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TWO PHOSPHATASE SITES ON THE Ca^{2+} CHANNEL AFFECTING DIFFERENT KINETIC FUNCTIONS

BY KATSUSHIGE ONO AND HARRY A. FOZZARD

From the Cardiac Electrophysiology Laboratories, Departments of Pharmacological and Physiological Sciences and Medicine, University of Chicago, Chicago, IL, USA

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SUMMARY

1. Changes in dihydropyridine-sensitive (L-type) Ca^{2+} channel kinetics were studied after prolongation of intrinsic phosphorylated time by the phosphatase inhibitor okadaic acid (OA) in cell-attached patches made from single isolated rabbit ventricular myocytes, using barium as the charge carrier.

2. At low concentrations (0·001–1 μM), OA decreased the number of sweeps without openings, while open duration was not changed. However, when cells were pretreated by a membrane-permeant cyclic AMP, 0·1 μM OA induced long-lasting channel openings as well.

3. At high concentrations (10–750 μM), OA additionally induced long-lasting openings, resulting in open time distributions that were best fitted by two exponentials.

4. The durations of an available state (T_S) and an unavailable state (T_F) were estimated by the numbers of non-blank sweeps per run and blank sweeps per run by applying repetitive 45 ms steps at 2 Hz to 0 mV from holding potentials of -80 mV. T_S was well fitted by an exponential curve, of which the time constant was increased from 0·67 to 1·60 sweeps by 0·1 μM OA, while T_F was 0·347 sweeps and remained unchanged.

5. OA activated brief openings and long-lasting, wide openings in a concentration-dependent manner. Namely, we find different dose–response relationships for the two kinetic effects of increased opening probability (mode 1) and prolongation of opening (mode 2). This behaviour suggests that there are at least two modulatory phosphorylation sites that are dephosphorylated by different phosphatases.

INTRODUCTION

Dihydropyridine-sensitive (L-type) Ca^{2+} channel current is increased by interventions that increase levels of cyclic AMP and activity of protein kinase A (PKA) and it is decreased by inhibition of PKA (Kameyama, Hescheler, Hofmann & Trautwein, 1986), leading to the conclusion that the channel is modulated by phosphorylation. Ca^{2+} current is also increased by block of protein phosphatases (Hescheler, Mieskes, Rüegg, Takai & Trautwein, 1988) or reduced by added phosphatases (Chad & Eckert, 1986; Hescheler, Kameyama, Trautwein, Mieskes &

Söling, 1987). This modulation can be seen at the single channel level (Osterrieder, Brum, Hescheler, Trautwein, Flockerzi & Hofmann, 1982; Reuter, 1983; Cachelin, de Peyer, Kokubun & Reuter, 1983; Flockerzi, Oeken, Hofmann, Pelzer, Cavalié & Trautwein, 1986; Gray & Johnston, 1987). Two kinetic effects of phosphorylation have been reported: an increase in probability that channels will open after exposure to lower doses of isoprenaline (Cavalié, Pelzer & Trautwein, 1986; Ochi & Kawashima, 1990), and a prolongation of channel open state after higher doses (Yue, Herzig & Marban, 1990; Ono & Fozzard, 1992). These kinetic changes are analogous to the 'mode' changes proposed by Hess, Lansman & Tsien (1984) to describe the effects of dihydropyridines on Ca^{2+} channel kinetics. According to their scheme, the channel is in mode 0 if, during depolarization, it fails to open. It is in mode 1 when it demonstrates brief openings, and in mode 2 when it shows long-duration openings. The occurrence of these two different kinetic effects of isoprenaline, each with a different dose–response relationship, suggests that two modulatory steps might be involved in the switching between modes.

Multiple putative phosphorylation sites are found in Ca^{2+} channels (Curtis & Catterall, 1985; Hosey *et al.* 1987; Nastainczyk *et al.* 1987; Tanabe *et al.* 1987; Catterall, 1988), so the two kinetic effects could involve two or more modulatory phosphorylations. In addition, cellular phosphatases may themselves be regulated by phosphorylation (Cohen, 1989; Neumann, Gupta, Schmitz, Scholz, Nairn & Watanabe, 1991). Using okadaic acid (Cohen, Holmes & Tsukitani, 1990) to influence phosphorylation times of cardiac Ca^{2+} channels, we find different dose–response relationships for the two kinetic effects of increased open probability and prolongation of opening. These observations provide evidence that modulation of cardiac L-type Ca^{2+} channels by phosphorylation is characterized by two distinct states: one of increased open probability (similar to mode 1), and another of prolonged mean open time (similar to mode 2). Furthermore, our results suggest that these are two different phosphorylation sites, one dephosphorylated by phosphatase 2A and the other by phosphatase 1. A preliminary account of part of this work has been presented in abstract form (Ono & Fozzard, 1991).

METHODS

Cell isolation procedure

Rabbit cardiac ventricular cells were isolated by enzymatic treatment and mechanical dispersion (Ono & Fozzard, 1992). The rabbits (2–3 kg) were anaesthetized with ketamine hydrochloride (80–100 mg kg^{-1}) and xylazine hydrochloride (3–5 mg kg^{-1}) prior to excision of the hearts. The hearts were retrogradely perfused via the aorta. The perfusion buffer contained (mM): 130 NaCl, 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), 10 glucose, 20 taurine, 10 creatine, 5·4 KCl, 3·5 $MgCl_2$ and 0·4 NaH_2PO_4 in ultra-pure double processed water (Milli Q instrument from Millipore Corp., USA), neutralized to pH 7·25 with NaOH and gassed with 100% O_2 (perfusion solution). The heart was perfused at 37 °C with the perfusion solution + $CaCl_2$ (0·75 mM) for 4 min, and then for 4 min with the perfusion solution + ethyleneglycol-bis-(*β*-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) of 0·1 mM. Finally the heart was perfused with the perfusion solution plus $CaCl_2$ (0·08 mM), collagenase (1 mg ml^{-1}) (Worthington, USA; Type II) and protease (0·1 mg ml^{-1}) (Sigma, USA; type 14) for 10 min. The digested heart was removed, chopped coarsely and shaken for four 5 min periods in a plastic flask containing the enzyme solution with the addition of 1% (w/v) bovine serum albumin. After each 5 min incubation the tissue was filtered through a nylon gauze (pore size 200 *μ*m), and undigested material shaken for a further period. The isolated cells filtering through the gauze were collected by centrifugation

