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Phosphorylation of presynaptic and postsynaptic calcium channels by cAMP-dependent protein kinase in hippocampal neurons

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Phosphorylation by cAMP-dependent protein kinase (PKA) and other second messenger-activated protein kinases modulates the activity of a variety of effector proteins including ion channels. Anti-peptide antibodies specific for the α1 subunits of the class B, C or E calcium channels from rat brain specifically recognize a pair of polypeptides of 220 and 240 kDa, 200 and 220 kDa, and 240 and 250 kDa, respectively, in hippocampal slices *in vitro*. These calcium channels are localized predominantly on presynaptic and dendritic, somatic and dendritic, and somatic sites, respectively, in hippocampal neurons. Both size forms of α_{1B} and α_{1E} and the full-length form of α_{1C} are phosphorylated by PKA after solubilization and immunoprecipitation. Stimulation of PKA in intact hippocampal slices also induced phosphorylation of 25–50% of the PKA sites on class B N-type calcium channels, class C L-type calcium channels and class E calcium channels, as assessed by a back-phosphorylation method. Tetraethylammonium ion (TEA), which causes neuronal depolarization and promotes repetitive action potentials and neurotransmitter release by blocking potassium channels, also stimulated phosphorylation of class B, C and E α1 subunits, suggesting that these three classes of channels are phosphorylated by PKA in response to endogenous electrical activity in the hippocampus. Regulation of calcium influx through these calcium channels by PKA may influence calcium-dependent processes within hippocampal neurons, including neurotransmitter release, calcium-activated enzymes and gene expression.

Key words: calcium channels/cAMP-dependent protein kinase/long-term potentiation/synaptic transmission

Introduction

Voltage-gated calcium channels are involved in the regulation of a variety of neuronal functions such as membrane excitability, gene expression and neurotransmitter release (see e.g. Miller, 1987). Physiological and pharmacological properties define at least five types of neuronal calcium channels (Bean, 1989; Llinas *et al.*, 1989; Hess, 1990; Tsien *et al.*, 1991; Sather *et al.*, 1993; Castillo *et al.*, 1994; Wheeler *et al.*, 1994): T-type channels are low-threshold calcium channels which are readily opened by a small depolarization, whereas a stronger depolarization is required to activate the high-threshold L-, N-, P- and Q-type channels. L-type calcium currents are blocked specifically by dihydropyridines and other organic calcium channel blockers (Glossmann and Striessnig, 1990) and N-type currents by ω-CTx GVIA (Aosaki and Kasai, 1989; Plummer *et al.*, 1989). ω-Agatoxin-IVA blocks P-type currents and, with significantly lower affinity, Q-type currents which can be further distinguished from P-type currents by their higher sensitivity to ω-CTx MVIIC (Mintz *et al.*, 1992a,b; Sather *et al.*, 1993; Stea *et al.*, 1994).

L-type channels from skeletal muscle are composed of five different subunits: α1, α2, β, γ and δ (Takahashi *et al.*, 1987; Campbell *et al.*, 1988; Catterall, 1988) and neuronal calcium channels contain a similar complex of α1, α2, β and δ subunits (Takahashi and Catterall, 1987; Ahlijanian *et al.*, 1990, 1991; McEnery *et al.*, 1991; Sakamoto and Campbell, 1991; Witcher *et al.* 1993; Hell *et al.*, 1994a). The main component is the α1 subunit which forms the ion-conducting pore. Five different α1 subunits have been identified by cloning and sequencing of cDNA from mammalian brain, constituting five different classes (A–E) of neuronal calcium channel α1 subunits (Snutch *et al.*, 1990, 1991; Chin *et al.*, 1991; Hui *et al.*, 1991; Mori *et al.*, 1991; Starr *et al.*, 1991; Dubel *et al.*, 1992; Niidome *et al.*, 1992; Seino *et al.*, 1992; Williams *et al.*, 1992a,b; Fujita *et al.*, 1993; Soong *et al.*, 1993). Class C and D α1 subunits are closely related to α1 subunits of skeletal muscle L-type channels. They form two different L-type channels which account for most, if not all, L-type calcium currents in the mammalian brain (Hell *et al.*, 1993a). Class B α1 subunits form N-type channels (Dubel *et al.*, 1992; Williams *et al.*, 1992b). The class A and E α1 subunits form calcium channels which have not been definitively identified as one of the physiologically distinct types of calcium currents in neurons, but class A α1 subunits are thought to be responsible for Q-type and possibly P-type calcium currents.

The purified skeletal muscle α1 subunit is phosphorylated by cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and other protein kinases (Curtis and Catterall, 1985; Flockerzi *et al.*, 1986; Nastainczyk *et al.*, 1987; Takahashi *et al.*, 1987; Jahn *et al.*, 1988; O'Callahan and Hosey, 1988). The ion conductance activity of purified and reconstituted skeletal muscle calcium channels is increased by PKA (Flockerzi *et al.*, 1986; Hymel *et al.*, 1988; Nunoki *et al.*, 1989; Mundiña-Weilenmann *et al.*, 1991). PKA phosphorylates the two size forms of the skeletal muscle α1 subunit differentially *in vitro*, as well as in intact skeletal muscle cells (De Jongh *et al.*, 1989; Lai *et al.*, 1990; Rotman *et al.*, 1992). Similar to the skeletal muscle L-type channel (De Jongh *et al.*, 1989, 1991), the class B, C and D α1 subunits exist in two size

forms which differ in each case at their COOH-terminal ends (Westenbroek *et al.*, 1992; Hell *et al.*, 1993a,b, 1994a,b). Immunopurified class B, C and E α1 subunits are substrates for several second messenger-regulated protein kinases, including PKA, PKC, cGMP-dependent protein kinase and calcium- and calmodulin-dependent protein kinase II (CaMKII; Hell *et al.*, 1993b, 1994b; Yokoyama, Westenbroek, Hell, Snutch and Catterall, submitted). Phosphorylation by PKA greatly increases the ion conductance activity of the full-length form of the class C α1 subunit when this subunit is expressed alone in CHO cells (Sculptoreanu *et al.*, 1993a). The full-length isoform of the class C α1 subunit is specifically phosphorylated by PKA *in vitro*, suggesting that only the longer form is regulated by PKA *in vivo* (Hell *et al.*, 1993b).

