

# Inhibition of Heart Calcium and Chloride Currents by Sodium Iodide

## *Specific Attenuation in cAMP-dependent Protein Kinase-mediated Regulation*

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**ABSTRACT** The enzymes cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) regulate the activity of cardiac ion channel proteins. In this study the whole-cell arrangement of the patch clamp technique was used to examine the effect of NaI on PKA-stimulated  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  channels in isolated guinea pig ventricular myocytes.  $\text{Cl}^-$  currents ( $I_{\text{Cl}}$ ) activated either by the  $\beta$ -adrenergic agonist isoproterenol or the membrane-soluble cAMP analogue, 8-chlorophenylthio (8-CPT) cAMP, were greatly reduced in amplitude after substitution of an external solution containing 140 mM NaCl with a solution containing 140 mM NaI. This reduction was accompanied by a shift of  $-7$  mV in the reversal potential ( $E_{\text{rev}}$ ) for  $I_{\text{Cl}}$  and could be reversed upon return to the NaCl external solution. Inhibition of  $I_{\text{Cl}}$  by NaI occurred in a concentration-dependent manner and was more pronounced for inward  $I_{\text{Cl}}$  ( $\text{IC}_{50} = 19$  mM at  $-60$  mV) than for outward  $I_{\text{Cl}}$  ( $\text{IC}_{50} = 60$  mM at  $+60$  mV). In contrast to  $I_{\text{Cl}}$  activated by PKA,  $I_{\text{Cl}}$  activated by PKC was slightly augmented in the presence of NaI and the  $E_{\text{rev}}$  was found to shift by  $-15$  mV. Based on these data, the relative permeability of  $\text{I}^-$  to  $\text{Cl}^-$  ( $P_{\text{I}}/P_{\text{Cl}}$ ) for this channel was calculated to be 1.79. NaI produced no change in the amplitude of inward calcium currents ( $I_{\text{Ca}}$ ) recorded under basal conditions, but strongly inhibited  $I_{\text{Ca}}$  augmented by isoproterenol and 8-CPT cAMP, and during dialysis of cells with the catalytic subunit of PKA (CS). The *in vitro* incorporation of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  into histone IIA and Kemptide, measured in the presence of PKA and cAMP, was not significantly different in assay mixtures containing salts of  $\text{Cl}^-$  and  $\text{I}^-$ . However, the ability of isoproterenol to augment basal  $I_{\text{Ca}}$  in whole-cell experiments was attenuated when experiments were carried out entirely in NaI external solution. Thus, the reduction in  $I_{\text{Cl}}$  and  $I_{\text{Ca}}$  observed in this study may result from a direct effect of  $\text{I}^-$  on the phosphorylation/dephosphorylation of cardiac ion channel proteins or associated regulatory proteins.

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## INTRODUCTION

The enzymes cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) regulate the activity of a large and diverse group of cellular proteins including ion channel proteins present in many excitable and nonexcitable tissues (for reviews see Levitan, 1985; Hartzell, 1988; Tsien, 1988; Shearman, Sekiguchi, and Nishizuka, 1989). In cardiac ventricular cells, stimulation of PKA by the neurohormone norepinephrine and the  $\beta$ -adrenergic receptor agonist isoproterenol modulates a number of ion channels that regulate the action potential plateau: the L-type  $\text{Ca}^{2+}$  channel (Brum, Osterrieder, and Trautwein, 1984; Cachelin, de Peyer, Kokubun, and Reuter, 1984; Kameyama, Hofmann, and Trautwein, 1985), a  $\text{Cl}^-$  channel (Bahinski, Nairn, Greengard, and Gadsby, 1989; Harvey and Hume, 1989a; Ehara and Ishihara, 1990; Matsuoka, Tsuguhisa, and Noma, 1990) and the delayed rectifier  $\text{K}^+$  channel (Bennett and Begenisich, 1987; Harvey and Hume, 1989b; Walsh, Begenisich, and Kass, 1989; Yazawa and Kameyama, 1990). Stimulation of PKC by phorbol ester compounds also increases the activity of delayed rectifier  $\text{K}^+$  channels (Tohse, Kameyama, and Irisawa, 1987; Walsh and Kass, 1988, 1991; Tohse, Kameyama, Sekiguchi, Shearman, and Kanno, 1990) and  $\text{Cl}^-$  channels (Walsh, 1991; Zhang, Barrington, and Ten Eick, 1992) in adult heart cells and  $\text{Ca}^{2+}$  channels in neonatal myocytes (Dosemeci, Dhallan, Cohen, Lederer, and Rogers, 1988; Lacerda, Rampe, and Brown, 1988). Numerous studies have suggested that regulation of  $\text{Ca}^{2+}$  channels by PKA and PKC occurs subsequent to a direct phosphorylation of the  $\alpha$  subunit of this protein (Curtis and Catterall, 1984; Hosey, Borsotto, and Campbell, 1986; Lai, Seagar, Takahashi, and Catterall, 1990; Chang, Gutierrez, Mundina-Weilenmann, and Hosey, 1991). In addition, amino acid sequence analysis of  $\text{Na}^+$  and  $\text{K}^+$  channels has revealed consensus sites for PKA- and PKC-dependent phosphorylation (Noda, Ikeda, Kayano, Suzuki, Takeshima, Kurasaki, Takahashi, and Numa, 1986; Tempel, Papazian, Schwarz, Jan, and Jan, 1987; Folander, Smith, Antanavage, Bennett, Stein, and Swanson, 1990).

The initial goal of this study was to characterize the anion permeability of PKA- and PKC-activated  $\text{Cl}^-$  currents ( $I_{\text{Cl}}$ ) in isolated guinea pig ventricular myocytes. Earlier results, indicating differences in the current-voltage ( $I$ - $V$ ) relationship for  $I_{\text{Cl}}$ , suggested that PKA and PKC might regulate different  $\text{Cl}^-$  channel subtypes in these cells (Walsh, 1991). Since  $\text{Cl}^-$  channels in a large number of secretory epithelial cells are more selective for  $\text{I}^-$  when compared with  $\text{Cl}^-$ , our experiments focused on determining the effect of substitution of this anion on  $I_{\text{Cl}}$ .

Some of these results have been reported in preliminary form (Walsh, 1992).

## MATERIALS AND METHODS

*Isolation of Ventricular Cells and Recording Procedures*

An enzymatic dissociation procedure modified from Mitra and Morad (1985) was used to isolate the myocytes. Briefly, hearts were removed from adult guinea pigs (250–400 g), mounted on a Langendorf-type column, and perfused for 10 min with a  $\text{Ca}^{2+}$ -free Tyrode's solution containing collagenase (0.25–0.32 U/ml) (type B; Boehringer Mannheim Corp., Indianapolis, IN) and protease (0.2 mg/ml) (type 14 or 25; Sigma Chemical Co., St. Louis, MO). After 20 min of perfusion with 0.2 mM  $\text{Ca}^{2+}$ -containing Tyrode's, the heart was dissected

into small pieces and single cells were obtained by gentle agitation. Cells were stored at room temperature (22–25°C) in normal Tyrode's solution (see below) and used 1–10 h after isolation.

The patch clamp method of Hamill, Marty, Neher, Sakmann, and Sigworth (1981) was used to record whole-cell ventricular currents using a Warner PC-501 amplifier (Warner Instrument Corp.). Pipettes were made from Gold Seal Accu-fill 90 Micropets (Clay Adams Inc.) and had resistances of 1–3 M $\Omega$  when filled with CsCl/aspartate internal solution. Series resistance compensation (1–2 M $\Omega$ ) was used in some of the  $I_{Ca}$  experiments. Data were sampled at 10 kHz, filtered at 2–5 kHz with a low pass Bessel filter, and stored using a Softek 386 computer.