at low speed for 120 s. The supernatant was then changed to a solution containing (mM): 150 potassium glutamate and 10 Hepes (pH 7·2). Cells were then stored in a solution containing (mM): 130 potassium glutamate, 5·7 $MgCl_2$, 0·1 EGTA, and 10 Hepes (pH 6·9). The preparation routinely yielded 50–90 % Ca^{2+}-tolerant cardiac myocytes with rod-shaped morphology. Small aliquots of cells were added to the bath solution (see below for contents) in a perfusion chamber of 200 μl volume constructed on a glass coverslip and mounted on the stage of an inverted microscope (Diaphot, Nikon, Japan).

Electrical recording and data analysis

Recordings (List EPC-7, List Electronics, Darmstadt-Eberstat, Germany) were made in the cell-attached configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) in order to maintain intact cellular metabolic conditions. The high potassium solution was assumed to collapse the membrane potential, so that applied potential was taken as the patch potential. Patch pipettes with resistances ranging between 1·4–1·8 MΩ were used to record from patches with small numbers of channels. Patches were depolarized for 195 ms every 1–2 s or for 45 ms every 0·5 s from a holding potential (V_H) of −80 mV.

Experimental protocols were controlled and data were acquired by a DOS-based 486 microcomputer programmed with ASYST (Asyst, Rochester, New York). Channel currents were passed through an 8-pole Bessel filter at 2 kHz and 12-bit digital samples were recorded at 10 kHz. Current sweeps were leak and capacity corrected using the average of sweeps with no activity (blanks) at each test potential. Transitions between closed and open levels were determined using a threshold detection algorithm that required two points above half the mean amplitude of the single-unit opening. Computer-detected openings were confirmed by visual inspection, and occasional sweeps were discarded because of excessive noise. Amplitude histograms were constructed from the corrected traces, and single channel current was taken as the mean value from a Gaussian function fitted to the amplitude histogram. The first bins were ignored for fits of open time histograms. All experiments were performed at room temperature (20–22 °C). Data are reported as means ± standard deviation (S.D.).

Stability of channel behaviour

In order to interpret changes in channel behaviour produced by okadaic acid and other reagents in the cell-attached recording mode, it is important to establish that the channel kinetics are stable during 15 min recording periods in the absence of intervention. Control measurements of channel behaviour were made for this and other concurrent studies and reported in detail recently (Ono & Fozzard, 1992). In summary, a diary of NP_o (proportion of time that the channels were open during each 195 ms duration depolarizing step from −70 to 0 mV) was made during 15 min recordings in cell-attached mode in fifteen cells. The average NP_o during the first 3 min epoch was compared with the average NP_o during the final 3 min. The ratio of NP_o values was 0·96 ± 0·08. A histogram of individual sweep NP_o values was well fitted by a single exponential, as expected for stationary kinetics. Open time distributions during similar early and late epochs were fitted by single exponentials and they did not change with recording time (0·33 ± 0·02 ms, thirteen cells). Single channel currents did not change during the 15 min periods. These results are consistent with no run-down or periodic behaviour during a 15 min recording period under these experimental conditions. Channel activity did cycle between mode 0 and mode 1 (as defined by Hess *et al.* 1984; and as shown in Fig. 4), but with time constants of seconds.

Solutions

The bathing solution contained (mM): 140 potassium aspartate and 10 Hepes (pH adjusted to 7·4 with tetraethylammonium hydroxide (TEA-OH)). This solution depolarized the ventricular cells to approximately 0 mV. The internal solution for the suction pipette contained (mM): 110 $BaCl_2$, 30 TEA-Cl, 0·05 tetrodotoxin (TTX), and 10 Hepes (pH adjusted to 7·4 with TEA-OH). TEA^+ and TTX were added to block K^+ and Na^+ channels, respectively. The agents used in the experiments were as follows: okadaic acid (OA), 8-(4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate (CPTcAMP) and *N*-[2-((3-(4-bromophenyl)-amino)-ethyl]-5-isoquinolinesulphonamide (H-89). Okadaic acid, a generous gift from Dr Y. Tsukitani (Fujisawa Pharmaceutical Co., Japan), was

stored as 10 mM with 100% ethanol. H-89 was stored as 10 mM with 100% dimethyl sulphoxide (DMSO). H-89 (*N*-[2-((3-4-bromophenyl)-amino)-ethyl]-5-isoquinolinesulphonamide) was from Calbiochem (San Diego, CA, USA), and other all chemicals were obtained from Sigma Chemical Co. Where appropriate, data are reported as means ± S.D.

RESULTS

Effect of okadaic acid on channel activity

Figure 1 shows sweeps of Ca^{2+} channel activity modulated by okadaic acid (OA). OA (0·1 μM) reduced the frequency of blank traces (Fig. 1*B*). OA (10 μM) additionally induced openings with much longer durations (Fig. 1*C*). Exposure to OA increased Ca^{2+} channel activity in two ways: (1) by reducing blank sweeps and thereby increasing the total number of openings, and (2) by inducing a new population of long-lasting channel openings. The former effect is similar to that reported for β-adrenergic agonists and related modulators (Cavalié *et al.* 1986; Ochi & Kawashima, 1990). The latter effect has been reported with high concentrations of isoprenaline or cyclic AMP (Yue *et al.* 1990; Ono & Fozzard, 1992) or high levels of the catalytic subunit of PKA (Ono & Fozzard, 1992). Here, we achieved these effects by blocking intrinsic phosphatases, thereby prolonging the durations of individual phosphorylations. In the presence of CTPcAMP, even 0·1 μM OA produced the long-opening behaviour (Fig. 1*E*). This result is not surprising because an intrinsic inhibitor of phosphatase 1 (inhibitor 1) can be activated by PKA-mediated phosphorylation (Cohen, 1989; Neumann *et al.* 1991). The reduction in blank sweeps and the reduced closing rate were studied further, but no additional studies were performed with combined cAMP and OA treatment.

