Cellular/Molecular

Regulation of an *Aplysia* Bag Cell Neuron Cation Channel by Closely Associated Protein Kinase A and a Protein Phosphatase

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Ion channel regulation by closely associated kinases or phosphatases has emerged as a key mechanism for orchestrating neuromodulation. An exemplary case is the nonselective cation channel that drives the afterdischarge in *Aplysia* bag cell neurons. Initial studies showed that this channel is modulated by both a closely associated PKC and a serine/threonine protein phosphatase (PP). In excised, inside-out patches, the addition of ATP (a phosphate source) increases open probability (P_O) through PKC, and this is reversed by the PP. Previous work also reported that, in certain cases, ATP can decrease cation channel P_O. The present study characterizes and provides a mechanism for this decreased P_O ATP response. The kinetic change for channels inhibited by ATP was identical to the previously reported effect of exogenously applied protein kinase A (PKA) (i.e., a lengthening of the third closed-state time constant). The decreased P_O ATP response was blocked by the PKA inhibitor peptide PKA_{6-22}, and its reversal was prevented by the PP inhibitor microcystin-LR. Furthermore, PKA_{6-22} did not alter the increased P_O ATP response. This suggests that both PKA and a PP are closely associated with these cation channels, but PKA and PKC are not simultaneously targeted. After an afterdischarge, the bag cell neurons are refractory and fail to respond to subsequent stimulation. The association of PKA with the cation channel may contribute to this decrease in excitability. Altering the constituents of a regulatory complex, such as exchanging PKA for PKC, may represent a general mechanism to precisely control ion channel function and excitability.

Key words: Aplysia; bag cell neurons; cation channel; phosphorylation; protein kinase A; excitability

Introduction

One of the primary means for initiating changes to neuronal excitability is phosphorylation-dependent ion channel regulation (Hille, 2001; Levitan and Kaczmarek, 2002; Magoski and Kaczmarek, 2003). Increasingly, the kinases and phosphatases that mediate this regulation are found closely associated with particular ion channels (Chung et al., 1991; Bielefeldt and Jackson, 1994; Rosenmund et al., 1994; Reinhart and Levitan, 1995; Holmes et al., 1996; Yu et al., 1997; Tibbs et al., 1998; Brandon et al., 1999; Tsunoda and Zucker, 1999; Davare et al., 2001; Huang et al., 2001; Marx et al., 2002; Nitabach et al., 2002; Zhou et al., 2002; Gingrich et al., 2004). The present study describes the regulation of a cation channel from *Aplysia* bag cell neurons by kinase and phosphatase activities closely associated with the channel in excised, inside-out patches.

The bag cell neurons initiate egg-laying behavior in *Aplysia californica* through a marked change in excitability called the afterdischarge (Kupfermann, 1967; Kupfermann and Kandel, 1970; Pinsker and Dudek, 1977; Rothmann et al., 1983; Conn and Kaczmarek, 1989). This ~30 min barrage of action potentials is triggered by synaptic input and results in the neurohemal secretion of egg-laying hormone. On termination of the afterdischarge, the bag cell neurons become refractory for ~18 hr, during which time additional afterdischarges cannot be induced. The inward current that drives the afterdischarge is provided by a nonselective cation channel (Wilson and Kaczmarek, 1993; Wilson et al., 1996). Previous work demonstrated that this channel was activated by a closely associated PKC, the effects of which could be reversed by a similarly targeted protein phosphatase (PP) (Wilson et al., 1998; Magoski et al., 2002). Both PKC and the PP were found to be constitutively active in excised, inside-out patches, and phosphorylation could be achieved by simply adding a phosphate source, such as ATP, to the cytoplasmic face of the channel.

However, more recent studies have shown that, in certain cases, rather than upregulating the cation channel, the application of ATP could, in fact, decrease activity (Magoski 2003; N. S. Magoski and L. K. Kaczmarek, unpublished observations). Interestingly, Wilson and Kaczmarek (1993) demonstrated that when exogenous protein kinase A (PKA) was applied to the cytoplasmic face of the channel, activity was decreased. Building on this, the present study shows that the ATP-induced decrease of cation channel activity is attributable to a closely associated PKA using

Received May 4, 2004; revised June 10, 2004; accepted June 14, 2004.

This work was supported by a Canadian Institutes of Health operating grant, a Canada Foundation for Innovation new opportunities grant, an Ontario Innovation Trust new opportunities grant, and a Queen's University research initiation grant to N.S.M. I am very grateful to S. L. Smith for excellent technical assistance and N.M. Magoski for critical evaluation of previous drafts of this manuscript.

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DOI:10.1523/JNEUROSCI.1694-04.2004

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ATP as a phosphate source to phosphorylate the channel or a nearby protein. Furthermore, the data will show that this phosphorylation is reversed by a similarly closely associated PP. Wilson and Kaczmarek (1993) proposed that PKA may be responsible for lowering channel activity at the end of the afterdischarge, to discourage bursting during the refractory period. As such, the channel–PKA association documented here would facilitate refractoriness. In general, by exchanging the enzymes targeted to an ion channel (e.g., switching PKA for PKC), it would be possible to regulate ion channel function and excitability in an exact and reliable manner over the long term.

Materials and Methods

Animals and cell culture. Adult *Aplysia californica* weighing 150–300 gm were obtained from Marine Specimens Unlimited (San Francisco, CA) or Marinus Inc. (Long Beach, CA). Animals were housed in an ~400 l aquarium containing continuously circulating, aerated sea water (Kent sea salt; Kent Marine, Acworth, GA) at 14–16°C on a 12 hr light/dark cycle and fed romaine lettuce three to five times per week.

For primary cultures of isolated bag cell neurons, animals were anesthetized with an injection of isotonic $MgCl_2$ (~50% of body weight), and the abdominal ganglion was removed and treated with neutral protease (13.33 mg/ml; catalog #165859; Roche Diagnositics, Indianapolis, IN) for 18 hr at 18–20°C, dissolved in normal artificial sea water (nASW; 460 mM NaCl, 10.4 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, 15 mM HEPES, 1 mg/ml glucose, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, pH 7.8 with NaOH). The ganglion was then transferred to fresh nASW, and the bag cell neuron clusters were dissected from their surrounding connective tissue. Using a fire-polished Pasture pipette and gentle titration, neurons were dispersed in nASW onto 35 × 10 mm polystyrene tissue culture dishes (catalog #430165; Corning, Corning, NY). Cultures were maintained in nASW for 1–3 d in a 14°C incubator, and experiments were performed on neurons that were *in vitro* for at least 1 d. Salts were obtained from Fisher (Ottawa, Ontario, Canada), ICN (Aurora, OH), or Sigma-Aldrich (St. Louis, MO).

