Protein Kinase C Regulates a Vesicular Class of Calcium Channels in the Bag Cell Neurons of *Aplysia*

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White, Benjamin H., Teresa A. Nick, Thomas J. Carew, and Leonard K. Kaczmarek. Protein kinase C regulates a vesicular class of calcium channels in the bag cell neurons of *Aplysia*. *J. Neurophysiol.* 80: 2514–2520, 1998. Protein kinase C (PKC) acutely increases calcium currents in *Aplysia* bag cell neurons by recruiting calcium channels different from those constitutively active in the plasma membrane. To study the mechanism of PKC regulation we previously identified two calcium channel α₁-subunits expressed in bag cell neurons. One of these, BC-α₁A, is localized to vesicles concentrated primarily in somata and growth cones. We used antibodies to BC-α₁A to analyze its expression in the bag cell neurons of juvenile *Aplysia* at a developmental stage at which PKC-sensitive calcium currents have previously been shown to be low. We find that vesicular BC-α₁A staining is generally reduced in juvenile bag cell neurons but that its expression level can vary among juvenile animals. In 17 bag cell clusters examined, the percentage of neurons that displayed punctate α₁BC- staining ranged from 0 to 85%. Sampling of calcium currents from cells of the same clusters by whole cell patch-clamp techniques revealed that the PKC-sensitive calcium current density is significantly correlated with the degree of vesicular staining. In contrast, no correlation of basal calcium current levels with aBC-α₁A staining was found. These results strongly suggest that BC-α₁A, a member of the ABE-subfamily of calcium channels, carries the PKC-sensitive calcium current in bag cell neurons. They are consistent with a model in which PKC recruits channels from the vesicular pool to the plasma membrane.

INTRODUCTION

The essential role of calcium in neurosecretion and gene regulation and in the regulation of diverse enzymes makes calcium channels a prime target for the modulation of neuronal function. In recent years, G-proteins have emerged as prominent negative regulators of calcium channel function (Dolphin 1996; Hille 1994), and protein kinases have been frequently, although not exclusively, identified as positive regulators of calcium channels (Dolphin 1996; Tsien et al. 1988). Adenosine 3',5'-cyclic monophosphate-dependent protein kinase (Armstrong and Eckert 1987; Artalejo et al. 1990), guanosine 3',5'-cyclic monophosphate-dependent protein kinase (Paupardin-Tritsch et al. 1986), and protein kinase C (PKC) (Swartz 1993; Yang and Tsien 1993) have all been shown to potentiate calcium currents in various neurons or neuroendocrine cells. A particularly interesting example of calcium current enhancement by PKC is found in the bag cell neurons of *Aplysia californica*, where PKC has been shown to enhance current not by modulating the activity of an already active calcium channel but by recruiting a previously inactive type of calcium channel (DeRiemer et al. 1985; Strong et al. 1987).

Patch-clamp analysis of bag cell neurons identified two types of calcium current, a basal current carried by a 12-pS channel constitutively present in the plasma membrane and a PKC-sensitive current carried by a 24-pS channel seen only after treatment of bag cell neurons with activators of PKC (Strong et al. 1987). A unique feature of calcium channel modulation in bag cell neurons is its sensitivity to whole cell recording conditions. Unlike kinase-mediated modulation of calcium channels observed in other cell types, recruitment is disrupted by the formation of whole cell patches before PKC activation (DeRiemer et al. 1985; Strong et al. 1987). The recruited channels themselves, however, are not sensitive to patch formation. Once they have been recruited, their activity can readily be recorded by either whole cell or cell-attached patch-clamp techniques. These observations suggest that the mechanism of recruitment is indirect. Strong et al. (1987) proposed that the PKC-sensitive channels may reside in a subcellular pool and be inserted into the membrane after PKC stimulation. This conclusion is consistent with calcium imaging studies, which show that PKC-sensitive calcium influx into bag cell neuron growth cones occurs at sites of apparent membrane addition (Knox et al. 1992). The identity of the channel regulated by PKC and its localization in bag cell neurons, however, remained undetermined.

