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Inhibition of Multiple Trans-sarcolemmal Cation Flux Pathways by Dichlorobenzamil in Cultured Chick Heart Cells

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SUMMARY

Dichlorobenzamil, an analog of amiloride, has been reported to inhibit Na-Ca exchange in sarcolemmal vesicles of guinea pig heart. To examine further the effect of the drug on Na-Ca exchange in intact cardiac cells and the pharmacological specificity of this action, we determined in cultured chick heart cells the effects of dichlorobenzamil on the following: 1) contractile state, 2) Na_i-dependent Ca uptake, 3) Ca uptake via slow Ca channels (defined as verapamil-inhibitable Ca uptake), 4) Ca efflux via the sarcolemmal Ca pump, 5) monovalent cation transport, and 6) cellular Ca and Na content. Dichlorobenzamil produced a concentration-dependent decrease in the amplitude of cell motion ($EC_{50} = 5 \times 10^{-7}$ M) and abolished the development of ouabain-induced rhythm disturbances and contracture. In normal or Na-loaded cells, dichlorobenzamil inhibited the Ca uptake rate, also in a concentration-dependent manner ($EC_{50} = 6 \times 10^{-7}$ M). Dichlorobenzamil (6×10^{-7} M) also caused a significant inhibition of the isoproterenol-induced elevation of Ca uptake. At 5×10^{-5} M, dichlorobenzamil blocked completely Ca influx via slow Ca channels. Ca efflux rate was also reduced by dichlorobenzamil ($EC_{50} = 10^{-6}$ M). Replacement of Na with choline in the efflux medium to prevent Ca efflux via Na-Ca exchange did not alter the ED_{50} of the drug's inhibition of Ca efflux rate. Dichlorobenzamil caused concentration-dependent inhibition of sodium pump activity as judged by ouabain-sensitive ^{42}K uptake (EC_{50} approximately 2×10^{-6} M), and, at concentrations above 5×10^{-7} M), produced an increase in steady state cellular Na content. These results indicate that dichlorobenzamil has several sites of action in intact heart cells and that the negative inotropic action of the drug is due, in part, to inhibition of Ca influx via both Na-Ca exchange and slow Ca channels.

The existence of Na-Ca exchange across the sarcolemmal membrane of cardiac muscle cells is well documented (1–6). The role of the Na-Ca exchange process in excitation-contraction coupling, however, remains poorly understood. To delineate the role of Na-Ca exchange in the handling of these ions by heart cells, the effects of changes in $[Ca]_o$, $[Na]_o$, or $[Na]_i$ on subsequent Ca influx or efflux, or Na efflux, have been examined using radioisotopic flux techniques. In experiments in which external Na was replaced with equimolar choline or Li, Ca influx and tonic tension were markedly enhanced (7–11). Elevation of $[Na]_i$ by ouabain exposure markedly augmented Ca uptake by cardiac myocytes (12–14). In other studies, Ca efflux was shown to decrease when Na-Ca exchange was inhibited by exposure of cardiac muscle to Na- and Ca-free medium (1, 15), suggesting a role of Na-Ca exchange in the maintenance of low $[Ca]_i$ by Ca extrusion. Others, however, found no evidence of a reduction in Ca efflux rate in cultured myocytes upon exposure to low Na_o medium (8, 10). Thus, although these and other related studies demonstrate the capability of Na-Ca exchange to be bidirectional, its physiologic role under conditions in which [Na] or [Ca] is not altered by artificial methods is not clear. Uncertainties regarding the stoichiometry and electrogenicity of Na-Ca exchange (16) add to the difficulty of defining the physiologic function of Na-Ca exchange.

Recently, dichlorobenzamil, an analog of amiloride, was reported to inhibit Na-dependent Ca uptake by sarcolemmal vesicles prepared from guinea pig ventricular muscle (17). In electrically stimulated isolated guinea pig papillary muscles, dichlorobenzamil produced a concentration-dependent decrease in developed tension. These findings led Siegl *et al.* (17) to conclude that Ca entry via Na-Ca exchange occurs under normal conditions. However, whether the negative inotropic action of dichlorobenzamil is related solely to inhibition of Na-Ca exchange is not clear, since it is not known whether the compound also affects other ion channels or transport systems that influence contractile state. To examine further the pharmacological specificity of dichlorobenzamil, in the studies reported here we examined the effect of dichlorobenzamil on 1) myocyte contractile state, 2) Ca uptake via Na-Ca exchange in intact cells, 3) Ca influx via slow Ca channels, 4) Ca efflux in the presence or absence of Na_o, 5) monovalent cation transport

ABBREVIATIONS: HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,,*N*′,*N*′-tetraacetic acid.

rate, and 6) cellular Ca and Na content. In particular, we attempted to establish conditions in which dichlorobenzamil exerts an effect on contractile state that can be attributed mainly or entirely to inhibition of Na-Ca exchange.

