# Two distinct functional effects of protein phosphatase inhibitors on guinea-pig cardiac L-type $Ca^{2+}$ channels

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1. The effects of the phosphatase inhibitors okadaic acid and calyculin A on single guinea-pig ventricular L-type $Ca^{2+}$ channels were studied. The inactive derivative norokadaone was used as a negative control.

2. The two known effects of cAMP-dependent stimulation are mimicked by the phosphatase inhibitors to a varying extent. Only okadaic acid promotes the high-activity gating mode ('mode 2'), while calyculin A increases channel availability to a larger extent. As revealed by kinetic analysis of slow gating, the two phosphatase inhibitors retard a slow rate constant, which is assumed to represent exit from the available state by dephosphorylation. Norokadaone was inactive in both regards.

3. Mode 2 gating elicited by very positive prepulses is augmented by okadaic acid, and mode 2 lifetime is prolonged. Calyculin A fails to affect these parameters. Thus, voltage-facilitated mode 2 gating reveals the same pharmacological properties as the mode 2 sweeps observed using conventional pulse protocols.

4. The results are interpreted in terms of the different sensitivity of protein phosphatase subtypes towards the inhibitors: channel availability appears to be controlled by a phosphorylation site dephosphorylated by a type 1-like phosphatase, while mode 2 gating is coupled to a distinct site, dephosphorylated by a type 2A-like phosphatase.

Stimulation of cardiac L-type $Ca^{2+}$ channels by cAMP-dependent phosphorylation regulates myocardial contractility and serves as a classical protocol for regulation of ion channels by phosphorylation. Yet the structural basis of upregulation remains unknown. Channels not only respond more frequently to depolarization (availability) but, in addition, the characteristics of the response are biased towards gating modes with high open probability (mode 1 and 2). It is highly conceivable that these two types of effects are coupled with distinct phosphorylation sites. This idea has been put forward previously (Yue, Herzig & Marban, 1990) and has been supported in the meantime: fast (modal) gating is kinetically independent of slow (availability) gating (Herzig, Patil, Neumann, Staschen & Yue, 1993), and the protein phosphatase inhibitor okadaic acid alters the two parameters in different concentration ranges (Ono & Fozzard, 1993). Therefore, we systematically compared the effects of two specific protein phosphatase inhibitors, calyculin A and okadaic acid, in an attempt to dissect slow and fast gating effects pharmacologically. Our study was prompted by a preliminary analysis of the effects of calyculin A (Neumann *et al.* 1994), which revealed some striking differences compared with conventional cAMP-dependent stimulation (Yue *et al.* 1990). In the present study, strictly similar conditions regarding drug concentration (~1 $\mu$M) and recording bandwidth etc. were used. Guinea-pig ventricular myocytes were chosen because modal gating of $Ca^{2+}$ channels was originally described in these cells (Hess, Lansman & Tsien, 1984). Furthermore, *multi-channel* patches were strictly excluded for the sake of a meaningful quantitative analysis of both modal gating and slow gating. Norokadaone served as a negative control. This is a close derivative of okadaic acid, but is >100-fold less active (Nishiwaki *et al.* 1990).

## METHODS

Ventricular myocytes were obtained from guinea-pigs killed by cervical dislocation. Cells were enzymatically isolated, using a Langendorff perfusion with collagenase and protease as described

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**Table 1. Effects of okadaic acid and norokadaone on fast gating parameters**

| | Open time (ms) | Closed time (ms) | First latency (ms) | Burst length (ms) | Inactivation (%) |
|---|---|---|---|---|---|
| Control | 0·69 ± 0·02 | 2·14 ± 0·22 | 23·5 ± 1·1 | 73·3 ± 6·6 | 50·2 ± 10·0 |
| Okadaic acid | 0·88 ± 0·05* | 1·73 ± 0·60 | 17·3 ± 1·0* | 81·1 ± 15·2 | 46·0 ± 13·5 |
| Control | 0·61 ± 0·07 | 4·36 ± 1·55 | 24·3 ± 5·1 | 55·4 ± 11·8 | 73·5 ± 4·4 |
| Norokadaone | 0·69 ± 0·12 | 3·16 ± 0·67 | 21·2 ± 4·0 | 56·1 ± 11·0 | 81·7 ± 2·5 |

Values are expressed as means ± S.E.M.; $n = 5$; * $P < 0·05$ *versus* control.

in detail previously (Herzig *et al.* 1993). They were kept in a bath solution containing (mM): potassium glutamate, 120; KCl, 25; $MgCl_2$, 2; CaATP, 1; EGTA, 2; Hepes, 10; pH 7·3. Recordings were made at 23–25 °C in a Petri dish (diameter 3·5 cm) placed on the stage of an inverted microscope.

Currents were recorded using an Axopatch 200 amplifier (sampling rate, 10 kHz; filter cut-off, 2 kHz; −3 dB; 4-pole Bessel), using the pCLAMP 5.5.1 software (both from Axon Instruments, Foster City, CA, USA) run on a 486 computer. Patch pipettes (resistance, 7–10 MΩ) were filled with a solution comprising (mM): $BaCl_2$, 70; sucrose, 110; Hepes, 10; pH adjusted to 7·4 with TEA-OH. L-type channels (identified by their typical unitary conductance and voltage dependence of activation) were usually found in one out of ten to fifteen patches using such pipettes. After digital subtraction of linear leak and capacity currents, channel openings and closures were identified by the half-height criterion. The original traces in Fig. 4 were digitally refiltered at 1 kHz for display.