Channel activity in a representative experiment at two concentrations of OA is illustrated in Fig. 2. Exposure to 0·1 μM OA reduced the number of blank sweeps (blank rate) from 66 to 34% in this patch, without changing the number of openings per sweep (Fig. 2*A*) or total open time per sweep (NP_0) (Fig. 2*B*). In this experiment the mean number of channel openings of the non-blank sweeps was 6·6 in control and 6·2 with 0·1 μM OA, and the mean NP_0 of non-blank sweeps was $29{\cdot}9 \times 10^{-3}$ in control and $26{\cdot}1 \times 10^{-3}$ with 0·1 μM OA. Cumulative open time increased modestly as the result of more sweeps with activity (Fig. 2*C*). OA (10 μM) greatly increased individual sweep NP_0 (Fig. 2*B*); the mean NP_0 of non-blank sweeps was $113{\cdot}8 \times 10^{-3}$. The slope of cumulative NP_0 (Fig. 2*C*) was increased, mainly by increasing the population of long openings, because blank rate during the application of 10 μM OA (29%) was not changed from that of 0·1 μM OA (34%). Five other cells showed similar effects with OA (0·1 μM, 10 μM). The mean normalized values for these experiments for 0·1 μM and 10 μM OA are included in Fig. 6*A*.

Effects of inhibition of phosphorylation on okadaic acid action

Phosphorylation was essential for these effects because they were prevented by prior exposure to a protein kinase inhibitor, H-89. The effects of OA on the rate of blank sweeps and mean open probability were studied under the condition of phosphorylation inhibition. Prior to the electrophysiological experiments, myocytes were treated with H-89, for 60 min. Open probability (NP_0) and cumulative NP_0 in the presence of H-89 with OA (10 μM) are shown in Fig. 3. Blank sweeps are marked by

vertical bars. Neither the blank sweep rate (68% in control; 67% with OA), nor the mean NP_0 of the non-blank sweeps (16×10^{-3} in control; 17×10^{-3} with OA) were changed by 10 μM OA. The mean open channel durations were 0·39 and 0·41 ms, before and after OA (10 μM) in this patch.

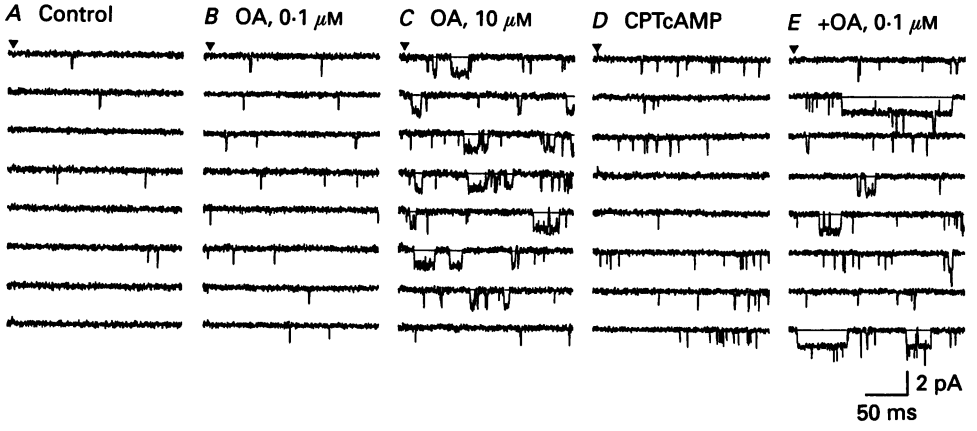

Fig. 1. Effect of okadaic acid (OA) and the membrane-permeant cyclic AMP analogue, 8-(4-chlorophenylthio)cyclic AMP (CPTcAMP) on Ca^{2+}-channel activity in on-cell patches. Eight consecutive sweeps are shown before (*A*), after application of 0·1 μM OA (*B*), and after addition of 10 μM OA (*C*). Panels *D* and *E* show effects of CPTcAMP alone (2 mM) and after addition of OA (0·1 μM). Onset of the voltage step is indicated. The single channel currents were activated by stepping to the test potential (V_T) of 0 mV for 195 ms from a holding potential (V_H) of −80 mV at 1 Hz. The number of channels were estimated as one (*A*,*B*,*C*) and two (*C*,*D*) from the maximum overlapping events.

The block of the OA effect by H-89 was studied in a total of five cells. Control null rate in the presence of H-89 was 54·8 ± 11%, and it was 53·6 ± 12·1% after exposure to 10 μM OA ($n = 5$). Mean NP_0 increased by only 6·8 ± 5·2% (not significant; $n = 5$). It is, therefore, apparent that OA must be stimulating Ca^{2+} channel activity through the modulation of protein phosphorylation. It is interesting that exposure to H-89 in the absence of OA caused a reduction of average NP_0 by 43 ± 18% ($n = 5$) (not shown), similar to the effect of PKA inhibition (Kameyama *et al.* 1986).