Materials and Methods

Tissue culture. Monolayer cultures of spontaneously contracting chick embryo ventricular cells were prepared as previously described (10). Briefly, hearts of 10-day-old chick embryos were removed and placed in Ca^{2+}- and Mg^{2+}-free Hanks' solution (Gibco Laboratories, Grand Island, NY). Ventricular tissue was cut into small fragments (less than 0.5mm^3), and individual cells were isolated by trypsinization at 37°. Cell suspensions were placed into 10 ml of cold trypsin inhibitor solution (50% heat-inactivated fetal calf serum and 50% Ca^{2+}- and Mg^{2+}-free Hanks' solution). This cell suspension was centrifuged at 400 × *g* for 10 min. The pellet was resuspended in culture medium containing 6% heat-inactivated fetal calf serum, 40% Medium 199 with Hanks' salts, 0.1% penicillin-streptomycin solution, and 54% balanced salt solution. Balanced salt solution contained 116 mM NaCl, 1.0 mM NaH_2PO_4, 0.8 mM $MgSO_4 \cdot H_2O$, 1.18 mM KCl, 26.2 mM $NaHCO_3$, 0.87 mM $CaCl_2$, and 5.5 mM glucose. The final concentrations of K^+, Na^+, and Ca^{2+} in culture media were 4.0 mM, 137 mM, and 0.97 mM, respectively. The cell suspension was diluted to 5 × 10^5 cells/ml and placed in plastic culture dishes (100 × 20 mm, Falcon) containing 25-mm circular glass coverslips. Cultures were incubated in a humidified 5% CO_2, 95% air atmosphere at 37°. Confluent monolayers in which an estimated 80% of cells exhibited spontaneous synchronous contractions developed by 3 days of incubation.

Contractility measurements. Changes in the contractile state of individual cells in the monolayers were assessed by the use of an optical-video system as previously described (10). A glass coverslip with attached monolayer of ventricular cells was continuously superfused in a chamber on the stage of an inverted phase contrast microscope with HEPES-buffered solution (pH 7.35) containing 5 mM HEPES, 0.9 mM $CaCl_2$, 4.0 mM KCl, 140 mM NaCl, and 0.5 mM $MgCl_2$ at a rate of 2 ml/min. A constant temperature of 37° was maintained by enclosing the microscope in a thermostated Lucite box. Following a 15-min equilibration period, cells were superfused with the desired solution. Changes in the amplitude of motion were monitored for one cell from each coverslip.

^{45}Ca, ^{42}K, and ^{24}Na fluxes and contents. For measurement of ^{45}Ca or ^{42}K uptake (10), monolayers of cells attached to glass coverslips were preincubated in the HEPES-buffered solution (see above) (pH 7.35) at 37° for 5 min and subsequently incubated in the same medium containing tracer amounts of ^{45}Ca (5 μCi/ml) or ^{42}K (5 μCi/ml) for desired periods of time. Coverslips were removed from the holder in the incubation bath and were immediately washed for 8 sec each in two 80-ml volumes of HEPES-buffered solution at 2–4°. To determine the effect of isoproterenol on ^{45}Ca uptake, cells were pretreated in Ca-free HEPES-buffered medium (0.1 mM EGTA) for 5 min and then incubated in HEPES-buffered medium containing 0.9 mM Ca and ^{45}Ca (18). Cells were then scraped off the coverslips and dissolved for 2 hr in 2 ml of solution containing 1% sodium dodecyl sulfate and 10 mM sodium borate. Aliquots of solution containing dissolved cells were assayed for radioactivity and protein content. For determination of cellular Ca content, cells were incubated in HEPES-buffered medium containing ^{45}Ca to steady state for 2 hr before washing as described above. For Ca efflux studies, cells were loaded to steady state with ^{45}Ca (2 hr) and subsequently exposed to medium with no radiolabeled ^{45}Ca for desired periods of time. ^{45}Ca remaining in the cells was then determined.

For measurements of cellular Na content, coverslips were incubated in HEPES-buffered solution (pH 7.35) containing tracer amounts of ^{24}Na (20 μCi/ml) for 30 min, at which time ^{24}Na content had reached equilibrium. Coverslips were washed in the manner described above and cellular content of ^{24}Na was determined.

Cell density correction. To normalize for cell density on each coverslip, the monolayers were grown in L[4,5-^{3}H(N)]leucine (0.1 μCi/ml) for 24 hr before each experiment. ^{3}H counts incorporated into protein permitted estimation of cell density on each coverslip, since the relationship between radioactive counts and protein concentration allowed accurate estimation of protein concentration for each coverslip. The simultaneous counting of ^{3}H and ^{45}Ca, ^{42}K, or ^{24}Na permitted normalization of ^{45}Ca, ^{42}K, or ^{24}Na content per mg of cell protein.

Miscellaneous. Protein content was assayed by the method of Lowry *et al.* (19) using crystalline bovine serum albumin as standard. $^{45}CaCl_2$, ^{42}KCl and ^{24}NaCl were purchased from New England Nuclear, Boston, MA. Dichlorobenzamil was the generous gift of Dr. P. K. S. Siegl of Merck, Sharp and Dohme. Nifedipine was a gift from Pfizer Laboratories and verapamil was a gift from G. D. Searle. Statistical analyses were performed using Student's *t* test and two-way analysis of variance.

