*A* are all at variance with the expected effects of Na^+ pump inhibition.

A more satisfactory explanation for the metabolic inhibitor-induced inward current is that it arises in a manner similar to the TI that occurs after repolarization of digitalis-intoxicated fibers (4–6). The digitalis-induced TI is believed to result from abnormal liberation of sequestered intracellular Ca^{2+}, which increases the permeability of the membrane to Na^+. The digitalis-induced TI and the metabolic inhibitor-induced current are similar in six respects: (*i*) Both are abolished by replacement of extracellular Na^+ with Tris (4, 6). (*ii*) Both exhibit similar voltage sensitivity (4, 6). (*iii*) Both are augmented by rapid pacing (4, 5). (*iv*) Both are sensitive to extracellular Ca^{2+} (4). (*v*) Both can be distinguished from I_{K2} (4, 5). (*vi*) Both can occur in the absence of digitalis toxicity but are augmented by it (5). Possible mediators of these currents include the Ca^{2+}-activated cation channels mentioned above (7) and electrogenic Na^+–Ca^{2+} exchange (22).

If the metabolic inhibitor-induced current is activated by intracellular Ca^{2+}, then the source and fate of the activator Ca^{2+} need to be determined. Jundt *et al.* (3) proposed that the Ca^{2+} released from atrial fibers by cyanide originates in mitochondria because cyanide caused Ca^{2+} release in isolated mitochondria. Metabolic inhibition might also cause loss of Ca^{2+} from the sarcoplasmic reticulum. Release of sarcoplasmic reticulum Ca^{2+} by abrupt exposure of myocardial cell aggregates to 10 mM caffeine is associated with a Na^+-sensitive TI that is similar in amplitude to the metabolic inhibitor-induced current, though its time course is more rapid (12).

If the activator Ca^{2+} is of intracellular origin, then the decay of the inward current could result from Ca^{2+} efflux across the sarcolemma. Rapid pacing, digitalis toxicity, and high extracellular Ca^{2+} all oppose net Ca^{2+} efflux and therefore could cause the inward current to become sustained. Alternatively, because the Ca^{2+}-activated inward current channels in cardiac cells "desensitize" (7), mere stabilization of intracellular free Ca^{2+} could cause the inward current to decay. Finally, it is possible that the inward current does not decay in Fig. 2*A* but is obscured by a slowly developing outward current of unknown origin.

An important implication of this study is that Ca^{2+}-dependent inward current might mediate the initial electrophysiological effects of ischemia. Pretreatment of ventricular myocardium with Ca^{2+} channel blockers retards depolarization during ischemia, as judged by the reduction of extracellular "injury" current (23). Early ischemic arrhythmias, which may be initiated by injury current (24), are also suppressed by these agents (25). Therefore, reduction of intracellular Ca^{2+} stores may ameliorate the adverse electrophysiological effects of metabolic inhibition *in vivo*.

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