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Modulation of a calcium-sensitive nonspecific cation channel by closely associated protein kinase and phosphatase activities

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ABSTRACT **Regulation of nonspecific cation channels often underlies neuronal bursting and other prolonged changes in neuronal activity. In bag cell neurons of *Aplysia*, it recently has been suggested that an intracellular messenger-induced increase in the activity of a nonspecific cation channel may underlie the onset of a 30-min period of spontaneous action potentials referred to as the "afterdischarge." In patch clamp studies of the channel, we show that the open probability of the channel can be increased by an average of 10.7-fold by application of ATP to the cytoplasmic side of patches. Duration histograms indicate that the increase is primarily a result of a reduction in the duration and percentage of channel closures described by the slowest time constant. The increase in open probability was not observed using 5′-adenylylimidodiphosphate, a nonhydrolyzable ATP analog, and was blocked in the presence of H7 or the more specific calcium/phospholipid-dependent protein kinase C (PKC) inhibitor peptide$_{(19–36)}$. Because the increase in activity observed in response to ATP occurred without application of protein kinase, our results indicate that a kinase endogenous to excised patches mediates the effect. The effect of ATP could be reversed by exogenously applied protein phosphatase 1 or by a microcystin-sensitive phosphatase also endogenous to excised patches. These results, together with work demonstrating the presence of a protein tyrosine phosphatase in these patches, suggest that the cation channel is part of a regulatory complex including at least three enzymes. This complex may act as a molecular switch to activate the cation channel and, thereby, trigger the afterdischarge.**

Modulation of ion channel activity by intracellular messengers underlies many prolonged changes in animal behavior (1). One dramatic example is found in *Aplysia* in which an increase in the excitability of bag cell neurons initiates behaviors that culminate in egg-laying (2). Specifically, brief stimulation of the pleuroabdominal connective nerve or application of reproductive tract peptides triggers a ≈30-min period of spontaneous repetitive action potentials known as the afterdischarge (3, 4). This is followed by a refractory period lasting 18 hr or more during which additional afterdischarges cannot be elicited (3, 5). Recent evidence suggests that activation of bag cell neuron nonspecific cation channels may provide the depolarizing drive underlying the afterdischarge (6, 7). As might be expected from the complex regulation of bag cell neuron activity, the regulation of cation channel activity also is complex. Cation channels are voltage- and Ca^{2+}-sensitive and, upon patch excision, can be found in several distinguishable modes of gating. The most dramatic distinction occurs between the bursting and the continuously active gating modes. Bursting, a term that often is applied to the grouping of action potentials, here refers to groupings of channels openings. These groupings are separated by an added population of long-lived channel closures in the 1–100 s range (6). Cation channels in the bursting mode transit to a continuous high activity mode after treatment with T-cell protein tyrosine phosphatase (PTPase), which eliminates the long-lived closures. Transition from the bursting to the continuous activity mode can also be produced by treatment with cAMP-dependent protein kinase (PKA), which increases the activity of a PTPase that is endogenous to excised patches (6).

We now report that the activity of the bag cell neuron cation channel also is regulated by intracellular ATP. Because the increase in channel activity requires hydrolyzable nucleotides and because the effect of ATP is blocked by the kinase inhibitors H7 and calcium/phospholipid-dependent protein kinase C (PKC) peptide$_{(19–36)}$, the effect appears to be mediated by a PKC-like protein kinase. Consistent with this hypothesis, the effect of ATP is reversed by exogenously applied protein phosphatase 1 (PP1) or by a microcystin-sensitive phosphatase also endogenous to excised patches. The prevalence of the PKC-like kinase and two types of phosphatases in excised patches containing cation channels suggests that these enzymes and cation channels are colocalized. The enzyme complex may serve as part of a molecular switch that contributes to activation of the cation channel, the onset of the bag cell neuron afterdischarge, and the ensuing refractory period.