A reference electrode made from an Ag-AgCl pellet was connected to the bath using an agar salt bridge saturated with Tyrode's solution. Data were adjusted for liquid junction potentials that arose between the pipette solution and bath solution. Liquid junction potential values were between –5 and +5 mV and were not significantly changed by substitution of 140 mM NaI for 140 mM NaCl.

#### *Measurement of $I_{Cl}$ and $I_{Ca}$*

Isolated cells were initially placed in a normal Tyrode's solution consisting of (mM): 132 NaCl, 5 KCl, 2 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 5 dextrose, and 5 HEPES, pH 7.4. After establishment of the whole-cell voltage clamp the solution was changed to a K-free external solution containing (mM): 140 NaCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 5 dextrose, 5 HEPES, 1 mM BaCl<sub>2</sub>, and 10–50  $\mu$ M tetrodotoxin, pH 7.4. In experiments where  $I_{Cl}$  was isolated,  $I_{Ca}$  was eliminated by addition of 200–500 nM nisoldipine (Miles Inc., West Haven, CT) to the external solution. Full substitution of NaI for NaCl was achieved by replacing NaCl with equimolar NaI in the K-free external solution. In the partial NaI substitution experiments, the external NaCl concentration was adjusted to keep the total halide concentration constant. The osmolarity of the NaCl and NaI external solutions was 275–280 mosM.

Patch electrodes were filled with an internal solution consisting of either (mM): 50 CsCl, 60 Cs-aspartate, 2 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 11 EGTA, 5 ATP (K<sup>+</sup> salt), 10 HEPES, and 30 CsOH, pH 7.3, or 50 KCl, 60 K-glutamate, 2 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 11 EGTA, 5 ATP (K<sup>+</sup> salt), 10 HEPES, and 30 KOH, pH 7.3. The ratio of EGTA/CaCl<sub>2</sub> in these solutions sets the free intracellular Ca<sup>2+</sup> concentration to ~10 nM (Fabiato, 1988). The KCl/glutamate solution was used in addition to the more appropriate (i.e., for measuring  $I_{Cl}$  and  $I_{Ca}$ ) CsCl/aspartate solution for two reasons. Use of the KCl/glutamate solution allowed measurement of the delayed rectifier K<sup>+</sup> current ( $I_K$ ) (using a 2–3-s voltage step to +50 mV). Since  $I_K$  is augmented by both PKA and PKC (Walsh and Kass, 1988; Yazawa and Kameyama, 1990), measurement of this current was useful in monitoring cellular protein kinase responsiveness in the  $I_{Cl}$  experiments. Second, it was important to determine whether reduction of  $I_{Cl}$  and  $I_{Ca}$  by NaI occurred under physiological conditions (i.e., with 140 mM K<sup>+</sup>). We found that quantitatively similar results with NaI could be obtained with the two internal solutions.

Ion currents were recorded during 40-ms voltage steps applied to various potentials from a holding potential of –40 mV. The typical recording protocol used in this study consisted of first recording background currents or  $I_{Ca}$  under basal conditions. Cells were then stimulated with isoproterenol or 8-CPT cAMP to activate  $I_{Cl}$  and augment  $I_{Ca}$ . When these currents reached peak amplitudes, cells were then exposed to NaI containing external solution (with isoproterenol or 8-CPT cAMP). It should be noted that no significant rundown in either  $I_{Cl}$  or  $I_{Ca}$  was observed in normal 140 mM NaCl containing external solution with isoproterenol or 8-CPT cAMP when monitored over a 5–10-min time period.

The reversal potential ( $E_{rev}$ ) for  $I_{Cl}$  was defined as the potential where the PKA- or PKC-sensitive background current was zero. In those cases where the exact zero current was not recorded, the  $E_{rev}$  was determined by fitting a straight line through the points on the  $I$ - $V$  curve directly above and below this potential. The relative permeability of I<sup>-</sup> to Cl<sup>-</sup> was determined

using the Goldman-Hodgkin-Katz equation:

$$E_{\text{rev,I}} - E_{\text{rev,Cl}} = RT/zF \ln (P_{\text{I}}[\text{I}^-]/P_{\text{Cl}}[\text{Cl}^-])$$

where  $P_{\text{I}}$  and  $P_{\text{Cl}}$  are the permeabilities,  $R$  is the gas constant,  $T$  is the temperature ( $^{\circ}\text{K}$ ),  $z$  is the valence, and  $F$  is Faraday's constant. Since increases in cationic conductance were not detected in the  $I_{\text{Cl}}$  experiments, permeability to  $\text{Na}^+$  and  $\text{K}^+$  was neglected in determining the permeability ratio for  $\text{I}^-$  to  $\text{Cl}^-$ .

#### *In Vitro Phosphorylation Assay*

The PKA assay was based on that of Roskoski (1983). PKA activity was assessed by the ability of the enzyme to phosphorylate histone II-A (0.5 mg/ml) or Kemptide (80  $\mu\text{g}/\text{ml}$ ) in a reaction mixture containing the PKA holoenzyme (63  $\mu\text{g}/\text{ml}$ ), bovine serum albumin (0.25 mg/ml), 100  $\mu\text{M}$  [ $\gamma\text{-}^{32}\text{P}$ ]ATP (300 cpm/pmol), 10 mM magnesium acetate, and 20 mM Tris buffer (pH 7.4) in the presence or absence of 10  $\mu\text{M}$  cAMP. Enzyme activity was assayed at  $30^{\circ}\text{C}$  in a final volume of 0.1 ml. The effect of  $\text{I}^-$  on PKA activity was determined by addition of NaI or KI to the assay mixture. The reaction was terminated after 10 min by spotting 65  $\mu\text{l}$  of the reaction mixture onto a  $1 \times 0.5\text{-in.}$  P-81 phosphocellulose strip (Whatman Inc., Clifton, NJ). The strips were washed four times in 75 mM phosphoric acid and dried under a warm air blower. Bound radioactivity was determined using a liquid scintillation counter. In some experiments PKA activity was determined in a modified whole-cell internal solution containing 10 mM HEPES, 10  $\text{MgCl}_2$ , 60 mM K-glutamate, 0.25 mM EGTA, bovine serum albumin (0.25 mg/ml), and 100  $\mu\text{M}$  [ $\gamma\text{-}^{32}\text{P}$ ]ATP (300 cpm/pmol) with either 50 mM KCl or 50 mM KI.

#### *Preparation of Drugs and Dialysis of Cells with Protein Kinases*

$\text{L}(-)$  Isoproterenol, phorbol-12,13-dibutyrate (PDB), adenosine-( $\gamma$ -thio)-triphosphate (ATP $\gamma\text{S}$ ), and the catalytic subunit of PKA (CS) used in whole-cell experiments were obtained from Sigma Chemical Co. (St. Louis, MO). The PKA holoenzyme, histone type II-A, Kemptide, and bovine serum albumin used in the [ $\gamma\text{-}^{32}\text{P}$ ]ATP assay were also purchased from Sigma Chemical Co. [ $\gamma\text{-}^{32}\text{P}$ ]ATP was obtained from Du Pont-New England Nuclear (Boston, MA) and 8-chlorophenylthio (8-CPT) cAMP was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Preparation and use of isoproterenol, PDB, 8-CPT cAMP, and CS has been described previously (Walsh et al., 1989; Walsh, 1991; Walsh and Kass, 1991).

In some experiments partially purified PKC was dialyzed into the myocytes in order to maximize the size of the PKC-sensitive  $I_{\text{Cl}}$  (Walsh, 1991). A mixed isozyme preparation of PKC, partially purified from rat brain, was generously supplied by Ms. Muriel C. Maurer and Dr. Julianne J. Sando (Department of Pharmacology, University of Virginia, Charlottesville, VA). The PKC preparation was purified using DE-52 cellulose and threonine Sepharose chromatography (Kikkawa, Go, Koumoto, and Nishizuka, 1986; Walker and Sando, 1988). For experiments using internal cellular dialysis, a solution of either CS or PKC was included in the internal pipette solution as described previously (Walsh et al., 1989; Walsh, 1991). After disruption of the cell membrane the enzymes move from the pipette into the cell by diffusion. Based on theoretical studies (Kameyama et al., 1985; Pusch and Neher, 1988), large molecular weight substances such as CS and PKC (molecular mass  $\sim 40,000$  and  $80,000$  D, respectively) should reach equilibrium at a slow rate ( $\tau = 10\text{--}15$  min) given the size of the cells and electrodes used in this study. This time course closely matches experimental rates determined for augmentation of  $I_{\text{Ca}}$  and  $I_{\text{Cl}}$ .