Phosphatase inhibitors (from Biotrend, Cologne, Germany, or Calbiochem, Bad Soden, Germany) were added in a 20 μl volume of a stock solution (0·1 mM in dimethyl sulphoxide (DMSO)) to the Petri dish (containing ~2 ml bath solution), yielding final concentrations of ~1 μM drug (0·56–1·5 μM) and about 1 % (v/v) DMSO. Five experiments were performed each with okadaic acid (0·7–1·4 μM), calyculin A (0·8–1·5 μM), and norokadaone (0·7–1·2 μM), using the pulse protocol depicted in Fig. 1 (pulses from −100 mV to +20 mV, duration 150 ms, applied every 600 ms). After a control period containing 480–600 sweeps, drug effects were recorded during another 294–1860 (in most cases 600–1100) sweeps. For the prepulse experiments shown in Fig. 4, twelve dishes containing cells were pre-incubated for 5–25 min with isoprenaline (0·6–1·0 μM, from a 0·1 mM stock solution stabilized with ascorbic acid (1 mg $ml^{-1}$)) and isobutylmethylxanthine (5·6–10 μM). After seal formation (11 single-channel patches and 1 multi-channel patch with only 1 channel active most of the time) and taking control data in the presence of the two cAMP-elevating agents, either okadaic acid (0·7–0·8 μM, $n = 5$) or calyculin A (0·6–1·0 μM, $n = 7$) was additionally applied. The effects on channel availability were checked in these experiments using the same pulse protocol as described above, but only 180–360 sweeps were sampled both before and after addition of the phosphatase inhibitor. The prepulse protocol is described in detail in Fig. 4.

All values are given as means ± S.E.M. Statistical significance is indicated by an asterisk, checked at the $P < 0·05$ level. A Student's paired two-tailed $t$ test comparing the individual pre- and postdrug values was used throughout. The data in Fig. 4 have been subjected to a Fisher's two-tailed exact test ($P < 0·05$).

# RESULTS

## Effects of phosphatase inhibitors on single-channel currents

The protein phosphatase inhibitor okadaic acid stimulates cardiac L-type $Ca^{2+}$ channels (Fig. 1*A* and *B*). As with β-agonists or cAMP derivatives (Yue *et al.* 1990), the probability that depolarization leads to channel opening (availability) is enhanced. Furthermore, within active sweeps, the time spent in the open state (open probability) seems longer compared with the control period (see below). Both effects contribute to the increase in the ensemble average current. We were puzzled initially by the totally different picture obtained using another phosphatase inhibitor, calyculin A (Fig. 1*C–G*); this compound enhanced availability dramatically, without any increase in open probability. On the other hand, as we have reported previously (Neumann *et al.* 1994), calyculin A speeds up the rate of inactivation, an effect not seen with okadaic acid. Since, in this study we compared effects of similar concentrations (~1 μM), this difference might be due to different potencies of the two phosphatase inhibitors and reflect concentration-dependent phenomena. To address this question, we further analysed the results of earlier experiments ($n = 4$) performed with 10-fold higher concentrations of okadaic acid (Neumann *et al.* 1993*a*), exactly equipotent with regard to channel availability (increase from 24·1 to 47·4 %, as compared with 27·4 to 46·7 % by calyculin A). However, even with this higher concentration of okadaic acid, inactivation was unaffected. This was gauged by the extent of decay of ensemble average currents (as a percentage of the peak current), which is not changed (from 47·7 ± 3·3 to 48·2 ± 6·8 %) by okadaic acid but is changed by calyculin A (from 50·0 ± 10·8 to 73·8 ± 7·2 %; $P < 0·05$). In the analysis of individual traces, inactivation was assessed by measuring mean burst length, i.e. the time from the first opening to the last closure within each sweep. While calyculin A shortened this value significantly (from 81·7 to 56·8 ms; Neumann *et al.* 1994), high concentrations of okadaic acid were still ineffective (no change, from 87·7 ± 5·6 to 86·2 ± 7·5 ms), like the lower concentrations used in the present experiments (Table 1). Thus, the lack of effect of okadaic acid on channel inactivation is not due to underdosing relative to calyculin A.

Alternatively, the differences between the two drugs could be due to the phosphatase subtype inhibition pattern of the two drugs, since okadaic acid is more selective for type 2A phosphatase (Hescheler, Mieskes, Rüegg, Takai & Trautwein, 1988), whereas calyculin A more potently inhibits type 1 phosphatase (Ishihara *et al.* 1989). In guinea-pig myocytes especially, okadaic acid is both more potent and more selective for type 2A phosphatases than calyculin A (Herzig, Meier, Pfeiffer & Neumann, 1995). The absence of any effects with