MATERIALS AND METHODS

Abdominal ganglia were excised from adult *Aplysia californica* (Alacrity Marine Biological Services, Redondo Beach, CA) that had been anesthetized by injecting isotonic $MgCl_2$. Bag cell neurons were dissociated from ganglia and maintained in artificial seawater (containing, in mM: 460 NaCl/10.4 KCl/11 $CaCl_2$/55 $MgCl_2$/10 Hepes, pH 7.8) supplemented with glucose (1 mg/ml), penicillin (100 units/ml), and streptomyocin (0.1 mg/ml) as described (7, 8). Channel currents were recorded by using a List EPC-7 (Adams–List, Wesbury, NY) amplifier and then low-pass filtered at 3 kHz and stored on videocassettes. Unless otherwise noted, pipettes contained artificial seawater and the bath contained, in mM: 500 K-aspartate, 70 KCl, 0.77 $CaCl_2$, 1.2 $MgCl_2$, 10 Hepes, 11 glucose, 0.77 EGTA, and 10 glutathione (a reducing agent), pH 7.3 (KOH). Pipettes were coated with Sylgard (Dow Corning) and had resistances ranging from 3–10 MΩ. Junction potentials were nulled immediately before seal formation. In most cases, test solutions were applied by using a gravity-driven multibar-

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Abbreviations: PKA, cAMP-dependent protein kinase; PKC, calcium/phospholipid-dependent protein kinase C; AMPPNP, 5′-adenylylimidodiphosphate; P_o, open probability; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PTPase, protein tyrosine phosphatase; CFTR, cystic fibrosis transmembrane conductance regulator; τ, time constants.

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rel perfusion system (9); in a few instances, reagents were bath-applied. Experiments were performed at room temperature (≈22°C).

Steady-state recordings were performed by using a holding potential of −60 mV. Open probabilities (P_o) were determined by using a minimum of 3 min of channel recording. Although single channel patches were preferred, the P_o for patches containing 2 or 3 channels was determined by adding the P_o at each current level and dividing by the number of current levels observed. The numbers given in the text refer to the number of patches, not channels. Kinetic analyses were performed on patches judged to contain only one channel on the basis of the single current level observed. Currents were sampled at 11 kHz and digitally filtered at 1.5 kHz (Gaussian; −3 dB). Durations were determined by using a half-amplitude threshold criterion and corrected for the filter response (ref. 10, Eq. 17). Time constants (τ) were obtained by using the binned maximum likelihood method (11) and a simplex search that was provided with the number of exponentials and an approximate starting value for each exponential as described (6). Means and standard errors are given where applicable.

PP1, isolated from rabbit skeletal muscle (12), was a gift from the laboratory of D. L. Brautigan, University of Virginia, Dept. of Microbiology. PKA inhibitor peptide$_{(6–22)}$, ATP (grade 2 disodium salt), GTP (type 3 disodium salt), 5′-adenylylimidodiphospate (AMPPNP), and most other nucleotides were obtained from Sigma. ATPγS, microcsytin-LR, and PKC inhibitor peptide$_{(19–36)}$ were obtained from Calbiochem. H7 was obtained from Alexis/LC Laboratories (San Diego, CA).

RESULTS AND DISCUSSION

To examine the regulation of cation channels, we used inside-out patches excised from the cell bodies of single cultured bag cell neurons in their resting unstimulated state. Cation channels were observed in ≈20% of these patches and, when observed, most often occurred in clusters. A nucleotide effect on channel activity was first suggested by differences in the level of activity when patches were excised into nucleotide free solutions or into intracellular solutions containing ATP and GTP at 5 and 0.1 mM concentrations, respectively. For cation channels that were in the continuously active mode after patch excision, the fraction of time channels spent in the open state, or the P_o, averaged 0.028 ± 0.007 (n = 24) in the absence of ATP and GTP. In contrast, the average P_o for continuously active cation channels in the presence of ATP and GTP was 0.75 ± 0.06 (n = 8). Cation channels in the bursting mode, identified by the presence of a population of exceptionally long closed times (6), were not observed in the absence of the two nucleotides.

To determine which nucleotide was responsible for the difference in P_o in the two solutions, the effect of application of either ATP or GTP to the intracellular face of cation channels was examined. No increase in P_o was observed after application of GTP (0.1 mM; n = 3; data not shown). In fact GTP appeared to produce a slight decrease in channel activity. In contrast, as shown in Fig. 1*A* (*Top*), addition of ATP (2 mM) in the presence of 1.2 mM Mg^{2+} increased the cation channel P_o from a control value of 0.163 to a final value of 0.616. ATP, at concentrations ranging from 0.25 to 5 mM, increased channel activity in all but two of 39 patches examined. Application of ATP never resulted in transitions to a bursting mode. The increases in activity observed in response to ATP were initiated within the first minute after application, although in some cases a steady plateau was not reached for ≈5 min (e.g., Fig. 4*B*). In the continued presence of ATP, the increases in activity persisted without rundown throughout the duration of the recordings (typically 15–30 min).