All whole-cell patch clamp experiments were conducted at room temperature ( $22\text{--}25^{\circ}\text{C}$ ) as indicated in the figure legends. Averaged values presented are means  $\pm$  SE. Where appropriate, statistical significance was estimated using Student's  $t$  test for unpaired observations.

## RESULTS

*Inhibition of  $I_{Cl}$  by Replacement of NaCl with NaI*

The left panel of Fig. 1 shows an example of  $I_{Cl}$  recorded from a guinea pig ventricular myocyte during a voltage step applied from a holding potential of  $-40$  to  $-60$  mV. No observable inward or outward currents can be recorded under basal conditions when cells are bathed in K-free external solution containing 1 mM  $BaCl_2$  and 200 nM nisoldipine (see sweep labeled ■). However, a large inward current can be activated after addition of 1  $\mu$ M of the  $\beta$ -adrenergic agonist isoproterenol to the

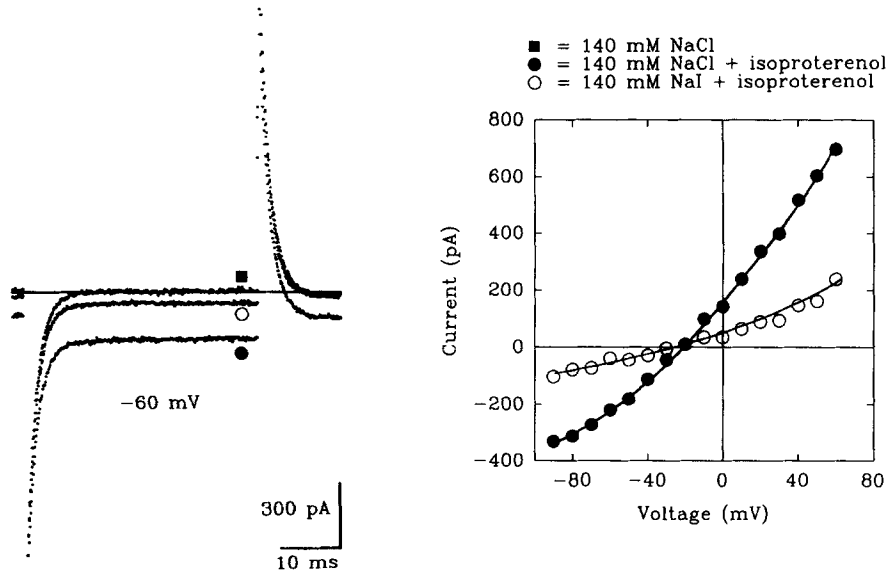


FIGURE 1. Inhibition of  $I_{Cl}$  by replacement of NaCl with NaI. (Left) Currents recorded during 40-ms voltage steps to  $-60$  mV in K-free external solutions containing 1 mM  $BaCl_2$  and 200 nM nisoldipine. Currents were recorded in the presence (● and ○) and absence (■) of 1  $\mu$ M isoproterenol in either 140 mM NaCl external solution (■ and ●) or 140 mM NaI external solution (○). (Right)  $I$ - $V$  relation for isoproterenol-sensitive current in NaCl and NaI external solutions. 146 mM external  $Cl^-$ / 56 mM internal  $Cl^-$  ( $E_{Cl} = -25$  mV). A curved line was drawn by hand to the data points to emphasize the outward rectification. Temperature = 25°C. Cell B26.

bath (see sweep labeled ●). The right panel of Fig. 1 displays the current versus voltage relationship for the isoproterenol-sensitive current recorded at potentials ranging from  $-90$  to  $+60$  mV. As has been reported previously (Bahinski et al., 1989; Harvey and Hume, 1989a; Matsuoka et al., 1990), this  $\beta$ -adrenergic-sensitive  $I_{Cl}$  displays outward-going rectification and has a reversal potential ( $E_{rev}$ ) close to the equilibrium potential for  $Cl^-$  ( $E_{Cl}$ ) ( $-23$  and  $-25$  mV, respectively, for the experiment displayed in Fig. 1). In agreement with earlier observations (Harvey, Clark, and Hume, 1990) we have found that this current can be reduced by monocarboxylic acids such as anthracene-9-carboxylic acid (results not shown), which are known to

block  $\text{Cl}^-$  channels in other tissues (Bryant and Morales-Aguilera, 1971; Welsh, 1986). In addition, the  $E_{\text{rev}}$  for this current was found to vary according to  $E_{\text{Cl}}$  when changes were made in the internal or external  $\text{Cl}^-$  concentration (results not shown) as previously reported (Bahinski et al., 1989; Harvey and Hume, 1989a; Matsuoka et al., 1990).

To begin characterizing the permeability properties of the heart  $I_{\text{Cl}}$ , the effect of equimolar replacement of external  $\text{Cl}^-$  with  $\text{I}^-$  was determined. As can be seen in the left panel of Fig. 1, substitution of 140 mM external NaCl with 140 mM NaI resulted in a large reduction in the amplitude of  $I_{\text{Cl}}$  (see sweep labeled O). This reduction in  $I_{\text{Cl}}$  was evident at all membrane potentials where  $I_{\text{Cl}}$  was measured (Fig. 1, right panel), although there was a tendency for greater inhibition to be present at negative potentials ( $-60$  to  $-90$  mV range) (see Fig. 2). Along with the inhibition of  $I_{\text{Cl}}$ ,

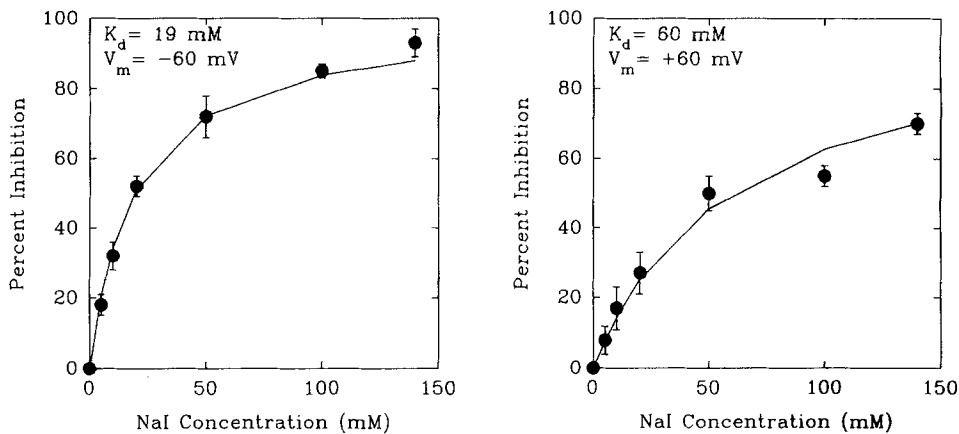


FIGURE 2. Concentration-dependent inhibition of  $I_{\text{Cl}}$  by NaI. Each point represents the mean  $\pm$  SE for three to six experiments. In most experiments two concentrations of NaI were tested on a single cell. The smooth curve is given by  $1/(1 + \text{IC}_{50}/[\text{NaI}])$  with an  $\text{IC}_{50}$  of 19 and 60 mM providing the best least-squares fit to inhibition at  $-60$  mV (left) and  $+60$  mV (right), respectively.

addition of NaI caused a small negative shift in the  $E_{\text{rev}}$  for the current. In six cells examined,  $E_{\text{rev}}$  decreased from  $-22 \pm 1$  to  $-29 \pm 1$  mV after substitution of 140 mM NaI. This change was statistically significant ( $P < 0.005$ ). Based on the  $E_{\text{rev}}$  data, the relative permeability of  $\text{I}^-$  to  $\text{Cl}^-$  ( $P_{\text{I}}/P_{\text{Cl}}$ ) for this channel was determined to be 1.31. Thus, although NaI decreased  $I_{\text{Cl}}$ , the anion  $\text{I}^-$  appeared to be more permeable to the channel. Similar results were obtained with NaI when  $I_{\text{Cl}}$  was activated by addition of a  $200\text{-}\mu\text{M}$  concentration of 8-CPT cAMP to the external solution.

