# Channel modulation by tyrosine phosphorylation in an identified leech neuron

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1. We have examined the effects of tyrosine phosphorylation on a spontaneously active cation channel that also participates in the modulation of pressure-sensitive (P) neurons in the leech. Cation channel activity in cell-attached or isolated, inside-out membrane patches from P cells in culture was monitored before and after treatments that altered the level of tyrosine phosphorylation.

2. In cell-attached recordings from intact P cells, bath application of genistein, an inhibitor of tyrosine kinases, resulted in a 6·6 ± 2·6-fold increase in channel activity with no change in the mean open time or amplitude. Daidzein, an inactive form of genistein, was without effect. Addition of pervanadate, a membrane-permeant inhibitor of tyrosine phosphatases, had no effect on its own and blocked the effect of subsequent addition of genistein.

3. In inside-out P cell membrane patch recordings, exposure to a catalytically active fragment of a tyrosine phosphatase resulted in a 10·3 ± 3·6-fold increase in channel activity with no change in the mean open time or amplitude. Orthovanadate had no effect on channel activity and, when added with the phosphatase, prevented the increase in activity.

4. Our results demonstrate that the basal activity of cation channels is increased by tyrosine dephosphorylation, suggesting a constitutive modulation of channel activity under resting conditions.

Tyrosine phosphorylation is a well-established signal for cellular differentiation (Cantley *et al.* 1991; Greenwald & Rubin, 1992). Membrane-spanning and cytosolic tyrosine kinases (Hanks, Quinn & Hunter, 1988) and phosphatases (Fischer, Charbonneau & Tonks, 1991) have been identified, but for most of these enzymes their physiological actions, and in particular their effects on intracellular targets, are only poorly understood. In the nervous system, a wide variety of tyrosine kinases and phosphatases have been cloned and are thought to signal the responses to growth factors (Schlessinger & Ulrich, 1992), to direct neurite outgrowth during development (Goodman, 1996), and may modulate ion channel activity (Siegelbaum, 1994). Interestingly, many are expressed at very high levels in post-mitotic neurons for reasons which are unclear (Brugge, Cotton, Queral, Barrett, Nonner & Keane, 1985; Hanley, 1988).

Recently, tyrosine phosphorylation of native and cloned ion channels, including ligand-gated (Hopfield, Tank, Greengard & Huganir, 1988; Wang & Salter, 1994; Moss, Gorrie, Amato & Smart, 1995; Valenzuela *et al.* 1995) and voltage-dependent channels (Wilson & Kaczmarek, 1993; Huang, Morielli & Peralta, 1993; Lev *et al.* 1995; Jonas, Knox, Kaczmarek, Schwartz & Solomon, 1996; Holmes, Fadool & Levitan, 1996), has been demonstrated and can result in either increased or decreased channel activity. Although these observations suggest that tyrosine phosphorylation may be a novel mechanism of modulation for a wide variety of channels (Siegelbaum, 1994), physiological roles for this signal transduction cascade have been established in only a few cases to date. The activity of several types of channels in *Aplysia* bag cell neurons is transiently regulated by tyrosine phosphorylation. The mode of gating of a cation channel is switched to a higher activity pattern by a tyrosine phosphatase activity that is regulated by protein kinase A (Wilson & Kaczmarek, 1993). In addition, an insulin receptor tyrosine kinase decreases the levels of calcium and potassium currents in bag cells (Jonas *et al.* 1996). Finally, run-down of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors in rat spinal dorsal horn neurons can be prevented by tyrosine phosphorylation (Wang & Salter, 1994). Interestingly, NMDA receptors are a major component of the tyrosine-phosphorylated proteins in postsynaptic densities in the mammalian brain (Moon, Apperson & Kennedy, 1994).

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In pressure-sensitive (P) neurons of the leech, we have described a spontaneously active cation channel that may help set the resting potential of the P cell (Drapeau, 1990). The activity of this cation channel is increased by membrane depolarization as well as by serotonin (Drapeau, 1990) via protein kinase C (PKC) (Catarsi & Drapeau, 1992), which contributes to the modulation of the receptive field properties (Mar & Drapeau, 1996). Serotonin also modulates the properties of other sensory neurons (Mar & Drapeau, 1996) and can trigger the initiation of complex behaviours in the leech and in other organisms (Leake, 1986). At sites of contact with serotonergic Retzius cells during (inhibitory) synapse formation (Ching, Catarsi & Drapeau, 1993), cation channel modulation by PKC is suppressed by a process that is dependent on tyrosine phosphorylation (Catarsi & Drapeau, 1993; Catarsi, Ching, Merz & Drapeau, 1995). As a first step in trying to unravel the details of these diverse, complex and interactive mechanisms of cation channel regulation, we examined whether tyrosine phosphorylation could modify the basal activity of cation channels. By altering the level of this process in intact P cells and, more directly, in isolated membrane patches, we found that tyrosine phosphorylation limited cation channel activity and could be a constitutive mechanism of channel modulation.