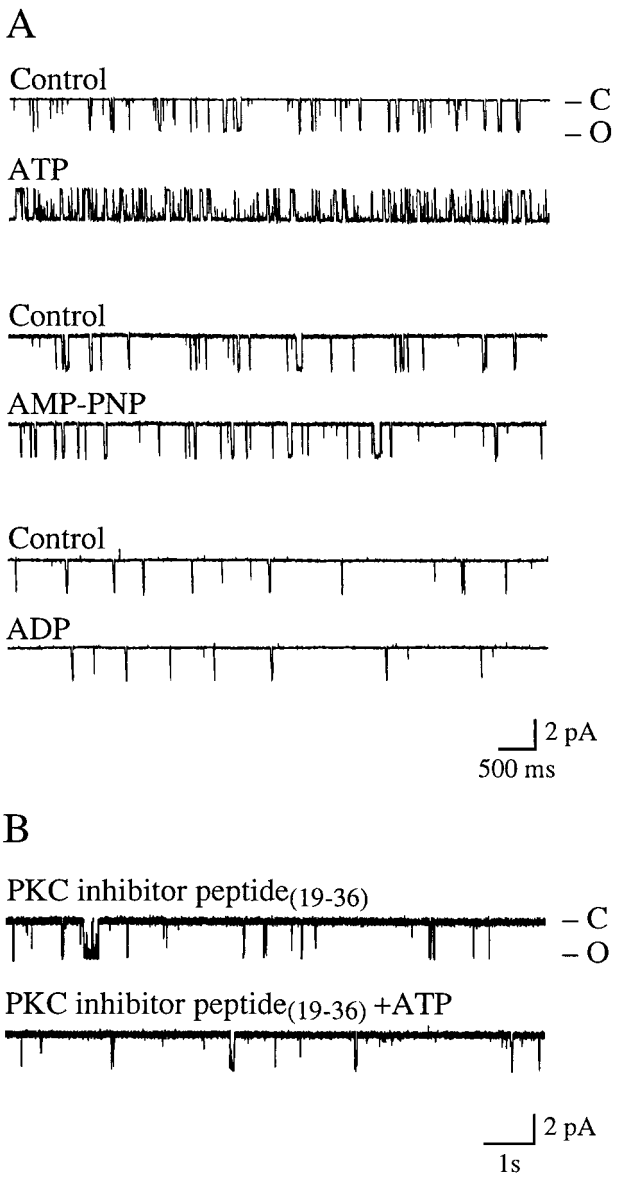

FIG. 1. Steady-state recordings of continuously active cation channels in excised inside-out patches from bag cell neurons. Holding potential = −60 mV. Channel openings are shown as downward deflections. (*A Top*) Single channel recordings immediately before and after application of 0.5 mM ATP. (*A Middle*) Single channel recordings showing the lack of an increase in activity after application of 1 mM AMPPNP. (*A Bottom*) Single channel recordings showing the lack of an increase in activity after application of 1 mM ADP. (*B*) Steady-state recordings showing block of the effect of ATP by 20 μM of the PKC inhibitor peptide$_{(19–36)}$.

Fig. 2 summarizes the data obtained for 24 patches containing from 1 to 3 cation channels, before and during application of ATP. The dashed lines represent the value expected if no change in P_o occurred. Both the starting P_o values and the magnitude of the effect of ATP varied considerably from patch to patch. Starting P_o values ranged from <0.001 to 0.163 and averaged 0.028 ± 0.008. The increase in P_o observed in response to ATP ranged from 1.7 to 41.9-fold and averaged 10.7 ± 2.2-fold. The magnitude of the ATP effect did not appear to correlate strongly with the concentration of ATP. In addition, when the P_o in control solution was <0.02, the average P_o in ATP did not exceed 0.2; however, even in these patches a substantial increase in activity was observed as evidenced by the fold increase in P_o (Fig. 2 *Bottom*). Although the source of the variability was not investigated further, some

