Functionally Distinct Phospho-Forms Underlie Incremental Activation of Protein Kinase–regulated Cl⁻ Conductance in Mammalian Heart

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ABSTRACT   The regulation of cardiac Cl^- conductance by cAMP-dependent protein kinase (PKA) and cellular phosphatases was studied in isolated guinea pig ventricular myocytes by using wide-tipped, perfused pipettes to record whole-cell currents. Exposure to forskolin (Fsk) or isoproterenol (Iso) elicits a Cl^- conductance that results exclusively from PKA-dependent phosphorylation because it can be completely abolished, or its activation fully prevented, by switching to pipette solution containing PKI, a synthetic peptide inhibitor of PKA. The Cl^- conductance activated by micromolar concentrations of either agonist reached its steady-state amplitude in 1–2 min and was deactivated promptly and entirely, usually within 2 min, upon washing out the agonist, implying a continuous high level of activity of endogenous protein phosphatases. Accordingly, intracellular application of okadaic acid or microcystin, both potent inhibitors of protein phosphatases 1 and 2A, during exposure to Fsk enhanced the steady-state Cl^- conductance and slowed its deactivation after washing out the Fsk. Maximal potentiation of the conductance, by $\sim 60\%$, was obtained with pipette concentrations of ~ 10 μM okadaic acid (or ~ 5 μM microcystin) and did not result from an increase in the apparent affinity for Fsk. In the presence of maximally effective concentrations of okadaic acid and/or microcystin, deactivation of the enhanced Cl^- conductance upon washout of agonist was incomplete, with about half of the conductance persisting indefinitely. That residual conductance did not reflect continued action of PKA because it was insensitive to PKI, but was identified as a fraction of the activated Cl^- conductance by its biophysical characteristics. The results suggest that complete deactivation of the PKA-regulated cardiac Cl^- conductance requires dephosphorylation by a type 1 and/or 2A phosphatase, but that partial deactivation can be accomplished by activity of some other phosphatase(s). These findings are consistent with sequential phosphorylation of a protein, probably the Cl^- channel itself, at two different kinds

of sites. The resulting phosphoproteins can be distinguished on the basis of their different contributions to whole-cell Cl^- conductance.

INTRODUCTION

In the myocardium, β-adrenergic agonists regulate a variety of ionic currents (for reviews, see Hartzell, 1988; Gadsby, 1990), including a Cl^- current identified recently in guinea pig and rabbit ventricular myocytes (Harvey and Hume, 1989; Bahinski, Nairn, Greengard, and Gadsby, 1989b; Matsuoka, Ehara, and Noma, 1990). Activation of that Cl^- conductance requires phosphorylation by cAMP-dependent protein kinase (PKA) because the Cl^- conductance can be elicited by direct intracellular application of either cAMP or the catalytic subunit of PKA (Bahinski et al., 1989b), and it can be abolished (Bahinski, Gadsby, Greengard, and Nairn, 1989a; Hwang, Horie, Nairn, and Gadsby, 1992c) by intracellular application of a specific peptide inhibitor of PKA (PKI; i.e., Walsh inhibitor, 5-24-amide; Cheng, Kemp, Pearson, Smith, Misconi, Van Patten, and Walsh, 1986). Moreover, regulation of the Cl^- conductance is mediated exclusively via PKA, and does not include any component of direct coupling between G_s proteins and Cl^- channels (cf. Brown and Birnbaumer, 1988), because isoproterenol (Iso) was found to have no effect when the cAMP-PKA pathway was either eliminated by intracellular application of PKI, or maximally activated by forskolin or intracellular cAMP (Hwang et al., 1992c).

Single-channel currents underlying this PKA-regulated cardiac Cl^- conductance have been recorded in cell-attached (Ehara and Ishihara, 1990; Ehara and Matsuura, 1993) and excised inside-out (Nagel, Hwang, Nastiuk, Nairn, and Gadsby, 1992) and outside-out (Ehara and Matsuura, 1993) patches from guinea pig ventricular myocytes. In inside-out patches, the Cl^- channels were activated by PKA catalytic subunit plus ATP, and with symmetrical ~ 150 mM $[Cl^-]$ the unitary currents varied linearly with membrane potential and reversed near 0 mV, yielding a single-channel conductance of ~ 12 pS ($25°C$); in addition, once phosphorylated by PKA, the channels required a hydrolyzable nucleoside triphosphate to open (Nagel et al., 1992). These are all distinguishing characteristics of cystic fibrosis transmembrane conductance regulator (CFTR), the epithelial Cl^- channel whose defective function in airway epithelial cells is believed to be responsible for the major debilitating symptoms of cystic fibrosis (reviewed, for example, by Collins, 1992; Welsh, Anderson, Rich, Berger, Denning, Ostedgaard, Sheppard, Cheng, Gregory, and Smith, 1992), and they strongly suggest that this PKA-regulated cardiac Cl^- channel is identical to CFTR. Indeed, Northern blots reveal CFTR RNA in the hearts of guinea pigs (Nagel et al., 1992; Levesque, Hart, Hume, Kenyon, and Horowitz, 1992), rabbits, and man (Levesque et al., 1992).

Although the general features of the activation pathway of the PKA-regulated cardiac Cl^- channels have been established (Bahinski et al., 1989a, b; Harvey, Clark, and Hume, 1990; Tareen, Ono, Noma, and Ehara, 1991; Hwang et al., 1992c; Nagel et al., 1992), little is known about the deactivation pathway that presumably involves dephosphorylation by protein phosphatases. Here we present evidence, from experiments using okadaic acid and microcystin, both relatively specific inhibitors of phosphatases 1 and 2A, that suggests that at least two functionally distinct phosphorylated forms of a protein underlie incremental regulation of this cardiac Cl^-

conductance. The cDNA sequence of human CFTR does indicate multiple consensus sites for PKA phosphorylation (Riordan, Rommens, Kerem, Alon, Rozmahel, Grzelczak, Zielenski, Lok, Plavsic, Chou et al., 1989), but a study of CFTR mutants suggested considerable, if not complete, redundancy between the phosphorylation sites examined (Cheng, Rich, Marshall, Gregory, Welsh, and Smith, 1991). Our findings suggest, on the other hand, that in heart, two or more phospho-forms differentially regulate CFTR Cl⁻ channel function, in accordance with a more recent report of results obtained with CFTR mutants (Chang, Tabcharani, Hou, Jensen, Kartner, Alon, Hanrahan, and Riordan, 1992).

Some preliminary results have been presented in abstract form (Horie, Hwang, and Gadsby, 1991).

METHODS

Isolation of Myocytes

Single ventricular myocytes were isolated by collagenase digestion of guinea pig hearts (see Hwang et al., 1992c). Briefly, guinea pigs of either sex (300–500 g) were fully anesthetized with pentobarbital ($\sim$100 mg/kg i.p.), the heart was quickly excised, the aorta was cannulated, and retrograde coronary perfusion at 36°C was begun with oxygenated normal Tyrode's solution. After the blood was washed out, the perfusate was switched to nominally Ca-free Tyrode's solution until contraction stopped, and then to Ca-free Tyrode's containing 0.1 mg/ml (Yakult, Co., Tokyo, Japan) or 1 mg/ml (type I; Sigma Chemical Co., St. Louis, MO) collagenase for $\sim$10 min. The enzyme was then washed out with a high K^+, low Ca^{2+} solution (Isenberg and Klöckner, 1982) at room temperature, and the partially digested heart was cut into small chunks, filtered through nylon mesh, and the resulting myocyte suspension stored at 4°C in the same solution.

Compositions of Solutions

Normal Tyrode's solution contained (mM): 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5 HEPES/NaOH (pH 7.4), and 5.5 glucose. The modified Tyrode's solution for superfusion of myocytes contained (mM): 145 NaCl, 1.5 $MgCl_2$, 5 HEPES/NaOH (pH 7.4), 1 $CdCl_2$, and 5.5 glucose. In Cl-free external solution, NaCl was replaced by Na-isethionate, $MgCl_2$ by $MgSO_4$, and $CdCl_2$ by $CdSO_4$. All superfusion solutions were prewarmed to 36°C. The standard pipette solution for intracellular dialysis contained (mM): 85 aspartic acid, 5 pyruvic acid, 10 EGTA, 20 TEACl, 5 $Tris_2$-creatine phosphate, 10 MgATP, 0.1 $Tris_{2.5}$-GTP, 2 $MgCl_2$, 5.5 glucose, and 10 HEPES (pH 7.4 with CsOH). Free $[Ca^{2+}]$ and $[Mg^{2+}]$ were estimated to be <1 nM and $\sim$1 mM, respectively (Fabiato and Fabiato, 1979; Tsien and Rink, 1980). Aspartate was replaced by Cl⁻ in the 109 mM Cl⁻ solution.