We recently identified two molecular species of calcium channel expressed in bag cell neurons and showed that one of them, BC-α₁A,¹ is localized to vesicles in the somata and growth cones of adult bag cell neurons (White and Kaczmarek 1997). We now show that BC-α₁A is present in the cultured bag cell neurons of a subpopulation of juvenile *Aplysia* and that its presence in vesicles correlates with the presence of PKC-sensitive calcium current. Correlation of the vesicularly localized class of calcium channels with the channels regulated by PKC suggests that PKC may act to enhance calcium currents by recruiting channels to the plasma membrane from a subplasmalemmal pool.

¹BC-α₁A designates the bag cell neuron calcium channel BCCa-I originally described by White and Kaczmarek (1997). Similarly, BC-α₁D in this paper designates the previously described BCCa-II. The new designations indicate the class of mammalian α₁-subunit to which each *Aplysia* channel appears to be most closely related.
**Methods**

**Animals**

Juvenile *Aplysia californica* were distinguished by weight, which has previously been shown to be a good predictor of afterdischarge capacity (Nick et al. 1996). All juveniles used weighed between 4 and 11 g and were obtained from the Aplysia Resource Facility (RSMAS, University of Miami).

**Immunocytochemistry**

Whole mount staining was carried out as follows. Animals were anesthetized with an injection of isotonic MgCl₂ (to 50% of body weight), and the pleural, pedal, and abdominal ganglia were excised together with their connective nerves into 0.5% Dispase in artificial seawater [ASW: 460 mM NaCl, 10.4 mM KCl, 55 mM MgCl₂, 11 mM CaCl₂, 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 1 g/l glucose, 100,000 units/l penicillin, 100 mg/l streptomycin, pH 7.8] for 30 min. Preparations were then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 h at 4°C before 20 min treatment with 0.1% trypsin in PBS at 23°C. Samples were thoroughly washed and exchanged into 4% Triton X-100 in PBS for 1 h before blocking in blocking solution (4% goat serum and 0.5% Triton X-100 in PBS) for 12 h at 4°C. Each sample was incubated 2 days at 4°C with rat primary antibodies in blocking solution. Affinity-purified antibodies to the two bag cell neuron calcium channels, BC-α₁A, and BC-α₁D, were used at 5 µg/ml as described by White and Kaczmarek (1997). A rabbit immunoglobulin fraction to human glial fibrillary acidic protein from Sigma Chemicals (St. Louis) Bag cell neurons were dissociated as described previously and Kaczmarek (1997). A rabbit immunoglobulin fraction to human antibodies to the ELH was the kind gift of Dr. Arlene Chiu and was used at a M

**Electrophysiology on juvenile bag cell neurons**

Bag cell neurons were dissociated as described previously and cultured at 15°C for 1 day before current measurements. Calcium currents were measured by the whole cell patch-clamp technique (Hamill et al. 1981) by using electrodes with resistances <1.5 MΩ. A 486DX computer with a Digidata 1200 A/D converter (Axon Instruments, Foster City, CA) was used to deliver current pulses in current-clamp mode and voltage steps in voltage-clamp mode with pClamp 6 software (Axon). Whole cell electrical signals were amplified with an EPC-7b patch/whole cell clamp (List-electronic, Germany). A 50-GΩ resistor was used for measurement of currents. Calcium currents were filtered at 3 kHz with an eight-pole Bessel filter and sampled at 600 Hz. Voltage step duration was 300 ms, except when measuring capacitance, in which case it was 120 ms. Series resistance and capacitance transients were compensated before current measurement, and currents were leak-subtracted with a P/8 protocol.

The pipette solution used was designed to block outward currents. It contained (in mM) 10 reduced glutathione, 5 MgCl₂, 20 ethylene glycol-bis(β-aminoethylether)-N,N,N',N’-tetraacetic acid, 4.14 CaCl₂, 470 CsOH, 100 TEA-Oh, 570 K-asaartapate, 5 ATP, 0.1 GTP, 1 mg/ml glucose, 10 HEPES, pH 7.3. The external solution was a modified ASW with choline chloride substituted for NaCl to remove sodium current. Pipettes were Sylgard coated to reduce capacitative noise. Iₘ was revealed by holding at −40 mV and stepping to +90 mV in 13 10-mV steps with a 5-s interpulse interval. PKC-sensitive calcium current was estimated as described subsequently from the whole cell currents elicited by the same protocol after addition of 20 nM phorbol 12-myristate 13-acetate (TPA).