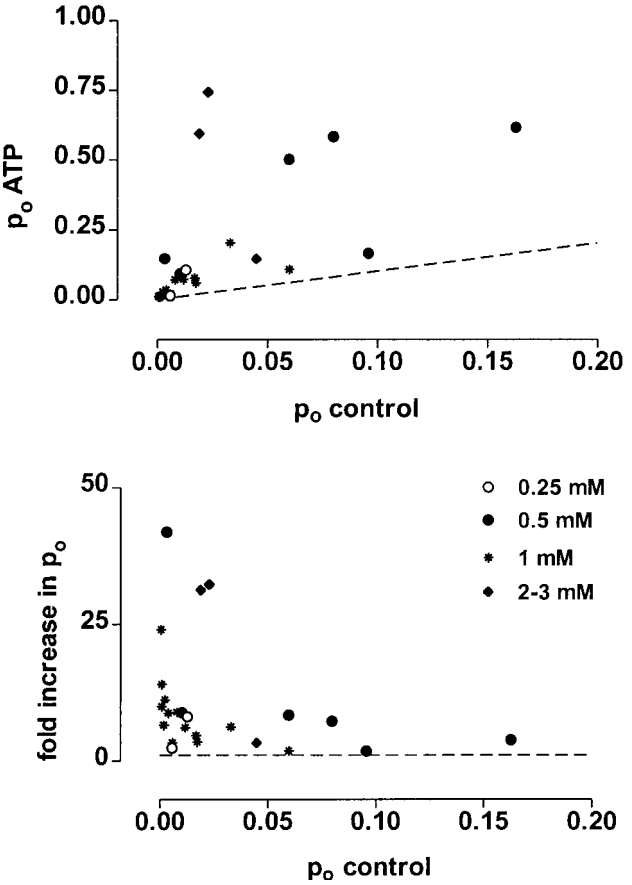

FIG. 2. Magnitude of the increase in P_o observed after application of various concentrations of ATP. Data obtained for 24 patches containing from 1 to 3 cation channels. In both plots, the dashed line represents the value expected if no increase in P_o were to occur. (*Top*) Comparison of the final average P_o as a function of the average starting P_o for each patch. Note that, in five of these patches, the final P_o reaches the same level initially observed when patches were excised into intracellular solution containing 5 mM ATP and 0.1 mM GTP. (*Bottom*) Comparison of the fold increase in P_o ($P_{o\ ATP}/P_{o\ control}$) as a function of the starting P_o for each patch. Although the final P_o in ATP observed for patches with a low initial P_o of ≈0.02 or less appears minimal, examination of the fold increase in P_o reveals a substantial increase in P_o after application of ATP.

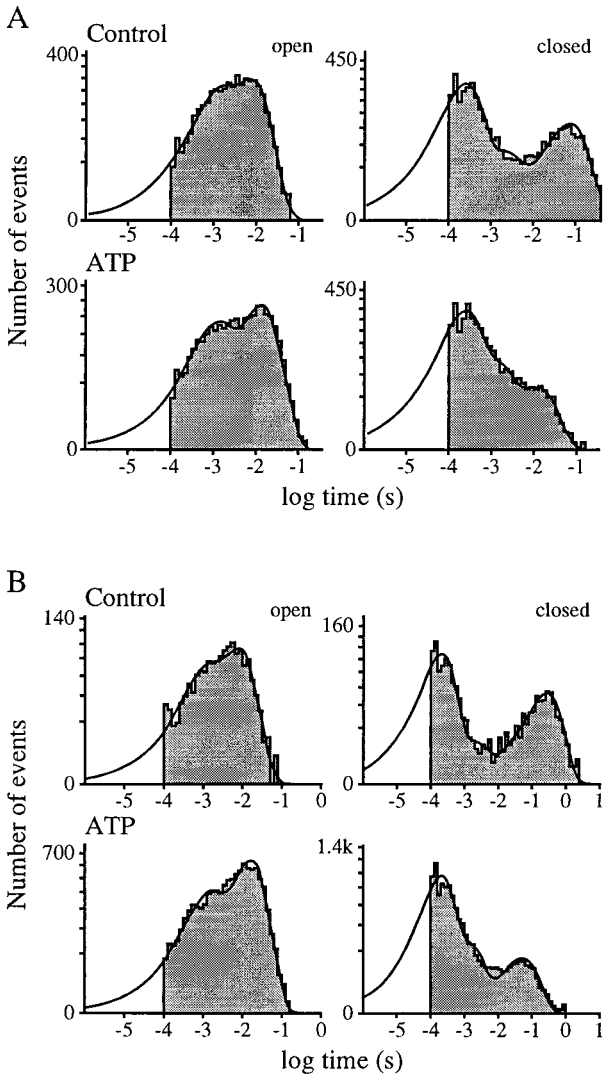

FIG. 3. Open and closed time histograms obtained for two cation channels before and after application of 1 mM ATP. The exponential fits used to determine the time constants are indicated by the superimposed curves (see Table 1 legend and *Materials and Methods*). (*A*) Histograms for the channel shown in Fig. 1 (*Top* traces). The average control P_o was 0.16, and the average P_o in ATP was 0.62. (*B*) Histograms for a channel that exhibited a smaller increase in P_o in response to ATP than that observed for the channel in *A*. For this channel, the average control P_o was 0.06 and the average P_o in ATP was 0.11. Note that ATP affected the same parameters for both channels.

differences in the starting P_o and the effect of ATP also may be due to differences in the modification of cation channels (or enzymes, see below) at the time of patch excision. For example, phosphorylation of the cation channel or a closely associated protein by PKA has been shown to decrease channel activity (6).