Stock solutions of isoproterenol-HCl (0.8 mM in isotonic Na-lactate buffer; Winthrop Pharmaceuticals, New York, NY), forskolin (10 mM in ethanol; Calbiochem Corp., La Jolla, CA), okadaic acid (1 mM in dimethylsulfoxide [DMSO]; purified from *Halichondria okadai* by Dr. Y. Tsukitani, Fujisawa Pharmaceutical Co., Tokyo, Japan), and microcystin (5 mM in H_2O; Calbiochem Corp.) were diluted to the desired final concentration immediately before use. Corresponding concentrations of the solvents alone were without effect. The peptide inhibitor, PKI (5-24-amide), was synthesized as described previously (Cheng et al., 1986) and weighed into pipette solutions to yield a 1 mM stock, subsequently diluted as required.

Whole-Cell Current Recording

Myocytes were dispersed into a small chamber (volume ~0.5 ml) on the microscope stage (Diaphot; Nikon, Garden City, NY) and, after the cells settled on the coverslip glass bottom, superfusion was begun with normal Tyrode's. Whole-cell currents were recorded via wide-tipped pipettes (pulled from borosilicate capillaries; Mercer Glass Works, Inc., New York, NY) with resistances of 0.9 ± 0.3 MΩ (SD; n = 57) when filled with Tyrode's solution, fitted with an intrapipette perfusion device (Soejima and Noma, 1984). After obtaining a giga-ohm seal near the center of the cell by gentle suction (~20 cm H_2O), the pipette contents were exchanged for standard pipette solution and the membrane was then ruptured by more vigorous suction controlled with a 10-ml syringe. Once the cell interior was equilibrated (~3 min) with the pipette solution, the extracellular solution was switched to modified Tyrode's solution and the holding potential was set to 0 mV to inactivate Na^+ and Ca^{2+} channel currents. K^+ channel currents were minimized by omitting K^+ from both intra- and extracellular solutions, and including 20 mM TEA^+ in pipette solutions. Omission of external K^+ and internal Na^+ also prevented currents generated by the Na^+/K^+ pump (Gadsby and Nakao, 1989). Na^+/Ca^{2+} exchange current (Kimura, Noma, and Irisawa, 1986) was prevented by lack of internal Na^+ and internal and external Ca^{2+}.

Two 3-M KCl half cells connected the clamp amplifier to the pipette interior and to the chamber to minimize liquid junction potentials. Membrane capacitance and access resistance were determined from the current relaxation elicited by a 10-ms step hyperpolarization of 10 mV. Whole-cell currents were recorded in response to 80-ms voltage pulses to potentials from +100 to −100 mV in 20-mV increments. Current and voltage signals were filtered at 2 kHz, digitized on-line at 8 kHz, and stored in an IBM PC-AT computer for later analysis with Asyst software (Keithley Instruments, Inc., Taunton, MA). Steady-state current–voltage (I-V) relationships were plotted from the steady current levels averaged over the final 12.5 ms of each pulse (see, for example, Fig. 3 B, below).

Summarized data are given as mean ± SD unless otherwise indicated.

RESULTS

Intracellular Okadaic Acid Enhances Fsk-activated, PKA-mediated Cl⁻ Conductance and Slows Its Deactivation

Fig. 1 A shows a typical ~200-pA outward shift of holding current at 0 mV, elicited by application of 3 μM forskolin (Fsk) to a myocyte dialyzed with pipette solution containing 24 mM [Cl⁻] and superfused with solution containing 150 mM [Cl⁻]. Under these conditions, in 108 cells the density of this holding current shift averaged 0.71 ± 0.62 pA/pF. The voltage dependence and reversal potential of the Fsk-activated current (Fig. 1 B, b-a), and its complete abolition (Fig. 1 B, c-a) upon switching to pipette solution containing 100 μM PKI, confirm that the current shift is caused exclusively by PKA-dependent activation of a Cl⁻ conductance.

The rapid and reversible activation and deactivation of this Cl⁻ conductance, by application and withdrawal of agonist (e.g., Figs. 2, 3, 5, 7, and 8) or by addition of PKI (Fig. 1), must reflect phosphorylation and dephosphorylation of the underlying Cl⁻ channels themselves, or of closely associated regulatory proteins, by highly active endogenous PKA and protein phosphatases. These results are consistent with the following simple two-state scheme:

$$D \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} P \qquad\qquad\qquad (Scheme\ 1)$$

where D and P represent dephosphorylated (deactivated) and phosphorylated (activated) states of the Cl^- channels, and α and β represent pseudo-first (or first) order rate constants for PKA-dependent phosphorylation and for dephosphorylation. α is expected to vary with the degree of stimulation of PKA, whereas β can be assumed approximately constant for a given cell. Accordingly, the steady-state fractional occupancy of the phosphorylated state, P, and therefore the relative amplitude of the Cl^- conductance, depends on the prevailing relative magnitudes of α and β, via $\alpha/(\alpha + \beta)$.

If α falls to zero, which it should do upon introduction of a maximally effective concentration of PKI, or on sudden withdrawal of agonist (unless there is a significant basal activation of PKA; see Discussion), the rate of deactivation of the conductance should reflect β, and the deactivation should proceed to completion. Consistent with this prediction, and hence with Scheme 1, intrapipette concentrations of PKI ≥ 40
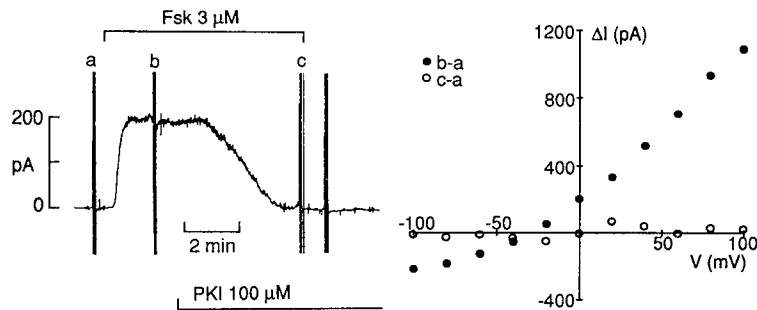

FIGURE 1. Phosphorylation and dephosphorylation regulate the Fsk-activated Cl^- current. (A) Chart record of changes in whole-cell current at the 0-mV holding potential caused by extracellular application of 3 μM Fsk and intrapipette application of 100 μM PKI. Total cell capacitance, 115 pF. By convention, in this and all other figures, bars above the current record mark extracellular maneuvers, and bars below it intracellular maneuvers. The vertical lines labeled a, b, and c, indicate application of voltage pulses to collect I-V data. (B) Steady-state difference I-V relationships from A showing Fsk-induced Cl^- conductance (b-a, ●) and its complete abolition by PKI (c-a, ○).

μM do indeed abolish the entire Cl^- conductance activated by extracellular Fsk (Fig. 1), by intracellular cAMP (Bahinski et al., 1989a), or by extracellular Iso (Hwang et al., 1992c).

To further test Scheme 1, we introduced okadaic acid into the pipette. Okadaic acid is a polyether fatty acid derivative ($C_{44}H_{66}O_{13}$) isolated from marine black sponges (Tachibana, Scheuer, Tsukitani, Kikuchi, Van Eugen, Clardy, Giopichand, and Schmitz, 1981) and is a potent inhibitor of protein phosphatases 1 and 2A (Takai, Bialojan, Troschka, and Rüegg, 1987; Bialojan and Takai, 1988; Hescheler, Mieskes, Rüegg, Takai, and Trautwein, 1988), two of the four major mammalian cytosolic serine and threonine phosphatases (Cohen, 1989). If β comprises activity of either type 1 or 2A phosphatase, then okadaic acid would be expected to reduce β and so cause both an increase in the steady-state level of protein phosphorylation,

and hence of Cl⁻ conductance, in the presence of Fsk, and a decrease in the rate of Cl⁻ conductance decline on Fsk removal.