## METHODS

### Cultures

P cells were isolated from the nervous system of the leech *Hirudo medicinalis* (purchased from Ricarimpex, Audenge, France) and cultured as described previously (Dietzel, Drapeau & Nicholls, 1986). Desheathed ganglia were exposed to collagenase (Type XI, Sigma) and the somata of the easily identified P neurons were removed by aspiration into a micropipette. P cells were plated for 3–5 days in the wells of polylysine-coated microtest culture dishes containing Leibovitz (L15) medium (Gibco) supplemented with 0·2 mg $ml^{-1}$ gentamicin, 0·1 mg $ml^{-1}$ ampicillin and 2% heat-inactivated fetal bovine serum (Gibco).

### Solutions

For cell-attached patch-clamp recordings, the culture medium was replaced with a 'recording saline solution' containing (mM): NaCl, 155; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 10; and Hepes, 10; adjusted to pH 7·4 with NaOH and to 330 mosmol $l^{-1}$. Inside-out

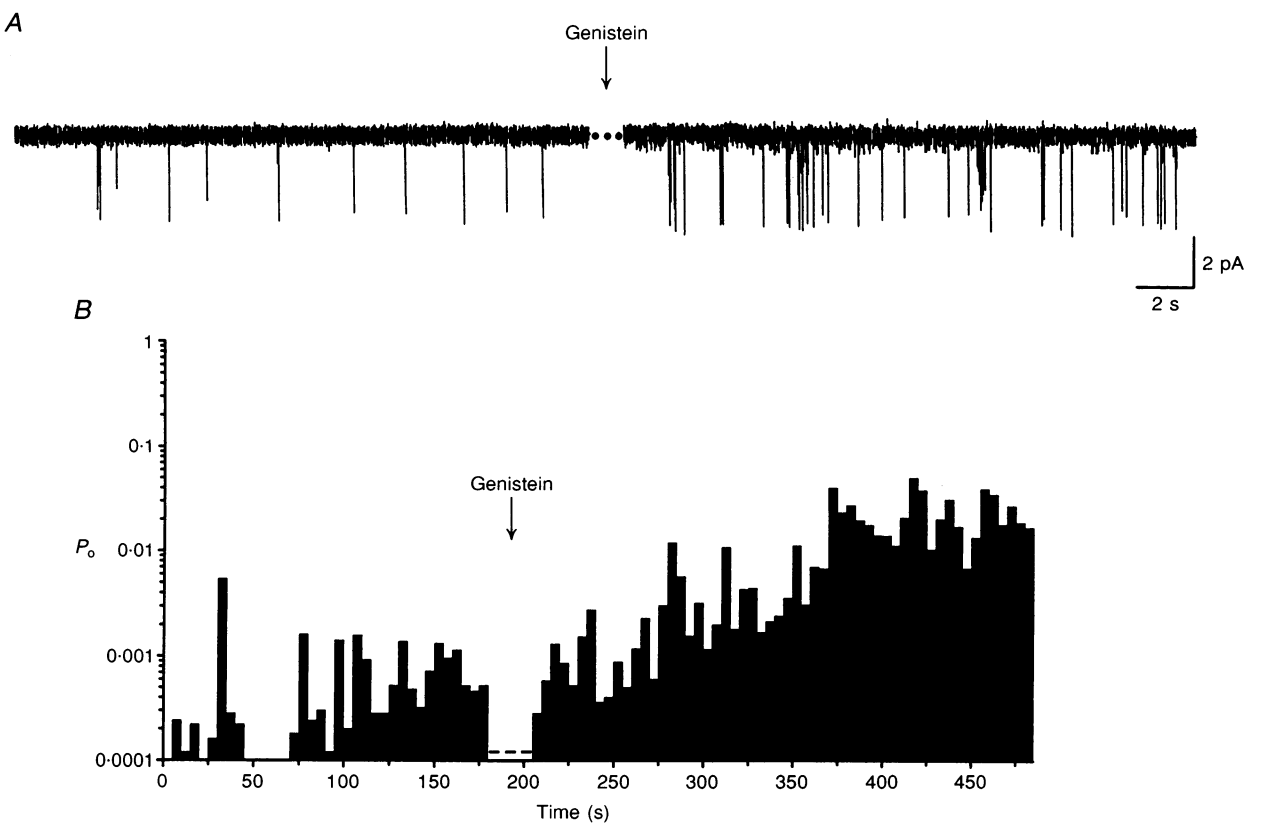


**Figure 1. Effect of genistein on the cation channel activity in cell-attached configuration**

*A*, 20 s current traces recorded before (left) and after (right) the application of 30 $\mu$M genistein (indicated by the arrow). No potential was applied to the pipette. Note that genistein increased the number of openings and did not affect the amplitude of the currents. The mean current amplitude was −2·64 pA during the control period and −2·38 pA after genistein application. *B*, continuous time histogram of the experiment depicted in *A* showing open probability ($P_o$) calculated in bins of 5 s duration and plotted on a logarithmic scale. Genistein was applied during the period indicated by the dashed line, a period during which $P_o$ was not recorded. Note the progressive increase in channel activity after genistein application. The values for $P_o$ and mean open time were: 0·00056 and 0·78 ms for the control period and 0·0124 and 0·71 ms after application of genistein.

patch-clamp recordings were carried out in the solution described above, to which was added 1·5 mM EGTA in order to yield a final concentration of free $Ca^{2+}$ of 0·1 μM ('low-$Ca^{2+}$ recording solution'). The recordings were performed in 10 μl wells of microtest dishes (Gibco). Test solutions were added by ejection of 1 μl of a 10-fold-concentrated solution to give the final concentration described in the text. Genistein and daidzein (LC Services, Woburn, MA, USA) were