Excised, inside-out patch-clamp recording. A single cation channel current was measured using an EPC-8 amplifier (HEKA Electronics, Mahone Bay, Nova Scotia, Canada) and the excised, inside-out patch-clamp method. Microelectrodes were pulled from borosilicate glass capillaries (1.5 mm internal diameter; model TW 150 F-4; World Precision Instruments, Sarasota, FL) and were fire polished to a resistance of 2–5 MΩ when filled with nASW (composition as above but lacking glucose, penicillin, and streptomycin). After excision, the cytoplasmic face was bathed with artificial intracellular saline (in mM: 500 K-aspartate, 70 KCl, 0.77 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 11 glucose, 5 EGTA, and 10 reduced glutathione, pH 7.3 with KOH; free $[Ca^{2+}] = 1\ \mu M$, calculated using the CaBuffer program, courtesy of Dr. L. Schlichter, University of Toronto, Toronto, Canada). Salts were from Fisher, ICN, or Sigma-Aldrich. Current was low-pass filtered at 1 kHz using the EPC8 Bessel filter and acquired at a sampling rate of 10 kHz using an IBM-compatible personal computer, a Digidata 1300 analog-to-digital converter (Axon Instruments, Union City, CA), and the Clampex acquisition program of pCLAMP (version 8; Axon Instruments). Data were gathered at room temperature (18–20°C) in 1–3 min intervals while holding the patch at −60 mV or, to avoid occasional contamination by Ca^{2+}-activated K^+ currents, −80 mV. All experiments were performed on the more commonly encountered continuously active cation channels as opposed to the rarely encountered burster channels (Wilson and Kaczmarek, 1993). Continuously active channels always show some level of activity when monitored for a minute or longer, whereas burster channels have additional, extremely long closures that last for several minutes. Thus, it is a straightforward matter to distinguish continuously active channels from burster channels, given that, even if the open probability (P_O) of a continuously active channel is low to begin with, there will always be periodic openings.

Reagents and drug application. Most drugs were introduced into the bath by pipetting a small volume (<10 μl) of concentrated stock solution into the culture dish (2 ml volume). Care was taken to pipette the stock near the side of the dish and as far away as possible from the patch at the tip of the microelectrode. ATP was obtained from either Sigma-Aldrich (grade 2, $2Na^+$ salt; catalog #A3377) or from ICN ($2Na^+$ salt; catalog #194613), PKA_{6-22} (P6062) and okadaic acid (K^+ salt; catalog #O7885) were both obtained from Sigma-Aldrich, and microcystin-LR (catalog #475815) was obtained from Calbiochem (San Diego, CA).

Analysis. To determine single-channel P_O and make statistical descriptions of channel kinetics, events lists were made from single-channel data files using the half-amplitude threshold criterion (Colquhoun and Sigworth, 1995) of the Fetchan analysis program of pClamp. Fetchan was also used to generate all-points histograms for determining channel amplitude. For analysis, most data did not require additional filtering below the 1 kHz used during acquisition; however, to avoid inclusion of noise-related "events" as channel openings, some data were filtered a second time using the Fetchan digital Gaussian filter to a final cutoff frequency of 500 Hz. For display in the figures, some data were filtered to a final cutoff frequency of 500 Hz or 250 Hz.

The Pstat analysis program of pClamp was used to read events lists and determine P_O, either automatically or manually, using the formula: $P_O = (t_1 + t_2 + \ldots t_n)/N \times t_{tot}$, where t is the amount of time that n channels are open, N is the number of channels in the patch, and t_{tot} is the time interval over which P_O is measured. The number of channels in the patch was determined by counting the number of unitary current levels, particularly at more positive voltages (typically −20 mV). Pstat was also used to generate single-channel open and closed dwell-time histograms and fit them with an exponential function describing the kinetic behavior of the channel. The time interval (*x*-axis) was binned logarithmically at 10 bins/decade, and histograms were fit with an exponential function using the maximum likelihood estimator method and a simplex search (Colquhoun and Sigworth, 1995), which was given the number of exponentials and estimated time constants (τs) at the start. Kinetic analysis was performed exclusively on patches that contained only one cation channel, as determined by a consistent display of a single open current level at more positive voltages (typically −20 mV). These channels also had to have a high enough initial P_O such that there were sufficient events to plot dwell-time histograms for reliable fitting. In practice, this translated into a minimum of 500 events for such histograms. Considering the recording periods used here (typically 3 min), along with an average open time of ~7 msec, the P_O had to be at least 0.02 to generate 500 events. Indeed, the P_O of the true single channels used for kinetic analysis was always above 0.02 and averaged ~0.1. Pstat was used yet again to determine the mean open and closed state current level by fitting all-points histograms with Gaussian functions using the least-squares method and a simplex search. Channel current amplitude was then calculated by subtracting the mean closed current level from the mean open current level at a given voltage.

To make P_O versus voltage relationships, P_O was first normalized by dividing by the maximal P_O (−20 mV), which was then plotted against a patch-holding potential using Origin (version 7; OriginLab, Northampton, MA). This relationship was then fit with a Boltzmann function using Origin to derive the half-maximal voltage ($V_{0.5}$) and the slope factor (k). Channel current versus voltage relationships were produced in Origin by plotting channel current amplitude against patch-holding potential, and single-channel conductance (g) was then determined by linear regression.

Data are presented as the mean and SEM. When appropriate, statistical analysis was performed using Instat (version 3; GraphPad Software, San Diego, CA). Student's *t* test (two-tailed and paired or unpaired) or the Wilcoxon matched-pairs test (two-tailed) was used to test whether the mean differed between two groups. A one-sample *t* test was used to test whether a mean differed from zero. Data were considered significantly different if the p value was <0.05.