Fig. 2 illustrates both of these effects in response to intracellular application of 10 μM okadaic acid. A brief exposure to 1 μM Fsk activated a Cl⁻ conductance (Fig. 2 A; Fig. 2 B, b-a, ●) that reached a steady state within 1–2 min and deactivated within 1–2 min on washing out the Fsk. A smaller (~50%) steady-state level of Cl⁻
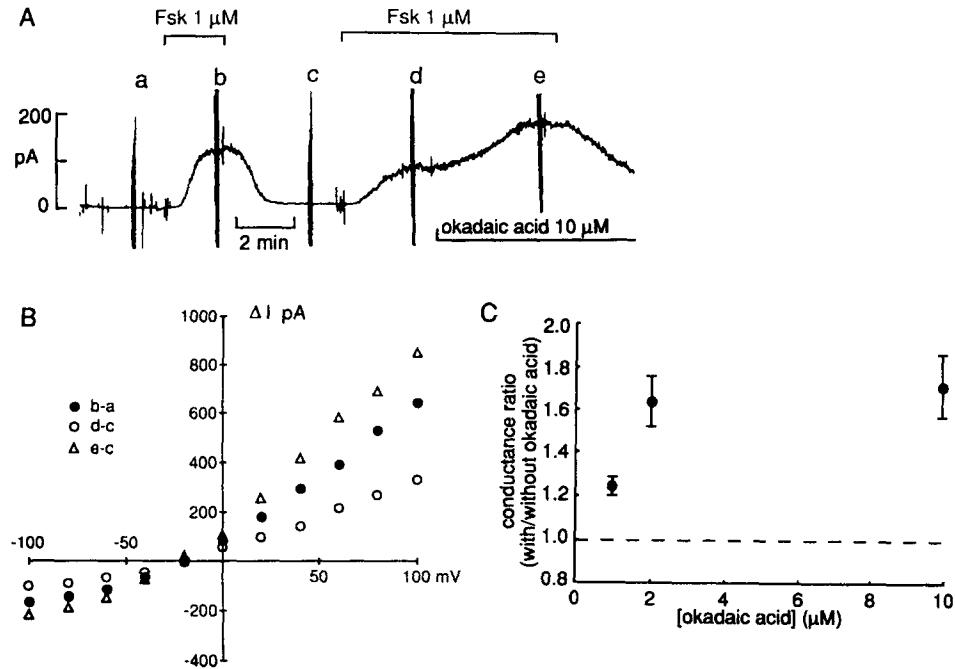

FIGURE 2. Intracellular okadaic acid enhances the Fsk-induced Cl⁻ conductance and slows its deactivation. (A) Chart record of current changes at 0 mV. (B) Steady-state difference I-V relationships from A showing the initial (b-a, ●) and second (d-c, ○) Fsk responses, and the augmented Fsk-elicited Cl⁻ conductance in the presence 10 μM okadaic acid (e-c, △). (C) Concentration dependence of the okadaic acid effect on Cl⁻ conductance. Mean (± SEM) ratios of the Fsk-induced Cl⁻ conductance with and without internal okadaic acid (e.g., |e-c|/|d-c| from A and B) plotted against pipette okadaic acid concentration. In each myocyte, at a given okadaic acid concentration, the conductance ratio was determined as the average ratio of Fsk-induced Cl⁻ current amplitudes, in the presence and absence of okadaic acid, measured at four different positive membrane potentials (usually +20, +40, +60, and +80 mV). Five, four, and four cells, respectively, contributed to the data at 1, 2, and 10 μM okadaic acid.

conductance (Fig. 2 A; Fig. 2 B, d-c, ○) was elicited, somewhat more slowly, during the second exposure to Fsk. The lower amplitude and activation rate signify a "rundown" of the Cl⁻ conductance response; the mechanism of the rundown is unknown and its rate varies among cells (cf. Horie, Hwang, and Gadsby, 1992). However, switching to a pipette solution containing 10 μM okadaic acid during the continued exposure to Fsk further enhanced the Cl⁻ conductance more than twofold,

to a new steady level (Fig. 2 *B*, *e-c*, △) greater than that attained during the prior application of Fsk. The reversal potential of the Fsk-induced current was not altered during rundown or during the action of okadaic acid (Figs. 2 *B*, 3, and 8 *B*). Okadaic acid similarly augmented the Cl⁻ conductance elicited by intrapipette application of cAMP, or persistently activated by Iso plus intrapipette GTPγS (not illustrated; cf. Hwang et al., 1992*c*).

The magnitude of this effect of okadaic acid was evaluated as the ratio of the steady-state level of Fsk-induced Cl⁻ conductance in the presence of okadaic acid to that in its absence, just before the change in pipette solution (e.g., $|e\text{-}c|/|d\text{-}c|$ in Fig. 2 *B*). For a given cell, this conductance ratio was estimated as the mean ratio of Fsk-induced currents in the presence and absence of okadaic acid, measured at each of four membrane potentials >0 mV (usually +20, +40, +60, and +80 mV). Fig. 2 *C* summarizes these ratios obtained from a total of 13 myocytes treated with three different concentrations of intrapipette okadaic acid. Because, despite the relatively short time lapse, a slight rundown of the Cl⁻ conductance between the measurements with and without okadaic acid cannot be ruled out, the ratios in Fig. 2 *C* must be assumed to underestimate the influence of okadaic acid. Nevertheless, Fig. 2 *C* confirms that okadaic acid caused an elevation of the steady-state Cl⁻ conductance in the presence of 1 μM Fsk, consistent with reduction of β in Scheme 1, and that the extent of this effect seemed saturated at 10 μM okadaic acid since it was not altered by the fivefold increase in concentration from 2 to 10 μM. The slower deactivation of the Cl⁻ conductance on withdrawal of Fsk in the presence of okadaic acid (Fig. 2 *A*) is also consistent with a reduction of β. However, if β had been reduced to zero, then all the Cl⁻ channels would remain in the phosphorylated, activated state even after washout of agonist. If the concentration of okadaic acid is effectively saturating, then the slow conductance decline implies that an additional, okadaic acid–insensitive phosphatase could play a role in the underlying dephosphorylation.

These effects of okadaic acid were examined further in experiments like that represented in Fig. 3, in which 1 μM Fsk was applied before and after addition of 10 μM okadaic acid to the pipette solution. Without okadaic acid, the Cl⁻ conductance deactivated promptly and completely after washout of the Fsk, as confirmed by the lack of difference currents over the entire voltage range examined (Fig. 3 *B*, *c-a*, and Fig. 3 *C*, *c-a*, □). Introduction of okadaic acid in the absence of Fsk caused no change in membrane conductance (Fig. 3 *C*, *d-c*, △), but the Cl⁻ conductance elicited by the subsequent exposure to Fsk was substantially enhanced (Fig. 3 *A*, *e* vs. *b*, Fig. 3, *B–D*, *e-d* vs. *b-a*), its deactivation after washing out the Fsk was delayed and slowed, and a component of the Cl⁻ conductance persisted indefinitely (Fig. 3 *A*, *f*, and Fig. 3, *B* and *D*, *f-d*). This sustained component was not dependent upon continuous activity of PKA because it was insensitive to intrapipette application of 100 μM PKI (Fig. 3 *A*, and Fig. 3 *D*, *f-d* = *g-d*).