Effects on mean phosphorylated and dephosphorylated time

Isoprenaline enhances single Ca^{2+} channel activity by increasing the probability of occurrence of a state with greater probability of opening. Ochi & Kawashima (1990) defined this state as an 'available state', and characterized its duration by the average number of consecutive non-blank sweeps (T_S). They proposed that this state was reached by phosphorylation. A dephosphorylated 'unavailable state' (T_F) was defined by the average number of consecutive blank sweeps. Increased rate of phosphorylation would be expected to abbreviate T_F, and decreased dephosphorylation would be expected to prolong T_S. In fifteen patches the effects of OA on the availability/unavailability of brief channel openings were studied at a low concentration where long openings were rarely observed. For these studies a cumulative 70 min of control and 54 min of OA exposure from the fifteen patches were analysed. OA (0·1 μM) increased T_S from 0·67 sweeps or 0·34 s (control, Fig. 4*A*)

to 1·60 sweeps or 0·80 s (Fig. 4*B*) with no effect on T_F (Fig. 4*C* and *D*), as expected if it only increased the average time the channels were phosphorylated. This is further evidence for the role of intrinsic channel phosphorylation for channel activity, and shows that low concentration of OA (< 1 μM) increases I_{Ca} without

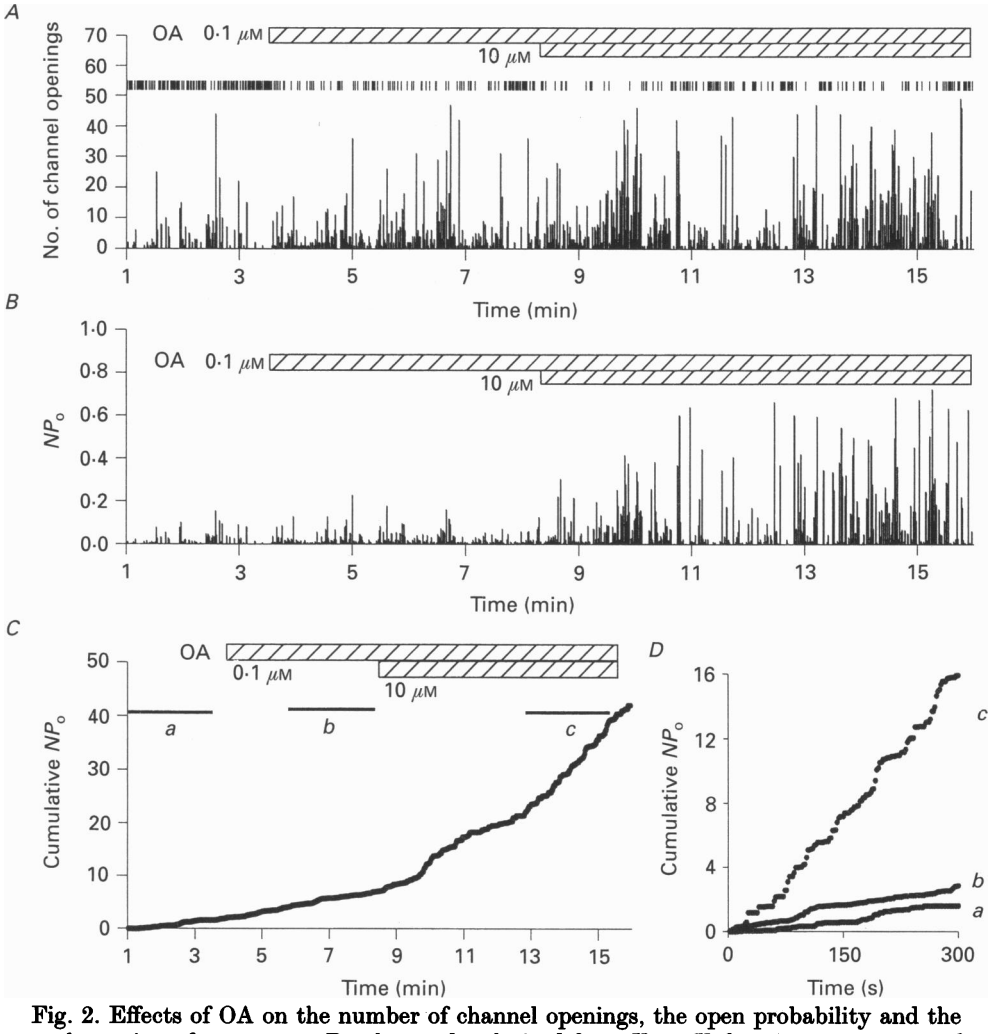

Fig. 2. Effects of OA on the number of channel openings, the open probability and the prolongation of open state. Patch was depolarized from V_H to V_T for 195 ms at a rate of 1 Hz. *A*, the number of channel openings during each 195 ms depolarization. Vertical bars indicate blank sweeps. *B*, open probability as the ratio of total open time to depolarization duration (NP_o) was plotted against the sweep number before and after OA (0·1, 10 μM) from the same experiment. *C*, NP_o values in *B* were also summed to give cumulative activities. *D*, normalized cumulative NP_o during the times indicated by solid lines of *a*, *b*, and *c* in *C*. There appeared to be only one channel in this patch.

affecting the on-rate of phosphorylation of the channel. This is in contrast to isoprenaline, which increases T_S in addition to decreasing T_F (Ochi & Kawashima, 1990). The effect of isoprenaline on T_S is probably because increased cyclic AMP activates a phosphatase inhibitor (Cohen, 1989; Neumann *et al.* 1991). Comparable