Results

ATP decreased cation channel activity in excised, inside-out patches

To record cation channels from cultured bag cell neurons, we used the excised, inside-out patch-clamp technique (Fig. 1*A*). With nASW bathing the extracellular face and artificial intracel-

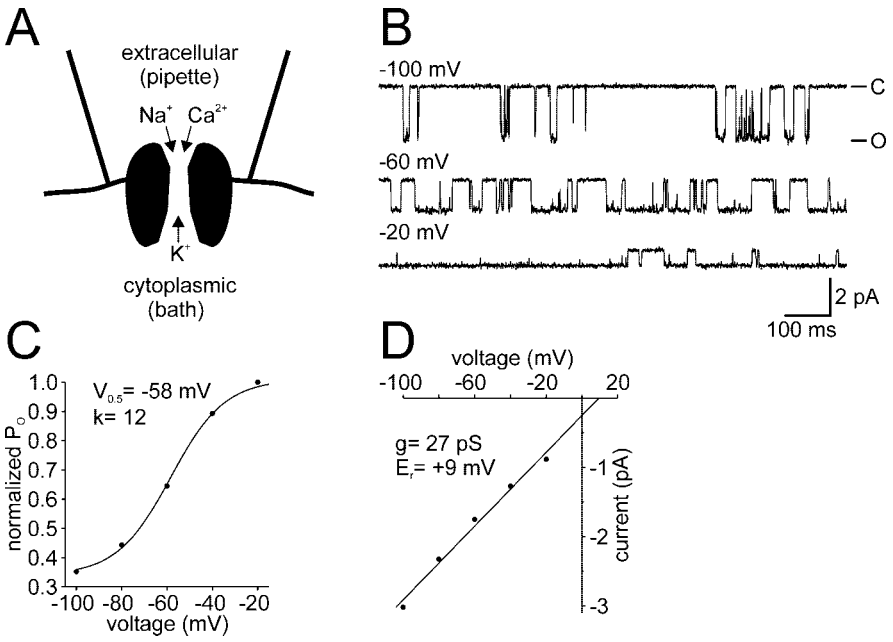

Figure 1. A cation channel in excised, inside-out patches. *A*, Diagrammatic representation of the bag cell neuron cation channel in an excised, inside-out patch (based on Wilson et al., 1996, 1998; Magoski et al., 2002). For the purposes of this study, the recording configuration is such that the extracellular face is within a pipette filled with nASW, whereas the cytoplasmic face is in a bath (tissue culture dish) containing artificial intracellular saline. Under these approximate physiological conditions, the channel is permeable to Na^+, K^+, and Ca^{2+} ions. *B*, Cation channel activity in an excised, inside-out patch at different steady-state holding potentials. Top trace, At −100 mV, the cation channel is seen as brief, unitary, inward current deflections of ~3 pA. The closed state is at the top of the trace and designated by −C, whereas the open state is at the bottom and designated by −O. Middle trace, At −60 mV, the channel opens and closes repeatedly. Bottom trace, At −20 mV, the channel is open much of the time. Note that at all holding potentials, the single-channel current shows no voltage-dependent inactivation. *C*, Normalized P_O versus voltage curve for the channel shown in *B*. P_O was calculated over the entire time (usually 1–3 min; see Materials and Methods) at the given holding potentials (−100, −60, −20 as well as −80 and −40). P_O was normalized by dividing by the P_O at −20 mV, plotted against voltage, and the points fit with a Boltzmann function. The Boltzmann provides half-maximal voltage ($V_{0.5}$) of activation and the slope factor (k) (i.e., the change in voltage required to move the P_O e-fold). *D*, Channel current versus voltage relationship for the channel shown in *B*. Channel current amplitude at a particular voltage was derived from Gaussian fits of all-points histograms (see Materials and Methods). This was plotted against patch-holding potential and fit with linear regression to determine single-channel conductance (g) and, based on the X-intercept, the predicted reversal potential (E_r).

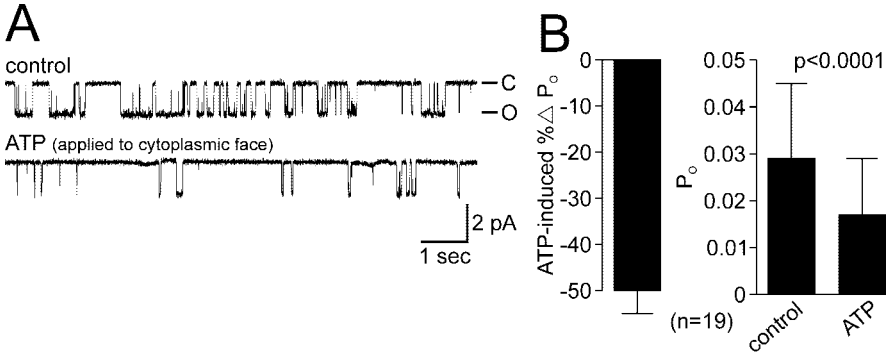

Figure 2. Inhibition of the cation channel by ATP. *A*, ATP inhibits the cation channel. Top trace, Control recording of a cation channel in an excised, inside-out patch. Bottom trace, Applying 1 mM ATP to the cytoplasmic face of the patch initiates a maintained drop in P_O. The patch was held at −60 mV. *B*, Summary of ATP-induced decrease in P_O. Left graph, In 19 cation channel-containing patches used as initial tests or parallel controls, application of 1 mM ATP causes an ~50% decrease in P_O. Right graph, ATP produces a statistically significant decrease in P_O from a control mean of −0.029 to a mean in ATP of −0.017 ($p < 0.001$; Wilcoxon matched-pairs test).

lular saline bathing the cytoplasmic face, currents recorded from cation channels showed characteristics essentially identical to those reported previously (Wilson and Kaczmarek, 1993; Wilson et al., 1996, 1998; Magoski et al., 2002). Specifically, the channels lacked voltage-dependent inactivation, displayed voltage-dependent opening (increased P_O with depolarization), had a conductance of 25–30 pS (~2 pA at −60 mV), and showed a predicted reversal potential of approximately +10 mV (Fig. 1*B-D*).

Previous studies demonstrated that the application of ATP to the cytoplasmic face of the cation channel resulted in an increased P_O (Wilson et al., 1998; Magoski et al., 2002). The enhanced activity was attributable to a closely associated PKC, the effects of which could be reversed by a similarly closely associated PP. Subsequent investigations reported that in ~30% of cation channel-containing patches excised from the bag cell neurons of many *Aplysia*, there was an obvious decrease in P_O after the application of 1 mM ATP (Magoski, 2003; Magoski and Kaczmarek, unpublished observations). In the experiments described here, the decreased P_O ATP response was characterized by a rapid and sustained drop in cation channel P_O that lasted for the remainder of the recording period (up to 15 min) (Fig. 2*A*). This inhibition resulted in an ~50% decrease in P_O with a P_O change from a mean of 0.029 in control to a mean of 0.017 in ATP ($n = 19$ patches) (Fig. 2*B*). The present study explores the mechanism underlying the decreased P_O ATP response.