Results

Contractility measurements. We studied the effect of dichlorobenzamil on the contractile state of spontaneously beating (110–130 beats/min) monolayers of cultured chick ventricular cells. Cells were superfused with concentrations of dichlorobenzamil ranging from 10^{-7} to 10^{-4} M, and changes in the amplitude of cell motion were measured. As shown in Fig. 1, dichlorobenzamil produced a concentration-dependent decrease in the amplitude of cell motion. At all concentrations of dichlorobenzamil studied, the amplitude of cell motion reached a new steady state level within 15 min with no significant change in beating rate up to the point where cell motion was no longer discernible. The negative inotropic action of dichlorobenzamil was completely reversible after washout periods not exceeding 30 min. Analysis of data in Fig. 1 (*inset*) using a log-logit plot indicated that the half-maximal concentration for the negative inotropic effect was 5 × 10^{-7} M.

Calcium fluxes. Since dichlorobenzamil has been reported to inhibit Na-Ca exchange in guinea pig heart sarcolemmal vesicles (17), we examined whether the negative inotropic effect of dichlorobenzamil was due to increased Ca influx via Na-Ca exchange in cultured chick ventricular cells. Cells were preincubated in normal HEPES-buffered medium with or without graded concentrations of dichlorobenzamil (5 × 10^{-7} to 5 ×

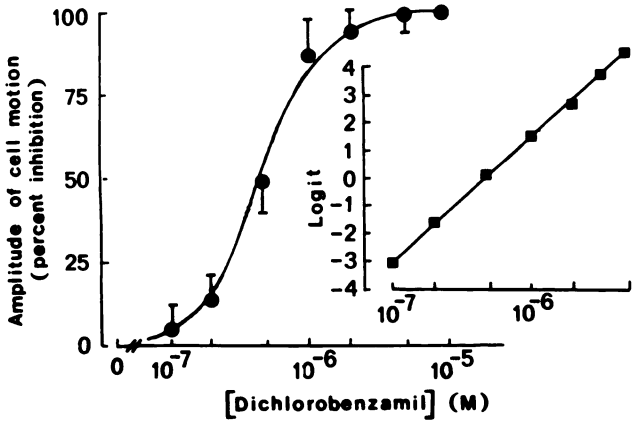

Fig. 1. Effect of dichlorobenzamil on contractile state. Monolayers of cultured chick embryo ventricular cells were superfused with HEPES-buffered medium (pH 7.4) for 10 min and then with medium containing dichlorobenzamil. Changes in the amplitude of cell motion were then monitored using an optical-video system. Each *point* represents the mean ± standard error of six determinations. The *inset* shows the log-logit plot of the concentration-effective curve. The EC_{50} is approximtely 5 × 10^{-7} M.

10^{-5} M) for 10 min and then incubated in Na-free medium containing 140 mM choline, 0.9 mM Ca, and tracer amounts of ^{45}Ca. Verapamil (10^{-6} M) was present in all media to completely inhibit Ca influx via slow Ca channels. Verapamil alone inhibited spontaneous contractile activity. Dichlorobenzamil produced a concentration-dependent decrease in ^{45}Ca uptake rate with an EC_{50} of approximately 6×10^{-7} M.

To explore further the effect of dichlorobenzamil on Na_i-dependent Ca uptake, cells were first loaded with Na by preincubation in medium containing 1 mM ouabain. This procedure increased cellular Na content from 56 ± 4 to 154 ± 12 nmol/mg of protein. Cells were then incubated in Na-free medium containing 140 mM choline, 0.9 mM Ca, and ^{45}Ca. Verapamil (10^{-6} M) was also added to all media to block Ca influx via slow Ca channels. Compared to ^{45}Ca uptake by non Na-loaded cells, ^{45}Ca uptake by Na-loaded cells was approximately 10-fold greater. Addition of dichlorobenzamil to preincubation and uptake medium resulted in a concentration-dependent decrease in ^{45}Ca uptake rate (Fig. 2). Under these conditions, the half-maximal inhibition of the ^{45}Ca uptake rate, with the effect of 5×10^{-5} M dichlorobenzamil taken as maximum, occurred at 6×10^{-7} M dichlorobenzamil (Fig. 3). Thus, the EC_{50} values of dichlorobenzamil for reduction of amplitude of cell motion and inhibition of Ca uptake via Na-Ca exchange were similar. These results suggest that the negative inotropic effect of dichlorobenzamil is associated with and, quite possibly, caused in large part by inhibition of Ca influx via Na-Ca exchange.