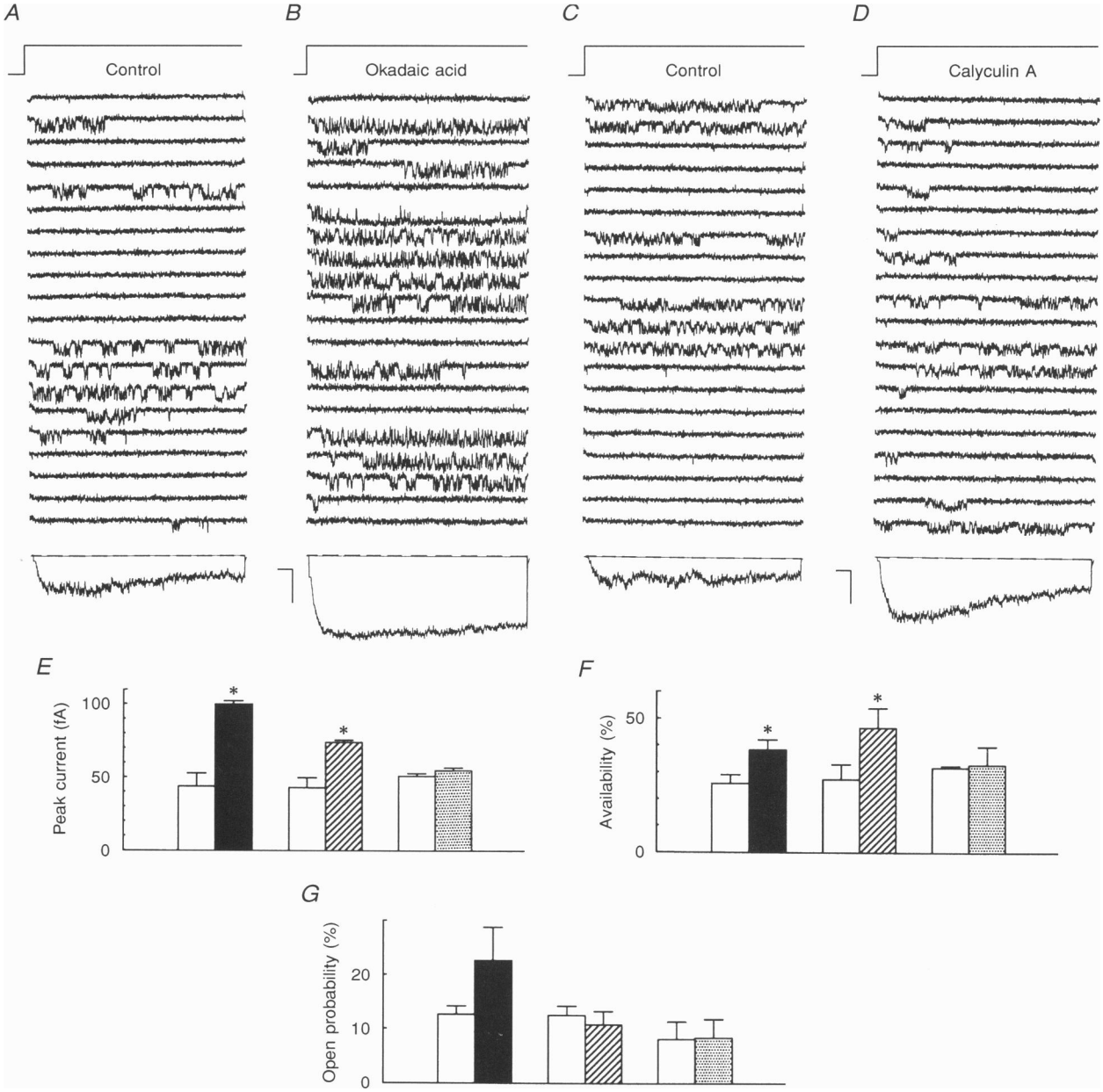


**Figure 1. Effect of phosphatase inhibitors on $Ba^{2+}$ currents through single cardiac L-type $Ca^{2+}$ channels**

Pulses delivered every 600 ms from −100 to +20 mV for 150 ms elicited channel activity before (*A*) and after (*B*) 0·72 μM okadaic acid, and before (*C*) and after (*D*) 0·86 μM calyculin A. The whole ensemble average current is shown below a number of consecutive individual traces. Scale bars denote 10 ms and 2 pA (single traces), or 0·03 pA (ensemble average), respectively. Drug effects on peak current (*E*), channel availability (fraction of active sweeps containing openings, *F*), and the open probability (fraction of time spent in the open state within active sweeps, *G*) are depicted together with the respective controls. □, control; ■, okadaic acid; ▨, calyculin A; ⊠, norokadaone. Okadaic acid enhanced open probability in 4 out of 5 cases. Calyculin A increased peak current and availability only (the data for calyculin A in *E*–*G* are taken from Neumann *et al.* 1994). * $P < 0{\cdot}05$ *versus* control.

norokadaone (Fig. 1*E–G*, Table 1) confirms that phosphatase inhibition is responsible for the observed changes of $Ca^{2+}$ channel behaviour. With this in mind, we analysed fast and slow channel gating more rigorously.

### Detailed analysis of fast and slow gating

The apparent increase in open probability by okadaic acid (Fig. 1*G*), although quite pronounced, is not statistically significant due to remarkable interindividual scatter. To investigate whether this compound really changes microscopic channel gating, we analysed various relevant parameters (Table 1). Indeed, significant increases of mean open times and of the latency to first opening were found. These effects are familiar consequences of cAMP-dependent phosphorylation of the channel (e.g. Yue *et al.* 1990). This gives a strong indication that with okadaic acid, as with elevation of cAMP, shifts in gating modes take place. Modal gating of cardiac L-type channels (Fig. 2) can be analysed by sorting sweeps according to the length of their respective longest openings ($t_{o,max}$) (Yue *et al.* 1990). This allows distinction among three active gating modes. In the briefest bin ($t_{o,max} \leqslant 0{\cdot}5$ ms), a number of sweeps with very low open probability and short open times were identified, called mode $0_a$ (typical example, Fig. 1*C*, trace 13). Most other sweeps were characterized by a bell-shaped $t_{o,max}$ distribution. They