However, this residual component showed the same reversal potential as the entire PKA-dependent Cl⁻ conductance (Fig. 3 *D*), and similar Cl⁻ sensitivity as demonstrated by the voltage dependence and size of the inward shift of current upon increasing pipette [Cl⁻] (Fig. 3 *A*; see also Bahinski et al., 1989*b*; Hwang et al., 1992*c*). Thus, assuming that the switch from 24 to 109 mM pipette [Cl⁻] resulted in the same change of intracellular [Cl⁻], and that only PKA-dependent Cl⁻ current was
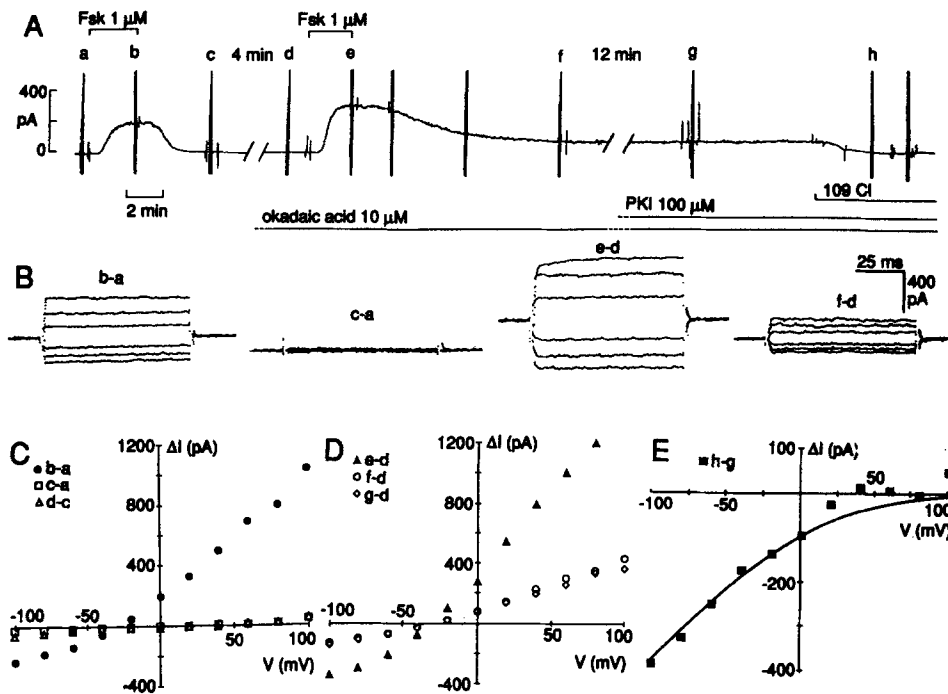
FIGURE 3.   Okadaic acid prevents complete deactivation of the Fsk-activated Cl⁻ conductance. (*A*) Whole-cell current at 0 mV; the two breaks indicate omission of 4- and 12-min sections of record. The lower lines mark changes of pipette solutions to include 10 μM okadaic acid, then add 100 μM PKI, and then raise pipette [Cl⁻] from 24 to 109 mM. (*B*) Superimposed sample traces of difference currents from *A*, determined by subtracting digitized records of currents elicited by voltage pulses to ±20, ±40, and ±60 mV under one condition from corresponding records obtained under another. Identifying letters correspond to those in *A*. Fsk-induced currents are shown before (*b-a*) and after (*e-d*) introduction of okadaic acid; *c-a* shows that Fsk action is completely reversible in the absence of okadaic acid, whereas *f-d* shows the residual currents in the presence of okadaic acid. (*C* and *D*) Steady-state whole-cell difference *I-V* relationships as indicated, from *A* and *B*. (*E*) Difference *I-V* relationship (*h-g*, ■) showing current change, Δ*I*, caused by raising pipette/intracellular [Cl⁻], Δ[Cl⁻]ᵢ; the smooth curve shows a nonlinear least-squares fit to the data of the constant field equation:

$$\Delta I = P_{Cl} \frac{VF^2}{RT} \cdot \frac{\Delta[Cl]_i}{[1 - \exp(VF/RT)]}$$

where P_{Cl} (cm·s⁻¹) is the Cl⁻ permeability coefficient (calculated assuming specific membrane capacitance = 1 μF·cm⁻²), V is membrane potential, and R, T, and F have their usual meanings.

affected, the fit of the constant-field equation to the difference currents (Fig. 3 *E*) yields an estimate for the Cl⁻ permeability coefficient, P_{Cl}, of 4.5 × 10⁻⁸ cm·s⁻¹ for this residual component of Cl⁻ conductance. This is roughly half the P_{Cl} value we previously estimated (~7 × 10⁻⁸ cm·s⁻¹; Hwang et al., 1992c) for the Cl⁻ conductance persistently activated by histamine (10 μM) in the presence of intrapipette

GTPγS (100 μM), approximately as expected on the basis of Fig. 4 B ($|c|/|b|$, ○) if the same population of Cl⁻ channels is responsible for both residual and Fsk-activated components of Cl⁻ conductance.

Fig. 4 summarizes results from 29 myocytes subjected to the protocol of Fig. 3 A to determine the influence of intrapipette okadaic acid concentrations from 1 to 20 μM. As schematically illustrated in Fig. 4 A, the effect to increase the steady-state amplitude of Fsk-induced Cl⁻ conductance was quantified as the ratio of the Cl⁻ conductance levels elicited by 1 μM Fsk in the presence and in the absence of okadaic acid (i.e., $|b|/|a|$, Fig. 4 A). The mean ratios are plotted against okadaic acid concentration in Fig. 4 B (●). Okadaic acid's effect to promote a residual component of Cl⁻ conductance after washout of Fsk was quantified as the ratio of that residual
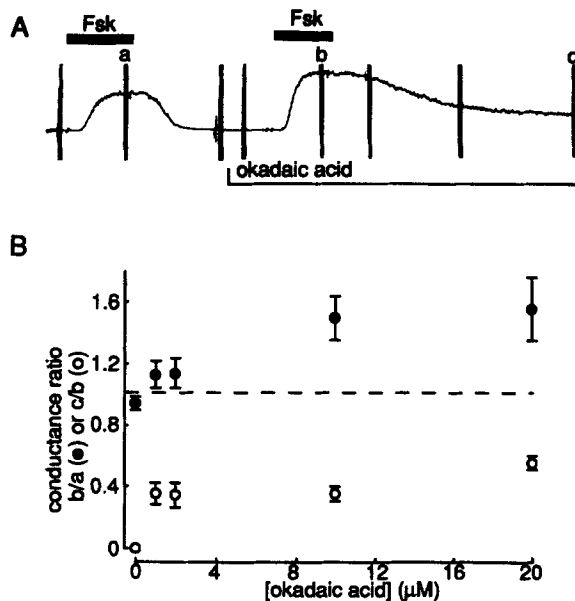

FIGURE 4. Summary of okadaic acid influence on Fsk-activated Cl⁻ conductance. (A) Schematized record showing protocol for evaluating okadaic acid effects. (B) Conductance ratios corresponding to $|b|/|a|$ (●) and $|c|/|b|$ (○) were obtained as in Fig. 2 from difference I-V curves of the Fsk-induced Cl⁻ conductance in the absence (a) or presence (b) of okadaic acid, and of the residual Cl⁻ conductance (c). The mean ratios (± SEM) are plotted versus okadaic acid concentration; the dashed line indicates a ratio of 1.0. The ratio $|b|/|a|$ is <1.0 in the absence of okadaic acid because of rundown; presumably, rundown causes all ratios of both kinds to be similarly slightly underestimated. For the ratio $|b|/|a|$, 6, 4, 3, 9, and 4 cells yielded data at 0, 1, 2, 10, and 20 μM okadaic acid, respectively, and for the ratio $|c|/|b|$ the corresponding numbers of cells were 135, 5, 7, 12, and 5.

Cl⁻ conductance level to the preceding steady-state Cl⁻ conductance level in the presence of both Fsk and okadaic acid (i.e., $|c|/|b|$, Fig. 4 A). The averages of these ratios are also shown in Fig. 4 B (○).

Despite the scatter in the measurements, they confirm that introduction of okadaic acid to the pipette solution enhanced Fsk-induced activation of the Cl⁻ conductance and prevented its full deactivation after withdrawal of Fsk. Measurable effects were obtained with 1 μM okadaic acid, and the effects were increased in a dose-dependent manner with apparently maximal effects occurring between 10 and 20 μM. However, even those highest concentrations of okadaic acid, although they maximally enhanced the Fsk-elicited Cl⁻ conductance, could not entirely prevent its deactivation

(i.e., maximum $|c|/|b|$ remained <1.0; Fig. 4 B). The results strengthen the conclusion from Fig. 2 that okadaic acid lowers β, but not to zero.

According to Scheme 1, if α were to fall to zero after removal of Fsk (that α does indeed become zero is confirmed by the lack of effect of 100 μM PKI), any non-zero value of β should lead eventually to complete deactivation of the Cl⁻ conductance. The incompatible facts that the conductance was not fully deactivated even after as long as 30 min (Figs. 3 and 4 B), and yet okadaic acid could not have reduced β to zero because some relatively rapid decline of the Cl conductance was still observed, suggest that Scheme 1 is oversimplified. Before accepting that conclusion, however, it was necessary to rule out an alternative explanation for how okadaic acid could enhance the Fsk-induced Cl⁻ conductance, and to ensure that a maximal inhibition of phosphatases 1 and 2A had been achieved.