Interaction with ouabain. To test further the inhibitory action of dichlorobenzamil on Na-Ca exchange, we examined the effect of the drug on the development of ouabain-induced arrhythmias and contracture. Since ouabain augments $[Na]_i$ and hence $[Ca]_i$ via Ca Na-Ca exchange (13, 14), a Na-Ca exchange inhibitor should blunt the ouabain-induced increase in $[Ca]_i$ via Na-Ca exchange and, consequently, the effect of ouabain on the contractile state. As shown in Fig. 4, ouabain (1 mM) alone produced an initial increase in the amplitude of cell motion followed soon by onset of contracture. When cells were exposed to 1 mM ouabain and 5×10^{-5} M dichlorobenzamil together, no positive inotropic effect and no contracture were observed. Instead, a decrease in the amplitude of cell motion occurred with no displacement of the end-diastolic position. When cells were exposed first to dichlorobenzamil, subsequent addition of 1 mM ouabain failed to elicit a positive inotropic effect on contracture. A concentration of dichlorobenzamil (5×10^{-7} M) that alone produced a substantial negative inotropic effect protected the cells from developing toxic rhythm disturbances in response to 4×10^{-6} M ouabain, a glycoside concentration that by itself produced rhythm disturbances and subsequent contracture. These results indicate that dichlorobenzamil exerts a negative inotropic effect in cultured chick heart cells and antagonizes the toxic effects of ouabain, i.e., the development of rhythm disturbances and contracture.

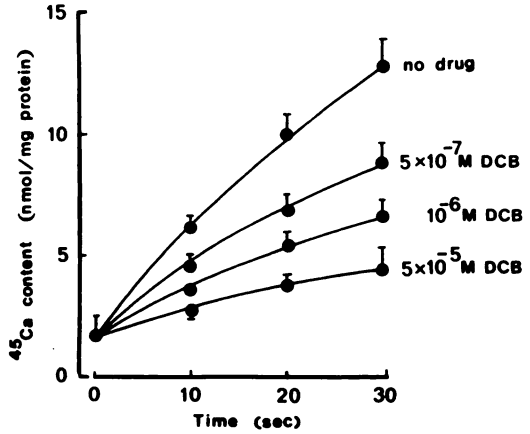

Fig. 2. Effect of dichlorobenzamil on Na_i-dependent Ca uptake. Cells were loaded with Na by preincubation in medium containing 1 mM ouabain for 15 min and then exposed to medium containing ouabain, dichlorobenzamil (*DCB*), ^{45}Ca, and no Na (Na replaced with choline). ^{45}Ca contents were determined after specified exposure intervals. Each *point* is the mean ± standard error of seven determinations. All values are significantly different from each other ($p < 0.05$).

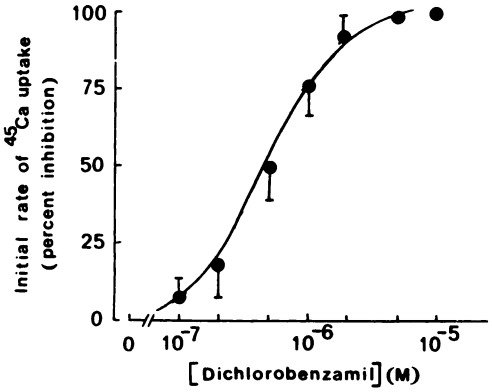

Fig. 3. Concentration-dependent effect of dichlorobenzamil on Ca uptake. The initial rates (0–20 sec) of ^{45}Ca uptake were calculated from the experiment shown in Fig. 2 and plotted against dichlorobenzamil concentrations. EC_{50} is approximately 6×10^{-7} M. Each *point* is the mean ± standard error of three determinations. Maximal inhibition of Ca uptake was produced by 5×10^{-5} M dichlorobenzamil and was taken as 100% inhibition.

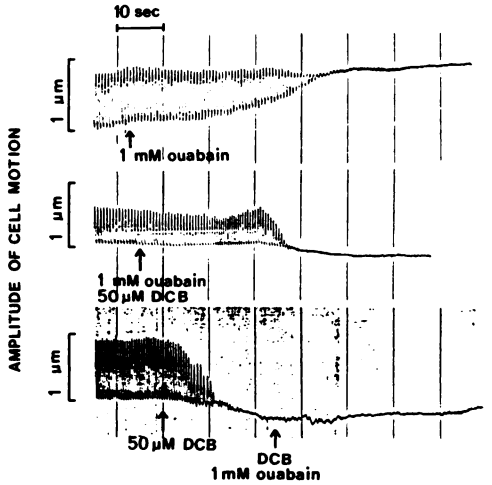

Fig. 4. Effects of ouabain and dichlorobenzamil (*DCB*) on contractile state. Changes in the amplitude of cell motion in response to 1 mM ouabain with or without 5×10^{-5} M dichlorobenzamil are shown in typical tracings from five experiments.

Slow Ca channels. Inhibition of Ca influx via slow Ca channels by Ca channel antagonists also reduces the amplitude of cell motion in cultured chick heart cells (10). To examine the possibility that inhibition of Ca influx via slow Ca channels may contribute to the negative inotropic action of dichlorobenzamil, we studied the effect of this compound on the isoproterenol-induced increase in Ca uptake. We have shown previously that isoproterenol enhances Ca uptake via slow Ca channels in

cultured chick heart cells.[1] Under similar experimental conditions, 10^{-7} M isoproterenol increased significantly the rate of ^{45}Ca uptake by a pathway that was completely blocked by 10^{-6} M nifedipine or 10^{-6} M verapamil. Addition of 6×10^{-7} M dichlorobenzamil resulted in a significant diminution of the isoproterenol-induced enhancement of Ca uptake (Fig. 5). Dichlorobenzamil at 5×10^{-5} M caused maximal inhibition of the isoproterenol-induced increase in Ca uptake similar to the effects of high concentrations of verapamil or nifedipine. These results are consistent with the view that dichlorobenzamil causes an inhibition of Ca influx via slow Ca channels at a concentration close to the EC_{50} for decreasing the amplitude of cell motion.