Kinetic analysis of channel behavior can provide insight regarding the biophysical and/or mechanistic basis of a change in phenotype. Accordingly, the closed- and open-state dwell times were examined before and after a decreased P_O ATP response in true, single-channel patches (for criteria, see Materials and Methods; $n = 7$ channels/patches) (Fig. 3*A*). The kinetic profile of the cation channel in control conditions (Fig. 3*B*, top graphs) was the same as reported previously (Wilson and Kaczmarek, 1993; Magoski et al., 2002), with the closed dwell-time histogram best fit by a three-exponential component (t_{C1}, t_{C2}, and t_{C3}) and the open dwell-time histogram best fit by a two-exponential component (t_{O1} and t_{O2}). The decreased P_O ATP response (Fig. 3*B*, bottom graphs) did not change the number of exponentials required to describe the closed or open times, nor did it lead to significant alterations in t_{C1}, t_{C2}, t_{O1}, and t_{O2}. However, a consistent increase in t_{C3} was observed after the introduction of ATP, which is best seen by noting the slight rightward shift and overall increase in the rightmost peak of the closed-time histogram. The summary data for these seven cation channels (Fig. 3*C*) showed that ATP induced an ~40% decrease in P_O without changing channel amplitude. The decrease in P_O was the result of a near 40% increase in t_{C3}, whereas the remaining closed- and open-state time constants were not altered appreciably.

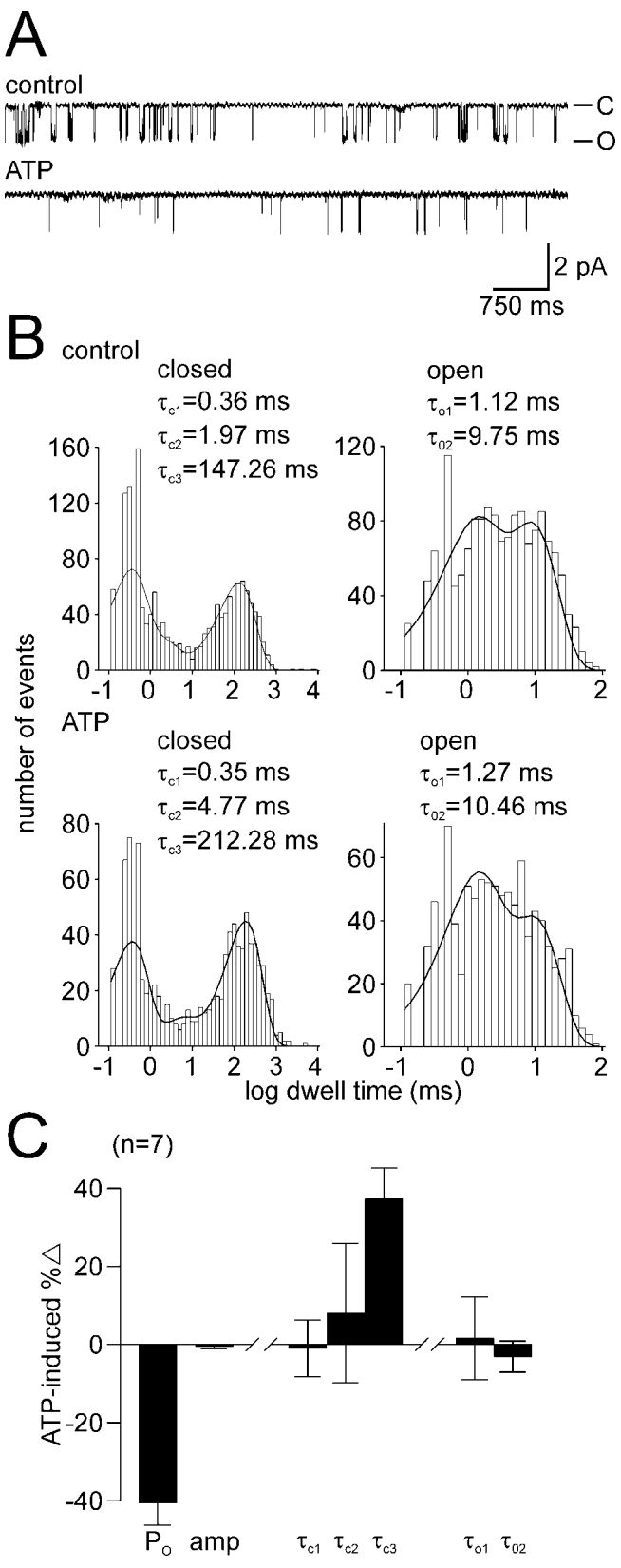

Figure 3. Kinetic analysis of the decreased P_0 ATP response. *A*, ATP inhibits a single cation channel. Top trace, Control recording of a true, single cation channel in an excised, inside-out patch. Bottom trace, Exposure of the cytoplasmic face of the patch to 1 mM ATP results in a decrease in P_0. The patch was held at −60 mV. *B*, Single channel closed and open dwell times are plotted as histograms along with a fit of a sum of exponentials (see Materials and Methods). The time constants for the exponential fits are given in the inset of each graph. During the control period (top graphs), channel closed times are best fit by three exponentials (t_{C1}, t_{C2}, and t_{C3}), and the open times by two exponentials (t_{01} and t_{02}). When ATP is added (bottom graphs), the t_{C1} or t_{C2} change only slightly, whereas t_{C3} is obviously larger (an over 40% increase from ~150 to ~215 msec). For the open times in ATP, neither t_{01} nor t_{02} show any overt change. *C*, Summary data for the decreased P_0 ATP response of true, single-cation channels. For these seven channels/patches, ATP reduces P_0 by ~40% with no change in channel current amplitude (amp). On average, although there is no real change in the first two closed-state time constants (t_{C1} and t_{C2}), the third (t_{C3}) shows an ~40% increase with ATP. For the open-state time constants (t_{01} and t_{02}), there is no net change with ATP.

A PKA inhibitor prevented the decreased P_O ATP response

Comparison of the kinetic analysis for the decreased P_O ATP response with previous studies of the cation channel pointed to a possible mechanism for the effect. Specifically, Wilson and Kaczmarek (1993) showed that application of exogenous PKA to the cytoplasmic face of the cation channel decreased P_O by ~40%, and this was the result of an increase of similar magnitude in the duration of t_{C3}. The similarity between the effect of exogenous PKA and the decreased P_O ATP response suggested that the latter was mediated by a PKA-like activity. Furthermore, given that the response was evident in excised, inside-out patches, the kinase must be closely associated with the channel. To test this, PKA_{6-22}, a very specific PKA inhibitor peptide (Glass et al., 1989) that is effective in *Aplysia* (Adams and Levitan, 1982) as well as the bag cell neurons (Conn et al., 1989), was tested on the decreased P_O ATP response. Parallel controls of standard decreased P_O ATP responses were performed on sister cultures (i.e., bag cell neurons isolated from the same animal and maintained *in vitro* for a similar period of time; $n = 6$ patches) (Fig. 4*A*). For the inhibitor, patches were excised into artificial intracellular saline containing 1 μM PKA_{6-22}, and after a recording period in peptide alone, ATP was applied. PKA_{6-22} consistently prevented the decreased P_O ATP response ($n = 7$ patches) (Fig. 4*B*). On average, the parallel controls showed an ~50% decrease in P_O with ATP, whereas in the presence of PKA_{6-22}, the effect of ATP on P_O was negligible (Fig. 4*C*).