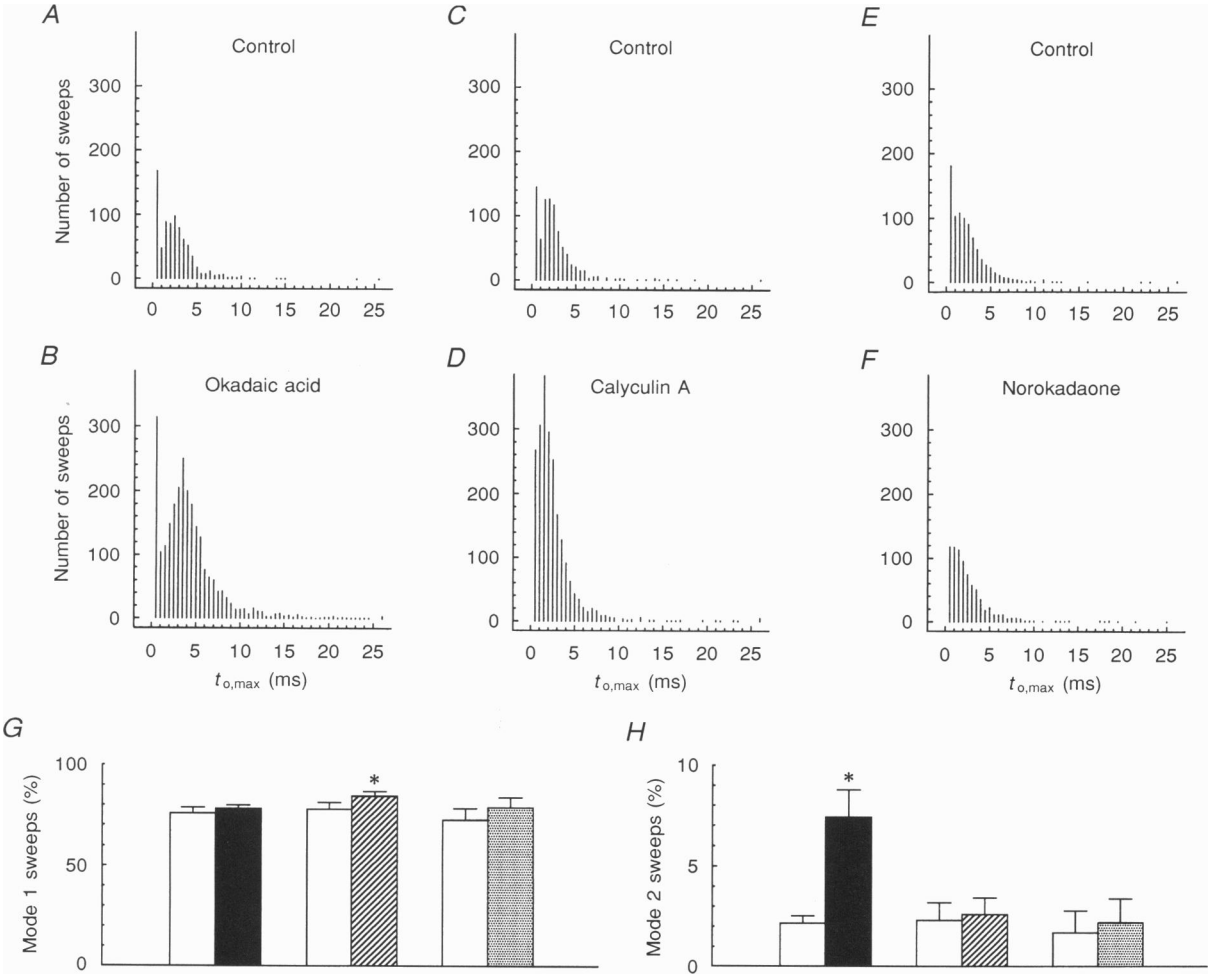


**Figure 2. Modal gating analysis**

In *A–F*, data from the 5 experiments in each group are pooled to give the $t_{o,max}$ frequency distributions before (*A*, *C* and *E*) and after okadaic acid (*B*), calyculin A (*D*) and norokadaone (*F*) (the outer right bins represent all sweeps with $t_{o,max} > 25$ ms). The respective longest opening of a sweep $t_{o,max}$ discriminates three distinct gating patterns: mode $0_a$ ($\leqslant 0{\cdot}5$ ms), mode 1 ($>0{\cdot}5$–8 ms) and mode 2 ($>8$ ms) (cf. Yue *et al.* 1990). To safely avoid artifacts, we ignored all mode $0_a$ sweeps in which no openings reached the full-amplitude level. In *G* and *H*, the frequency (relative to all active sweeps) of mode 1 and 2 was determined in each individual experiment. □, control; ■, okadaic acid; ▨, calyculin A; ⁘, norokadaone. Calyculin A slightly but significantly increased mode 1 over mode $0_a$, but possibly more mode $0_a$ sweeps escaped identification during the later (and more noisy) period of the experiments. Okadaic acid, on the other hand, significantly increased the frequency of mode 2 sweeps. Norokadaone was without effect. $*P < 0{\cdot}05$ *versus* control.

reflect the conventional gating pattern, typically containing many repetitive but brief openings. Occasionally, mode 2 sweeps (defined as $t_{o,max} > 8$ ms) were observed (Fig. 1*B*, trace 6). As seen in the statistical analysis (Fig. 2*H*), mode 2 is rare under control conditions (~2% of active sweeps), but is markedly augmented by okadaic acid. This amplification explains why open probability tends to increase (Fig. 1*G*). The large variability of this effect is also explained by a closer look at modal gating; while the relative effect of okadaic acid on mode 2 sweep frequency is highly reproducible (3·6-, 3·0-, 3·2-, 3·4- and 4·1-fold increases) between the five