Sarcolemmal Ca pump. The two major pathways for Ca efflux from myocardial cells are believed to be the sarcolemmal ATP-dependent Ca pump and Na-Ca exchange (20). If Ca efflux occurs in part via Na-Ca exchange, an inhibitor of this exchange would be expected to reduce the rate of Ca efflux. Accordingly, we examined the effect of this drug on Ca efflux in cultured chick heart cells. Cells were equilibrated in medium containing ^{45}Ca for 2 hr and then exposed to medium with the same total Ca concentration (0.9 mM), but without ^{45}Ca, in the presence or absence of dichlorobenzamil. In Fig. 6, ^{45}Ca content remaining in the cells following various efflux periods is plotted as a percentage of the initial Ca content. Dichlorobenzamil treatment diminished the rate of Ca efflux in a concentration-dependent fashion (Fig. 6; EC_{50} approximately 10^{-6} M). At 5×10^{-5} M dichlorobenzamil, 88% of the ^{45}Ca still remained in the cells after a 30-sec efflux period compared to 50% in control cells.

To determine whether the dichlorobenzamil-induced change in Ca efflux is due to reduced Ca efflux via Na-Ca exchange or via the sarcolemmal Ca pump, the ^{45}Ca efflux experiment was repeated with Na-free efflux medium. Since Na-free medium effectively precludes Ca efflux via Na-Ca exchange, the observed Ca efflux under this condition is assumed to occur via

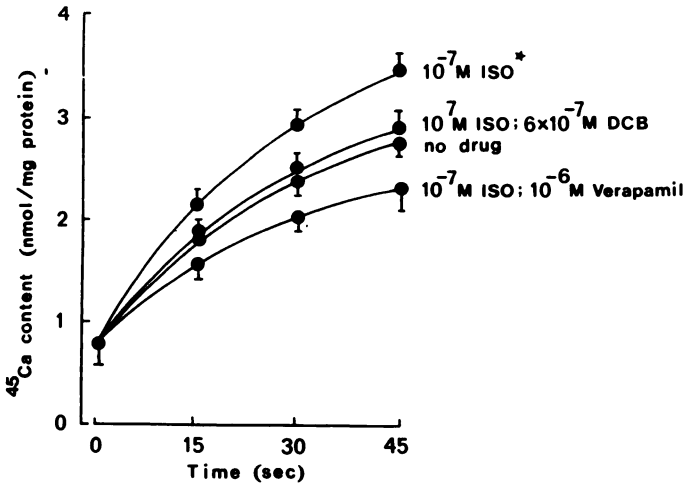

Fig. 5. Effect of dichlorobenzamil (*DCB*) and verapamil on isoproterenol (*ISO*)-induced increase in Ca uptake. Cells were preincubated in Ca-free medium (+0.1 mM EGTA) for 5 min and then incubated in normal medium (0.9 mM Ca) with ^{45}Ca. Dichlorobenzamil or verapamil was added to preincubation and uptake media. Each *point* is the mean ± standard error of eight determinations. ★, significantly greater than all three other curves ($p < 0.05$).

[1] D. Kim, unpublished observations.

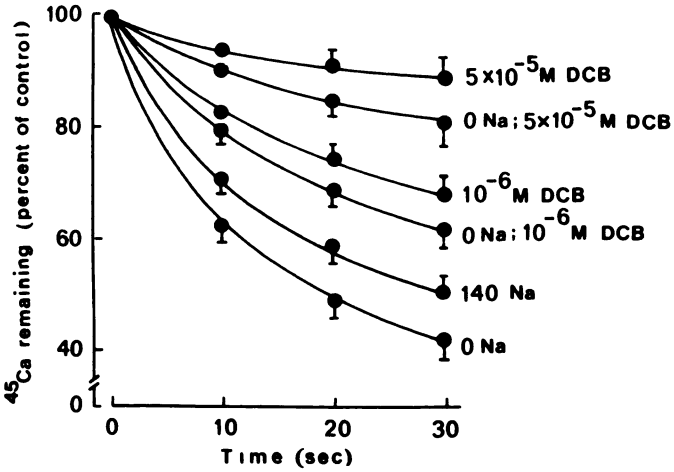

Fig. 6. Effect of dichlorobenzamil (*DCB*) on Ca efflux. Cells were incubated with ^{45}Ca to steady state (2 hr) and then incubated in medium with or without dichlorobenzamil for an additional 10 min. Cells were then exposed to efflux medium with or without dichlorobenzamil, or to efflux medium with no Na (Na replaced with choline) with or without dichlorobenzamil. ^{45}Ca content in cells was then assayed and expressed as a percentage of the ^{45}Ca content observed prior to efflux. Each *point* is the mean ± standard error of eight determinations. All *curves* are significantly different from each other ($p < 0.05$).