The time course for reduction of  $I_{\text{Cl}}$  by NaI usually varied from one cell to another. In some myocytes, inhibition occurred rapidly (within seconds) after addition of NaI, whereas in other myocytes, several minutes were required to achieve a steady-state reduction of  $I_{\text{Cl}}$ . In a number of cells where washout of NaI was attempted, inhibition of  $I_{\text{Cl}}$  could be completely reversed on return to NaCl-containing external solution ( $n = 3$ ).

Partial substitution of NaCl with NaI also caused a reduction in  $I_{Cl}$ . Fig. 2 displays the concentration versus inhibition curve for NaI reduction of  $I_{Cl}$  obtained during voltage steps to membrane potentials of  $-60$  mV (left panel) and  $+60$  mV (right panel). Inhibition of  $I_{Cl}$  could be observed at all potentials with a concentration of NaI as low as 5 mM. Smooth curves with inhibition constants ( $IC_{50}$ ) of 19 mM (at  $-60$  mV) and 60 mM (at  $+60$  mV) are superimposed on the data points in Fig. 2.

*NaI Does Not Inhibit  $I_{Cl}$  Activated by Protein Kinase C*

Impermeant cations such as  $Cs^+$  can enter the pore of voltage-activated  $K^+$  channels and serve as blocking agents since these ions do not permeate the entire channel

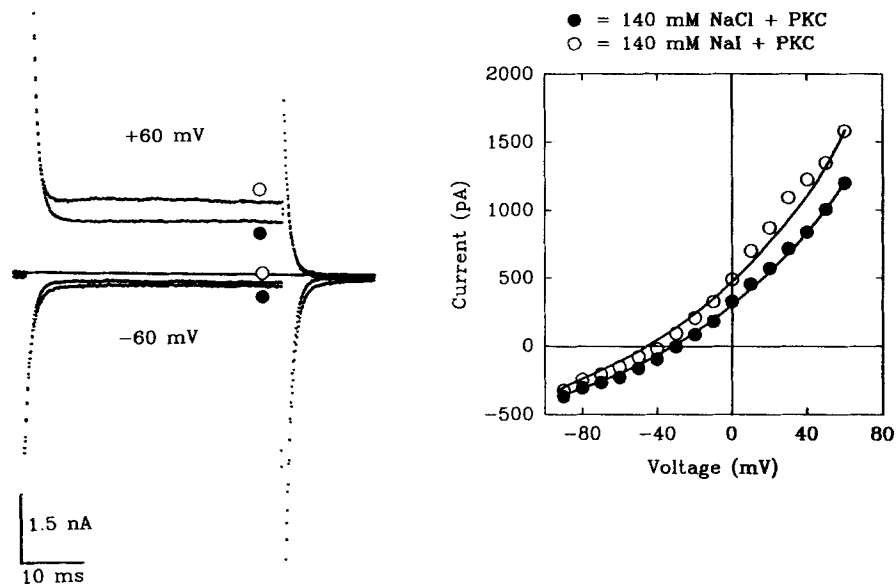


FIGURE 3. NaI does not inhibit  $I_{Cl}$  activated by PKC. (Left) Currents recorded during 40-ms voltage steps to  $-60$  and  $+60$  mV in the presence of PDB during internal dialysis with PKC (activity = 0.8 nmol  $P_i$ /min per ml). Currents were recorded in either NaCl (●) or NaI (○) external solutions 20 and 22 min, respectively, after membrane breakthrough with the patch pipette. (Right)  $I-V$  relation for PKC-sensitive  $I_{Cl}$ . PKC-sensitive current was determined by subtracting currents recorded during the first minute after breakthrough of the pipette into the cell from currents recorded 19 and 21 min later. 146 mM external  $Cl^-$ / 58 mM internal  $Cl^-$  ( $E_{Cl} = -24$  mV). Curved lines were drawn by hand to the  $I-V$  relations. Temperature = 25°C. Cell B33.

pore (Bezania and Armstrong, 1972; Hagiwara, Miyazaki, and Rosenthal, 1976; Coronado and Miller, 1979). One explanation for our results could be that  $I^-$  acts in an analogous manner on heart  $Cl^-$  channels. Since we have recently found that stimulation of PKC can also activate a heart  $I_{Cl}$  (Walsh, 1991), it was of interest to determine whether both the PKA- and PKC-activated currents were inhibited by NaI.

Fig. 3 shows  $I_{Cl}$  recorded in NaCl and NaI external solutions during dialysis of a ventricular cell with partially purified PKC (0.8 nmol  $P_i$ /min per ml). With exogenous

PKC in the cell, phorbol esters such as PDB activate large inward and outward currents (Walsh, 1991). As was the case for the isoproterenol (PKA)-activated  $I_{Cl}$  shown in Fig. 1, the PKC-sensitive current in this experiment had an  $E_{rev}$  ( $-30$  mV) close to  $E_{Cl}$  ( $-24$  mV) and an outward rectifying  $I$ - $V$  relationship in 140 mM NaCl external solution (58 mM internal  $Cl^-$ ) (Fig. 3). However, in contrast to  $I_{Cl}$  activated by PKA, the PKC-sensitive  $I_{Cl}$  was not reduced after substitution of NaCl with NaI, but was actually augmented at positive potentials. It should be noted that in some cells the PKC-activated current displayed a linear  $I$ - $V$  relationship when measured between  $-90$  and  $+60$  mV, as reported previously (Walsh, 1991). Regardless of the shape of the  $I$ - $V$  relation, NaI produced no reduction in  $I_{Cl}$  but caused  $E_{rev}$  to shift to more negative potentials. The mean shift of  $-15 \pm 2$  mV in the  $E_{rev}$  ( $n = 4$ ) indicated that  $I^-$  was more permeable than  $Cl^-$  for the PKC-activated channel with a  $P_I/P_{Cl}$  equaling 1.79. Similar results were obtained when  $I_{Cl}$  was activated by PDB in the absence of exogenous PKC in the pipette. In this case, activation of  $I_{Cl}$  resulted from a stimulation of endogenous PKC present in the ventricular cells (Capogrossi, Kaku, Filburn, Pelto, Hansford, Spurgeon, and Lakatta, 1990).

#### *Inhibition of $\beta$ -adrenergic Augmented $I_{Ca}$ by NaI*

The results shown in Figs. 1–3 suggest that NaI inhibits  $I_{Cl}$  activated by PKA, but not  $I_{Cl}$  activated by PKC. Since multiple  $Cl^-$  channel subtypes have been identified in other cells (Blatz and Magleby, 1985; Cliff and Frizzell, 1990; Welsh, 1990), it is possible that the ventricular cells contain separate PKA- and PKC-activated channels. Alternatively, the results might be explained if NaI, through some unknown mechanism, inhibits the  $\beta$ -adrenergic/PKA phosphorylation pathway involved in activating the  $Cl^-$  channel. Since the cardiac  $I_{Ca}$  is regulated during  $\beta$ -adrenergic stimulation (for review see Reuter, 1983), the  $Ca^{2+}$  channel served as a valuable model for testing the latter hypothesis.