A PKA inhibitor also reversed the decreased P_O ATP response

The previous description of cation channel modulation by a closely associated protein kinase was that of PKC-dependent upregulation; furthermore, the effects of PKC could be reversed by a PP that was also targeted to the channel (Wilson et al., 1998; Magoski et al., 2002). As an initial step in determining whether a PP, capable of reversing the decreased P_O ATP response, was associated with the cation channel, PKA_{6-22} was applied after ATP. The anticipated outcome of this would be that inhibition of the kinase would allow any phosphatase activity present in the patch to dephosphorylate the channel and return P_O to its former level. The decrease in P_O produced by ATP during these experiments was reversed back to control values with the subsequent introduction of PKA_{6-22} ($n = 5$ patches) (Fig. 5*A*). The change in P_O amounted to an ~45% drop, which was followed by a ~150% increase with PKA_{6-22} (Fig. 5*B*). It is expected that the percentage change seen after the addition of PKA_{6-22} would involve a seemingly large increase, because the activity must be elevated from a relatively low value in the presence of ATP compared with control.

PP inhibition prevented reversal of the decreased P_O ATP response

If reversal of the decreased P_O ATP response by PKA_{6-22} was attributable to the inhibition of PKA allowing for the activity of a PP to be observed, it follows that previous inhibition of the PP

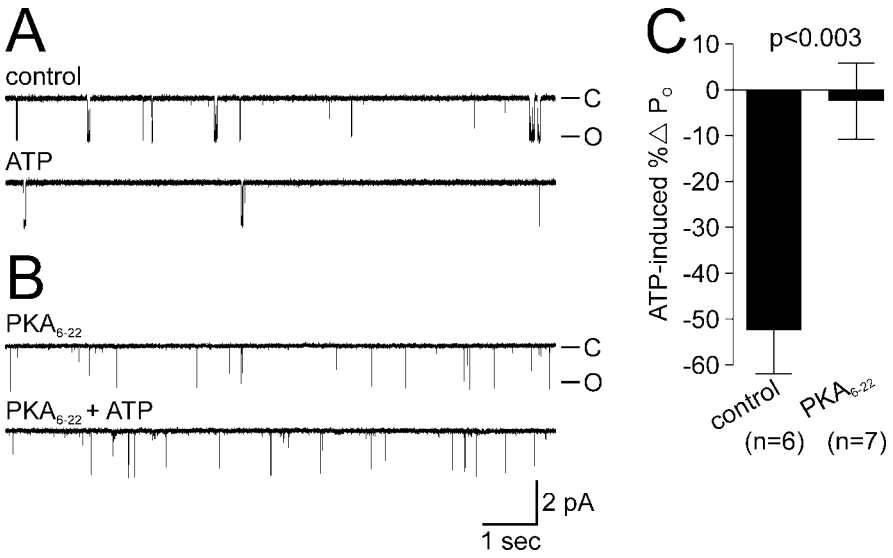

Figure 4. The decreased P_0 ATP response is blocked by pretreatment with PKA_{6-22}. *A*, A parallel control decreased P_0 ATP response. Top trace, A control cation channel recorded in an excised, inside-out patch. Bottom trace, Addition of 1 mM ATP to the cytoplasmic face of the patch produces a decrease in P_0. The patch was held at −60 mV. *B*, The presence of PKA_{6-22} prevents the decreased P_0 ATP response. Top trace, Recording of a cation channel with 1 μM PKA_{6-22} bathing the cytoplasmic face of the patch. Bottom trace, Introduction of 1 mM ATP along with PKA_{6-22} does not result in a P_0 change. The patch was held at −60 mV. *C*, Summary data for the effect of ATP on cation channels in the absence or presence PKA_{6-22}. Under control conditions, ATP elicits an ~50% decrease in P_0 ($n = 6$ patches), whereas in the presence of PKA_{6-22}, the mean change is just below 0% ($n = 7$ patches; $p < 0.003$; unpaired Student's *t* test).

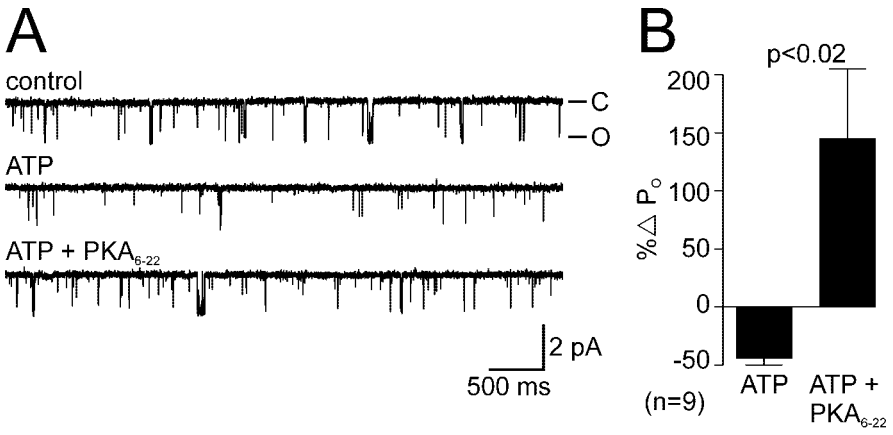

Figure 5. The decreased P_0 ATP response is reversed by subsequent application of PKA_{6-22}. *A*, Reversal of the decreased P_0 ATP response by PKA_{6-22}. Top trace, A cation channel recorded in the excised, inside-out configuration. Middle trace, ATP (1 mM) applied to the cytoplasmic face of the patch decreases P_0. Bottom trace, Addition of 1 μM PKA_{6-22} in the maintained presence of ATP results in restoration of P_0 back to that of control. The patch was held at −60 mV. *B*, Summary data for the effect of introducing PKA_{6-22} after ATP inhibits cation channels. In this group, ATP produces an ~45% decrease in P_0, but with the addition of PKA_{6-22}, there is an ~150% increase in P_0 as activity returns to control levels ($n = 9$ patches; $p < 0.02$; paired Student's *t* test).

would prevent reversal. The first PP blocker tested in this manner was okadaic acid, an inhibitor that is believed to be relatively more specific for PP2 than PP1 (Bialojan and Takai, 1988). Patches were excised into artificial intracellular saline containing 100 nM okadaic acid, which was followed by ATP, and finally PKA_{6-22}. Surprisingly, in the presence of okadaic acid, PKA_{6-22} was still capable of initiating recovery in cation channel activity after a decreased P_O ATP response ($n = 4$ patches) (Fig. 6*A*). On average, ATP lowered P_O by ~50%, which was reversed markedly by PKA_{6-22} with an almost 250% enhancement (Fig. 6*B*).