the ATP-dependent Ca pump. As shown in Fig. 6, choline substitution of Na in the efflux medium enhanced the rate of ^{45}Ca efflux, as has been demonstrated previously (21). Further addition of dichlorobenzamil, however, resulted in a concentration-dependent inhibition of Ca efflux ($EC_{50} = 10^{-6}$ M) similar to that observed in Na-containing medium. At 5×10^{-5} M, dichlorobenzamil markedly inhibited Ca efflux such that only small decreases in ^{45}Ca content were observed. These results are consistent with the view that, in cultured chick heart cells, dichlorobenzamil at higher concentrations is an effective inhibitor of Ca efflux via the sarcolemmal Ca pump.

Cellular Ca content. To determine the effect of dichlorobenzamil on net Ca flux, we examined the effect of the drug on cellular ^{45}Ca content. Cells were incubated in medium containing ^{45}Ca (total $[Ca]_o$ = 0.9 mM) for 2 hr to steady state and then were further incubated for an additional 10 min in medium containing the same specific activity of ^{45}Ca and, also, graded concentrations of dichlorobenzamil. At relatively low concentrations (5×10^{-7} M), dichlorobenzamil did not alter measurably the cellular ^{45}Ca content, whereas at a higher concentration (2×10^{-5} M) it produced a small decrease in cellular ^{45}Ca content from a mean of 4.25 ± 0.19 to 3.76 ± 0.21 nmol/mg of protein (±SE; $p < 0.05$). Thus, the inhibitory effects of dichlorobenzamil (2×10^{-5} M) on Ca fluxes across the sarcolemmal membrane are accompanied by a small decrease in cellular pool size.

Cellular Na content. Since Ca uptake via Na-Ca exchange is Na_i dependent, as demonstrated in Fig. 4, it is necessary to exclude the possibility that dichlorobenzamil reduced Ca uptake via Na-Ca exchange as a consequence of a primary effect on $[Na]_i$. Therefore, we examined the effect of dichlorobenzamil on cellular Na content in the presence or absence of ouabain. Cells were incubated to equilibrium in ^{24}Na-containing medium with no drug, with dichlorobenzamil in graded concentrations (5×10^{-7} to 5×10^{-5} M), with 1 mM ouabain, or with both dichlorobenzamil and ouabain for 30 min. As expected, 1 mM ouabain increased cellular Na content substantially. Dichlorobenzamil also increased cellular Na content in a concentration-

dependent manner (Fig. 7). Thus, the inhibitory effect of dichlorobenzamil on Ca uptake via Na-Ca exchange could not be accounted for by altered cellular Na content since dichlorobenzamil reduced Ca uptake via Na-Ca exchange, despite substantial increases in cellular Na content that would favor Ca influx. This raises the possibility that we may be underestimating the true potency of the drug as an Na-Ca exchange inhibitor.

^{42}K fluxes. The observed increase in cellular Na content produced by dichlorobenzamil suggests that the drug may have an inhibitory effect on the sodium pump in the cell membrane. To test this hypothesis, we determined the effect of dichlorobenzamil on the rate of ^{42}K uptake in the presence and absence of 1 mM ouabain. Cells were preincubated in medium containing concentrations of dichlorobenzamil ranging from 5×10^{-7} to 5×10^{-5} M for 10 min and were then further incubated in medium containing dichlorobenzamil and ^{42}K in the presence or absence of 1 mM ouabain. As shown in Fig. 8, dichlorobenzamil produced a concentration-dependent decrease in the rate of ^{42}K uptake ($EC_{50} = 2 \times 10^{-6}$ M). A half-maximal concentration for the negative inotropic effect of dichlorobenzamil, 5×10^{-7} M, reduced the ouabain-sensitive ^{42}K uptake rate by approximately 12% ($p < 0.05$). The effect of 5×10^{-5} M dichlorobenzamil on ^{42}K uptake was close to that of 1 mM ouabain, indicating that this concentration of dichlorobenzamil caused a near-maximal inhibition of the rate of ouabain-sensitive ^{42}K uptake. At 5×10^{-5} M, dichlorobenzamil also reduced significantly the ouabain-insensitive ^{42}K uptake. These results demonstrate that dichlorobenzamil is capable of inhibiting the Na-K pump in intact cultured chick heart cells.

