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...
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 Marimus 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 h light/dark cycle and fed romaine lettuce three to five times per week.

For primary cultures of isolated bag cells, animals were anesthetized with an injection of isotonic MgCl2 (~50% of body weight), and the abdominal ganglion was removed and treated with neutral protease (13.33 mg/ml; catalog #16589; Roche Diagnostics, Indianapolis, IN) for 18 h at 18–20°C, dissolved in normal artificial sea water (nASW; 460 mM NaCl, 10.4 mM KCl, 11 mM CaCl2, 5 mM MgCl2, 15 mM HEPES, 1 mg/ml glucose, 100 Units/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 Pasteur pipette and gentle titration, neurons were dispersed in nASW onto 35 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, Marlboro 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 CaCl2, 1.2 MgCl2, 10 HEPES, 11 glucose, 5 EGTA, and 10 reduced glutathione, pH 7.3 with KOH; free [Ca2+]i = 1 μM, calculated using the CaBuffer program, courtesy of Dr. L. Schlachter, 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 Digidoc 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 Ca2+-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 (Po) 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), PKAα22 (P6062) and okadaic acid (K+ salt; catalog #07885) were both obtained from Sigma–Aldrich, and microcystin-LR (catalog #475815) was obtained from Calbiochem (San Diego, CA).

Analysis. To determine single-channel Po 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 opening, 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 Pstst analysis program of pClamp was used to read events lists and determine Po, either automatically or manually, using the formula: 

\[ P_O = \frac{t_1 + t_2 + \ldots + t_n}{N \times t_{tot}} \quad \text{where } t \text{ is the amount of time that } n \text{ channels are open, } N \text{ is the number of channels in the patch, and } t_{tot} \text{ is the time interval over which } P_O \text{ 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). Pstst 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 (τ) 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 Po 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 Po had to be at least 0.02 to generate 500 events. Indeed, the Po of the true single channels used for kinetic analysis was always above 0.02 and averaged ~0.1. Pstst 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 Po versus voltage relationships, Po was first normalized by dividing by the maximal Po (~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 (Vo,50) and the slope factor (k).

Channel current versus voltage relationships were produced in Origin by subtracting the mean open and closed state current level from the patch–holding potential, and plotting channel current amplitude against patch–holding potential, and finally, 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 decreases cation channel activity in excited, inside–out patches

To record cation channels from cultured bag cell neurons, we used the excised, inside–out patch–clamp technique (Fig. 1A). With nASW bathing the extracellular face and artificial intracel-
Methods) at the given holding potentials (Wilson et al., 1996, 1998; Magoski et al., 2002). Specifically, the channels lacked voltage-dependent inactivation, displayed voltage-dependent 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 open-channel (increased $P_o$, with depolarization), had a conductance of 25–30 pS ($\sim$ 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 $\sim$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 (Magosi, 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 $\sim$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; 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 $\sim$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.

Figures 1 and 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 $\sim$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).
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. 4A). 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. 4B). 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. 4C).

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 up-regulation; 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. 5A). The change in $P_O$ amounted to an ~45% drop, which was followed by a ~150% increase with PKA$_{6-22}$ (Fig. 5B). 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

$\tau_{C1}$, and the open times by two exponentials ($t_{O1}$ and $t_{O2}$). When ATP is added (bottom graphs), the $t_{O1}$ or $t_{O2}$ change only slightly, whereas $t_{C1}$ is obviously larger (an over 40% increase from ~150 to ~215 msec). For the open times in ATP, neither $t_{O1}$ nor $t_{O2}$ show any overt change. C, Summary data for the decreased $P_O$ ATP response of true, single-cation channels. For these seven channels/patches, ATP reduces $P_O$ 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 ($t_{O1}$ and $t_{O2}$), there is no net change with ATP.
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, increase \( P_O \) 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. 7A). 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. 7B).

**Discussion**

The inward current that depolarizes the bag cell neurons during the afterdischarge arises from a nonselective, Ca\(^{2+}\)-permeable, voltage-dependent, noninactivating cation conductance (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 Linas, 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; Finsler 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.

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$_{8-22}$ and PKA$_{6-20}$, 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 (Hilliker 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, pre-treating 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-

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**Figure 6.** Reversal of the decreased $P_O$ 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 $P_O$ 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 $P_O$. Bottom trace, Introduction of 1 $\mu$M PKA$_{6-22}$ in the combined presence of ATP and okadaic acid, initiates a return of $P_O$ toward control levels. The patch was held at $60 \pm 0.03$ mV. B, Summary data for the lack of an effect of okadaic acid on PKA$_{6-22}$-mediated reversal of the decreased $P_O$ ATP response. The application of ATP in the presence of okadaic acid decreases $P_O$ by ~50%, and after introduction of PKA$_{6-22}$, channel activity returns to pre-ATP levels with a near 25% increase in $P_O$ ($n = 4$ patches; $p < 0.03$; Wilcoxon matched-pairs test). C, Pretreatment with microcystin-LR prevents reversal of the decreased $P_O$ 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 $P_O$ decrease. Bottom trace, When 1 $\mu$M PKA$_{6-22}$ is applied along with ATP and microcystin-LR, there is no reversal of activity, and $P_O$ remains lowered. The patch was held at $60 \pm 0.03$ mV. D, Summary data for the effect of microcystin-LR on PKA$_{6-22}$-mediated reversal of the decreased $P_O$ ATP response. While in the presence of microcystin-LR, the addition of ATP causes a slightly >50% decrease in $P_O$. This drop in activity is not reversed with the subsequent application of PKA$_{6-22}$ and, in fact, the $P_O$ drops even further by ~35% ($n = 5$ patches).
phosphatase dephosphorylates the cation channel after the actions of PKA or PKC.

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 channel undergoes 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 PKC could bind directly to the channel. Transition between different regulators would then be achieved by PKA dissociation and PKC association. For example, both the PP and PKC could bind directly to the channel. Transition between different regulators would then be achieved by PKA dissociation and PKC association. 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; Nita-bach 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.

References


Post thumbnail image

Title: Modulating ion channel function and excitability, particularly over long time periods

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Magoski NS • PKA Inhibits a Cation Channel

Abstract: This review discusses the regulation of ion channel function and excitability in neurons, focusing on the role of protein kinases and phosphatases.

Keywords: Protein kinases, phosphatases, ion channels, excitability, neurons
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