The inability of okadaic acid to prevent PKA_{6-22}-induced reversal of the decreased P_O ATP response could be attributable to its ineffectiveness as a PP inhibitor in bag cell neurons, and/or the PP responsible may be more similar to PP1 than PP2. Thus, microcystin-LR, an inhibitor with more equal specificity between PP1 and PP2 (MacKintosh et al., 1990), was used next. With 200 nM microcystin-LR bathing the cytoplasmic face of cation channels, the reduction in activity elicited by ATP was not reversed with application of PKA_{6-22} ($n = 5$ patches) (Fig. 6*C*). The mean decrease in P_O brought about by ATP was just over 50%, and instead of showing recovery, the P_O actually went down slightly by ~35% after PKA_{6-22} (Fig. 6*D*).

PKA inhibition did not alter the increased P_O ATP response

Previous work on the cation channel demonstrated that the application of ATP to the cytoplasmic face could, in some cases, increased P_O through the actions of closely associated PKC (Wilson et al., 1998; Magoski et al., 2002). If PKA were associated with the cation channel at the same time as PKC, there may, in fact, be a competition between the two kinases for modulation of the activity of the channel in the presence of ATP. This would also have implications for how the regulatory complex may be organized during different states of excitability. To examine the possibility that PKA and PKC are simultaneously closely associated with certain cation channels, PKA_{6-22} was added to the cytoplasmic face of channels displaying an increased P_O ATP response. If PKA was associated with the channel at the same time as PKC, inhibition of PKA should result in an even greater increase in P_O; however, cation channel activity did not rise further after the introduction of PKA_{6-22} (Fig. 7*A*). The increased P_O ATP response amounted to an ~250% increase in activity, which was followed by a small, ~20% decrease, with PKA_{6-22} ($n = 12$ patches) (Fig. 7*B*).

Discussion

The inward current that depolarizes the bag cell neurons during the afterdischarge arises from a nonselective, Ca^{2+}-permeable, voltage-dependent, noninactivating cationconductance (Kaczmarek and Strumwasser, 1984; Wilson and Kaczmarek, 1993; Wilson et al., 1996). Currents with similar properties have been shown to maintain prolonged, repetitive, and/or burst firing in a large number of functionally diverse neurons from multiple species (Wilson and Wachtel, 1974; Partridge et al., 1979; Green and Gillette, 1983; Stafstrom et al., 1985; Swandulla and Lux, 1985; Alonso and Llinas, 1989; Rekling and Feldman, 1997; Beurrier et al., 1999; Morisset and Nagy, 1999; Raman et al., 2000; Egorov et al., 2002; Perrier and Hounsgaard, 2002). Cation channel activation can also initiate activity-dependent changes to intrinsic excitability (Egorov

et al., 2002; Zhang and Linden, 2003), which for the bag cell neurons leads to clear long-term changes in neuronal activity and animal behavior (Kupfermann, 1967; Kupfermann and Kandel, 1970; Pinsker and Dudek, 1977; Wilson and Kaczmarek, 1993; Wilson et al., 1996; Magoski et al., 2000, 2002). The upregulation of this channel during the afterdischarge is attributable to the sustained input of several second messengers and kinases (Conn and Kaczmarek, 1989; Magoski and Kaczmarek, 2003), including a persistent increase in PKC (Wayne et al., 1999). A close, physical association between PKC and the cation channel facilitates the afterdischarge by guaranteeing a precise timing and localization of increased enzyme activity leading to increased P_O.

In excised, inside-out patches, the closely associated PKC manifests itself as an increased P_O after application of ATP to the cytoplasmic face. However, as shown in the present study, P_O can also be decreased by ATP in a rapid and sustained manner. This suggests that the decreased P_O ATP response is also attributable to a closely associated kinase activity phosphorylating the channel or a nearby protein. Alterations to channel phenotype, such as a change in P_O with phosphorylation, are correlated with often unique changes in either the duration or number of exponentials required to fit the dwell times representing the kinetic profile of the channel (Colquhoun and Sigworth, 1995). Although the decreased P_O ATP response is not associated with a change in the number of exponentials required to fit either the open- or closed-state dwell times, it is accompanied by a consistent ~40% elevation to the third closed-state time constant, t_{C3}. An increase in t_{C3} suggests that the channel favors remaining closed, resulting in the reduced activity characteristic of the decreased P_O ATP response. Wilson and Kaczmarek (1993) showed that, in the presence of ATP, application of the catalytic subunit of bovine PKA to the cytoplasmic face caused a 40% decrease in P_O and a similar increase in t_{C3} duration. The degree of similarity between the decreased P_O ATP response and the effects of exogenous PKA raises the possibility that the ATP-induced drop in activity is mediated by a closely associated PKA-like activity.