Okadaic Acid Does Not Alter the Apparent Affinity for Fsk Action

An alternative explanation for the increased magnitude and slowed deactivation of Fsk-induced Cl⁻ conductance (although not for the residual component) is that okadaic acid might have markedly increased the apparent affinity with which Fsk activates adenylate cyclase, thereby effectively increasing α; the slowed deactivation would reflect the fact that diffusion barriers delay Fsk dissociation. It is known, for example, that the sensitivity to Fsk can be modulated by activation of G proteins (Parsons, Lagrutta, White, and Hartzell, 1991; Tareen et al., 1991; Hwang, Horie, Dousmanis, and Gadsby, 1992a). We therefore determined Fsk concentration–response relationships both in the absence of okadaic acid, and with 10 μM okadaic acid in the pipette. Fig. 5 A shows a chart record from one of these experiments. Before and after applying okadaic acid, 0.01 and 0.1 μM Fsk activated practically no Cl⁻ current and 1 μM Fsk elicited a substantial Cl⁻ conductance even though, as usual, okadaic acid greatly enhanced this effect. The implied lack of influence of okadaic acid on the sensitivity to Fsk is confirmed by the summarized data in Fig. 5 B. For each myocyte, full I-V relationships were obtained for the Cl⁻ currents activated by each Fsk concentration with and without okadaic acid. The size of the conductance activated under each of these conditions was then normalized with respect to that activated by 1 μM Fsk, in the same cell, in the absence of okadaic acid; these conductance ratios were calculated as in Figs. 2 C and 4 B. The curves show least-squares fits to the data of the Hill equation, yielding half-maximally activating Fsk concentrations ($K_{0.5}$) of 0.45 ± 0.04 μM (± SEM) in the absence of okadaic acid, and 0.36 ± 0.04 μM (± SEM) in its presence, and Hill coefficients of 2.8 ± 0.5 and 2.1 ± 0.5 (± SEM), respectively. These $K_{0.5}$ values are comparable to those previously reported for Fsk stimulation of L-type Ca channel currents in frog (0.4 μM; Hartzell and Fischmeister, 1987) and guinea pig (~0.4 μM; Asai, Pelzer, McDonald, and Pelzer, 1991) ventricular myocytes, and of Cl⁻ current in guinea pig myocytes (~ 1 μM; Tareen et al., 1991).

Thus, the apparent affinity for Fsk action is not substantially altered by a high concentration of okadaic acid, and so the latter's influence on Fsk-induced Cl⁻ conductance can be attributed to reduction of β via phosphatase inhibition, rather than to an increase in effective phosphorylation rate, α.

Intrapipette versus Extracellular Application of Okadaic Acid

A further concern, however, is whether inclusion of 10 or 20 μM okadaic acid in the pipette solution fully inhibits okadaic acid–sensitive phosphatases. Because okadaic acid is lipophilic and readily permeates cell membranes (Hescheler et al., 1988;
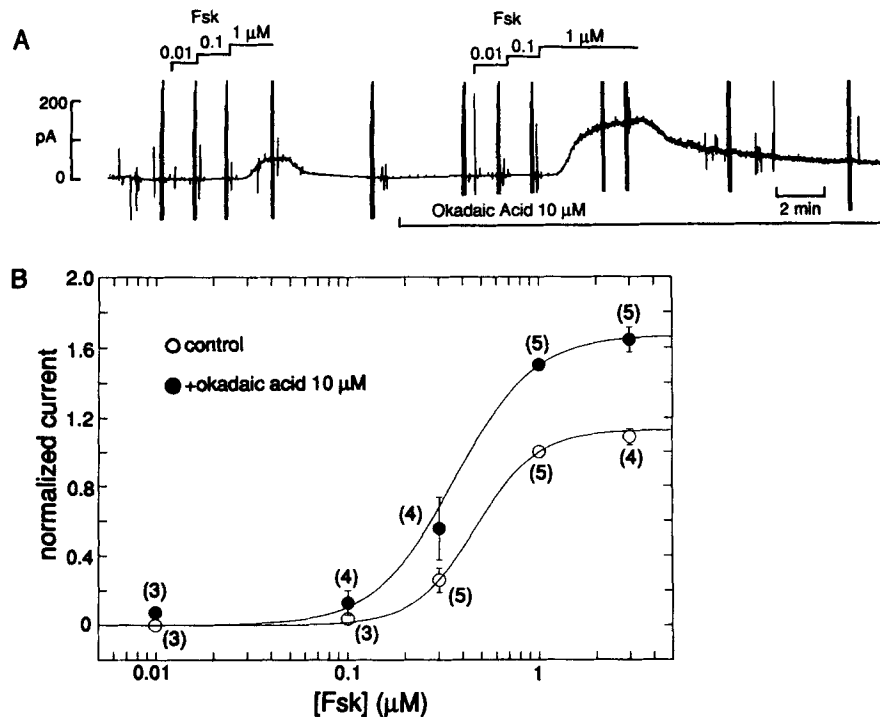

FIGURE 5. Influence of okadaic acid on Fsk dose–response relationship. (*A*) Chart record of current changes at 0 mV, demonstrating the protocol for obtaining cumulative dose–response data. Full Fsk-induced *I-V* relationships were determined for at least three concentrations of Fsk, with and without 10 μM okadaic acid, in each cell. Fsk-induced Cl⁻ conductance ratios were determined at positive potentials, as in Fig. 2, for each Fsk concentration, relative to that elicited by 1 μM Fsk in the absence of okadaic acid. (*B*) Semilog plot of mean (± SEM) normalized Cl⁻ conductance in the absence (○) and presence (●) of 10 μM okadaic acid versus Fsk concentration; the numbers of cells are given in parentheses. The smooth curves show nonlinear least-squares fits of the Hill equation,

$$\text{normalized conductance} = g_{max}/\{1 + (K_{0.5}/[\text{Fsk}])^n\}$$

where $K_{0.5}$ is the half-maximally activating Fsk concentration, g_{max} is the normalized Cl⁻ conductance at infinite [Fsk], and n is the Hill coefficient. g_{max} was 1.1 ± 0.1 without, and 1.7 ± 0.1 with okadaic acid; see text for other fit parameters.

Cohen, Holmes, and Tsukitani, 1990), the steady-state concentration of okadaic acid inside the cell, near the membrane, could be far below that in the pipette solution if the effective permeability of the pipette tip, governing diffusion into the cell, is lower than that of the cell membrane (in combination with any other "sink"), which governs

loss from the cytoplasm. So it is conceivable that, in the experiments of Figs. 2–4, the partial deactivation of Cl⁻ current on washout of Fsk was simply an indication that intracellular concentrations of okadaic acid were insufficient to fully inhibit sensitive phosphatases. However, if that were the case, extracellular application of a given concentration of okadaic acid would be expected to result in a higher intracellular concentration (barring a high-capacity intracellular sink), and hence stronger effect, than that achieved by applying the same concentration via the pipette. On the contrary, in three myocytes we observed effects of 5 μM extracellular okadaic acid similar to, or possibly smaller than, effects with 2 μM intrapipette okadaic acid: namely, the steady-state Fsk-induced Cl⁻ conductance was increased by 23 ± 6% over the control level (cf. $|b|/|a|$ in Fig. 4 B) and the residual component amounted to 12 ± 2% of that increased conductance (cf. $|c|/|b|$ in Fig. 4 B).

That intrapipette concentrations >5 μM yielded maximal effects is in keeping with biochemical studies, which have shown complete inhibition of both type 1 and 2A protein phosphatases from rabbit skeletal muscle by okadaic acid concentrations in the range 0.1–10 μM (Hescheler et al., 1988; Cohen et al., 1990).

Microcystin Mimics the Effects of Okadaic Acid

Further indication that pipette perfusion with >5 μM okadaic acid achieved maximal inhibition of okadaic acid–sensitive phosphatases came from experiments with microcystin (derived from a hepatotoxic cyclic peptide of cyanobacteria; Rinehart, Harada, Namikoshi, Chen, and Harvis, 1988), a hydrophilic membrane-impermeant inhibitor of phosphatases 1 and 2A (Honkanen, Zwiller, Moore, Daily, Khatra, Dukelow, and Boynton, 1990). Fig. 6 shows that 5 μM microcystin, applied via the pipette, mimicked the effects of okadaic acid in that the steady level of Fsk-induced Cl⁻ conductance was increased by about one-third (Fig. 6 A, and Fig. 6 B, c-a vs. b-a) and the deactivation of the conductance after washing out Fsk was slow and incomplete, leaving a residual sustained component of the conductance that was insensitive to PKI (Fig. 6 A, and Fig. 6 B, d-e, ○).