We next examined single channel open and closed times to identify the changes in kinetics that underlie the increase in P_o. Fig. 3*A* shows the open and closed time distributions, plotted on logarithmic scales, for the single cation channel of Fig. 1 (*Top*) before and after application of 1 mM ATP. As reported previously (6), open time distributions were best fit by a sum of two exponentials, τ_{o1} and τ_{o2}, and the majority of open times were described by the second, slower time constant. Closed times were well fit by a sum of three exponentials, τ_{c1}–τ_{c3}. The average values for these kinetic features are presented in Table 1 and represent the averages for five patches containing only a single cation channel. Because the number of exponentials is assumed to reflect the number of states a channel may enter, these results suggest that cation channels are complexly gated and can assume a minimum of two open and three closed states. Further examination of the event distributions reveals that ATP produced dramatic changes in the open and closed times (Fig. 3 and Table 1). The most prominent change was a reduction in the duration and percentage of channel closures described by τ_{c3}, the slowest time constant. After application of ATP, channel closures were shorter and the closures described by τ_{c3} occurred less frequently. In addition, channel openings described by both τ_{o1} and τ_{o2} were longer in the presence of ATP, although the relative percentage of openings described by τ_{o1} and τ_{o2} did not appear to change. These changes in kinetic parameters were observed in response to ATP in all channels examined, regardless of the starting P_o level or the magnitude of the effect (see Fig. 3*B* and Table 1).

To characterize the mechanism underlying the effect of ATP on cation channel activity, we investigated the nucleotide specificity of the effect. The average P_o obtained for patches containing from 1 to 3 channels before and during application of each nucleotide is given in Table 2. Examples of responses to AMPPNP and ADP are shown in the middle and bottom

Table 1. Summary of the kinetic features of cation channels observed in single channel patches before and after application of ATP

Condition	*n*	P_o	τ_{o1} (%)	τ_{o2} (%)	τ_{c1} (%)*	τ_{c2} (%)†	τ_{c3} (%)
Control	5	0.07 ± 0.02	1.02 ± 0.20 (43)	9.1 ± 1.5 (57)	0.46 ± 0.13 (42)	3.8 ± 1.2 (15)	384 ± 130 (44)
ATP	5	0.40 ± 0.11	1.37 ± 0.37 (43)	22.7 ± 8.5 (57)	0.58 ± 0.22 (51)	3.3 ± 1.5 (19)	69 ± 23 (25)

All τ values are expressed in milliseconds. Means are presented ± SEM. The percentage of events described by each exponential is given in parentheses. The single channel patches used for kinetic analysis were chosen on the basis of noise levels and the duration of recording; the average P_o for all single channel patches of the present study ($n = 14$) was 0.04 ± 0.01 (control) and 0.25 ± 0.08 (in ATP). The concentration of ATP used in the five patches was, in mM, 0.5, 0.5, 1, 2, and 3.

*The briefest closures are poorly resolved at the employed filter frequency leading to an overestimation of τ_{c1}, and potentially obscuring any differences between the two conditions in gating events on the microsecond timescale.

†A somewhat improved fit was obtained if the closed durations described by τ_{c2} (corresponding to the trough between the two major peaks) were fit with two exponentials instead of one. Variability in the τ_{c2} column may be due to the conservative fit chosen.

traces of Fig. 1*A*, respectively. No appreciable change in activity was noted in response to either ADP (1–2 mM, $n = 8$) or AMP (1 mM, $n = 4$). Similarly, we observed no increase in channel activity in the presence of up to 3 mM of AMPPNP ($n = 8$), a nonhydrolyzable ATP analog (13). In contrast, the activity of cation channels did increase in the presence of ATPγS (1–3 mM), a poorly hydrolyzable ATP analog that can substitute for ATP in many kinase reactions ($n = 8$) (14). As might be expected given the slower rates at which ATPγS is used in phosphotransferase reactions (14), in three of the eight patches examined, the initiation of the response to ATPγS occurred more slowly than the response to ATP, requiring between 2 and 5 min, and a final $P_o > 0.50$ was observed in only one case.