The left panel of Fig. 4 shows  $I_{Ca}$  recorded during voltage steps to  $+10$  mV in the presence and absence of isoproterenol. As expected, isoproterenol produced a large increase in the peak  $I_{Ca}$  and caused the voltage dependence for activation of the  $Ca^{2+}$  channels to shift to more negative potentials (Bean, Nowycky, and Tsien, 1984; Bean, 1989). Substituting 140 mM NaI for 140 mM NaCl caused a drastic reduction in  $I_{Ca}$  augmented in the presence of isoproterenol (Fig. 4). Overall, in five experiments NaI decreased the peak  $I_{Ca}$  by  $54 \pm 5\%$ . However,  $I_{Ca}$  was not completely reduced to the control amplitude in any of these experiments. NaI inhibited the isoproterenol-sensitive component of  $I_{Ca}$ , determined by subtracting the control records from the records obtained in the presence of isoproterenol, by  $75 \pm 6\%$  ( $n = 5$ ). In addition to decreasing the size of the isoproterenol-enhanced current, NaI caused the  $I$ - $V$  relationship for  $I_{Ca}$  to shift back in the direction of the control values (see right panel of Fig. 4). In the presence of NaI, the membrane potential required for peak activation of  $I_{Ca}$  during  $\beta$ -adrenergic stimulation was increased by  $10 \pm 3$  mV ( $n = 5$ ) when compared with peak activation in NaCl.

Despite the large reduction in the isoproterenol-augmented  $I_{Ca}$ , NaI produced no obvious decline in the basal  $I_{Ca}$  (Fig. 5) when monitored for up to 10 min after substitution of NaI for NaCl. In five experiments the peak  $I_{Ca}$  amplitude changed by  $1 \pm 5\%$  in the presence of NaI. Surprisingly, in some experiments there was a small



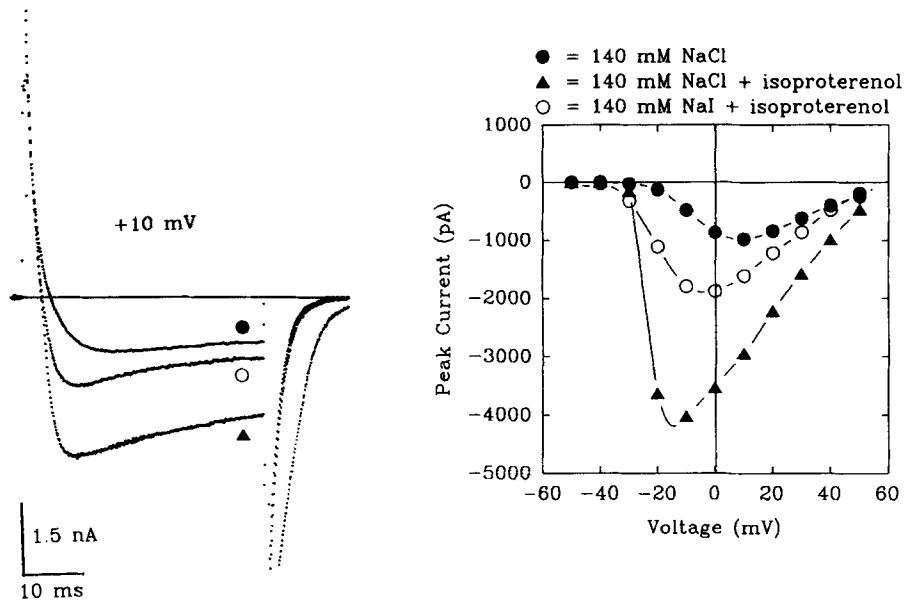


FIGURE 4. Inhibition of  $\beta$ -adrenergic augmented  $I_{Ca}$  by NaI. (Left)  $I_{Ca}$  recorded during 40-ms voltage steps to +10 mV in K-free external solutions containing 1 mM  $CaCl_2$ . Currents were recorded in the presence (○ and ▲) and absence (●) of 2  $\mu$ M isoproterenol in either 140 mM NaCl external solution (● and ▲) or 140 mM NaI external solution (○). (Right)  $I$ - $V$  relation for  $I_{Ca}$  recorded in NaCl and NaI external solutions. Temperature = 22°C. Cell B57.

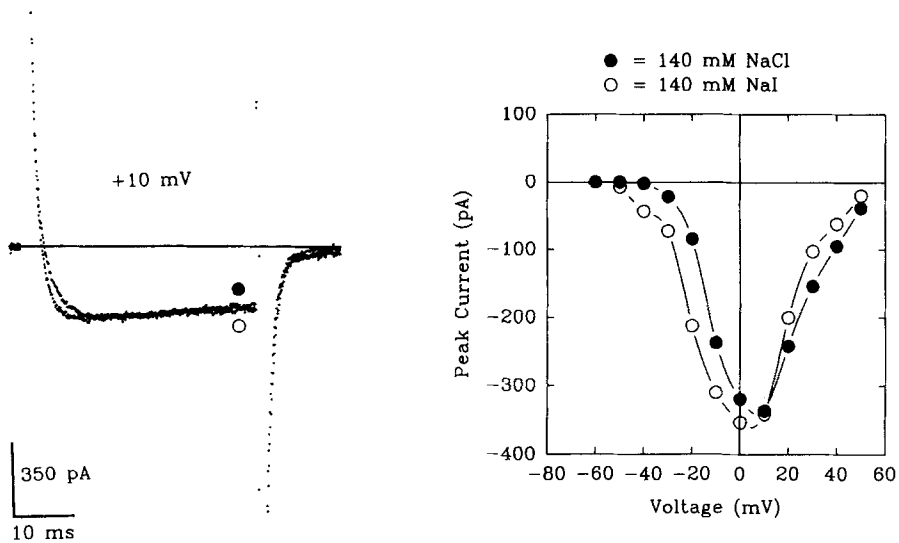


FIGURE 5. NaI does not inhibit basal  $I_{Ca}$ . (Left)  $I_{Ca}$  recorded during 40-ms voltage steps to +10 mV in either 140 mM NaCl external solution (●) or 140 mM NaI external solution (○). (Right)  $I$ - $V$  relation for  $I_{Ca}$  recorded in NaCl and NaI external solutions. Temperature = 24°C. Cell B53.

increase noted at some potentials in the peak amplitude of  $I_{Ca}$  with NaI. As shown in Fig. 5, this effect was associated with a slight speeding in the activation kinetics of  $I_{Ca}$  (Fig. 5, left panel) and a decrease in the membrane potential threshold required for activation of  $I_{Ca}$  (Fig. 5, right panel).

*NaI Inhibits  $I_{Ca}$  Increased by cAMP and CS*

Inhibition of isoproterenol-regulated  $Cl^-$  and  $Ca^{2+}$  channels by NaI could result from a direct action of this chemical on the  $\beta$ -adrenergic receptor, the enzyme adenylate