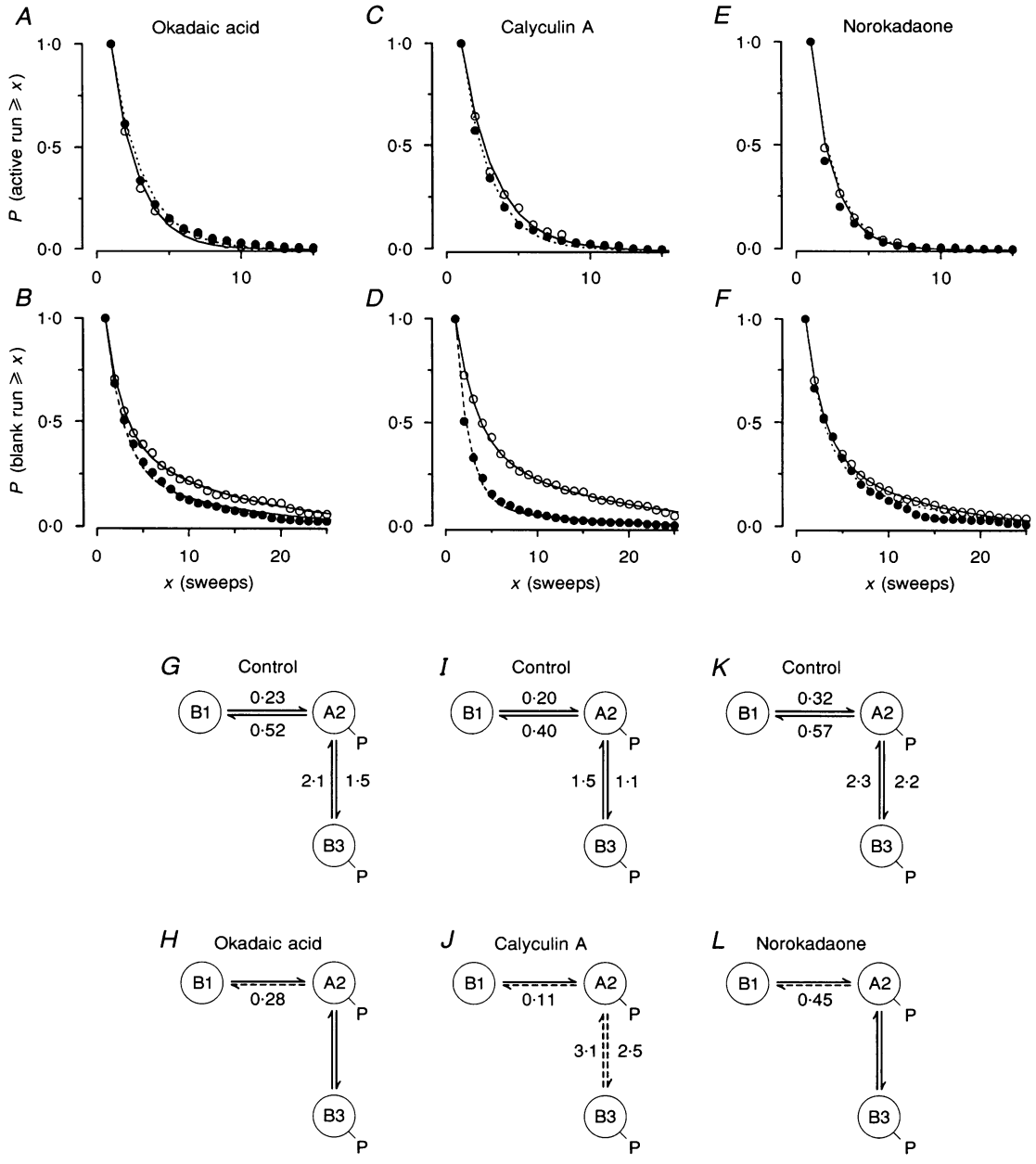


**Figure 3. Effects of phosphatase inhibitors on slow gating**

To obtain histograms for the duration of runs of active sweeps (*A*, *C* and *E*) and blank sweeps (*B*, *D* and *F*), the data were pooled from all 5 experiments in each group and transformed into the probability format. Fits were obtained using discrete-time Markov analysis, assuming the three-state model shown in *G*–*L* and as further explained in the text. Results for the rate constants with the three sets of controls (○, continuous lines in *A*–*F* and continuous arrows in *G*–*L*) are comparable. Values of rate constants are given as $s^{-1}$. The drug effects (●, dashed lines and arrows in *H*–*L*) in the case of okadaic acid (*H*) could be accounted for by a single change in the rate from A2 to B1. Calyculin A has additional effects on the transitions between A2 and B3. Rate constants not indicated in *H*, *J* and *L* are identical to the corresponding controls (*G*, *I* and *K*). P indicates phosphorylation.

**Table 2. Slow gating parameters directly derived from sweep histogram analysis**

| | Active run length (s) | Blank run length (s) |
|---|---|---|
| Control | 1·16 ± 0·17 | 2·91 ± 0·82 |
| Okadaic acid | 1·58 ± 0·29* | 2·68 ± 0·68 |
| Control | 1·39 ± 0·24 | 2·80 ± 0·69 |
| Calyculin A | 1·16 ± 0·23 | 1·51 ± 0·12 |
| Control | 0·95 ± 0·17 | 3·52 ± 0·82 |
| Norokadaone | 0·77 ± 0·13 | 2·78 ± 0·66 |

Values represent means ± S.E.M. from 5 experiments in each group; *$P < 0·05$.

experiments, the absolute effect varies widely (increases by 2·1, 4·0, 5·7, 6·6 and 7·9% of active sweeps). This is due to a large scatter of the baseline values of mode 2 (between 0·9 and 2·6% of active sweeps). In fact, we found a highly significant correlation when plotting individual values for the increase in open probability *versus* the respective absolute increase in mode 2 (correlation coefficient ($r$) = 0·98, not shown). This finding confirms how strongly mode 2 sweeps may determine the average open probability of a whole ensemble of sweeps. At the same time, it re-emphasizes that the open probability is not a particularly well-suited parameter to use to screen for such effects on fast gating. Norokadaone was ineffective in altering the proportion of the various gating modes (Fig. 2*G* and *H*), and calyculin A only slightly enhanced mode 1 sweeps (Fig. 2*G*). Thus, the modal gating analysis ascertained that calyculin A, in contrast to okadaic acid, affects only one of the two mechanisms of cAMP-dependent upregulation, namely the availability of the channel to respond to a voltage step.

In addition to measuring the percentage of active sweeps (Fig. 1*F*), slow gating – the transition between an available (active sweeps) or unavailable (blank sweeps) channel state – can be analysed kinetically. This should provide further information about the mechanism by which the channels are rendered more active. Slow gating has been usually assessed by sweep histogram analysis (e.g. Ochi & Kawashima, 1990; Ono & Fozzard, 1993). In this analysis, the number of adjacent sweeps which are alike – either active or blank – is counted as an active or blank run and binned, which yields histograms of run duration, like those in Fig. 3*A–F*. Initially, we constructed conventional sweep histograms from every single experiment and obtained the average durations of active runs by fitting a mono-exponential curve. The mean duration of blank runs was obtained by simple averaging, due to the more complex behaviour of this parameter (see below). The results of this analysis are indicated in Table 2. The only striking effect we observed was an increase by okadaic acid of the duration of active runs, which is highly compatible with the presumed inhibition of dephosphorylation. However, there are several problems with the conventional approach to analysis of slow $Ca^{2+}$ channel gating. First, the patch-to-patch variability is very large, even when, within an optimal experiment, ensembles of more than a thousand sweeps are obtained. Therefore, in the following, we pooled the data from all five experiments of each group. Second, blank-run histograms are consistently more complex than a mono-exponential function (see Fig. 3*B*, *D* and *F* and Herzig *et al.* 1993). This means that a two-state model (active state ⇌ blank state) is oversimplified, and fitted exponential functions cannot yield the transition rate constants directly. Third, due to the discrete nature of the observed data, governed by the pulse protocol, transitions occurring between depolarizing voltage steps will be missed. To account for the latter two problems, we used an analysis which has been described in detail previously (Herzig *et al.* 1993). After converting the data into the probability format, we plotted active (Fig. 3*A*, *C* and *E*) or blank (Fig. 3*B*, *D* and *F*) runs at least $x$ sweeps long against the run length $x$. A Markov formalism applicable to these discrete-time observations was used to generate the best-fit curves shown in Fig. 3. The simplest Markov model to accomplish a mono-exponential active-run lifetime and a bi-exponential blank-run lifetime is a three-state model with two blank states (B1 and B3) and one active state (A2), like the one sketched in Fig. 3*G–L*. The geometry of this particular model, and the assignment of phosphorylation of the regulatory site was derived and discussed extensively in our previous work on $Ca^{2+}$ channel regulation (Herzig *et al.* 1993), including the delineation of all the necessary model equations. The essential features of the model are the two exits from an active state A2, one towards B1 by dephosphorylation, and another – a more rapid exit – towards B2, mediated by a second process, possibly by inactivation. The three sets of control data (○, Fig. 3*A–F*) were derived in this study from a total of 2827, 2104 and 2760 sweeps, respectively. The sets of rate constants obtained independently for these controls are indicated in Fig. 3*G*, *I* and *K*. They agree reasonably well, which confirms that pooling the data indeed yields a more robust estimation of slow gating kinetics. The data obtained after drug treatment (●) with okadaic acid (*A* and *B*), calyculin A (*C* and *D*) and norokadaone (*E* and *F*) represent 6210, 5080