diluted 1000-fold from a stock solution in DMSO. Sodium pervanadate was prepared by adding 1 part of 500 mM $H_2O_2$ to 50 parts of 10 mM sodium orthovanadate (Sigma) dissolved in recording saline solution (see Wallace, 1995). The mixture was incubated for 10 min at room temperature (20–22 °C) and then added to the P cells at a final concentration of 100 μM 10–30 min before the recordings. Truncated CD45 T-cell protein tyrosine phosphatase (PTPase) was a gift from Dr E. Fisher (University of Washington, Seattle, WA, USA).

### Recordings

P cells were patch clamped using 5–10 MΩ electrodes coated to within 50 μm of the tip with dental wax and filled with low-$Ca^{2+}$ recording solution, diluted by 10% to improve seal formation. Seal resistances were typically in the range 2–10 GΩ and the r.m.s. current noise was around 0·4 pA at a 1 kHz bandwidth. Recordings were made using an Axopatch-1A patch-clamp amplifier (Axon Instruments) and were low-pass filtered at 1 kHz (−3 dB) before being digitized (NeuroData Instruments Corp., NY, USA) and stored on videotape. Analog signals from the recording device were digitized at 10 kHz, transferred to a hard disk and analysed with an IBM PC-compatible computer. Analysis of the experiments was performed using pCLAMP 6 software (Axon Instruments). Channel openings were determined using a 50% threshold cursor set at the main conductance state of 60 pS (Drapeau, 1990). Data were reported as means ± S.E.M., their significance was tested using an ANOVA test. Results were considered statistically different when $P < 0·05$.

## RESULTS

### Cell-attached recording: inhibition of tyrosine kinases increases cation channel activity

Cation channels can be easily identified in P cell-attached patches by their characteristic properties already described in a previous study (Drapeau, 1990). The most obvious characteristics are rare and spontaneous inward openings, larger than those of any other channel type, as illustrated in Fig. 1*A* in which no potential was applied to the pipette, i.e. the P cell patch was at its resting potential of about −50 mV. Single channels open approximately once per second with a mean open time of ~1 ms (probability of opening ($P_o$), ~0·0002) under these recording conditions, and have a conductance of ~60 pS and reversal potential ~60 mV depolarized from rest. Most patches in this study contained several channels ($P_o$ ranging from 0·0006 to 0·0017).