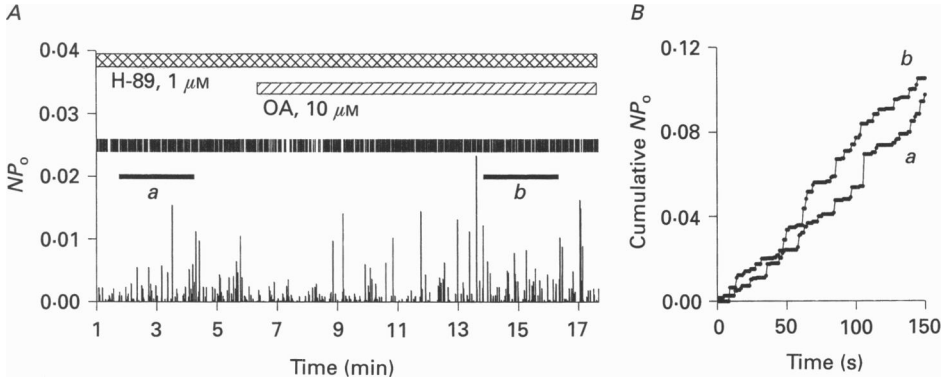

Fig. 3. Effect of protein kinase inhibitor (H-89) on the OA effect. *A*, the pattern of the open probability was not changed by 10 μM OA when the cell was pretreated by the membrane-permeant protein kinase inhibitor (H-89) of 1 μM for 60 min. The vertical bars indicate the blank sweeps. *B*, the cumulative NP_o in control (*a*) and after the effect of OA (*b*) during the time indicated by continuous lines in *A*.

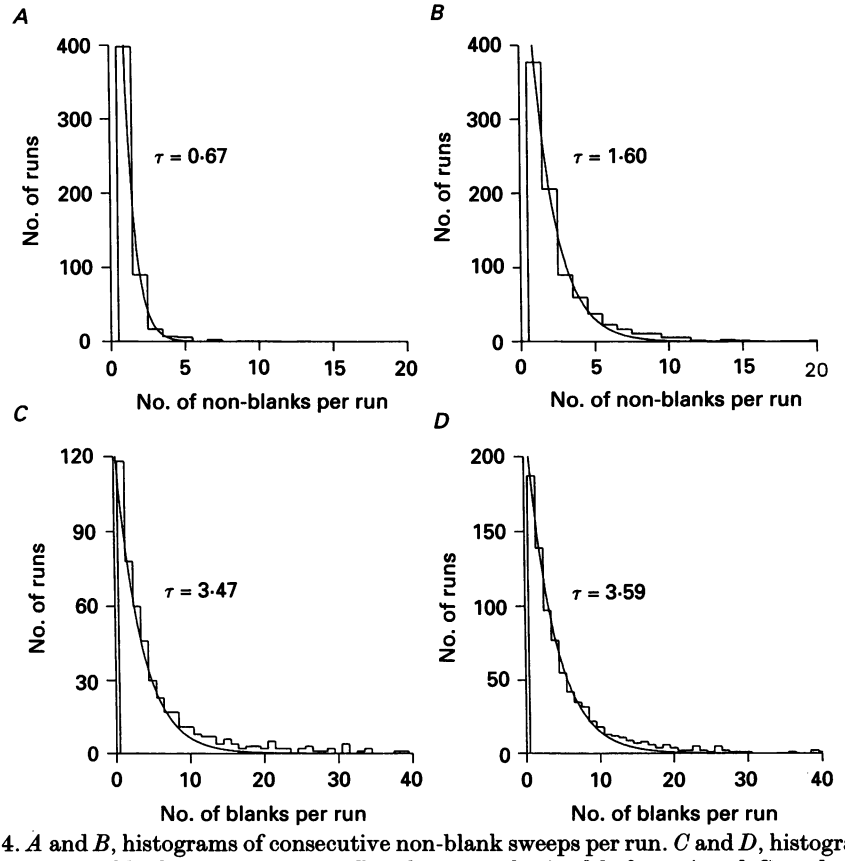

Fig. 4. *A* and *B*, histograms of consecutive non-blank sweeps per run. *C* and *D*, histograms of consecutive blank sweeps per run. Results were obtained before (*A* and *C*) and after application of 0·1 μM OA (*B* and *D*). Patches were depolarized from V_H to V_T for 45 ms at a rate of 2 Hz. Histograms of non-blank and blank sweeps were obtained from fifteen patches exposed to OA (8601 control sweeps; 6561 sweeps for 0·1 μM OA) and were described by single exponentials with time constants (in sweeps) shown.

determination of the mean duration of the state associated with delayed channel closure was difficult because of the requirement for single channel patches and the relatively short (~ 1 s) mode duration of this state.

Dose–response relationship of the two kinetic effects

The prolongation of open-channel duration was further studied by measuring open-channel duration distributions at various concentrations of OA. Open-channel