and 2274 sweeps, respectively. The effect of okadaic acid can be modelled by changing a single rate constant (from A2 to B1) from 0·52 to 0·28 $s^{-1}$, in accordance with an inhibitory action on dephosphorylation (all rate constants not indicated in Fig. 3*H*, *J* and *L* are similar to those in the respective controls in Fig. 3*G*, *I* and *K*). Whereas norokadaone leaves slow gating essentially unaltered, calyculin A has a very complex action; the active-run histogram (Fig. 3*C*) is shortened rather than prolonged, and only our simultaneous analysis of the bi-exponential blank-run histogram resolves this apparent paradox: the rate constants between A2 and B3 are markedly increased, such that active runs more often become briefly interrupted. Occupancy of the active state thus deviates even more from that of the 'phosphorylated' state(s). The model-derived 'dephosphorylation' rate A2 to B1 is in fact markedly retarded by calyculin A, the effect being larger than that of okadaic acid. Thus the kinetic effects of both drugs – when unravelled by discrete-time Markov analysis – are in quantitative agreement with their

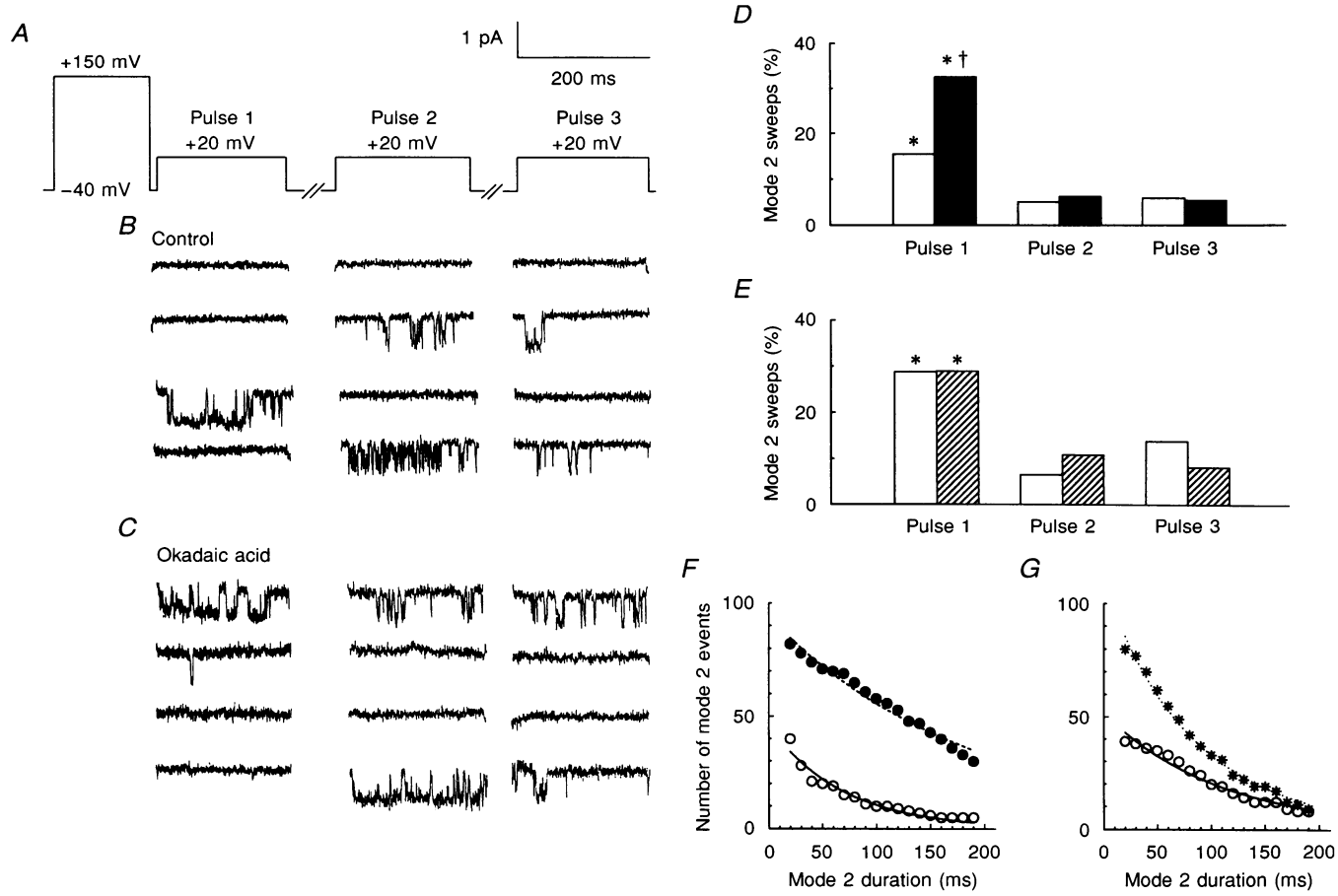


**Figure 4. Prepulse experiments designed to assess the relation between voltage-dependent mode 2 activity and phosphorylation-dependent potentiation**