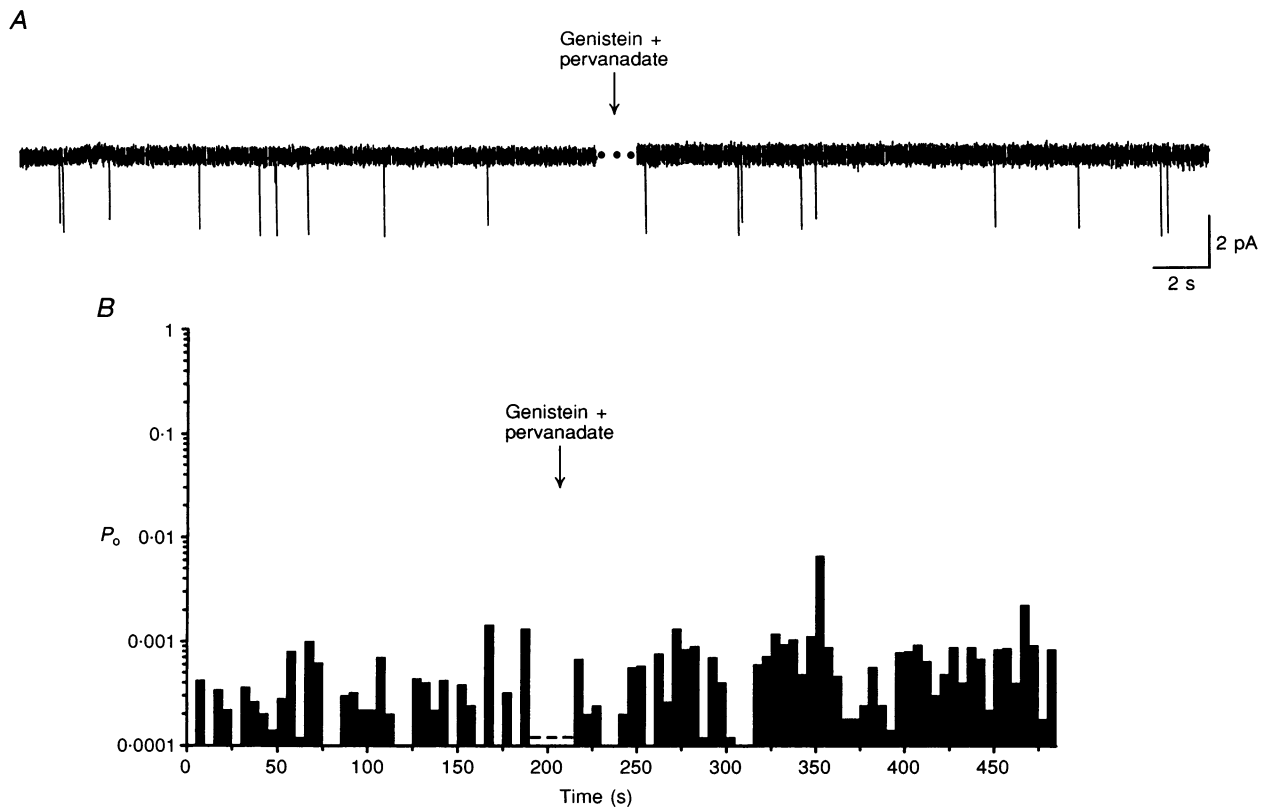


**Figure 2. Effect of genistein on the cation channel activity in pervanadate-treated P cells**

*A*, 20 s current traces recorded in the presence of 100 μM pervanadate before (left) and after the application of genistein (right). Note that in contrast to the traces depicted in Fig. 1*A*, the number of openings was relatively unaffected by genistein. *B*, continuous time histogram of the experiment depicted in *A* showing $P_o$ calculated in bins of 5 s duration. The values for $P_o$ and mean open time were: 0·00133 and 0·87 ms for the control period and 0·00134 and 0·71 ms after application of genistein.

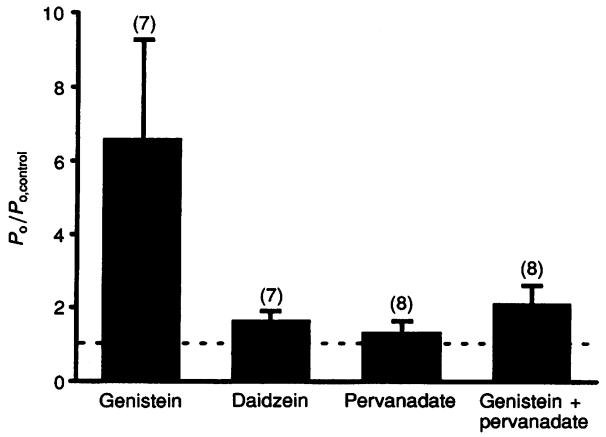


**Figure 3. Summary of the experiments in cell-attached configuration**

$P_o/P_{o,control}$ represents the average of the ratio between the open probability calculated after and before the treatment for each experiment. The number of experiments is indicated in parentheses. The error bars are + S.E.M. The ratio of 1 indicated by the dashed line represents basal activity.