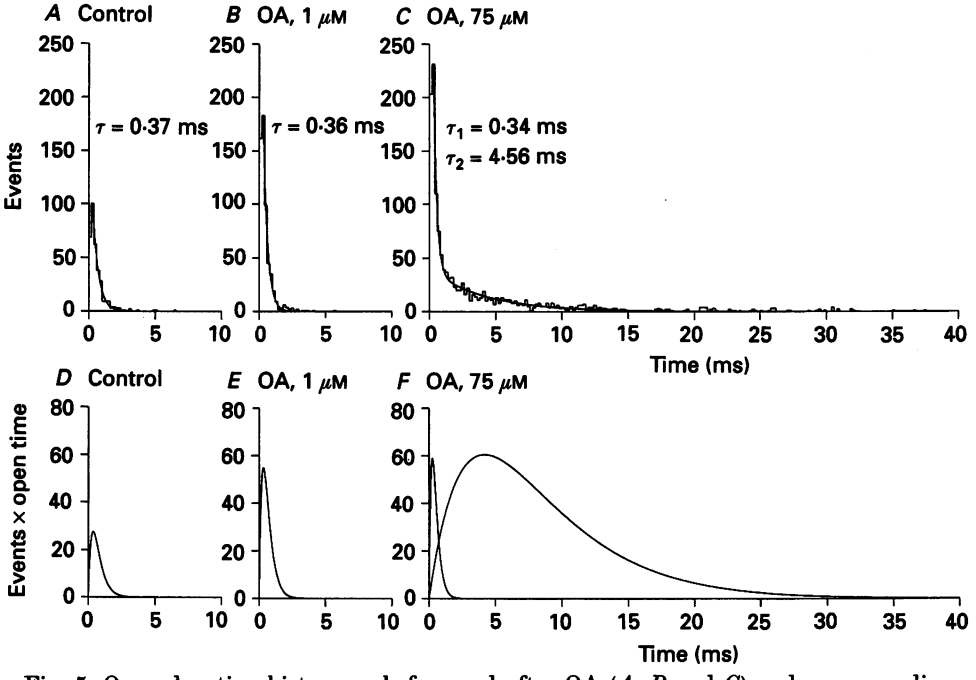

Fig. 5. Open duration histogram before and after OA (*A*, *B* and *C*) and corresponding (events × open time) figures (*D*, *E* and *F*). Each histogram was obtained using 270 sweeps. Open–close tables were previously prepared by requiring two data points beyond the half-amplitude level. Open duration histograms before (*A*) and after application of 1 μM OA (*B*) were well fitted by the single exponential functions,

$$f(t) = \exp\left(-\frac{1}{\tau}t + c\right),$$

where τ is the time constant and $\exp(c)$ is the intercept for each panel *A* and *B* with τ values of 0·37 and 0·36 ms, respectively. The open duration histogram after 75 μM OA required summation of two exponentials, with a faster τ of 0·35 ms, and slower one of 4·56 ms (*C*). The curves in *D*, *E* and *F* were obtained by the following equation:

$$Q(t) = t\,f(t),$$

which multiplies the points from each fitting curve ($f(t)$) for the brief opening mode (*D* and *E*) and long-lasting opening mode (*F*) by corresponding open channel durations (t). The areas under the curves represent the ionic charge through each opening mode, for determination of the response in the separate dose–response relationships.

duration histograms from the same number of sweeps were obtained in the control condition (Fig. 5*A*), after 1 μM OA (Fig. 5*B*), and after 75 μM OA (Fig. 5*C*). The application of 1 μM OA increased the number of individual openings without

affecting the mean open duration (τ = 0·37 ms in control; τ = 0·36 ms with OA). OA (75 μM) additionally induced a channel population that has long-lasting openings. The open duration distribution with 75 μM can be fitted with a two-exponential relationship, where the time constant of the short component was substantially

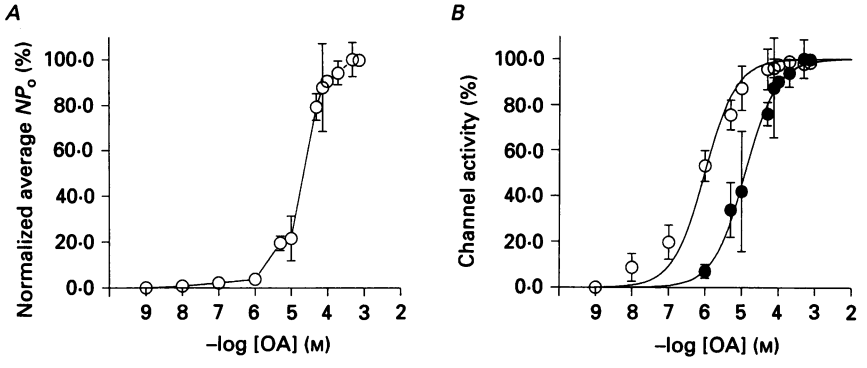

Fig. 6. Dose–response relationship of OA for average NP_o of overall channel activity (*A*), and channel activity increase for brief (○) and long-lasting (●) openings (*B*). For dose–response relationships, each data point is the average of three to seven cells. In panel *B*, data were best fitted using the equation

$$y/y_{max} = 1/(1+(k_{\frac{1}{2}}/C)),$$

where y is measured charge, y_{max} is charge at saturating concentrations (750 μM) and C is OA concentration. This yielded a half-maximal concentration $k_{\frac{1}{2}}$ = 1·0 μM for brief openings, and $k_{\frac{1}{2}}$ = 12·4 μM for long-lasting openings. For the measurement of charge movement through each opening mode, open duration histograms were obtained and analysed by the integration of each $Q(t)$ curve for a 195 ms duration of test pulse as shown in Fig. 5*D*, *E* and *F*.

unchanged from the control and the long-lasting component was new (τ = 0·34 ms, 4·56 ms). The total current movement through the channel openings was evaluated by products of the channel duration and its events numbers before (Fig. 5*D*) and after OA (Fig. 5*E* and *F*). The summation of current by time (ionic charge movement) could be obtained by integration of events numbers multiplied by their open time for the duration of test pulse (195 ms). The total ionic charge movements produced by the short or the long openings were obtained by summing the products of the exponential fits of the short or the long openings multiplied by their durations.

The effects of OA on NP_o and at higher doses on the development of a second population of long openings were seen in every experiment in seven cells. A separate dose dependence of each kinetic effect was obtained by application of various concentrations of OA and computation of the charge movements attributed to the two components, as illustrated in Fig. 5. Because of the time required to collect sufficient data for the computations, it was not possible to obtain measurements with each dose in every cell, but each dose value in Fig. 6*B* is the mean and standard deviation from three to seven cells. These data resemble two single site dose–response curves (Fig. 6*B*) with K_D (dissociation constant) for enhancement of brief opening (○) of 1·0 μM and K_D for long openings (●) of 12·4 μM. The dose dependence of OA on normalized average increase in NP_o showed effects over 4 log units (Fig. 6*A*).