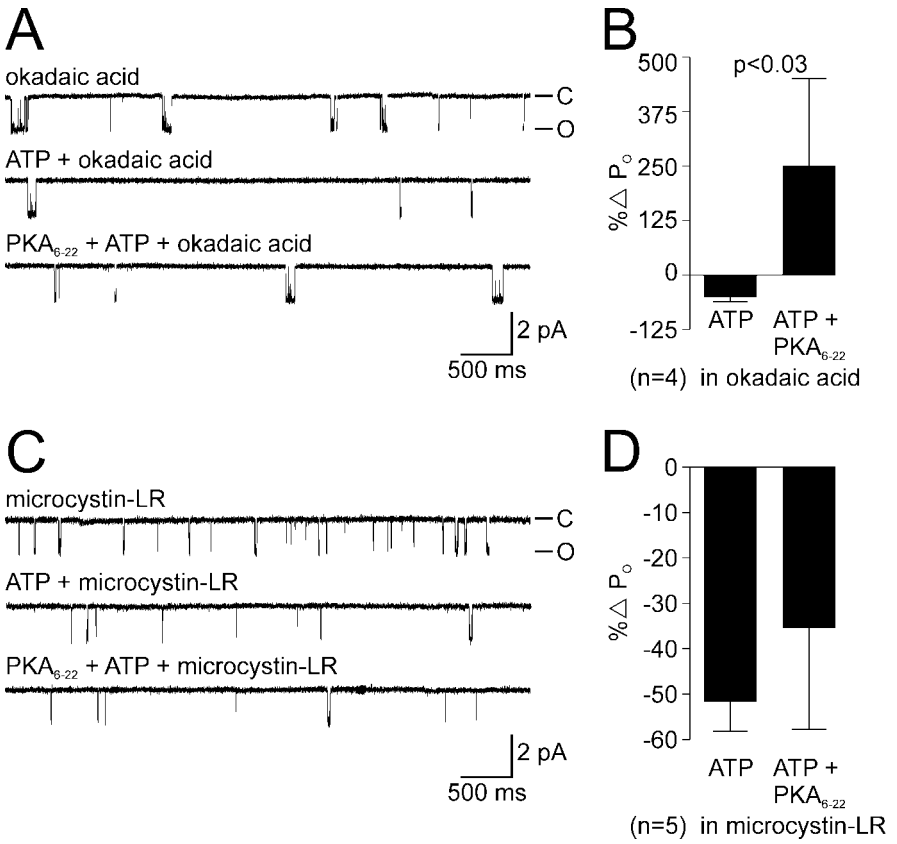

Figure 6. Reversal of the decreased P_0 ATP response by PKA_{6-22} is prevented by microcystin-LR but not okadaic acid. *A*, Pretreatment with okadaic acid does not alter the ability of PKA_{6-22} to reverse the decreased P_0 ATP response. Top trace, A recording of a cation channel in an excised, inside-out patch with the cytoplasmic face bathed by 100 nM okadaic acid. Middle trace, Application of 1 mM ATP initiates a decrease in P_0. Bottom trace, Introduction of 1 μM PKA_{6-22}, in the combined presence of ATP and okadaic acid, initiates a return of P_0 toward control levels. The patch was held at −60 mV. *B*, Summary data for the lack of an effect of okadaic acid on PKA_{6-22}-mediated reversal of the decreased P_0 ATP response. The application of ATP in the presence of okadaic acid decreases P_0 by ~50%, and after introduction of PKA_{6-22}, channel activity returns to pre-ATP levels with a near 250% increase in P_0 ($n = 4$ patches; $p < 0.03$; Wilcoxon matched-pairs test). *C*, Pretreatment with microcystin-LR prevents reversal of the decreased P_0 ATP response by PKA_{6-22}. Top trace, Excised, inside-out patch recording of a cation channel with the cytoplasmic face bathed by 200 nM microcystin-LR. Middle trace, Delivery of 1 mM ATP results in a P_0 decrease. Bottom trace, When 1 μM PKA_{6-22} is applied along with ATP and microcystin-LR, there is no reversal of activity, and P_0 remains lowered. The patch was held at −60 mV. *D*, Summary data for the effect of microcystin-LR on PKA_{6-22}-mediated reversal of the decreased P_0 ATP response. While in the presence of microcystin-LR, the addition of ATP causes a slightly >50% decrease in P_0. This drop in activity is not reversed with the subsequent application of PKA_{6-22}, and, in fact, the P_0 drops even further by ~35% ($n = 5$ patches).

A cation channel–PKA association is further reinforced by the ability of PKA_{6-22} to prevent the decreased P_O ATP response. PKA_{6-22} is a very specific blocker of PKA, based on the endogenous PKA inhibitor protein (PKI) (Walsh et al., 1971; Ashby and Walsh, 1972; Glass et al., 1989). PKI was shown by Adams and Levitan (1982) to be extremely effective at inhibiting *Aplysia* brain PKA in a biochemical assay. Furthermore, PKI and/or its derived peptides, PKA_{6-22} and PKA_{5-24}, have been used to demonstrate a role for PKA in the control of excitability and action potential height in the bag cell neurons themselves (Conn et al., 1989), as well as phenomena in other marine preparations, including *Aplysia* sensory neuron spike broadening (Castellucci et al., 1982), serotonin-induced increase of K^+ current in *Aplysia* neuron R15 (Adams and Levitan, 1982), and transmitter release from the squid giant synapse (Hilfiker et al., 2001). Finally, in excised, inside-out patch-clamp recordings from several preparations, these peptides have established that PKA, through a close, physical association, can regulate Ca^{2+}-activated K^+ channels (Bielefeldt and Jackson, 1994; Esguerra et al., 1994; Wang and Kotlikoff, 1996).

When a kinase is found to be closely associated with a channel, a phosphatase is often present to act as a balance (Bielefeldt and Jackson, 1994; Reinhart and Levitan, 1995; Wilson et al., 1998; Davare et al., 2001; Magoski et al., 2002; Marx et al., 2002), and the PKA-mediated decrease of cation channel P_O is no exception. When PKA_{6-22} is applied after the ATP-induced decrease in P_O occurs, activity recovers toward control levels. In addition, pretreating patches with microcystin-LR, a PP inhibitor, prevents PKA_{6-22} from reversing the response. These data suggest that a PP is closely associated with the channel, and when PKA is inhib-

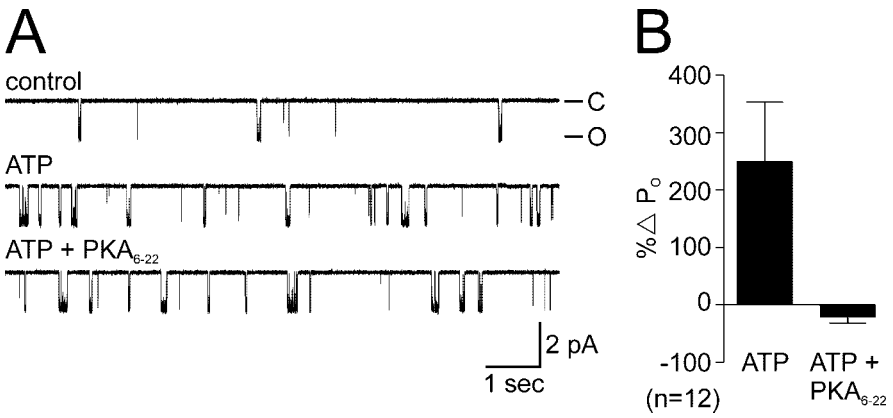

Figure 7. The increased P_O ATP response is not augmented by PKA_{6-22}. *A*, PKA_{6-22} does not alter the magnitude of the increased P_O ATP response. Top trace, An excised, inside-out patch recording of a cation channel. Middle trace, Application of 1 mM ATP to the cytoplasmic face produces a robust P_O increase. Bottom trace, The P_O remains unchanged after the addition of 1 μM PKA_{6-22}. The patch was held at −60 mV. *B*, Summary data for the lack of an effect of PKA_{6-22} on the increased P_O ATP response. This set of responsive channels shows an ~250% elevation in P_O with the addition of ATP. The enhancement is not altered further when PKA_{6-22} is added, because the P_O shows only an ~20% drop that is not significantly different from zero ($n = 12$ patches; $p > 0.05$; one-sample *t* test).