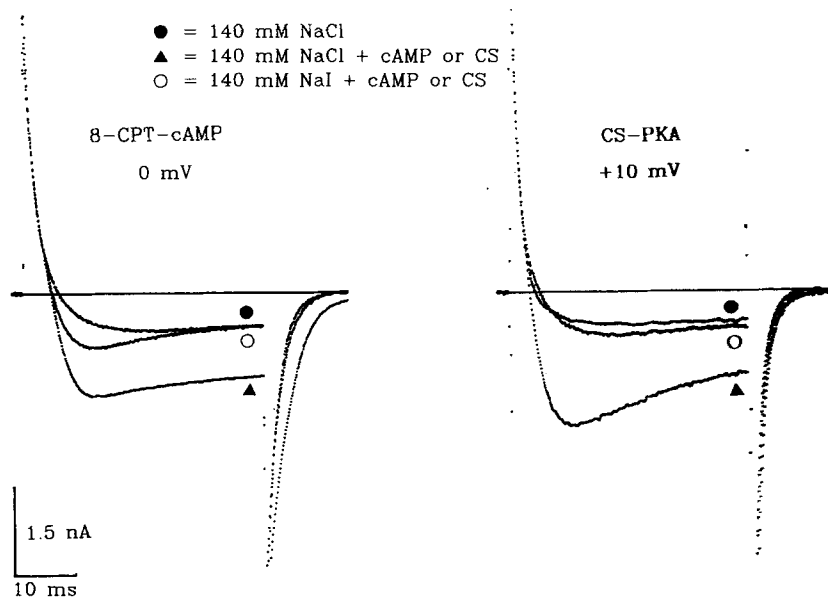


FIGURE 6. NaI inhibits  $I_{Ca}$  increased by cAMP and CS. (Left)  $I_{Ca}$  recorded during 40-ms voltage steps to 0 mV in K-free external solutions containing 1 mM  $CaCl_2$ . Currents were recorded in the presence (○ and ▲) and absence (●) of 200  $\mu$ M 8-CPT cAMP in either 140 mM NaCl external solution (● and ▲) or 140 mM NaI external solution (○). Temperature = 24°C. Cell B39. (Right)  $I_{Ca}$  recorded during 40-ms voltage steps to +10 mV during internal dialysis with CS (activity = 7.5 nmol  $P_i$ /min per ml). Currents were recorded in either NaCl (● and ▲) or NaI (○) external solutions during the first minute (●) after membrane breakthrough with the patch pipette and 16 (▲) and 18 (○) min later. Temperature = 22°C. Cell B65. Calibration bars are the same for both panels.

cyclase, or the  $G_s$  protein pathway that links  $\beta$ -receptor activation with cAMP production. To eliminate these possibilities, the effect of NaI was examined on  $I_{Ca}$  augmented by addition of the membrane soluble cAMP derivative 8-CPT cAMP (200  $\mu$ M). As shown in the left panel of Fig. 6, addition of 140 mM NaI reduced the 8-CPT cAMP-augmented  $I_{Ca}$  to a level close to that of the control. Overall, in four experiments NaI reduced the cAMP-stimulated component of  $I_{Ca}$  by  $81 \pm 10\%$ . This inhibition was not significantly different ( $P > 0.2$ ) from that observed for the isoproterenol-sensitive  $I_{Ca}$  (see above).

The right panel of Fig. 6 shows  $I_{Ca}$  recorded during dialysis of a cell with 3  $\mu$ M of

the catalytic subunit of PKA (CS). A high concentration of CS was used in these studies in order to cause maximal stimulation of  $I_{Ca}$  (Kameyama et al., 1985). As was the case with isoproterenol and 8-CPT cAMP, substitution of NaI produced a large reduction in the CS-augmented  $I_{Ca}$ . Concomitant with this reduction, NaI caused a decrease in the amplitude of  $I_{Cl}$  measured during voltage steps to  $-90$  mV (not shown). A similar reduction in  $I_{Ca}$  and  $I_{Cl}$  was observed in another cell dialyzed with the same concentration of CS. Thus, it is unlikely that inhibition of  $I_{Cl}$  and  $I_{Ca}$  results from the ability of NaI to prevent the dissociation of the regulatory and catalytic subunits of PKA.

*Effect of NaI on the In Vitro Phosphorylation of Histone-IIA and Kemptide*

One simplistic explanation for the results thus far presented is that NaI or  $I^-$  alone diffuses into the cardiac cell and inhibits PKA-mediated phosphorylation of various

TABLE I  
*Effect of  $I^-$  on PKA-mediated Phosphorylation*

Enzyme*	Substrate	Condition <sup>†</sup>	Activity <sup>‡</sup>
PKA + cAMP	Histone-IIA	Tris + 140 mM NaCl	24 ± 1
		Tris + 140 mM NaI	23 ± 1
PKA + cAMP	Histone-IIA	HEPES + 50 mM KCl	61 ± 4
		HEPES + 50 mM KI	59 ± 2
PKA + cAMP	Kemptide	Tris + 50 mM KCl	194 ± 3
		Tris + 50 mM KI	165 ± 6
PKA + cAMP	Kemptide	HEPES + 50 mM KCl	236 ± 5
		HEPES + 50 mM KI	228 ± 8
CS	Kemptide	HEPES + 50 mM KCl	203 ± 7
		HEPES + 50 mM KI	204 ± 7

\*Enzyme activity was assayed with either histone-IIA (0.5 mg/ml) or Kemptide (80 µg/ml) as described in Materials and Methods. The holoenzyme of PKA (63 µg/ml) and the catalytic subunit of PKA (CS) (2 µg/ml) were used in these assays.

<sup>†</sup>The Tris buffer solution consisted of 20 mM Tris, 10 mM MgAcetate, 100 µM [ $\gamma$ -<sup>32</sup>P]ATP (300 cpm/pmol), and bovine serum albumin (0.25 mg/ml). The HEPES buffer solution consisted of 10 mM HEPES, 10 mM MgCl<sub>2</sub>, 60 mM K-glutamate, 0.25 mM EGTA, 100 µM [ $\gamma$ -<sup>32</sup>P]ATP (300 cpm/pmol), and bovine serum albumin (0.25 mg/ml).

<sup>‡</sup>The results represent the mean ± SE of three determinants.

proteins including ion channels. As a first step in testing this hypothesis we determined the effect of NaI on the in vitro phosphorylation of the acidic protein histone. In the presence of PKA, incorporation of [ $\gamma$ -<sup>32</sup>P]ATP into histone-IIA was measured in assay mixtures supplemented with either 140 mM NaCl or 140 mM NaI. As can be seen in Table I, [ $\gamma$ -<sup>32</sup>P]ATP labeling of histone was not significantly different in NaI- and NaCl-containing Tris buffer solutions. Phosphorylation assays were also performed under conditions designed to more closely mimic the whole-cell patch clamp experiments and to provide a more "physiological" substrate in the phosphorylation reaction. For this purpose a modified whole-cell internal solution containing either 50 mM KCl or 50 mM KI was used to measure [ $\gamma$ -<sup>32</sup>P]ATP incorporation in the peptide Kemptide. Despite these modifications in the assay

mixture, substitution of  $I^-$  for  $Cl^-$  caused no apparent inhibition of PKA-mediated phosphorylation (Table I).

*Attenuation of the  $I_{Ca}$  Response to Isoproterenol in NaI External Solution*

NaI might act through a substrate-specific mechanism and inhibit the in situ phosphorylation of ion channel proteins. If so, regulation of  $I_{Ca}$  by isoproterenol should be attenuated in 140 mM NaI external solution. In the experiment displayed in Fig. 7,  $I_{Ca}$  was recorded in the presence of NaI both before and after addition of isoproterenol. With 140 mM external NaI,  $\beta$ -adrenergic stimulation caused almost no

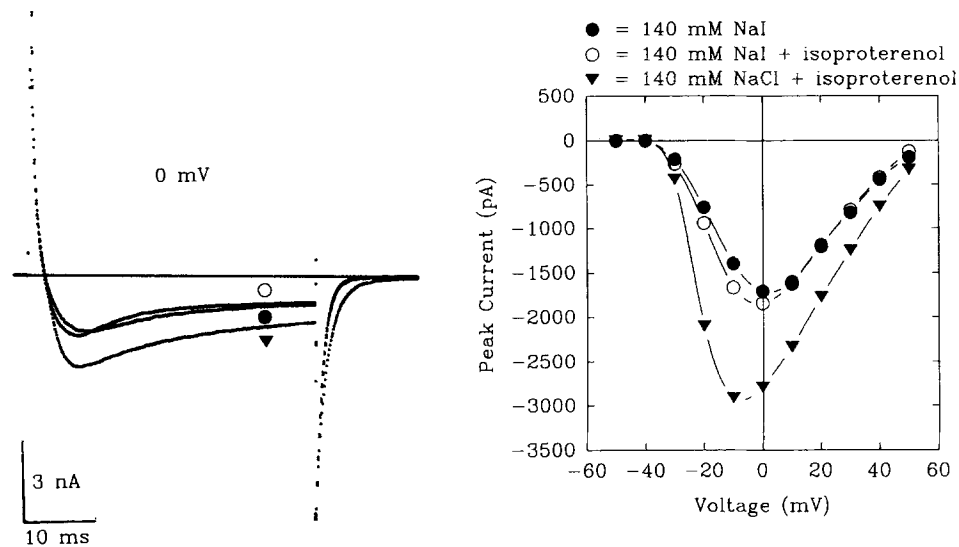


FIGURE 7. Attenuation of the  $I_{Ca}$  response to isoproterenol in NaI external solution. (Left)  $I_{Ca}$  recorded during 40-ms voltage steps to 0 mV in K-free external solutions containing 1 mM  $CaCl_2$ . Currents were recorded in the presence (○ and ▼) and absence (●) of 2  $\mu$ M isoproterenol in either 140 mM NaI external solution (● and ○) or 140 mM NaCl external solution (▼). (Right)  $I$ - $V$  relation for  $I_{Ca}$  recorded in NaCl and NaI external solutions. Temperature = 25°C. Cell B68.