Discussion

We investigated in the present study the pharmacological properties of a putative Na-Ca exchange inhibitor, dichlorobenzamil, using spontaneously contracting cultured chick ventricular cells. Although Na-Ca exchange has been proposed to play an important role in regulation of intracellular Ca concentration, the physiologic role of Na-Ca exchange in the heart is still not well known. It appears that the exchange can be bidirectional under appropriate experimental conditions. For example,

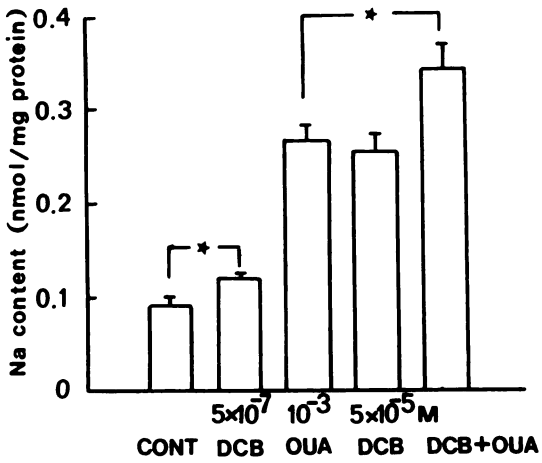

Fig. 7. Effect of dichlorobenzamil (*DCB*) and ouabain (*OUA*) on cellular Na content. Cells were incubated in medium containing ^{24}Na and no drug, dichlorobenzamil, ouabain, or both drugs (5×10^{-5} M DCB + 10^{-3} M OUA) to steady state (30 min) and cellular Na contents were determined. Each *bar* represents the mean ± standard error of 10 determinations. ★, significantly different ($p < 0.05$).

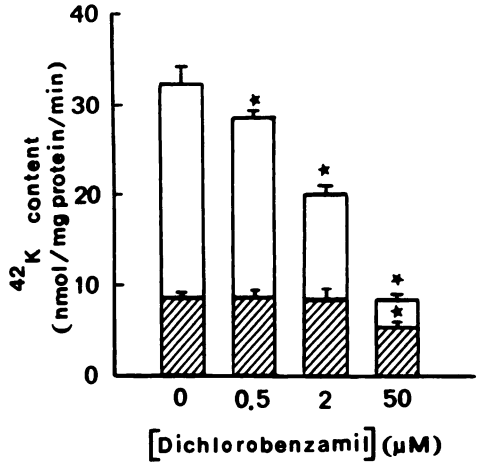

Fig. 8. Effect of dichlorobenzamil and ouabain on ^{42}K uptake. Cells were preincubated in medium containing no drug, dichlorobenzamil, ouabain, or dichlorobenzamil plus ouabain for 10 min and then incubated in medium containing identical combinations of drugs and ^{42}K. *Whole bars* represent total ^{42}K uptake. □, ouabain-sensitive ^{42}K uptake; ▨, ouabain-insensitive ^{42}K uptake. Each *bar* represents the mean ± standard error of seven determinations. ★, significantly lower than the corresponding control values observed in the absence of drugs ($p < 0.05$).

Glitsch *et al.* (6) demonstrated that an increase in $[Na^+]_i$ resulted in an enhancement of Ca influx in isolated guinea pig atrial muscle. Langer *et al.* (8) showed that, in cultured neonatal rat heart cells, reduction of $[Na]_o$ was associated with a gain in Ca content. It was also found that Ca efflux via Na-Ca exchange can occur when $[Ca]_i$ is raised and can be prevented when the exchanger is inhibited by removal of Na_o and Ca_o (1, 15). Whether the net flux of Ca via Na-Ca exchange under physiologic conditions is inward or outward is uncertain and may vary with tissue source and experimental conditions. A specific inhibitor of Na-Ca exchange would be of considerable potential usefulness. We have therefore examined the effect of dichlorobenzamil, which has been shown to inhibit Na_i-dependent Ca uptake in sarcolemmal vesicles prepared from guinea pig heart (17), on contractile state and Ca fluxes in intact cultured chick ventricular cells.

In cultured chick ventricular cells, dichlorobenzamil produced a concentration-dependent decrease in the amplitude of cell motion with an EC_{50} of 5×10^{-7} M and concentration-dependent decrease in Na^+_i-dependent Ca uptake with a similar EC_{50} of 6×10^{-7} M. These results suggest that the negative inotropic effect of dichlorobenzamil may be due primarily to its inhibitory effect on Na-Ca exchange. In guinea pig papillary muscle, Siegl *et al.* (17) observed a similar relationship between the dichlorobenzamil-induced negative inotropic effect and inhibition of Ca uptake by sarcolemmal vesicles from the same source. In contrast to our finding that concentrations above 10^{-6} M completely inhibited spontaneous contractile activity of cultured chick heart cells, Siegl *et al.* (17) found that, in guinea pig heart, high concentrations of dichlorobenzamil (40–100 μM) produced smaller negative inotropic effects than lower concentrations (10–20 μM). This biphasic response was attributed to the inhibitory effect of high concentrations of dichlorobenzamil on sarcolemmal Ca-ATPase, resulting in decreased Ca extrusion and, consequently, increased intracellular Ca content.