Three possible mechanisms have been described for the modulation of ion channels by ATP. First, ATP regulates ATP-sensitive channels in heart and other tissues by a mechanism involving binding to an allosteric site (15). Secondly, in the case of the cystic fibrosis transmembrane conductance regulator (CFTR), ATPase-like activity appears to mediate the effect of ATP. Although the nucleotide specificity is broad, ATPγS cannot substitute for ATP and the effect appears to require nucleotide hydrolysis (16). Finally, ATP has been shown to modulate Ca^{2+}-activated potassium channels by a phosphorylation-dependent mechanism involving a kinase closely associated with the channel proteins (17, 18). Our results with ATP analogs suggest that, as observed for CFTR and Ca^{2+}-activated potassium channels, ATP hydrolysis is required for the modulation of bag cell neuron cation channels. In contrast to CFTR however, there appears to be a high specificity for ATP similar to that observed for a number of kinases (e.g., refs. 19 and 20) and ATPγS was able to substitute for ATP, albeit with a possible decreased efficiency. Together, these observations suggest that the effect of ATP is mediated by a kinase endogenous to excised patches.

To test the hypothesis that phosphorylation underlies the effect of ATP on cation channels, we examined the channel response to ATP in the presence of several kinase inhibitors. When cells were preincubated (40 min to 2 hr) in a bath solution containing the broad spectrum kinase inhibitor H7 (100 μM), ATP failed to increase the cation channel P_o in each of the four patches examined (Table 2). Because, in bag cell neurons, H7 has been shown to block responses mediated by PKC, but not responses mediated by PKA (21), we next examined the effect of the highly specific, synthetic PKC inhibitor $peptide_{(19–36)}$. This peptide corresponds in sequence to those amino acids which comprise the pseudosubstrate autoinhibitory domain of PKC (22). ATP (1 mM) failed to increase the P_o when applied in the presence of the PKC inhibitor $peptide_{(19–36)}$ in five of eight patches tested (5 and 20 μM; Table 2). Moreover, the overall mean P_o in the presence of ATP was not noticeably different than the P_o for these patches in control conditions ($n = 8$). In contrast, a normal response to ATP was observed in the presence of 1 μM of the PKA inhibitor $peptide_{(6–22)}$. These results indicate that ATP serves as a phosphate donor in a phosphorylation reaction mediated by a kinase endogenous to excised patches. Moreover, the kinase is PKC-like based on the inhibition produced by the PKC inhibitor $peptide_{(19–36)}$.

Table 2. Effect of necleotides on cation channel P_o and block of the effect

Condition		*n* (N)	P_o control	P_o nucleotide	P_o wash
Nucleotides	mM				
ATP	0.25-3	24 (39)	0.028 ± 0.007	0.184 ± 0.047	
ADP	1	5 (8)	0.003 ± 0.001	0.005 ± 0.002	
AMP	1	4 (4)	0.006 ± 0.005	0.006 ± 0.002	
AMP-PNP	1	3 (8)	0.042 ± 0.017	0.035 ± 0.024	
ATPγS	1	4 (8)	0.005 ± 0.003	0.020 ± 0.010	
	3	1 (8)	0.033	0.511	
Inhibitors	μM			ATP	
H7	100	4	0.040 ± 0.010	0.017 ± 0.007	
PKC (19-36)	5	3	0.023 ± 0.005	0.018 ± 0.007	
PKC (19-36)	20	5	0.032 ± 0.015	0.026 ± 0.008	
PKA (6-22)	1	5	0.009 ± 0.004	0.075 ± 0.021	
Control	-	6 (9)	0.053 ± 0.029	0.168 ± 0.086	0.038 ± 0.020
Microcystin	0.010	3 (4)	0.001 ± 0.000	0.012 ± 0.001	0.004 ± 0.001
Microcystin	0.200	4	0.007 ± 0.002	0.019 ± 0.005	0.016 ± 0.004

The values given are the average P_o values (± S.E.M) obtained before and after application of each nucleotide at the concentrations indicated. Inhibitors were present for all conditions (control, ATP, and wash). The concentration of ATP used with kinase inhibitors was 1 mM; the concentration of ATP used with phosphatase inhibitors was 2 mM. *n* is the number of patches contributing to each determination of P_o; the N given in parenthesis is the number of patches for which qualitatively similar results were obtained regardless of the number of channels present and at concentrations ranging from 1 to 3 mM, except in the case of ATP in which the concentration ranged from 0.25 to 5 mM. P_o determinations were limited to patches containing from 1 to 3 channels (see *Materials and Methods*).