To investigate whether the cationic channel is regulated by endogenous tyrosine kinases in P cells, we examined the effect of inhibiting tyrosine kinases as this should result in a progressive dephosphorylation of the substrate by endogenous phosphatases. P cells were exposed to genistein, an inhibitor of tyrosine kinases (Akiyama *et al.* 1987) and of tyrosine phosphorylation in P cells (Catarsi *et al.* 1995). After a 3–5 min control period, 30 $\mu$M genistein was applied, resulting in a progressive increase in the probability of opening of the channel ($P_o$) reaching its maximal effect 2–3 min after the drug application (Fig. 1*A* and *B*). On average genistein caused a 6·6 ± 2·6-fold increase in the

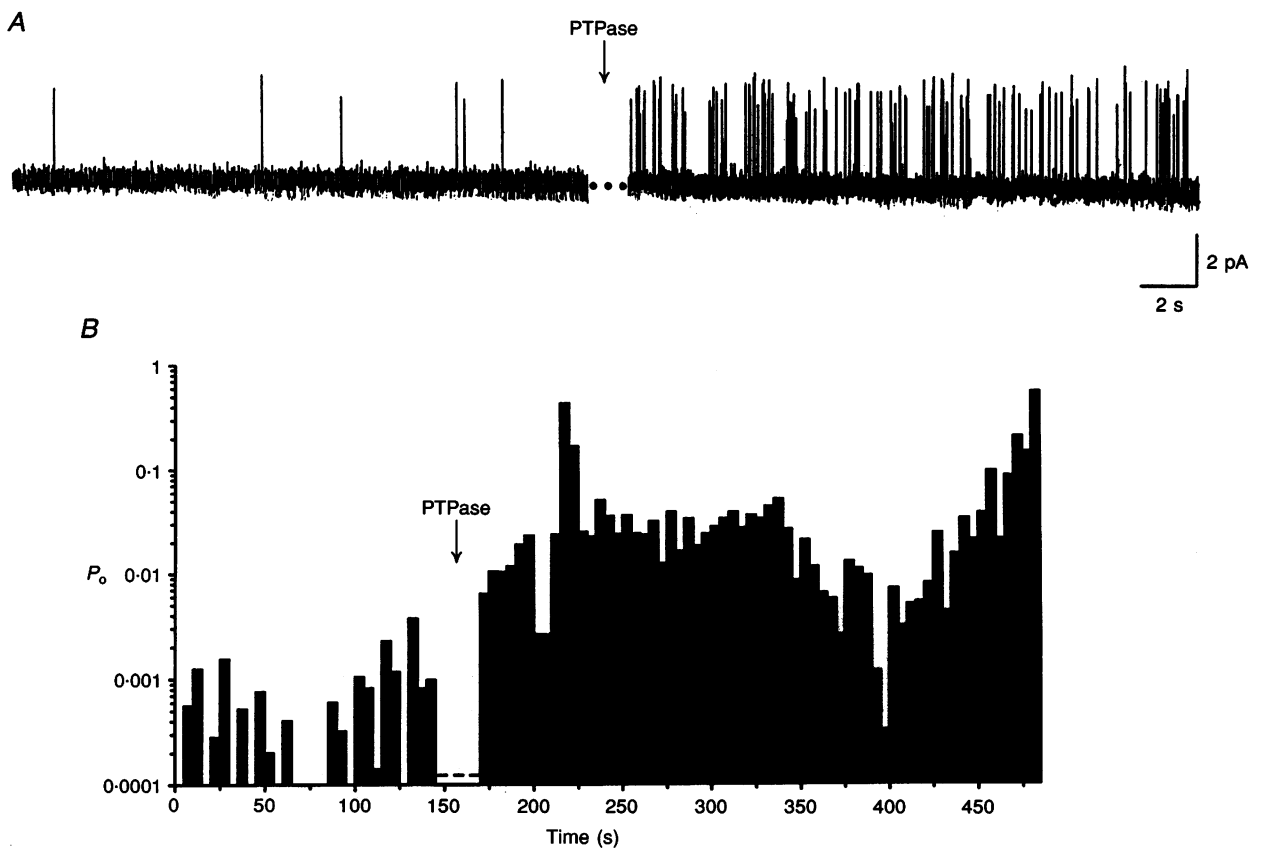


**Figure 4. Effect of PTPase on channel activity in inside-out configuration**

*A*, 20 s current traces recorded before (left) and after the application of 50 nM PTPase (right). The patch was depolarized by 50 mV. Note that PTPase dramatically increased the number of openings with no change in the amplitude of the current. The mean current amplitude was 2·81 pA in the control period and 2·80 pA after PTPase application. *B*, continuous time histogram of the experiment depicted in *A* showing $P_o$ calculated in bins of 5 s duration. Note that PTPase rapidly increased the channel activity, the maximal effect appearing a few seconds after the application. The values for $P_o$ and mean open time were: 0·00032 and 1·22 ms for the control period and 0·0094 and 1·23 ms after the PTPase treatment.