To test further the ability of dichlorobenzamil to inhibit Na-Ca exchange, we examined the effect of the drug on the development of ouabain-induced rhythm disturbances and contrac-

ture. Ouabain has been shown to augment $[Ca]_i$ via Na-Ca exchange as a consequence of sodium pump inhibition (13, 14, 22). Therefore, an Na-Ca exchange inhibitor should antagonize the effect of ouabain. Our results, that dichlorobenzamil abolished the rhythm disturbances or contracture produced by 4×10^{-6} M or 10^{-3} M ouabain, respectively, support the view that dichlorobenzamil is an inhibitor of Na-Ca exchange. These results are in agreement with those of Seigl *et al.* (17), who reported that dichlorobenzamil caused significant inhibition of the positive inotropic effect of veratridine, which also augments $[Ca]_i$ via Na-Ca exchange (23). Bush *et al.* (24) also observed that dichlorobenzamil suppressed ouabain-induced arrhythmias in isolated cat papillary muscles or in intact anesthetized dogs. The preliminary findings of Hume *et al.* (25), that in single frog atrial myocytes dichlorobenzamil inhibited "creep-current" thought to occur as a result of electrogenic Na-Ca exchange, is also consistent with the above results.

In order to relate the decrease in amplitude of cell motion to inhibition of Ca uptake via Na-Ca exchange, involvement of Ca uptake via slow Ca channels must be assessed as well. To test the effect of dichlorobenzamil on slow Ca channel permeability, we determined the effect of the compound on isoproterenol-induced augmentation of Ca uptake. A concentration of dichlorobenzamil that reduced the amplitude of cell motion by 50% caused approximately 35% inhibition of the isoproterenol-induced increase in Ca uptake, indicating that dichlorobenzamil reduced slow Ca channel permeability. Since a decrease in Ca influx via the slow Ca channel would be expected to cause a negative inotropic effect (26), a significant part of dichlorobenzamil's negative inotropic action at concentrations of 5×10^{-7} M and above appears to be due to partial blockade of Ca influx via the slow Ca channel.

The inhibitory effect of dichlorobenzamil on Ca uptake via Na-Ca exchange could in principle be due to a reduction in $[Na]_i$ rather than a direct action on the exchanger, since Ca uptake via Na-Ca exchange is dependent on $[Na]_i$. For example, Frelin *et al.* (27) concluded that amiloride decreased Ca uptake via Na-Ca exchange as a consequence of its effect on intracellular Na concentration, rather than a direct action on Na-Ca exchange. In our experiments, however, dichlorobenzamil actually increased cellular Na content. The effect of dichlorobenzamil does not appear to be on Na-H exchange alone, since inhibition of this exchanger would lower $[Na]_i$ (28). Thus, under our experimental conditions, the inhibition by dichlorobenzamil of Na-Ca exchange appears to be due to a direct action on the exchanger.

The marked enhancing effect of dichlorobenzamil on cellular Na content indicates that Na entry must be augmented or that Na efflux pathway(s) must be blocked by the drug. The best characterized Na efflux pathway is the NaK-ATPase (Na-K pump) in the sarcolemmal membrane; we therefore examined ouabain-sensitive ^{42}K uptake as an indicator of Na-K pump activity (29). Dichlorobenzamil was similar in potency to ouabain in inhibiting monovalent cation active transport (EC_{50}: 4×10^{-6} M for ouabain and 2×10^{-6} M for dichlorobenzamil). Addition of dichlorobenzamil to 1 mM ouabain, which alone caused a maximal inhibition of the Na-K pump and increased cellular Na content by approximately 3-fold, produced a further significant elevation of cellular Na content to 3.8-fold of the control level. We speculate that the difference in cellular Na content is due to additional inhibition by dichlorobenzamil of Na efflux via Na-Ca exchange. Coupled exchange of Na and Ca requires that influx of Ca occur simultaneously with efflux of Na (30, 31). Indeed, Bridge *et al.* (31) have shown that Na efflux is stimulated by elevation of $[Ca]_o$. The similar effects of 5×10^{-5} M dichlorobenzamil and 1 mM ouabain on cellular Na content support this view since 5×10^{-5} M dichlorobenzamil alone did not fully inhibit Na pump activity and would be expected, if there were no effect on Na-Ca exchange, to raise cellular Na content to a level lower than that produced by 1 mM ouabain, which causes complete inhibition of Na pump activity.

In addition to inhibition of ion transport across the sarcolemmal membrane, dichlorobenzamil may also affect Ca transport involving sarcoplasmic reticulum and mitochondria since the drug may accumulate within the cell (17). Indeed, dichlorobenzamil has been reported to inhibit paired pulse-induced increases in tension in guinea pig papillary muscle, suggesting an effect on sarcoplasmic reticulum Ca release and/or Ca sequestration (17). Thus, the negative inotropic action of dichlorobenzamil is probably the sum of the effects of the drug on Ca transport across the sarcolemmal membrane as well as within the cell.

In summary, in cultured chick heart cells, dichlorobenzamil produced concentration-dependent decreases in the amplitude of cell motion, in Ca influx via Na-Ca exchange and slow Ca channels, and in Ca efflux via the sarcolemmal Ca pump. Dichlorobenzamil also inhibited sodium pump activity and elevated cellular Na content. Comparison of the concentration-dependent effects indicates that the negative inotropic action of the drug is due primarily to inhibition of Ca influx via both Na-Ca exchange and slow Ca channels. Due to the multiple sites of action of dichlorobenzamil, however, this compound does not uniquely determine the physiologic role of Na-Ca exchange in excitation-contraction coupling in cultured chick heart cells.

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