The pulse protocol (*A*) was applied to compare prepulse-potentiated activity in the adjacent test pulse 1 with activity observed during the following test pulses 2 and 3. The interval between test pulses (+20 mV, 200 ms) was 200 ms, with a holding potential of −40 mV (holding potential was set between −60 and −100 mV for the first 50 ms of this interval when necessary, to facilitate recovery from inactivation). Some consecutive traces obtained before (*B*) and after (*C*) addition of okadaic acid are shown. In *D* and *E*, mode 2 activity (defined as a period of high open probability containing at least one opening >8 ms duration) is plotted as a percentage of all active sweeps (cf. Fig. 2*H*), comparing test pulses 1–3. □, control; ■, okadaic acid; ▨, calyculin A. Mode 2 data were obtained in 4 out of 5 experiments with okadaic acid, and in all 7 experiments with calyculin A. Test pulse 1 contained significantly more mode 2 periods than pulse 2 or 3, both in the absence and presence of okadaic acid or calyculin A (*$P < 0{\cdot}05$). Furthermore, this 'coupling' to the prepulse was significantly enhanced by okadaic acid, but not by calyculin A (†$P < 0{\cdot}05$). In *F*, the lifetime of mode 2 is analysed and compared before (○, $\tau = 68$ ms) and after (●, $\tau = 193$ ms) okadaic acid. The corresponding values of $\tau$ in *G* are 107 ms before (○) and 84 ms after (✱) calyculin A.

steady-state effects on channel availability. In addition, they are in qualitative agreement with our expectations for a phosphatase inhibitor.

**Effects on prepulse-facilitated mode 2 gating**

Control of channel availability may be the most obvious effect with physiological levels of $\beta$-adrenergic stimulation (Ochi & Kawashima, 1990), but is there also an important role for mode 2 gating? We suspected that, as in adrenal medulla (Artalejo, Mogul, Perlman & Fox, 1991; Artalejo, Rosie, Perlman & Fox, 1992), mode 2 gating could be the functional manifestation of a joint regulation of the channel by voltage (Pietrobon & Hess, 1990) and phosphorylation by cAMP-dependent protein kinase (Sculptoreanu, Rotman, Takahashi, Scheuer & Catterall, 1993). To test this idea, we studied the influence of okadaic acid ($n = 5$) on channels exposed to strongly depolarizing prepulses in the presence of saturating concentrations of isoprenaline and isobutylmethylxanthine (Fig. 4). In this instance, calyculin A served as a negative control ($n = 7$), since it was expected not to alter modal gating (see above). When channel availability is raised by the micromolar concentrations of the cAMP-elevating agents, the phosphatase inhibitors fail to exert any additive effects on this parameter ($61{\cdot}6 \pm 12{\cdot}0$ and $55{\cdot}6 \pm 12{\cdot}4\%$ before and after okadaic acid, respectively, and $52{\cdot}0 \pm 10{\cdot}5$ and $45{\cdot}3 \pm 9{\cdot}6\%$ before and after calyculin A, respectively). Channels very often displayed mode 2 activity during that test pulse which directly followed the prepulse (Fig. 4*B* and *C*). This 'coupling' is quantitatively analysed in Fig. 4*D* and *E*. It is markedly enhanced by okadaic acid (Fig. 4*D*), but not by calyculin A (Fig. 4*E*). In addition, okadaic acid prolongs the lifetime of mode 2 – normally some tens of milliseconds – about 3-fold (Fig. 4*F*). Again, calyculin A is ineffective (Fig. 4*G*). In summary, modal gating is again selectively affected by okadaic acid, but not by calyculin A, when studying prepulse potentiation of mode 2.

## DISCUSSION

At first glance, protein phosphatase inhibitors simply mimic the effects of cAMP-dependent phosphorylation (Yue *et al.* 1990) of cardiac L-type $Ca^{2+}$ channels (see Neumann *et al.* 1993*a*, 1994), and the lack of effect of the inactive derivative norokadaone confirms the idea that specific effects on protein phosphatases are encountered. A closer look at the extent of the various components of stimulation, however, strongly suggests that phosphatase inhibitors can help to refine our knowledge about $Ca^{2+}$ channel regulation by phosphorylation. Okadaic acid increases mean open time (Table 1) and tends to increase open probability within active sweeps (Fig. 1*G*). The effects can be readily explained by a marked augmentation of mode 2 gating (Fig. 2). All these phenomena are not seen with calyculin A. Conversely, calyculin A has been shown to hasten the rate of inactivation (Neumann *et al.* 1994), and this effect is not shared by okadaic acid.

The qualitative differences between okadaic acid and calyculin A are probably not just due to a different potency of inhibition of phosphatase activity of the drugs. Although in the present study we examined the action of micromolar concentrations only, there are several hints that different effects on the diverse gating parameters are not merely a reflection of a concentration-dependent increasing inhibition of channel dephosphorylation. First, micromolar amounts of okadaic acid are more effective with regard to modal gating than calyculin A, but at the same time less effective with regard to channel availability. Second, when higher concentrations of okadaic acid are analysed, which are equipotent with micromolar calyculin A regarding availability, there is still no effect on the rate of inactivation. And third, after pretreatment with cAMP-elevating agents, both agents are ineffective in increasing channel availability further, but again okadaic acid – and not calyculin A – alters modal gating behaviour.