To further verify the involvement of ATP in a phosphorylation reaction, we next examined the reversibility of the effect of ATP. Surprisingly, when patches treated with ATP were returned to a control intracellular solution, a rapid and complete reversal was observed for all patches examined ($n = 9$; Fig. 4*A Left* and Table 2 *Bottom*). Given that no phosphatase had been added, we hypothesized that a phosphatase, specific for serine and threonine residues and also endogenous to excised patches, might be responsible for the reversal. Indeed, when the experiment was repeated in the presence of microcystin (200 nM), a potent inhibitor of PP1 and protein phosphatase 2A (PP2A) (23), the P_o failed to return to baseline levels upon removal of ATP ($n = 4$; Fig. 4*A Right* and Table 2). Lowering the concentration of microcystin from 200 to 10 nM resulted in a 30% inhibition of the return to baseline levels ($n = 4$; Table 2). The reported specificity of microcystin for PP1 and PP2A (23) and previous observations indicating that PP2A, applied in the presence of ATP, increases, rather than decreases, the P_o of cation channels (6), suggest that the endogenous phosphatase is PP1-like.

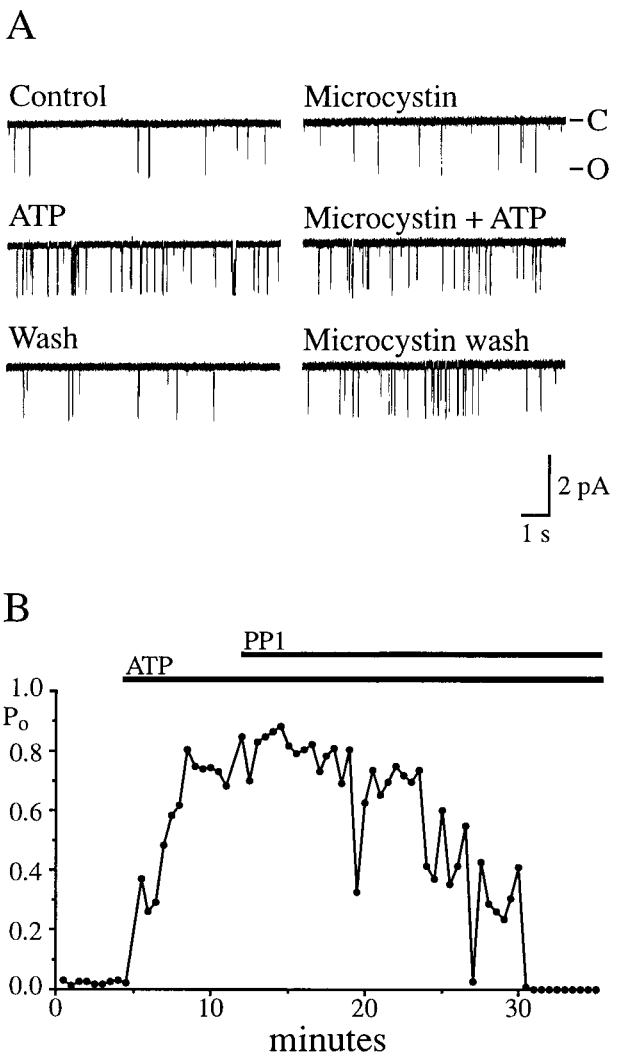

FIG. 4. Reversal of the effect of ATP. (*A*) Steady-state recordings of cation channels showing reversal of the effect of ATP after return of the patch to a zero-ATP, control intracellular solution (*Left*), and block of the reversal in the presence of 200 nM microcystin-LR (*Right*). Holding potential, −60 mV. (*B*) Time course of the change in P_o observed during treatment with ATP (2 mM) and, subsequently, PP1 (22 nM). Each data point represents the average P_o obtained for one 30-s interval. Application of ATP and PP1 is indicated by the bars at the top of the figure. The effect of ATP was noticeable almost immediately, although ≈4 min were required for the P_o to stabilize at the new higher value. In response to PP1, which was applied in the continued presence of ATP, the P_o oscillated for several minutes before a complete reversal.