increase in the peak amplitude of  $I_{Ca}$  and no alteration was seen in the  $I$ - $V$  relationship (Fig. 7). After this exposure to NaI, addition of NaCl external solution resulted in almost a doubling in the peak amplitude of  $I_{Ca}$  and the  $I$ - $V$  relationship was shifted to more negative potentials (Fig. 7). In some experiments (three of six cells) moderate increases in  $I_{Ca}$  could be obtained in NaI external solution. However, in these cells the  $2.7 \pm 0.3$ -fold augmentation caused by a maximal concentration of isoproterenol (2  $\mu$ M) (Kameyama et al., 1985; Walsh et al., 1989) was less than that obtained in NaCl external solution ( $4.4 \pm 0.7$ -fold,  $n = 4$ ). Thus, these results sharply contrast with those obtained in the in vitro [ $\gamma$ - $^{32}P$ ]ATP labeling experiments.

*NaI Inhibits  $I_{Cl}$  in the Presence of Intracellular ATP $\gamma$ S*

The experiments shown in Figs. 6 and 7 provide evidence that NaI inhibits cardiac  $Cl^-$  and  $Ca^{2+}$  channels by modulating the phosphorylation/dephosphorylation of cellular proteins. To determine whether NaI might be stimulating the dephosphorylation of PKA target proteins, ventricular cells were dialyzed with an internal solution in which the normal 5 mM ATP was replaced with 5 mM ATP $\gamma$ S (Fig. 8). Under these conditions, stimulation of PKA by isoproterenol results in the formation of a thio-phosphoprotein, which is more resistant to dephosphorylation (Yount, 1975; Kameyama et al., 1985). Thus, if NaI is decreasing  $I_{Ca}$  and  $I_{Cl}$  by augmenting dephosphorylation, this action should be greatly diminished with ATP $\gamma$ S in the internal solution. Despite the large inward and outward  $I_{Cl}$  activated in the presence of ATP $\gamma$ S, substitution of external NaCl with NaI resulted in a substantial decrease in  $I_{Cl}$  (Fig. 8). In four experiments with internal ATP $\gamma$ S,  $I_{Cl}$  recorded at  $-60$  and  $+60$

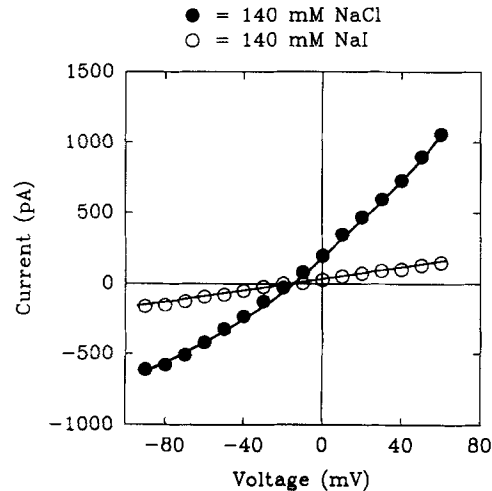


FIGURE 8. NaI inhibits  $I_{Cl}$  in the presence of intracellular ATP $\gamma$ S. *I-V* relation for isoproterenol-sensitive current in NaCl and NaI external solutions during dialysis of a cell with internal solution containing 5 mM ATP $\gamma$ S. 146 mM external  $Cl^-$ / 56 mM internal  $Cl^-$  ( $E_{Cl} = -25$  mV). A curved line was drawn by hand to the data points to emphasize the outward rectification. Temperature = 24°C. Cell B91.

mV was reduced by  $78 \pm 5$  and  $80 \pm 4\%$ , respectively. This inhibition caused by NaI was not significantly different than that observed in the presence of internal ATP (Fig. 2) ( $P > 0.05$ ).

## DISCUSSION

*Effect of NaI on  $\beta$ -adrenergic Regulation of  $I_{Cl}$  and  $I_{Ca}$* 

The principal new finding summarized in this study is that NaI inhibits  $\beta$ -adrenergic-mediated regulation of heart  $Cl^-$  and  $Ca^{2+}$  channels. This inhibitory effect probably results from the diffusion of free  $I^-$  across the sarcolemmal membrane and into the myocyte. Reduction of  $I_{Cl}$  and  $I_{Ca}$  was found to occur in the presence of the membrane-soluble cAMP analogue 8-CPT cAMP and during dialysis of cells with the catalytic subunit of PKA (CS), indicating that this inhibition occurs at a mechanistic

step beyond the release of CS from the regulatory subunit of the PKA enzyme. NaI caused no significant reduction in the basal  $I_{Ca}$ , suggesting that NaI acts specifically on  $Ca^{2+}$  channels activated during  $\beta$ -stimulation. Although the in vitro phosphorylation of histone and Kemptide was not inhibited by  $I^-$  when compared with  $Cl^-$ , the finding that normal augmentation of  $I_{Ca}$  by isoproterenol was attenuated in NaI external solution suggests that NaI may inhibit the in situ phosphorylation of cellular proteins.

NaI was found to inhibit the heart  $I_{Cl}$  in a concentration-dependent manner when substituted for NaCl. In agreement with these findings, Overholt and Harvey (1992) in a recent abstract have reported that substitution of external  $Cl^-$  with  $I^-$  decreases the slope conductance of the isoproterenol-stimulated  $I_{Cl}$ . Miller and White (1980) have also shown that  $I^-$  blocks  $Cl^-$  channels obtained from *Torpedo* electroplax membrane and recorded in lipid bilayers. This block occurs in a concentration-dependent manner ( $IC_{50} = 30$  mM) (Miller and White, 1980). Since isethionate ( $SCN^-$ ) was found to have a similar blocking action, it was suggested that anions such as  $I^-$  and  $SCN^-$  might directly occlude the *Torpedo*  $Cl^-$  channel (Miller and White, 1980). In the present study reduction of  $I_{Cl}$  by NaI was greater at relatively negative potentials ( $-60$  to  $-90$  mV,  $IC_{50} = 19$  mM) when compared with the reduction at more positive potentials ( $+40$  to  $+60$  mV,  $IC_{50} = 60$  mM). If the inhibition of  $I_{Cl}$  resulted from an occlusion of the channel by  $I^-$  during passage of the anion through the pore, a greater inhibition would have been expected at positive potentials where movement of  $I^-$  was inward. Since our experiments were conducted in asymmetrical  $Cl^-$  solutions (146 mM external and 56 mM internal  $Cl^-$ ), the greater inhibition of  $I_{Cl}$  observed at negative potentials may be related to the lower  $Cl^-$  concentration within the cell.