Values for raw currents were obtained with the Clampfit subprogram of pClamp 6. Current values near the peak (20–40 ms after pulse initiation) were averaged from four traces for each voltage step to lower the signal to noise ratio. Currents were normalized for cell size by dividing by the cell capacitance to give current densities. Mean current densities are presented with error bars indicating the SE of the mean.

The basal and PKC-sensitive calcium currents for the population
of cells on each coverslip were estimated as follows. Calcium current densities were measured in two cells before the addition of TPA and averaged to obtain $I_{Ca}$. Calcium current densities were then measured in two different cells (see RESULTS) 30 min after addition of 20 nM TPA. Subtraction of the current-voltage ($I-V$) curve obtained from the first set of measurements from the $I-V$ curve obtained from the second set then yielded an estimate of the calcium current stimulated by TPA ($I_{SR}$). The $I-V$ relationships for both $I_{Ca}$ and $I_{PKC}$ peaked near +30 nV and the current density at this voltage was used for comparisons of current and calcium channel staining.

RESULTS

Whole-mount staining of bag cell clusters from juvenile Aplysia with antibodies directed against two calcium channels previously identified in adult bag cell neurons revealed strong differences in the levels of labeling. Affinity-purified antibodies against BC-α1A, a calcium channel α1-subunit of the ABE subfamily, produced no significant staining of bag cell clusters above background (Fig. 1, A and C). In contrast, antibodies to BC-α1D, a calcium channel α1-subunit of the SCD subfamily, prominently stained bag cell clusters from juvenile Aplysia (Fig. 1B). These results contrasted with those previously obtained in adult animals (200–300 g), which showed specific staining of the bag cell neurons with both antibodies (White and Kaczmarek 1997). Because previous studies have shown that juvenile bag cell neurons in culture have significantly more basal calcium current than PKC-sensitive calcium current (Nick et al. 1996), the staining results suggested that BC-α1A may represent the PKC-sensitive calcium channel.

To more closely examine expression levels of BC-α1A, we stained neurons from juvenile bag cell clusters that were triturated and cultured in ASW. We found that αBC-α1A staining of the cultured juvenile neurons was low and exhibited considerable variability (Fig. 2, A and B). Many cells stained only lightly above background levels (Fig. 2, C and D), and robust staining, when observed, tended to be restricted to the bag cell neuron somata, sometimes localized to one side, as seen in Fig. 2A (see arrow). Closer examination of this staining often showed it to consist of punctate staining of the type seen in adults with this antibody, although such staining was more easily resolved in cells where αBC-α1A staining was less intense and more broadly distributed (Fig. 3). We could not determine if all the αBC-α1A staining was associated with vesicles, but its specificity was evident from its inhibition by preincubation with the fusion protein against which αBC-α1A was made (Fig. 2C). Preincubation with this fusion protein reduced staining to levels seen with secondary antibody alone (Fig. 2D), whereas preincubation with the fusion protein against which αBC-α1D was made caused no inhibition of staining (Fig. 2B). Punctate staining with the αBC-α1A antibody was not overtly altered by treatment with phorbol esters, as previously reported for adult bag cell neurons (White and Kaczmarek 1997).

Expression levels of BC-α1A varied not only among bag cell neurons in the same cluster but also exhibited substantial differences from cluster to cluster. Of the 11 cultured bag cell neuron clusters initially examined three showed virtually no punctate staining of bag cell neurons with the αBC-α1A antibody, whereas three others showed some degree of punctate staining in more than three-quarters of the bag cell neurons. Frequency of punctate staining was intermediate in the other five preparations examined. Cluster-to-cluster differences and the generally low level of staining are likely to account for our failure to detect BC-α1A in the three whole mount preparations we examined. We presume that such differences derive from the variable onset of BC-α1A expression in the bag cell neurons, but we did not systematically investigate the developmental time course of expression.