DISCUSSION

These phosphatase inhibition studies demonstrate two kinetic effects of phosphorylation of Ca^{2+} channels, increased opening probability and decreased channel closing rate, similar to the two kinetic effects produced by activation of PKA (Yue *et al.* 1990; Ono & Fozzard, 1992) and analogous to the mode switching identified by Hess *et al.* (1984) for dihydropyridine effects. The phosphorylation that increases opening probability is achieved at lower levels of cyclic AMP and dephosphorylation is achieved by a phosphatase that is blocked at lower levels of OA. The phosphorylation that decreases closing rate requires higher levels of cyclic AMP and dephosphorylation is achieved by a phosphatase blocked only at higher levels of OA. Hescheler *et al.* (1987) reported that intracellular perfusion with either phosphatase 2A and phosphatase 1 reduced or abolished the enhancement of Ca^{2+} current by isoprenaline. They also perfused with inhibitor 2 of phosphatase 1, and alone it increased the Ca^{2+} current somewhat, leading them to suggest that both phosphatases 1 and 2A were involved in Ca^{2+} channel modulation.

Although both putative sites can be phosphorylated by PKA under experimental conditions, this does not indicate which protein kinase is normally involved. Biochemical studies have shown that several protein kinases can phosphorylate the channel protein *in vitro* (Nastainczyk *et al.* 1987; O'Callahan & Hosey, 1988).

Single Ca^{2+} channel kinetics have not previously been studied during blockade of dephosphorylation, but they have been studied in planar bilayers where the dephosphorylation pathway of the channel does not exist (Flockerzi *et al.* 1986; Mundiña-Weilenmann, Ma, Ríos & Hosey, 1991). The similarity of the long-lasting channel openings obtained by high concentration ($> 10\ \mu M$) of OA in our study and those obtained after stimulation by cyclic AMP-dependent protein kinase where no intrinsic/extrinsic phosphatase was present indirectly supports our hypothesis that there is a second functioning phosphorylation site that prolongs channel-open durations. These two modes of modulation of L-type Ca^{2+} channel current are probably responsible for the cyclic slow gating process (Fig. 4) (Cavalié *et al.* 1986; Ochi & Kawashima, 1990). They resemble the mode switching described by Hess *et al.* (1984) for dihydropyridine agonist and antagonist effects, but it is not clear if the dihydropyridine effects are the same as phosphorylation effects. Of the two kinetics processes, the delayed closing process (mode 2) is of greater importance in increasing the size of the current. Our experiments also demonstrate the potentially important role of intrinsic phosphatases in the channel phosphorylation time, emphasizing that there are several means of control of phosphorylation/dephosphorylation of Ca^{2+} channels.

H-89 alone reduced NP_0 values to almost half of the control values, suggesting that under our control conditions the Ca^{2+} channels were partially modulated by phosphorylation. This result agrees with the observations of Kameyama *et al.* (1986) that inhibition of PKA reduced whole-cell Ca^{2+} currents. Armstrong & Eckart (1987) and Ono & Fozzard (1992) have suggested that some level of phosphorylation is absolutely necessary for Ca^{2+} channel activity in inside-out patches. The difference between the intact cell results and those with inside-out patches suggests that some cytoplasmic process can sustain some channel activity, perhaps by a phosphorylation mediated by some protein kinase not inhibited by protein kinase inhibitor or H-89.

The protein kinase blocker H-89 is thought to block preferentially PKA (Chijiwq *et al.* 1990), but effects of these high doses on PKC are not excluded. Alternatively, it is possible that Ca^{2+} channels can be modulated by other means, such as by GTP-binding proteins, through a process independent of phosphorylation (Yatani *et al.* 1987).

Phosphatases 1, 2A, 2B and 2C are all present in heart muscle (Cohen, 1989). OA has been shown to block these phosphatases with different affinities (Bialojan & Takai, 1988; Cohen *et al.* 1990; Hescheler *et al.* 1988). Phosphatase 2B is active only when stimulated by Ca^{2+}–calmodulin (Cohen, 1989), and inhibition of calmodulin by W-7 (100 μM) in these experiments had no effect on the OA-induced changes in Ca^{2+} channel kinetics ($n = 5$, data not shown). Phosphatase 2C is not affected by OA, leaving phosphatases 1 and 2A as candidates for OA effects in these experiments. When phosphatase 2A is tested *in vitro* with phosphorylated phosphorylase or phosphorylated myosin light chain protein as substrates, it is much more sensitive to be blocked by OA than phosphatase 1. Although phosphatases are not usually thought to be specific, it seems likely that the site increasing opening probability may be dephosphorylated by phosphatase 2A, and the site reducing closing rate may be dephosphorylated by phosphatase 1. The *in vitro* K_Ds for inhibition of phosphatases 2A and 1 are much lower than in these *in vivo* studies. This difference has been seen in other *in vivo* OA studies, and attributed to problems of delivery of the inhibitor into the cytoplasm and the relatively high cytoplasmic concentration of phosphatases (Cohen *et al.* 1990). The effects of OA were likely to be specific because they could be prevented by H-89. No toxic effects of the relatively high doses of OA were seen. In addition any possible modulatory effect of intracellular Ca^{2+} could not have been responsible for the OA results because the bathing solutions contained no Ca^{2+} and the currents were measured with Ba^{2+}.

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REFERENCES

ARMSTRONG, D. & ECKERT, R. (1987). Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proceedings of the National Academy of Sciences of the USA* **84**, 2518–2522.

BIALOJAN, C. & TAKAI, A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochemical Journal* **256**, 283–290.

CACHELIN, A. B., DE PEYER, J. E., KOKUBUN, S. & REUTER, H. (1983). Ca^{2+} channel modulation by 8-bromocyclic AMP in cultured heart cells. *Nature* **304**, 462–464.

CATTERALL, W. A. (1988). Structure and function of voltage-sensitive ion channels. *Science* **242**, 50–61.

CAVALIÉ, A., PELZER, D. & TRAUTWEIN, W. (1986). Fast and slow gating behaviour of single calcium channels in cardiac cells. *Pflügers Archiv* **406**, 241–258.