That 5 μM microcystin is indeed a maximally effective pipette concentration is demonstrated by the results in Fig. 7, A and B. As found for okadaic acid, introduction of 5 μM microcystin between Fsk applications enhanced the subsequent Fsk-induced Cl⁻ conductance level (in this case, by almost 50%; Fig. 7 A, and Fig. 7 B, d-c vs. b-a). However, doubling the pipette microcystin concentration in the presence of Fsk did not increase the Cl⁻ conductance further (Fig. 7 B, e-d, △), indicating that a maximal effect had been attained; yet, as with okadaic acid, this maximal concentration of microcystin did not completely abolish deactivation of the Cl⁻ conductance on withdrawal of Fsk.

Because microcystin and okadaic acid are believed to inhibit the same group of phosphatases, a maximal concentration of either inhibitor should occlude any effect of the other, a prediction confirmed by Fig. 7, C and D. Introduction of 10 μM microcystin had no effect on membrane currents in the absence of Fsk (Fig. 7 C, d-c, ▲), but caused the Cl⁻ conductance elicited by the subsequent exposure to 2 μM Fsk to be 60% greater than that during the previous exposure. More important, that Cl⁻ conductance was not increased further when 10 μM okadaic acid was added to the microcystin already in the pipette (Fig. 7 C, f-e, ○). The same result was obtained

when these two phosphatase inhibitors were applied in the reverse order (not illustrated). The noisiness of the PKA-activated whole-cell current (evident in all the figures) reflects slow gating of the channels (cf. Bahinski et al., 1989*b*; Nagel et al., 1992) and indicates that the channel open probability was less than unity, so that an increase in whole-cell current should have been visible if dephosphorylation had been further inhibited. We conclude that, as expected, okadaic acid and microcystin cause their similar effects by a common mechanism, i.e., inhibition of protein phosphatases 1 and/or 2A.

Multiple Phosphorylated States of Cl⁻ Channels

The effects of okadaic acid and microcystin to increase the level of activated Cl⁻ conductance and reduce the rate of its deactivation are both consistent with phosphatase inhibition. But our results show that partial deactivation of the Cl⁻ conductance follows removal of Fsk even in the presence of maximally effective concentrations of okadaic acid (Figs. 2*A*, 3*A*, 4*B*, and 5*A*), microcystin (Figs. 6*A*
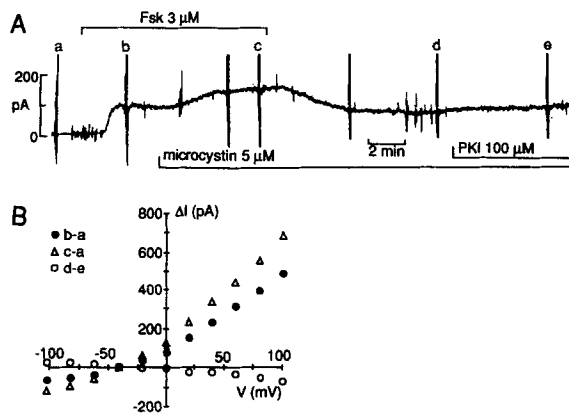

FIGURE 6. Microcystin, a membrane-impermeant inhibitor of protein phosphatases 1 and 2A, mimics the effects of okadaic acid on Fsk-activated Cl⁻ conductance. (*A*) Chart record of current changes at 0 mV. (*B*) Difference *I-V* relationships showing Fsk-induced Cl⁻ current in the absence (●, *b-a*) or presence of 5 μM microcystin (△, *c-a*), and lack of influence of 100 μM PKI on the residual Cl⁻ conductance (○, *d-e*).

and 7*A*), or both (Fig. 7*D*). In other words, β in Scheme 1 cannot be zero under these conditions, and some phosphatase(s) other than those sensitive to okadaic acid and microcystin must be able to dephosphorylate the Cl⁻ channels (or associated regulatory proteins). Because Scheme 1 then predicts eventual full deactivation of the Cl⁻ conductance, which is not observed, the scheme has to be expanded to include at least two phosphorylated states; e.g.,

$$D \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} P_1 \underset{\beta'}{\overset{\alpha'}{\rightleftharpoons}} P_1P_2 \qquad \text{(Scheme 2)}$$

where dephosphorylation of state P_1, but not of state P_1P_2, requires a phosphatase that is sensitive to okadaic acid and microcystin. (An alternative possibility, that there are two populations of channels, is considered in the Discussion). According to Scheme 2, in the presence of Fsk and sufficient okadaic acid to reduce β to zero, only states P_1 and P_1P_2 should be occupied, with a distribution determined by the relative magnitudes of α' and β'; but removal of Fsk should then lead to complete

dephosphorylation of state P_1P_2, leaving only state P_1 occupied. The fact that the residual component of Cl^- conductance (state P_1) amounts, on average, to only half of the Fsk-activated Cl^- conductance level (states $P_1 + P_1P_2$) argues that state P_1P_2 is associated with a higher Cl^- conductance than is state P_1.
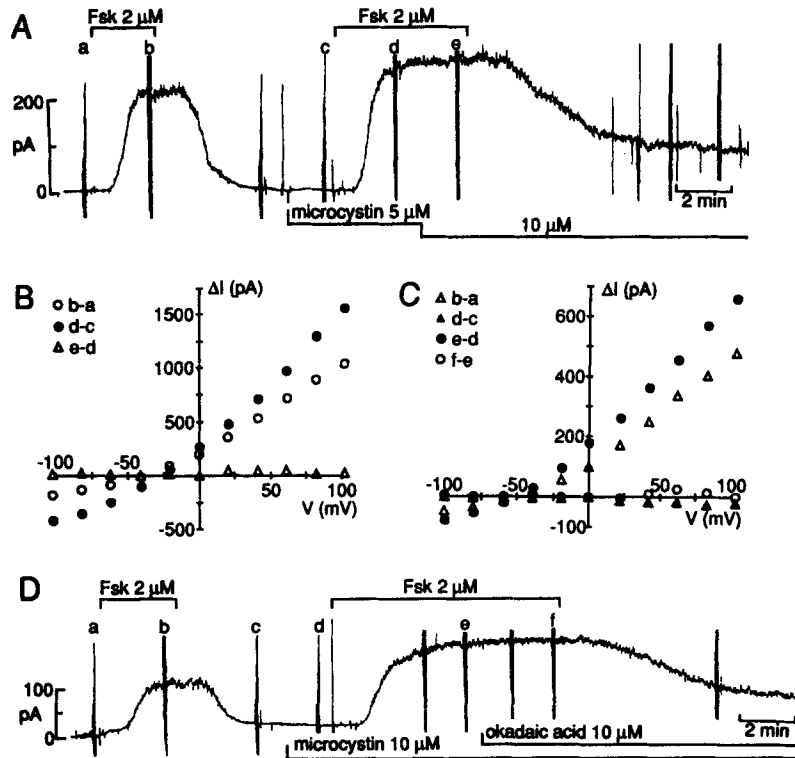

FIGURE 7.   Concentration dependence of microcystin action and occlusion of okadaic acid effect by microcystin. (*A*) Chart record of current at 0 mV showing enhancement of Fsk-induced Cl^- current by 5 μM microcystin, with no further effect of 10 μM. (*B*) Difference *I-V* relationships showing Fsk-activated Cl^- conductance in the absence (○, *b-a*) and presence (●, *d-c*) of 5 μM microcystin, and lack of effect of doubling the [microcystin] (△, *e-d*). (*D*) Chart record showing occlusion of the influence of okadaic acid by a maximally effective concentration of microcystin. (*C*) Difference *I-V* relationships from *D*.

An alternative, nonsequential scheme in which each of two phosphorylated states is accessible from the dephosphorylated state, e.g.,

$$P_1 \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} D \underset{\beta'}{\overset{\alpha'}{\rightleftharpoons}} P_2 \qquad\qquad (\text{Scheme } 3)$$

where, again, β is assumed to be the dephosphorylation rate constant affected by okadaic acid, can be ruled out by the results illustrated in Fig. 8. Scheme 3 requires that, in the presence of sufficient okadaic acid to reduce β to effectively zero, P_1 should become an absorbing state. Hence, after stimulation of PKA (here, by Iso; Fig.

$8A$, b), P_1 should remain populated even after withdrawal of Iso, whereas state P_2 should become dephosphorylated. The observed persistent Cl$^-$ conductance (Fig. $8A$, f; Fig. 8 C, f-d) is consistent with that interpretation.