To determine which of the two electrophysiologically characterized channel types might be the functional correlate of the BC-α1A channel we examined levels of punctate staining in populations of bag cell neurons from which basal and PKC-
sensitive calcium currents were recorded. Due to their small size, measurement of the voltage-sensitive calcium currents in juvenile bag cell neurons required use of the whole cell patch-clamp technique. Because of this, basal and PKC-sensitive currents could not be measured in the same cell since, as previously described in adults (Strong et al. 1987), addition of TPA had no acute effect when currents were recorded in the whole cell mode (data not shown). It was also not possible to examine punctate staining in the same cells from which currents were sampled because the cells did not survive patch pipette removal. Therefore, for each cluster, the average basal calcium current in the cultured bag cell neurons was estimated from whole cell currents measured in two cells before TPA addition. The average PKC-sensitive calcium current was estimated from the currents measured in two more cells after TPA addition. As a measure of the level of punctate staining, we determined the percentage of punctately stained cells in a cluster (see METHODS). Calcium current densities and frequencies of punctate staining were measured for 17 independent clusters. Scoring of cells for punctate staining was carried out blind (i.e., without knowledge of the previous current measurements).

As expected from our earlier results, the 17 clusters showed considerable differences in the degree of $\alpha_{\text{BC}-\alpha_{1A}}$ staining, with the percentage of punctately stained neurons ranging from 0 to 85%. Interestingly, the average calcium current densities for the 17 clusters also revealed cluster-to-cluster differences, with the PKC-sensitive current densities varying over twice as large a range as the basal currents. To determine whether the differences in PKC-sensitive calcium current density were likely to correlate with differences in punctate staining we sorted the 17 clusters according to their PKC-sensitive calcium current densities and divided the clusters into two groups along the median ($2.7 \, \text{nA/nF}$). These two groups represented clusters with low and high average PKC-sensitive current densities, respectively (Fig. 4B, top panel), and for each group we averaged the frequency of punctate staining.

We found that the group of clusters with the high PKC-sensitive calcium current density also had a strikingly higher proportion of neurons with vesicular $\alpha_{\text{BC}-\alpha_{1A}}$ staining (Fig. 4B, bottom panel). Indeed, the approximately sixfold difference in peak current densities observed for the two groups was accompanied by a nearly fourfold difference in the frequency of punctate staining. This difference was found to be very significant by the two-tailed $t$-test ($P < 0.01$). To determine whether basal calcium currents might vary together with the PKC-sensitive calcium currents, we also calculated the average basal calcium current densities for the two groups of clusters (data not shown). In contrast with sixfold difference in peak current density exhibited by the PKC-sensitive currents (Fig. 4B, top panel), the peak basal current densities were virtually the same for the groups with low and high PKC-sensitive current densities ($-3.8 \pm 0.6 \, \text{nA/nF}$ and $-4.3 \pm 0.6 \, \text{nA/nF}$ at $+30 \, \text{mV}$, respectively). This suggested that the basal current densities were correlated with neither the PKC-sensitive currents nor with levels of $\alpha_{\text{BC}-\alpha_{1A}}$ staining.

We confirmed that there was no correlation between basal calcium current densities and levels of punctate staining by
repeating the sorting of the 17 clusters, this time according to the magnitude of their basal calcium current densities. The clusters were divided, as before, into two groups, which now represented clusters with low and high basal current densities. In addition to the average basal calcium current density of each group, the average PKC-sensitive calcium current density and the average level of punctate staining of each group were again determined. The basal calcium current densities for the groups above and below the median (data not shown) differed by a factor of two with peak values at +30 mV of −2.5 ± 0.3 nA/nF and −5.3 ± 0.3 nA/nF, respectively. There was, however, no significant difference in the average PKC-sensitive calcium current densities for the two groups (Fig. 4A, top panel) and, more importantly, no difference in the average percentage of punctately stained neurons (Fig. 4A, bottom panel).

To confirm the correlation results with the entire range of data, we plotted the frequency of punctate staining for each cluster as a function of its basal and PKC-sensitive calcium current densities and analyzed the resulting scatterplots by linear regression (Fig. 5). As expected, the regression analysis found no significant correlation between the basal calcium current densities of the clusters and their frequencies of punctate staining ($r = -0.11$). In contrast, the observed cluster-to-cluster differences in PKC-sensitive calcium current densities correlated significantly with their differences in punctate staining ($r = 0.55, P = 0.02$). It is instructive to note that some of the clusters with current density and staining values that correlated poorly may reflect necessary methodological biases more than an absence of correlation between αBC-α1A staining and PKC-sensitive currents. For example, the cluster with 4% punctate staining and −6.4 nA/nF PKC-sensitive current density (Fig. 5, right panel) exhibited robust αBC-α1A staining, but because the staining could not generally be resolved as punctate the cluster scored low for the percentage of neurons punctately stained.