The picture emerging from the study on fast gating parameters is nicely complemented and confirmed by the analysis of slow gating. In each case, the increase in channel availability is reflected by a corresponding change in the rate constant, which we ascribe to 'dephosphorylation' in the model. Furthermore, our earlier hypothesis (Herzig *et al.* 1993) of a non-active state which is nevertheless phosphorylated at the regulatory site (B3 in Fig. 3) receives further support; when saturating concentrations of cAMP-elevating agents are present, availability is not only considerably below 100% but it also fails to be further augmented by phosphatase inhibition. The action of calyculin A on the two rate constants which link the 'phosphorylated' states A2 and B3 seems unexpected at first glance. However, it is reasonable to speculate that the increased rate constant from A2 to B3 is just another reflection of the hastened process of inactivation, already found in the analysis of fast gating. At present, we are quite uncertain whether this action of calyculin A is a genuine drug effect, linked to a separate calyculin A-sensitive phosphorylation site. Alternatively, it could merely be a reflection of current-dependent inactivation (Mazzanti, DeFelice & Liu, 1991), caused by the increase of channel availability in the absence of any additional stimulatory influence on modal gating. In any case, if indeed more phosphorylated channels become and remain inactivated, the effect of calyculin A on availability underestimates its influence on channel phosphorylation. Taking this into account, the quantitative difference between calyculin A and okadaic acid would in fact be even more striking.

In conclusion, each of the drugs can dissect out one of the two cAMP-dependent regulatory mechanisms. According to present biochemical evidence, we attribute the calyculin A effect on availability to a site dephosphorylated by a type 1 phosphatase. This is supported by previous work on cAMP effects on slow gating, in which inhibition of channel dephosphorylation by endogenous phosphatase inhibitor 1 takes place (Herzig *et al.* 1993). Conversely, a type 2A phosphatase-sensitive site is probably coupled to modal gating, given its high sensitivity towards okadaic acid. For a firm interpretation of this kind, however, we would also have to know how intensely each of the sites is phosphorylated by protein kinase A, because the net effect of both phosphorylation and dephosphorylation reactions governs the observable channel gating. In fact, in the study of Ono & Fozzard (1993), the difference in the concentrations of okadaic acid required to increase availability and open times, respectively, was abolished in the presence of a cAMP derivative. Similarly, in our prepulse experiments with cAMP-elevating agents, mode 2 was enhanced by okadaic acid, but availability remained unaffected. Thus, strikingly different effects can be seen with a given concentration of a phosphatase inhibitor, evidently depending on the 'background' level of protein kinase activity. Testing *several* phosphatase inhibitors may thus be a very useful alternative to concentration–response studies for identifying different phosphorylation sites pharmacologically. When ascribing functional effects to the action of a certain phosphatase, one has to encounter still other methodological problems. For instance, the extent to which the inhibitors reach their site of action is unknown. Discrepancies, e.g. between phosphatase inhibitory potency *in vitro* and effects on protein phosphorylation in intact cells, can be tentatively explained by different membrane penetration (for comparison of okadaic acid and calyculin A, cf. Neumann *et al.* 1993*a*, 1994). Furthermore, biochemical data should not be directly extrapolated from a different tissue. Calyculin A, for example, is several orders of magnitude less potent in inhibiting type 2A (and type 1) phosphatases of guinea-pig ventricular myocytes *in vitro* (Herzig *et al.* 1995) compared with skeletal or smooth muscle enzymes (Ishihara *et al.* 1989). One would clearly like to know the exact inhibitory profile regarding those particular isozymes which are functionally relevant for the channel, and which might be closely associated with it (Ono & Fozzard, 1992; Wang, Townsend & Rosenberg, 1993). Direct application of purified phosphatases to inside-out patches would certainly be another interesting, but technically demanding, approach. We would predict from the present results that a type 1 phosphatase would reduce availability, whereas the site which controls fast modal gating would be more sensitive to a highly okadaic acid-sensitive phosphatase, i.e. type 2A-like phosphatase.

While mode 2 behaviour is rarely identifiable under standard conditions, strong prepulses lead to a high frequency of this type of gating (Pietrobon & Hess, 1990). The idea – originally proposed for chromaffin cells (Artalejo *et al.* 1991, 1992) – that phosphorylation underlies prepulse-induced facilitation has been recently extended to cardiac channels (Sculptoreanu *et al.* 1993). Our findings (Fig. 4) accorded nicely with the prepulse-induced whole-cell currents measured through cloned cardiac $Ca^{2+}$ channels in the presence of protein kinase A and/or okadaic acid (Sculptoreanu *et al.* 1993) It is clear now that the molecular gating mechanism of this voltage- and phosphorylation-dependent current is mode 2. The observation that calyculin A also failed to affect mode 2 gating in these experiments further validates our approach to identifying phosphorylation sites by the use of *several* inhibitors.

The molecular mechanisms which link mode 2 gating, its dependence on voltage prepulses and channel phosphorylation (Bean, 1990) in heart are certainly attractive targets for further research. Besides this, however, the mode 2-mediated large currents elicited by strong voltage pulses could have important functional implications. Besides conventional $\beta$-adrenergic stimulation, for instance, the frequency dependence of $Ca^{2+}$ current (Lee, 1987) could be linked to such a phosphorylation-linked mechanism (Tiaho, Piot, Nargeot & Richard, 1994). Furthermore, it will be very interesting to see how the two regulatory mechanisms of $Ca^{2+}$ channel regulation are altered in diseased heart, given the increased content of protein phosphatase activity in failing myocardium (Neumann, Boknik, Kaspareit, Schmitz & Zimmermann, 1993*b*), the altered force–frequency relationship (Mulieri, Hasenfuss, Leavitt, Allen & Alpert, 1992), and its disturbed $\beta$-adrenergic modulation (Böhm, La Rosee, Schmidt, Schulz, Schwinger & Erdmann, 1992). The localization of the phosphorylated amino acids also remains to be determined (Yoshida, Takahashi, Nishimura, Takeshima & Kokubun, 1992; Haase, Karczewski, Beckert & Krause, 1993; DeJongh, Colvin, Murphy, Takahashi & Catterall, 1994). However, to address this question, our results provide a clear-cut guideline for a pharmacological test of channel molecules altered by site-directed mutagenesis of the candidate serine (or threonine) residues.

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