channel activity (average of the ratio of $P_o/P_{o,control}$ of each individual experiment) (Fig. 3). This effect was not associated with a significant change in either the mean open time ($P = 0{\cdot}2$) or the conductance ($P = 0{\cdot}1$) of the channel. Because the patches normally contained several channels, we could not accurately determine the channel closed time durations, which were expected to be reduced in order to account for the increased activity.

In order to determine whether the action of genistein was related to the inhibition of endogenous tyrosine kinases, we tested the effect of daidzein (30 $\mu$M), a structural analogue of genistein which fails to inhibit tyrosine kinases (Akiyama *et al.* 1987). As summarized in Fig. 3, on average daidzein caused only a $1{\cdot}7 \pm 0{\cdot}2$-fold increase in channel activity, a result significantly different from that of genistein ($P = 0{\cdot}02$) but not ($P = 0{\cdot}9$) from that obtained using 0·1% DMSO alone which caused an insignificant $1{\cdot}4 \pm 0{\cdot}3$-fold increase in channel activity ($P = 0{\cdot}9$; data not shown).

To demonstrate that the increase in channel activity by genistein is mediated by a progressive dephosphorylation by endogenous tyrosine phosphatases, P cells were incubated for 10–30 min with membrane-permeant sodium pervanadate (100 $\mu$M), a protein tyrosine phosphatase inhibitor which has been shown to cause a maximal increase in tyrosine phosphorylation in several preparations (Heffetz, Bushkin, Dror & Zick, 1990; Meier, Perez & Wallace, 1995; Wallace, 1995). While sodium pervanadate alone did not produce a noticeable effect on $P_o$ ($1{\cdot}3 \pm 0{\cdot}3$-fold increase), it significantly reduced the action of genistein ($2{\cdot}1 \pm 0{\cdot}5$-fold increase; $P = 0{\cdot}02$; Figs 2 and 3).

Therefore these results demonstrate that genistein increases the channel activity through the inhibition of tyrosine kinases and subsequent dephosphorylation by endogenous tyrosine phosphatases. They also suggest that in P cells the tyrosine kinases modulating $P_o$ are constantly active and overcome the action of endogenous tyrosine phosphatases.

### Inside-out patches: tyrosine phosphatase treatment increases cation channel activity

In order to demonstrate that the target of the endogenous tyrosine kinases is membrane associated (perhaps even the channel itself) and not a diffusible messenger, the channel activity was recorded using the inside-out patch