**DISCUSSION**

It has been known for nearly a decade that PKC potentiates calcium currents in Aplysia bag cell neurons by recruiting a voltage-sensitive calcium channel that is not normally observed in the plasma membrane. The molecular identity of this channel and the mechanism of recruitment have, however, remained unknown. The evidence presented here suggests that BC-α1A, a member of the ABE subfamily of voltage-sensitive calcium channels whose localization in bag cell neurons is predominantly intracellular, is the carrier of the PKC-sensitive current. Expression of both BC-α1A and PKC-sensitive calcium currents is attenuated in the bag cell neurons of juvenile *Aplysia*, and the presence of vesicular BC-α1A channels in cultured juvenile bag cell neurons correlates significantly with the presence of PKC-sensitive currents. In addition, vesicular BC-α1A expression fails to correlate with basal calcium current densities in juvenile bag cell neurons.

Previous physiological characterization of bag cell neuron calcium currents indicates that the basal and PKC-sensitive calcium currents derive from only two types of voltage-sensitive calcium channel, a 12-pS channel constitutively present on the membrane surface and thought to be responsible for the basal current and a 24-pS channel recruited by
PKC (Strong et al. 1987). These observations constrain the interpretation of the correlation data presented here. In particular, our observation that BC-α1A expression fails to correlate with basal calcium current densities in juvenile bag cell neurons appears to preclude identification of BC-α1A with the 12-pS channel. Conversely, the positive correlation of αBC-α1A staining with PKC-sensitive currents strongly supports the conclusion that BC-α1A is the 24-pS calcium channel of bag cell neurons recruited by PKC.

It was previously suggested that the PKC-sensitive channels of bag cell neurons are translocated to the plasma membrane from an intracellular pool (Strong et al. 1987). Our finding that a vesicular class of calcium channels correlates with the presence of PKC-sensitive calcium currents in bag cell neurons is consistent with this hypothesis. Our inability to directly detect redistribution of BC-α1A in response to phorbol ester treatment of juvenile neurons is not surprising given that only 1,000 24-pS channels may be sufficient to produce a 3-nA/nF change in calcium current density, which is the mean change we observe on PKC stimulation. Such a small number of channels would be difficult to resolve by immunohistochemical techniques, particularly if the translocatable pool is large compared with the number of channels translocated. Although we cannot rule out other mechanisms of modulation, the vesicular localization of BC-α1A and its correlated presence with PKC-sensitive currents in bag cell neurons make translocation a plausible mechanism of channel modulation by PKC.

It also has been suggested that the PKC-sensitive channels serve to potentiate secretion of the peptide hormone ELH from bag cell neurons (Loechner et al. 1992). Secretion of ELH is associated with a prolonged period of electrical activity known as the afterdischarge, which is characterized by potentiation of calcium currents, reflected in enhancement of bag cell neuron action potentials. Inhibition of PKC during the afterdischarge has been shown to block both action potential enhancement (Conn et al. 1989) and reduce ELH release (Loechner et al. 1992), suggesting a plausible role for BC-α1A in these processes. This possibility is particularly interesting because BC-α1A is a member of the ABE subfamily of voltage-sensitive calcium channels. Two mammalian members of this subfamily (α1A and α1B) have been implicated in the release of classical neurotransmitters, but less is known about their possible role in peptide release (Dunlap et al. 1995).

It is also possible, however, that BC-α1A plays a different role in peptide release, independent of possible translocation in response to PKC stimulation. Recent evidence suggests that calcium entry through voltage-gated calcium channels may play a limited role in sustaining ELH release in mature bag cell neurons because release can outlast the period of the afterdischarge (Wayne and Wong 1994). Moreover, insulin, which stimulates calcium release from a novel intracellular store in bag cell neurons, can stimulate ELH secretion from mature bag cell clusters in the absence of an afterdischarge (Jonas et al. 1997). These results suggest that calcium release from intracellular stores plays an important role in sustaining ELH release, and it is possible that the intracellular calcium channel BC-α1A may reside on calcium-containing vesicles and mediate release of calcium from that pool. Further work is clearly necessary to determine the role of BC-α1A in bag cell neuron function, but the evidence presented here strongly suggests that the physiological correlate of this channel is the previously identified, 24-pS channel recruited by PKC.

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