However, a second application of Iso would be expected to further increase occupancy of state P_1, leading to a larger persistent component of Cl$^-$ conductance when Iso is again withdrawn. Indeed, Scheme 3 predicts that repeated (or prolonged) exposures to any agonist should eventually lead to 100% occupancy of state P_1, with no discernible deactivation of the Cl$^-$ conductance on removal of the agonist. On the contrary, Fig. 8 shows that the residual component of the Cl$^-$ conductance had the
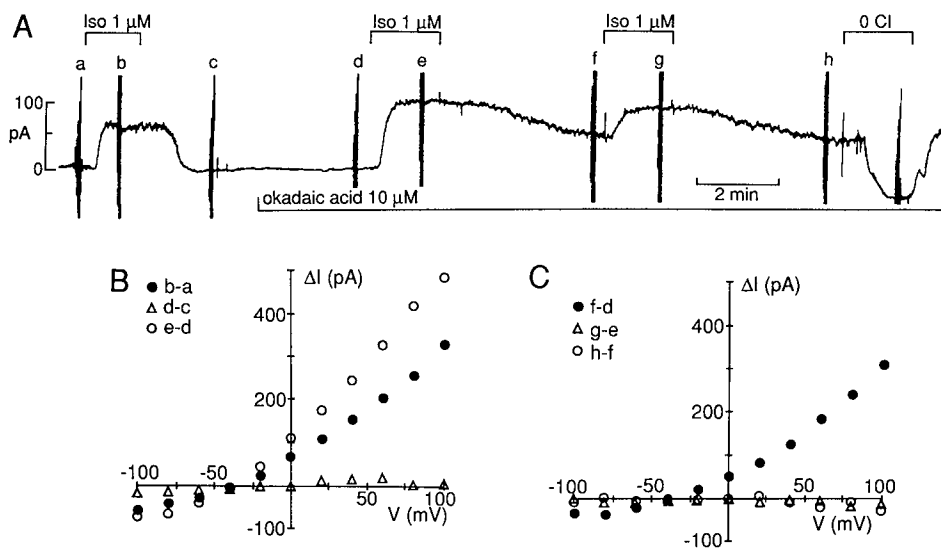

FIGURE 8. A repeat application of Iso in the presence of okadaic acid elicits the same level of activated Cl$^-$ conductance, and leaves the same level of residual Cl$^-$ conductance, as obtained during the previous application. (A) Chart record of current at 0 mV, indicating exposures to Iso and brief period of replacement of external Cl$^-$ with isethionate. (B) Steady-state difference I-V relationships showing enhanced Iso-activated conductance in the presence of okadaic acid (e-d vs. b-a), and lack of effect of okadaic acid alone (d-c). (C) Steady-state difference I-V relationships showing the residual Cl$^-$ conductance (●, f-d), and confirming no difference between the levels of Iso-activated Cl$^-$ conductance (△, g-e) or residual Cl$^-$ conductance (○, h-f) associated with repeat applications of Iso.

same magnitude after the second exposure to Iso as it had after the first. We conclude that PKA-dependent activation of the cardiac Cl$^-$ conductance proceeds via a sequential, multiple phosphorylation scheme of the kind represented by Scheme 2.

That the residual conductance after Iso withdrawal is Cl$^-$ selective is confirmed by the ~80-pA inward current shift (Fig. $8A$; cf. Fig. 8 C, f-d, ●) on sudden replacement of extracellular Cl$^-$ with isethionate ions (cf. Bahinski et al., 1989b), as well as by the outwardly rectifying nature of the resulting difference current (not illustrated).

DISCUSSION

Lack of Basal Activation of Cardiac Cl⁻ Conductance

In cardiac myocytes, equilibration of the intracellular milieu with pipette solutions containing ≥ 50 μM PKI fully prevents Cl⁻ conductance activation by Iso or Fsk (Hwang et al., 1992c). This finding confirms that PKI greatly diminishes the PKA phosphorylation rate (represented by α and α' in Schemes 1–3), presumably to effectively zero. The fact that PKI abolishes exactly that component of membrane conductance activated by intrapipette cAMP (Bahinski et al., 1989a), or by extracellular Iso (Hwang et al., 1992c) or Fsk (Fig. 1), no more and no less, demonstrates both that activation of the Cl⁻ conductance occurs exclusively via PKA-dependent protein phosphorylation, and that basal activation of the Cl⁻ conductance, i.e., in the absence of agonists (Hwang et al., 1992c), must be negligibly small (cf. Bahinski et al., 1989b).

This lack of basal activation of Cl⁻ conductance could reflect a basal PKA phosphorylation rate close to zero and/or a relatively high basal dephosphorylation rate. If a high dephosphorylation rate were solely responsible for the negligible basal activation, application of okadaic acid or microcystin (both of which evidently reduce phosphatase activity) in the absence of Iso or Fsk would be expected to cause activation of a measurable Cl⁻ conductance. On the contrary, up to 5 min of treatment with 10 μM okadaic acid (2.3 ± 0.3 min; n = 9) or 10 μM microcystin (2.5 ± 0.2 min; n = 4) in the absence of agonists had no effect on membrane conductance (e.g., Figs. 3, 5, 7–9), implying that the basal phosphorylation rate is extremely low and, hence, that the relevant kinase is inactive until the cell is stimulated (cf. Cohen et al., 1990). This conclusion is supported by the observation that PKI (which clearly reduces PKA phosphorylation rate) did not alter membrane conductance in the absence of agonists (Hwang et al., 1992c). It remains to be seen whether this negligible, basal, PKA-dependent phosphorylation rate of the proteins regulating cardiac Cl⁻ conductance is also a property of intact myocytes in situ, or can be entirely attributed to our vigorous dialysis of the cell contents against the pipette solution, and consequent lowering of the basal cellular [cAMP] by diffusion into the pipette, or to G protein–mediated partial inhibition of adenylyl cyclase (via activation of G_i by pipette GTP; Hwang et al., 1992a, c).

More General Scheme for Phosphorylation/Dephosphorylation of PKA-regulated Cardiac Cl⁻ Channels

Because maximally effective concentrations of okadaic acid and microcystin, alone or in combination, prevented complete deactivation of the Cl⁻ conductance but allowed its slow, partial deactivation, we conclude that there are at least two functionally distinct phospho-forms of the regulatory protein which, by analogy to biochemical results obtained with CFTR, is likely to be the Cl⁻ channel itself (see below). We suggest that, in the presence of okadaic acid (or microcystin), the residual component of Cl⁻ conductance reflects the properties of Cl⁻ channels phosphorylated at a site (or sites) that can be dephosphorylated only by type 1 and/or 2A phosphatases, whereas the roughly twofold larger conductance during PKA stimulation is attributable to the same population of Cl⁻ channels, some of which are additionally

phosphorylated at a further site that can be dephosphorylated by a phosphatase other than type 1 or 2A.

However, the present data do not allow us to rule out an alternative explanation: namely, that there are two populations of Cl⁻ channels with virtually the same properties except that dephosphorylation of one population, but not the other, requires an okadaic acid–sensitive phosphatase. Those closely similar, if not identical, properties would have to include absolute requirement for phosphorylation by PKA, approximate rate of PKA-dependent activation, approximate time independence of ensemble Cl⁻ conductance (Fig. 3 B), sensitivity to changes of internal (Fig. 3, A and E; cf. Hwang et al., 1992c) and external (Fig. 8 A; cf. Bahinski et al., 1989b) [Cl⁻] by substitution with aspartate and isethionate, respectively, and approximate permeability coefficient for Cl⁻. Additional experiments have shown (Hwang et al., 1992b) that neither the Fsk-activated Cl⁻ conductance (in the absence or presence of microcystin) nor the residual component of conductance (in the presence of microcystin) is altered by 100 μM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS); some kinds of Cl⁻ channel, but not CFTR (Cliff, Schoumacher, and Frizzell, 1992), are blocked by such stilbenes. These similarities between activated and residual conductance components, the likelihood that PKA-regulated cardiac Cl channels are in fact CFTR (Levesque et al., 1992; Nagel et al., 1992), and the knowledge that, during its activation, CFTR becomes phosphorylated at multiple sites (Cheng et al., 1991; Picciotto, Cohn, Bertuzzi, Greengard, and Nairn, 1992) lead us to pursue the explanation based on functionally distinct phospho-forms of the same population of proteins.

Although the three-state sequential phosphorylation model (Scheme 2) has the smallest number of states needed to explain our data, it arbitrarily imposes the constraint of ordered phosphorylation. A more general version is the following four-state scheme with random phosphorylation of the two sites:

$$\begin{array}{ccc} & P_1 & \\ \alpha \diagup \beta & \beta' \diagdown \alpha' & \\ D & & P_1 P_2 \\ \beta' \diagdown \alpha' & \alpha \diagup \beta & \\ & P_2 & \end{array}$$

(Scheme 4)

where, as before, β and β' represent, respectively, okadaic acid–sensitive and –insensitive dephosphorylation rate constants.