Recent findings concerning the effects of external  $Na^+$  replacement on cardiac ion channels strikingly parallel the results of our experiments. Harvey, Jurevicius, and Hume (1991) have reported that replacement of external  $Na^+$  with compounds such as tetramethylammonium and *N*-methyl-D-glucamine results in a reduction of  $I_{Cl}$  and  $I_{Ca}$  during stimulation by isoproterenol, 3-isobutyl-1-methylxanthine, 8-bromoadenosine cAMP, and CS. These results supported earlier findings indicating that external  $Na^+$  was required for  $\beta$ -adrenergic activation of  $I_{Cl}$  (Egan, Noble, Noble, Powell, Twist, and Yamaoka, 1988; Harvey and Hume, 1989a; Matsuoka et al., 1990). Based on experiments in which  $Na^+$  was included in the pipette solution, Harvey et al. (1991) postulated that a certain concentration of internal  $Na^+$  may be necessary for PKA-mediated regulation of  $Ca^{2+}$ ,  $Cl^-$ , and  $K^+$  channels. Thus, taken together with the present study, these results suggest that alterations in either external  $Na^+$  or  $Cl^-$ , especially when occurring subsequent to stimulation of PKA, lead to an inhibition in PKA-regulated channel activity. In support of this role for external  $Cl^-$ , preliminary experiments have indicated that substitution of NaBr for NaCl also causes a reduction in isoproterenol-stimulated  $I_{Ca}$  and  $I_{Cl}$  (Walsh, K. B., unpublished results).

#### *Permeability of PKA- and PKC-activated $Cl^-$ Channels for $I^-$*

Despite the large reduction observed in the PKA-activated  $I_{Cl}$  during substitution of NaCl with NaI, there was no reduction in the PKC-activated  $I_{Cl}$ . In actuality, there

was an increase in the conductance of the  $Cl^-$  channel in the presence of external  $I^-$ . This increase was accompanied by a shift of  $-15$  mV in the  $E_{rev}$  for PKC-sensitive current, suggesting that the channel has a higher permeability for  $I^-$  than for  $Cl^-$ . A smaller negative shift in  $E_{rev}$  was observed in the presence of  $I^-$  during  $\beta$ -adrenergic stimulation. Thus, although NaI reduces PKA-mediated regulation of  $I_{Cl}$ , those  $Cl^-$  channels that remain active in the presence of NaI may be more selective for  $I^-$ .  $Cl^-$  channels found in numerous cells, including human (Frizzell, Reckemmer, and Shoemaker, 1986; Li, McCann, and Welsh, 1990) and canine (Li et al., 1990) airway epithelium, human colonic tumor (Hayslett, Gogelein, Kunzelmann, and Greger, 1987), rabbit urinary bladder (Hanrahan, Alles, and Lewis, 1985), and hippocampal neurons (Hamill, Bormann, and Sakmann, 1983) have a greater permeability for  $I^-$  than for  $Cl^-$ . The permeability ratio of  $I^-/Cl^-$  of 1.79 for the PKC-activated  $I_{Cl}$  is very close to the value of 1.75 reported for the outward-rectifying  $Cl^-$  channel of human respiratory epithelium cells (Li et al., 1990), which can be activated by PKA and PKC (Welsh, 1990). Future studies examining the permeability of the PKC-activated  $Cl^-$  channel to anions including  $Br^-$ ,  $F^-$ , and  $SCN^-$  will help in assessing the similarity of the heart channel to those found in these other tissues.

#### *Possible Sites of NaI Inhibition of $I_{Cl}$ and $I_{Ca}$*

A number of important observations were made in this study concerning the action of NaI on heart  $Cl^-$  and  $Ca^{2+}$  channels. Reduction of  $I_{Cl}$  and  $I_{Ca}$  during substitution of NaCl with NaI occurred relatively quickly, and in some cells could be completely reversed upon return to normal NaCl external solution. The time course and reversibility of the inhibitory effects of NaI could be consistent with an action of  $I^-$  at a site on or near the  $Cl^-$  and  $Ca^{2+}$  channels. When studied during voltage steps to potentials negative to  $E_{Cl}$ , a concentration of NaI as low as 5 mM produced a small decrease in  $I_{Cl}$ , and almost complete inhibition of  $I_{Cl}$  was observed with 140 mM NaI. The inhibitory effects of NaI on  $I_{Cl}$  were specific for channel activation by PKA when compared with activation by PKC. Thus, the effect of NaI may result from the binding of  $I^-$  to a specific and saturable phosphorylation site within the cell.

Both the  $\alpha$  and  $\beta$  subunits of the dihydropyridine-sensitive  $Ca^{2+}$  channel from skeletal muscle are in vitro substrates for PKA (Curtis and Catterall, 1984; Hosey et al., 1986; Imagawa, Leung, and Campbell, 1987). Phosphorylation of a serine residue on the  $\alpha$  subunit (Rohrkasten, Meyer, Nastainczyk, Sieber, and Hofmann, 1988) may be associated with the increased  $Ca^{2+}$  channel activity seen during  $\beta$ -adrenergic stimulation (Chang et al., 1991). In contrast to skeletal muscle, the  $\alpha_1$  subunit of the cardiac  $Ca^{2+}$  channel may not be a substrate for PKA. Klockner, Itagaki, Bodi, and Schwartz (1992) have reported that  $Ba^{2+}$  currents recorded in *Xenopus* oocytes expressing the  $\alpha_1$  subunit from cardiac muscle are not increased during stimulation of PKA. Furthermore, PKA-mediated augmentation of the  $Ba^{2+}$  current was found to require the coexpression of the  $\beta$  subunit (Klockner et al., 1992). Thus, one possible explanation for our results is that  $I^-$  inhibits the phosphorylation of the  $Ca^{2+}$  channel  $\beta$  subunit and the  $Cl^-$  channel. Alternatively, if the effects of PKA on the cardiac  $Ca^{2+}$  and  $Cl^-$  channels are mediated through the same or similar regulatory phosphoprotein(s), it is possible that  $I^-$  prevents the phosphorylation of these proteins. These conclusions are supported by our finding that in some cells increases in  $I_{Ca}$  during

$\beta$ -stimulation were prevented by NaI. However, we found no evidence for an inhibition by  $I^-$  of the in vitro phosphorylation of histone and Kemptide by PKA. In addition, moderate increases in  $I_{Ca}$  with isoproterenol were sometimes observed in the presence of NaI.

As an alternative explanation for our results,  $I^-$  might cause inhibitory effects by increasing the dephosphorylation of the  $Cl^-$  and  $Ca^{2+}$  channels through stimulation of protein phosphatase activity. This might account for the rapid (seconds) decrease in  $I_{Ca}$  and  $I_{Cl}$  that often occurred upon NaI substitution. Divalent cations such as  $Mn^{2+}$  and  $Ni^{2+}$ , as well as various polycations (polysine and protamine), increase the activity of protein phosphatases 2A and 2B (King and Huang, 1984; Pelech and Cohen, 1985). Both of these phosphatases have been implicated in the regulation of cardiac  $I_{Ca}$  (Hescheler, Mieskes, Ruegg, Takai, and Trautwein, 1988). For this reason it was somewhat surprising that the inhibition of  $I_{Cl}$  caused by NaI was not significantly affected by substitution of ATP $\gamma$ S for ATP in the internal solution (see Fig. 8). Kameyama et al. (1985) have shown that replacement of internal ATP with ATP $\gamma$ S causes an augmentation in the size of the isoproterenol-stimulated  $I_{Ca}$  and slows the return of  $I_{Ca}$  to control levels after washout of isoproterenol. These results were interpreted to suggest that the thio-phosphorylated  $Ca^{2+}$  channel is a poor substrate for ventricular phosphatases (Kameyama et al., 1985). However, no biochemical evidence was presented in this study to support a conclusion that the  $Ca^{2+}$  channel protein is a target of thio-phosphorylation. Thus, further experiments using specific inhibitors of protein phosphatases, such as okadaic acid, will be required to provide more definitive information on the effect of  $I^-$  on the dephosphorylation of cardiac  $Cl^-$  and  $Ca^{2+}$  channels.

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