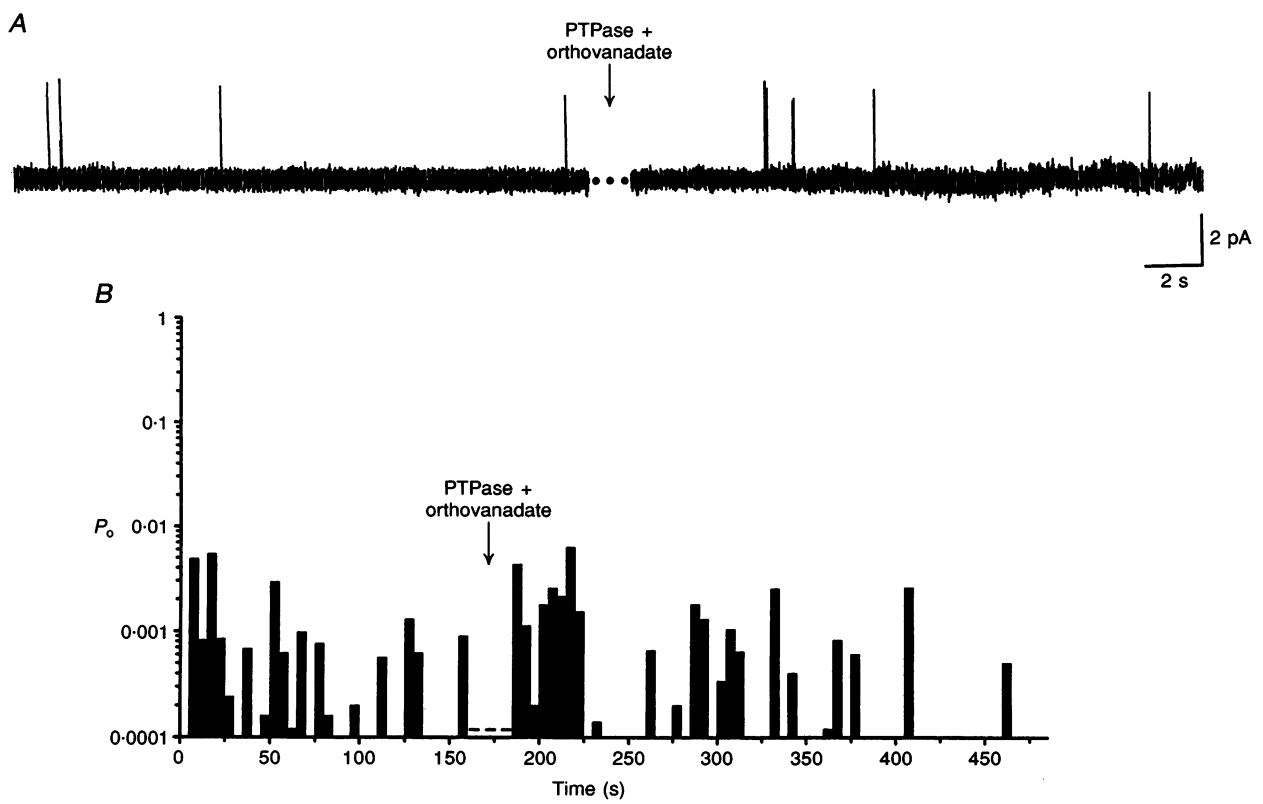


**Figure 5. Effect of concomitant application of PTPase and sodium orthovanadate on channel activity**

*A*, 20 s current traces recorded before (left) and after the application of 50 nM PTPase and 100 $\mu$M sodium orthovanadate (right). Note that the number of openings was unaffected by the treatment. *B*, continuous time histogram of the experiment depicted in *A* showing $P_o$ calculated in bins of 5 s duration. Note that PTPase failed to increase the channel activity when applied in the presence of sodium orthovanadate. The values for $P_o$ and mean open time were: 0·00059 and 1·38 ms for the control period and 0·00059 and 1·13 ms after the treatment.

configuration that allows agents to be applied directly to the cytoplasmic surface of the membrane. For this purpose, after identifying the channel in cell-attached configuration, the patch was isolated in low-$Ca^{2+}$ solution (see Methods). After excision, a −50 mV potential was applied to the pipette, i.e. the patch was depolarized by 50 mV. Under these conditions the current is outward with a mean open time varying between 1·5 and 2 ms (see Drapeau, 1990; Catarsi & Drapeau, 1992) and $P_o$ was in the range 0·0003–0·0035. Application of 50 nM PTPase (Zander *et al.* 1991) to the cytoplasmic face of the membrane produced an immediate increase of $P_o$ (Fig. 4). On average PTPase caused a 10·3 ± 3·6-fold increase in channel activity (Fig. 6). As for genistein, PTPase increased the frequency of opening without affecting the mean open time ($P = 0·10$) or the conductance ($P = 0·07$) of the channel. This effect was presumably due to a reduction in channel closed durations, which could not be determined accurately due to the presence of multiple channels in the patches.

To test for the specificity of the PTPase effect, we repeated this treatment in the presence of orthovanadate, an inhibitor of PTPases (Swarup, Cohen & Garbers, 1982). The increase in channel activity was prevented when PTPase was ejected concomitantly with sodium orthovanadate (100 μM; Fig. 5), an inhibitor of tyrosine phosphatases, as the average of the ratio of $P_o/P_{o,control}$ ($n = 6$) was 0·95 ± 0·08 (Fig. 6), statistically different from the value obtained with the treatment of PTPase alone ($P = 0·007$). Sodium orthovanadate alone did not induce any noticeable effect on channel activity (0·8 ± 0·1-fold effect; Fig. 6). Therefore the increase in channel activity produced by PTPase is related to a process of dephosphorylation and the target of endogenous tyrosine kinases is membrane associated.