Exogenously applied PP1, purified from rabbit, also reversed the effect of ATP. PP1 was applied in the continued presence of ATP in an attempt to shift the balance between existing kinase and phosphatase activities. Fig. 4*B*, which plots the average P_o obtained for successive 30-s intervals, shows the results of an experiment examining the effects of PP1. Upon patch excision, the cation channel displayed an initial P_o ranging between 0.01 and 0.03. In response to ATP (2 mM), the P_o increased, reaching a final plateau during which the average P_o was 0.742. Application of PP1 (22 nM) in the continued presence of ATP caused the P_o to return to baseline levels, albeit over a protracted time course. Although complete reversal required 20 min, dramatic oscillations in P_o were observed ≈7 min after the addition of PP1. The oscillations, as well as the protracted time course of the reversal, are consistent with an ongoing competition between opposing kinase and phosphatase activities. PP1 also reversed the effect of ATP in the two other patches tested. No change in the average P_o was observed when PP1 was applied to patches immediately after patch excision and before exposure to ATP (P_o in PP1 = 0.031 ± 0.011; $n = 7$) suggesting that phosphorylation by the endogenous kinase is a necessary prerequisite to the effect of PP1. The simplest interpretation of these data are that purified PP1, like the endogenous phosphatase, dephosphorylates the residues phosphorylated by the endogenous kinase. Other interpretations that we cannot rule out, however, are that PP1 may dephosphorylate the endogenous, PKC-like kinase to down-regulate its activity, or that PP1 may dephosphorylate and increase the activity of the endogenous phosphatase.

In summary, our results indicate that ATP serves as a phosphate donor in a phosphorylation reaction mediated by a kinase endogenous to excised patches. This conclusion is based on evidence indicating a narrow nucleotide specificity, a requirement for hydrolyzable nucleotides, block of the effect by kinase inhibitors, and the ability of phosphatases to reverse the effect. Moreover, the kinase is PKC-like, as indicated by the inhibition produced by H7 and the PKC inhibitor peptide$_{(19–36)}$. Our data also indicate that a phosphatase present in excised patches serves to reverse the effect of ATP. The results obtained by using microcystin, combined with earlier results obtained by using PP2A (6), suggest that the endogenous phosphatase is PP1-like. In addition, purified PP1 also reversed the effect of ATP.

Perhaps the most striking observation is that not one, but three, enzymes appear consistently localized with cation channels in excised patches. Dendrotoxin-binding potassium channels purified from rat brain (24), type 2 Ca^{2+}-activated potassium channels reconstituted in lipid bilayers (17, 25), and Ca^{2+}-activated potassium channels present in excised patches of posterior pituitary nerve terminals (18, 26) also are modulated by consistently colocalized kinase, and in some cases, phosphatase activities. In the case of the cation channel, previous work has demonstrated that a PTPase activity colocalizes with these channels in patches excised from bag cell neurons (6). In the present study, a response to ATP was observed in all but two of the patches containing cation channels and reversed without the addition of phosphatase in every instance examined, demonstrating the presence of two additional enzymes. Close association between the channel and these enzymes could be the result of a broad distribution of enzymes throughout the bag cell neuron membrane or cytoskeletal network. An exceptionally high density of enzymes would however be required to account for their consistent presence in cation channel-containing patches. Alternatively, close association between the channel and these enzymes could be the result of protein-protein interactions. These interactions could be direct, or indirect and mediated by

a "scaffolding" protein. Finally, the activity of at least one of these enzymes could be a property intrinsic to the channel protein. The coexistence of enzyme and ion channel in the same protein also has been suggested for some Ca^{2+}-activated potassium channels (27, 28), adenylyl cyclase (29, 30), CFTR (16), and for *Shaker* potassium channels whose beta subunits belong to the aldo–keto reductase superfamily (31, 32). Given that the cation channel appears to colocalize with three enzymes, all three of these mechanisms may be involved.

The PKA-regulated PTPase previously shown to be endogenous to isolated patches removes exceptionally long-lived fourth and fifth closed states that are responsible for the bursting gating mode of the channel (6). The primary action of the PKC-like kinase of the present study is to reduce the favorability of the third-longest closed state. Both types of regulation can dramatically increase the activity of cation channels. Moreover, both types of regulation appear to occur when crammed patches are exposed to peptides that trigger the afterdischarge of bag cell neurons (7) and the activity of both PKA and PKC appears to increase during the afterdischarge (33, 34). These observations suggest that the cation channel may serve to integrate input from several signaling pathways. The enzyme complex associated with cation channels may serve as part of a molecular switch that contributes to activation of the cation channel and the onset of the bag cell neuron afterdischarge.

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