ited, the phosphatase is free to dephosphorylate the substrate. The effectiveness of microcystin-LR is in contrast to another PP inhibitor, okadaic acid, which failed to prevent reversal of the response by PKA_{6-22}. Okadaic acid has a greater specificity for PP2 over PP1 (Bialojan and Takai, 1988), whereas microcystin-LR has a similar specificity between PP1 and PP2 (MacKintosh et al., 1990). In *Aplysia*, the major neuronal plasma membrane-bound phosphatase is PP1-like (Endo et al., 1995), and both the PP1- and PP2-like enzymes share a similar sensitivity to microcystin-LR (Ichinose et al., 1990). Moreover, microcystin-LR attenuates the FMRFamide-induced K^+ current (Endo et al., 1995) and prolongs serotonin-induced spike broadening (Ichinose et al., 1990) in pleural sensory neurons, as well as prevents the reversal of PKC-dependent phosphorylation of the bag cell neuron cation channel (Wilson et al., 1998). Thus, it is probable that a PP1-like phosphatase dephosphorylates the cation channel after the actions of PKA or PKC.

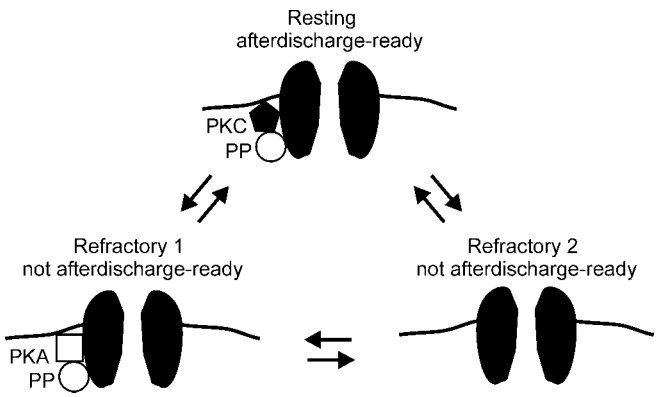

Figure 8. Model for altering the constituents of the cation channel regulatory complex under different states of excitability. Top, At rest, when the bag cell neurons are ready to afterdischarge, the regulatory complex consists of a closely associated, stimulatory PKC along with a counterbalancing PP. This would represent channels that display the increased P_O ATP response. Bottom left, After an afterdischarge, the bag cell neurons become refractory and, despite additional stimulation, are unable to afterdischarge further. This first refractory scenario has an inhibitory PKA, as well as a counterbalancing PP, associated with the cation channel. The down-regulation of cation channel activity by PKA may contribute to the inexcitability seen during refractoriness. Bottom right, A second refractory scenario has a cation channel that lacks a regulatory complex altogether. Arrows indicate the possibility of transition between different states of the complex by altering the component enzymes.

The decrease or increase in P_O observed after ATP seems to depend on whether PKA or PKC is closely associated with the channel. Given that introducing PKA_{6-22} after an increased P_O ATP response does not result in additional elevation of cation channel activity suggests the association of PKC precludes association of PKA. In addition to describing that ~30% of channels/animals displayed a decreased P_O with ATP, Magoski (2003), as well as Magoski and Kaczmarek (unpublished observations), also reported that ~40% of cation channels excised from refractory bag cell neurons displayed a decreased P_O ATP response, compared with none of the channels from resting neurons. The bag cell neurons become refractory after termination of the afterdischarge and remain in a state in which bursting cannot be initiated for ~18 hr (Conn and Kaczmarek, 1989). Whereas Ca^{2+} entry is key to initiating refractoriness (Kaczmarek and Kauer, 1983; Magoski et al., 2000), a clear, mechanistic understanding of the refractory period is incomplete (Zhang et al., 2002). However, it has been established that electrical or pharmacological stimulation of refractory neurons does not elicit the cumulative depolarization that drives the afterdischarge (Kaczmarek and Kauer, 1983; Kauer and Kaczmarek, 1985; Wilson et al., 1996). Because the cation channel underlies this depolarization, its regulation may be altered under such circumstances. Indeed, based on their finding that exogenous PKA lowers channel activity, Wilson and Kaczmarek (1993) concluded that refractoriness may involve PKA-dependent inhibition of the cation channel. If this is the case, then the cation channel–PKA association documented here provides a means to promote or maintain refractoriness. In addition, the constituents of the cation channel regulatory complex could be altered to further different states of excitability. Before and throughout the afterdischarge, PKC is associated with the channel, whereas during the transition to the refractory period, PKC dissociates and PKA potentially associates with the channel (Fig. 8, schematic).

Several mechanisms may contribute to organizing the cation channel regulatory complex. For example, both the PP and either PKC or PKA could bind directly to the channel. Transition between different regulators would then be achieved by PKA displacing PKC and vice versa. Alternatively, the association or upregulation of one kinase could simply sterically hinder the actions of another, permanently associated kinase. As suggested by Magoski et al. (2002), the cation channel and its regulatory enzymes could also be brought together by a scaffolding protein. Exchange of PKA for PKC could be regulated by altering the affinity of the scaffold for one kinase over the other, perhaps with steric hindrance preventing dual kinase occupation. Once in close proximity, presumably PKA or PKC regulates the channel by phosphorylating it on distinct sites, both of which could be dephosphorylated by the PP. In contrast, a secondary channel-associated protein could be the actual target of phosphorylation, and in turn this protein alters channel gating phenotype.

Complexes of kinases and/or phosphatases with ion channels

have been documented for a large number of voltage-gated and ionotropic channels from various species (Chung et al., 1991; Bielefeldt and Jackson, 1994; Rosenmund et al., 1994; Reinhart and Levitan, 1995; Holmes et al., 1996; Yu et al., 1997; Tibbs et al., 1998; Brandon et al., 1999; Tsunoda and Zucker, 1999; Davare et al., 2001; Huang et al., 2001; Marx et al., 2002; Nitabach et al., 2002; Zhou et al., 2002; Gingrich et al., 2004). For the bag cell neurons, the cation channel can be considered an integration point at which appropriate signaling molecules converge in a complex to control excitability. The association of different regulators at different times (e.g., PKA/PP vs PKC/PP) suggests that a general mechanism for diversifying modulation can be found in rearranging the constituents of a given channel complex. This strategy of "regulating the regulators" is well suited for precisely modulating ion channel function and excitability, particularly over long time periods.

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