## DISCUSSION

In cell-attached recordings from intact P cells, inhibition of tyrosine kinase activity by genistein was found, within a few minutes of its application, to increase cation channel activity without affecting its conductance. The mean open time of the channels was also unaffected, indicating a higher rate of opening upon inhibition of tyrosine kinase activity. No increase of the channel actvity was found in the presence of daidzein, an inactive analogue of genistein. The action of genistein was prevented by pre-incubation with pervanadate, a membrane-permeant inhibitor of tyrosine phosphatases, consistent with a requirement for dephosphorylation in order to increase channel activity. However, treatment with pervanadate alone had no effect, suggesting that a maximal suppression of channel activity was present under resting conditions, i.e. that further phosphorylation in the presence of the phosphatase inhibitor was without any additional effect.

The results with intact P cells could not distinguish between a modulation of the channel through tyrosine phosphorylation of cytosolic components or a more direct tyrosine phosphorylation of membrane associated proteins, possibly including the channel itself. In inside-out patch recordings, treatment with a catalytically active tyrosine phosphatase (but not in the presence of the inhibitor orthovanadate) also resulted in channel activation. These results indicate a membrane delimited if not a direct tyrosine phosphorylation of the channels. Only a biochemical demonstration of channel phosphorylation can prove a direct regulation, as has been observed for several other channels (Hopfield *et al.* 1988; Huang *et al.* 1993; Valenzuela *et al.* 1995; Moss *et al.* 1995; Lev *et al.* 1995; Holmes *et al.* 1996). As with intact P cells, channel activation by tyrosine dephosphorylation was due to an increased rate of opening

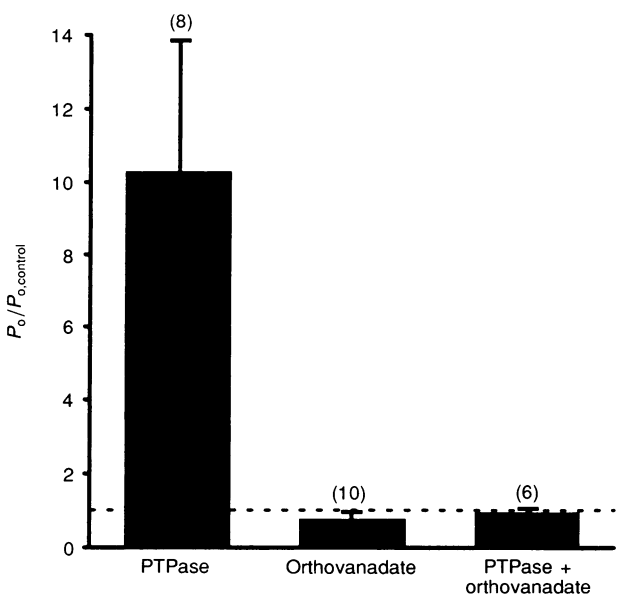


**Figure 6. Summary of the experiments in inside-out configuration**

$P_o/P_{o,control}$ represents the average of the ratio between the open probability calculated after and before the treatment for each experiment. The number of experiments is indicated in parentheses. The error bars are + S.E.M. The ratio of 1 indicated by the dashed line represents basal activity.

rather than prolonged openings. The lack of effect (activation) in the presence of orthovanadate alone and the lack of an increase in the channel activity after excision ('run-up') suggest that, in contrast with *Aplysia* cation channels (Wilson & Kaczmarek, 1993), the patches did not contain an endogenous phosphatase activity.

The large increase in cation channel activity that we observed with either genistein or PTPase application suggests that the channels are tightly regulated (inhibited) by tyrosine phosphorylation under resting conditions. The basal activity of the cation channels may help set the resting potential of the P cell, which is depolarized from the potassium equilibrium potential (Drapeau, 1990). It will be interesting to determine if, in addition to limiting the basal activity, this regulation is related to cation channel modulation by protein kinase C (Catarsi & Drapeau, 1992) and its loss during synapse formation (Catarsi & Drapeau, 1993). The cation channel is sensitive to amiloride (Sanchez-Armass, Merz & Drapeau, 1991) and recently an invertebrate amiloride-sensitive cation channel has been cloned (Lingueglia, Champigny, Lazdunski & Barbry, 1995). This other cation channel contains multiple tyrosine phosphorylation consensus sites, suggesting that this process may regulate cation channel activity. Interestingly, the nematode degenerin gene, which is essential for survival of a class of neurons (Driscoll & Chalfie, 1991), codes for a protein resembling the mammalian amiloride-sensitive cation channel expressed in epithelial cells (Canessa, Horisberger & Rossier, 1993). Tyrosine phosphorylation may thus be a multimodal modulatory mechanism for this type of channel in neurons and perhaps other tissues.


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