In the absence of agonists, basal Cl⁻ conductance is negligible (presumably α and α' are nominally zero; see above) and so all channels can be assumed to be in state D. Strong PKA activation, on the other hand, should elicit population of all states, D, P_1, P_2, and P_1P_2, in proportions governed by the relative sizes of the four rate constants. Introduction of okadaic acid would then reduce β to zero, selectively abolishing dephosphorylation of site 1, and thus shift channels from state D to P_1 and from P_2 to P_1P_2, until a new steady state is reached with only states P_1 and P_1P_2 occupied, just as described for Scheme 2. Also as for Scheme 2, on turning off PKA stimulation in the presence of okadaic acid (i.e., α and α' both restored to zero) β' would dephosphorylate P_1P_2, but only at site 2, leaving all channels trapped in the P_1 state.

As already mentioned, the conductance decrease on removal of Fsk during inhibition of types 1 and 2A phosphatases indicates that state P_1P_2 must be associated with either a higher single channel conductance or a higher open probability than associated with state P_1. To date, only a single conductance amplitude has been noted in histograms of unitary currents in Cl^- channels activated by PKA plus MgATP in giant, inside-out, excised patches (Nagel et al., 1992; Nagel, G., T.-C. Hwang, and D. C. Gadsby, unpublished results), or in outside-out patches excised from myocytes with raised intracellular [cAMP] (Ehara and Matsuura, 1993). However, channel open probability may vary (Nagel et al., 1992) and a steplike increase of open probability is occasionally observed in excised inside-out patches containing only one channel after its activation by PKA and ATP, consistent with an influence of phosphorylation state on channel gating (Nagel, G., T.-C. Hwang, and D. C. Gadsby, unpublished results; but cf. Ehara and Matsuura, 1993).

Further information to support Schemes 2 and 4, and to permit distinction between them, will require determination of the gating properties of the various postulated phosphorylated states, as well as identification of the phosphatase(s) insensitive to okadaic acid and microcystin. Mammalian protein-serine/threonine phosphatases have been classified as types 1, 2A, 2B, and 2C (for review, see Cohen, 1989). Under our experimental conditions (nominally Ca^{2+}-free pipette solutions containing 10 mM EGTA and ~1 mM free Mg^{2+}) in the presence of okadaic acid, only type 2C phosphatases are likely to be active since type 2B phosphatases are Ca^{2+}/calmodulin dependent and show an absolute requirement for Ca^{2+} (Cohen, 1989).

Functional Significance of Multiple Phosphorylation Sites in CFTR Cl^- Channels

The demonstration that PKA-regulated cardiac Cl^- channels closely resemble CFTR (Levesque et al., 1992; Nagel et al., 1992) provides a reasonable biochemical basis for their regulation by PKA-dependent phosphorylation of multiple sites. The cytoplasmic regulatory domain of CFTR comprises several serine residues in consensus PKA phosphorylation sequences (Riordan et al., 1989), and phosphopeptide mapping has shown that PKA phosphorylates four or five serines in CFTR both in vitro and in vivo (Cheng et al., 1991; Picciotto et al., 1992). However, qualitative functional analysis of CFTR mutants (with one to four serine/alanine point mutations) expressed in HeLa cells led to the suggestion that the multiple phosphorylations are degenerate, and that phosphorylation at a single serine might suffice to activate a Cl^- channel (Cheng et al., 1991; cf. Chang et al., 1992). Our data are not inconsistent with activation of CFTR Cl^- channels by phosphorylation at a single site, but they argue that macroscopic conductance is enhanced by phosphorylation at an additional site (or sites) as very recently reported for CFTR mutants (Chang et al., 1992). Because ATP or another hydrolyzable nucleoside triphosphate is required to open the PKA-phosphorylated Cl^- channel (Anderson, Berger, Rich, Gregory, Smith, and Welsh, 1991; Nagel et al., 1992), pointing to critical roles for CFTR's two nucleotide binding folds (cf. Anderson and Welsh, 1992), an important future task will be to understand how the different PKA-phosphorylated states influence channel gating by ATP and analogues.

Comparison with PKA Regulation of Cardiac Ca^{2+} Currents

Among several cardiac ion channels modulated by PKA-mediated phosphorylation (e.g., Hartzell, 1988), voltage-gated L-type Ca^{2+} channels have so far been investigated in most detail. For example, Kameyama, Hofmann, and Trautwein (1985) showed that the nonhydrolyzable ATP analogue, ATPγS, introduced into guinea pig ventricular myocytes via the pipette, enhanced both basal and Iso-stimulated whole-cell Ca^{2+} currents (Kameyama et al., 1985; Kameyama, Hescheler, Hofmann, and Trautwein, 1986; Hescheler, Kameyama, and Trautwein, 1987), and slowed (Kameyama et al., 1985) or prevented (Hescheler et al., 1987) the current decline after washing out Iso, results consistent with thiophosphorylation of Ca^{2+} channels rendering them relatively resistant to the normally strong activity of endogenous phosphatases.

Evidence was found for involvement of endogenous phosphatase 1, since direct introduction of exogenous catalytic subunit of phosphatase 1 abolished the stimulation of Ca^{2+} current caused by maintained exposure to Iso, and introduction of the regulatory subunit (called inhibitor 2) of phosphatase 1 enhanced the stimulation by Iso and slowed approximately twofold, but did not prevent, recovery of the basal Ca^{2+} current level after washout of Iso (Hescheler et al., 1987). Extracellular application of 5 μM okadaic acid enhanced Iso-stimulated current by about one-third and slowed approximately threefold its return to control level upon withdrawal of Iso (Hescheler et al., 1988). However, 5 μM extracellular okadaic acid was evidently not maximally effective because it barely increased ($\leq 10\%$) basal Ca^{2+} current, which was increased further by ≥ 10 μM extracellular okadaic acid and was roughly doubled by intracellular application of 50 μM okadaic acid (Hescheler, et al., 1988). Although saturation was not demonstrated for either inhibitor, the highest concentration of phosphatase 1 inhibitor (10,000 U/ml) strongly enhanced the Iso-stimulated Ca^{2+} current but increased basal Ca^{2+} current only $< 30\%$ (Hescheler et al., 1987), far less than the largest effect of okadaic acid (Hescheler et al., 1988), which should have inhibited both phosphatases 1 and 2A, implying a possible role for endogenous phosphatase 2A in dephosphorylation of Ca^{2+} channels.

Some limited comparison with our results is possible. First, as already discussed, cell dialysis with as much as 20 μM okadaic acid did not activate any Cl^- current (Figs. 3, 5, 7, and 8), in contrast to its activation of basal Ca^{2+} channel current (Hescheler et al., 1988) which presumably reflects phosphorylation by PKA or other kinase(s) (cf. Artalejo, Rossie, Perlman, and Fox, 1992) in the absence of agonists. The most likely explanation for this difference is a lower resting intracellular [cAMP] in our experiments due to more complete cell dialysis via our lower resistance pipettes (< 1 MΩ). Another possibility is that phosphorylation and/or dephosphorylation of Ca^{2+} channels involves different enzyme types, or isoforms, than those that regulate Cl^- channels.

Second, intracellular application of 10,000 U/ml phosphatase 1 inhibitor or extracellular application of 5 μM okadaic acid (apparently a submaximal concentration) slowed the decline of Ca^{2+} current after washout of Iso, but did not prevent its return to the basal level (Hescheler et al., 1988). In contrast, a similar concentration of okadaic acid led to the appearance of a residual component of Cl^- conductance.

The implication is that, unlike Cl^- channels, Ca^{2+} channels might be fully dephosphorylated by either okadaic acid–sensitive or –insensitive phosphatase(s) and, hence, that a simple two-state scheme (like Scheme 1) might be adequate to account for kinase regulation of Ca^{2+} channels.

Finally, because ATP or another hydrolyzable nucleoside triphosphate is required for Cl^- channel gating after PKA-mediated phosphorylation, and because we find that complete replacement of pipette ATP with ATPγS results in ATP-lack contracture of myocytes, we have been unable to examine the influence of thiophosphorylation of cardiac Cl^- channels (but cf. Harvey, Jurevicius, and Hume, 1991).

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