CHAD, J. E. & ECKERT, R. (1986). An enzymatic mechanism for calcium current inactivation in dialysed *Helix* neurons. *Journal of Physiology* **378**, 31–51.

CHIJIWQ, T., MISHIMA, A., HAGIWARA, M., SANO, M., INOUE, T., NAITO, K., TOSHIOKA, T. & HIDAKA, H. (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(*p*Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), of PC12D pheochromocytoma cells. *Journal of Biological Chemistry* **265**, 5267–5272.

COHEN, P. (1989). The structure and regulation of protein phosphatases. *Annual Review of Biochemistry* **58**, 453–508.

COHEN, P., HOLMES, C. F. B. & TSUKITANI, Y. (1990). Okadaic acid: a new probe for the study of cellular regulation. *Trends in Biochemical Sciences* **15**, 98–102.

CURTIS, B. M. & CATTERALL, W. A. (1985). Phosphorylation of the calcium antagonist of the voltage-sensitive calcium channel by cAMP-dependent protein kinase. *Proceedings of the National Academy of Sciences of the USA* **82**, 2528–2532.

FLOCKERZI, V., OEKEN, H., HOFMANN, F., PELZER, D., CAVALIÉ, A. & TRAUTWEIN, W. (1986). Purified dihydropyridine-binding site from skeletal muscle t-tubules is a functional calcium channel. *Nature* **323**, 66–68.

GRAY, R. & JOHNSTON, D. (1987). Noradrenaline and β-adrenoceptor agonists increase activity of voltage-dependent calcium channels in hippocampal neurons. *Nature* **327**, 620–622.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HESCHELER, J., KAMEYAMA, M., TRAUTWEIN, W., MIESKES, G. & SÖLING, H. D. (1987). Regulation of the cardiac calcium channel by protein phosphatases. *European Journal of Biochemistry* **165**, 261–266.

HESCHELER, J., MIESKES, G., RÜEGG, J. C., TAKAI, A. & TRAUTWEIN, W. (1988). Effects of a protein phosphatase inhibitor, okadaic acid, on membrane currents of isolated guinea-pig cardiac myocytes. *Pflügers Archiv* **412**, 248–252.

HESS, P., LANSMAN, J. B. & TSIEN, R. W. (1984). Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. *Nature* **311**, 538–544.

HOSEY, M. M., BARHANIN, J., SCHMID, A., VANDAELE, S., PTASIENSKI, J., O'CALLAHAN, C., COOPER, C. & LAZDUNSKI, M. (1987). Photoaffinity labelling and phosphorylation of a 165 kilodalton peptide associated with dihydropyridine and phenylalkylamine-sensitive calcium channels. *Biochemical and Biophysical Research Communications* **147**, 1137–1145.

KAMEYAMA, M., HESCHELER, J., HOFMANN, F. & TRAUTWEIN, W. (1986). Modulation of Ca current during the phosphorylation cycle in the guinea-pig heart. *Pflügers Archiv* **407**, 123–128.

MUNDIÑA-WEILENMANN, C., MA, J., RÍOS, E. & HOSEY, M. M. (1991). Dihydropyridine-sensitive skeletal muscle Ca channels in polarized planar bilayers; effects of phosphorylation by cAMP-dependent protein kinase. *Biophysical Journal* **60**, 902–909.

NASTAINCZYK, W., RÖHRKASTEN, A., SIEBER, M., RUDOLPH, C., SCHÄCHTELE, C., MARMÈ, D. & HOFMANN, F. (1987). Phosphorylation of the purified receptor for calcium channel blockers by cAMP kinase and protein C. *European Journal of Biochemistry* **169**, 137–142.

NEUMANN, J., GUPTA, R. C., SCHMITZ, W., SCHOLZ, H., NAIRN, A. C. & WATANABE, A. M. (1991). Evidence for isoproterenol-induced phosphorylation of phosphatase inhibitor-1 in the intact heart. *Circulation Research* **69**, 1450–1457.

O'CALLAHAN, C. M. & HOSEY, M. M. (1988). Multiple phosphorylation sites in the 165-kilodalton peptide associated with dihydropyridine-sensitive calcium channels. *Biochemistry* **27**, 6071–6077.

OCHI, R. & KAWASHIMA, Y. (1990). Modulation of slow gating process of calcium channels by isoprenaline in guinea-pig ventricular cells. *Journal of Physiology* **424**, 187–204.

ONO, K. & FOZZARD, H. A. (1991). Modulation of L-type calcium channel kinetics by phosphatase inhibitor. *Circulation* **84**, II–442.

ONO, K. & FOZZARD, H. A. (1992). Phosphorylation restores activity of L-type calcium channels after rundown in inside-out patches from rabbit cardiac cells. *Journal of Physiology* **454**, 673–688.

OSTERRIEDER, W., BRUM, G., HESCHELER, J., TRAUTWEIN, W., FLOCKERZI, V. & HOFMANN, F. (1982). Injection of subunits of cyclic AMP-dependent protein kinase into cardiac myocytes modulates Ca^{2+} current. *Nature* **298**, 576–578.

REUTER, H. (1983). Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature* **301**, 569–574.

TANABE, T., TAKESHIMA, H., MIKAMI, A., FLOCKERZI, V., TAKAHASHI, H., KANGAWA, K., KOJIMA, M., MATSUO, H., HIROSE, T. & NUMA, S. (1987). Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature* **328**, 313–318.

YATANI, A., CODINA, J., IMOTO, Y., REEVES, J. P., BIRNBAUMER, L. & BROWN, A. M. (1987). A G protein directly regulates mammalian cardiac calcium channels. *Science* **238**, 1288–1292.

YUE, D. T., HERZIG, S. & MARBAN, E. (1990). β-Adrenergic stimulation of calcium channels occurs by potentiation of high-activity gating modes. *Proceedings of the National Academy of Sciences of the USA* **87**, 753–757.