In the experiments described in this report, we have investigated phosphorylation of the class B, C and E calcium channels in intact hippocampal neurons. We chose to investigate these three channels due to their distinct subcellular localization. Class B N-type channels play a crucial role in calcium-dependent neurotransmitter release and subsequent synaptic transmission (e.g. Takahashi and Momiyama, 1993; Turner *et al.*, 1993; Wheeler *et al.*, 1994). This channel type is localized in dendrites and presynaptic terminals of many neurons, including the large mossy fiber terminals which form synapses on the CA3 pyramidal neurons in the hippocampus (Westenbroek *et al.*, 1992). Class C L-type channels were found in patches on cell bodies and proximal dendrites in most areas of the brain but, in the dentate gyrus and CA3 regions of the hippocampal formation, most of the immunoreactivity was observed in dendritic fields extending along the dendrites of the CA3 neurons (Hell *et al.*, 1993a). Electron microscopic immunocytochemistry reveals that patches of class C L-type channels are concentrated in the cell surface membrane of postsynaptic cells and, in some cases, are located on the postsynaptic side of asymmetric excitatory synapses (Westenbroek and Catterall, in preparation). Class E calcium channels are nearly exclusively seen on cell bodies of hippocampal neurons with hardly any immunoreactivity visible in dendritic areas (Yokoyama, Westenbroek, Hell, Snutch and Catterall, submitted). Thus, our experiments examine the phosphorylation of calcium channels which are localized in three distinct subcellular compartments—dendrites, cell body and presynaptic terminals.

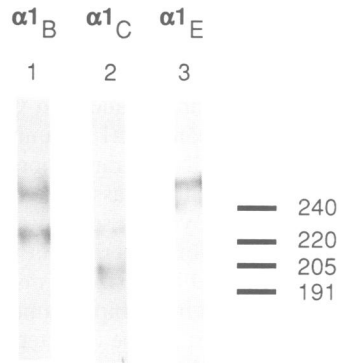

Fig. 1. Identification of hippocampal calcium channel α1 subunits by immunoblotting. Class B N-type channels were immunoisolated with anti-CNB1 (lane 1), class C L-type channels with anti-CNC1 (lane 2) and class E calcium channels with anti-CNE2 (lane 3) from different hippocampal slices. After SDS–PAGE and transfer onto nitrocellulose, two size forms of the class B, C and E α1 subunits were detected with anti-CNB1 (lane 1), anti-CNC1 (lane 2) and anti-CNE2 (lane 3) as probing antibody, respectively.

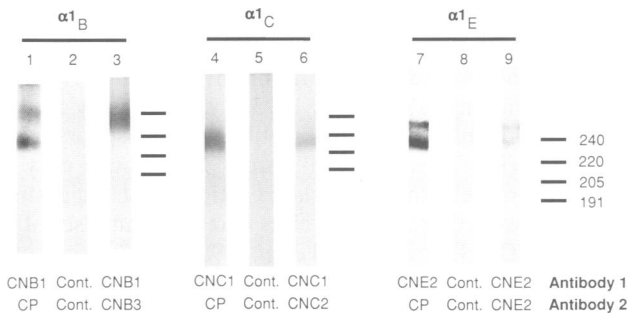

Fig. 2. Double immunoprecipitation of hippocampal calcium channel α1 subunits phosphorylated by PKA. Calcium channels in different samples of solubilized hippocampal slices were immunoprecipitated with anti-CNB1 (lanes 1 and 3), -CNC1 (lanes 4 and 6), -CNE2 (lanes 7 and 9) or control antibodies (lanes 2, 5 and 8). The immunocomplexes were incubated with PKA, washed and dissociated. The second immunoprecipitation was performed with anti-CP(1382–1400) (lanes 1, 4 and 7), -CNB3 (lane 3), -CNC2 (lane 6), -CNE2 (lane 9) or control antibodies (lanes 2, 5 and 8). PKA phosphorylated both size forms of the class B and E α1 subunit (lanes 1 and 7, respectively). Only the larger size form of the class C α1 subunit was phosphorylated by PKA (lane 4), even though both size forms are present after double immunoprecipitation with anti-CNC1 and -CP(1382–1400), as observed after phosphorylation with PKC (not shown). Note that the COOH-terminal antibody anti-CNB3 recognized only the longer size form of the class B α1 subunit (lane 3).

Results

Characterization of calcium channels from hippocampus

To identify and characterize the gene products of different classes of neuronal calcium channels in the hippocampus, site-directed polyclonal anti-peptide antibodies have been produced which are directed towards amino acid sequences specific to the class B, C or E α1 subunits. Anti-CNB1 was made against a sequence in the intracellular loop between domains II and III of the class B α1 subunit, and anti-CNB3 against a COOH-terminal sequence. Both antibodies specifically recognized N-type channels (Dubel *et al.*, 1992; Westenbroek *et al.*, 1992; Hell *et al.*, 1994b). Anti-CNB1 identified two size forms of the class B α1 subunit of ~220 and 240 kDa in rat brain in both immunoprecipitation and immunoblotting experiments. Anti-CNB3 reacted only with the 240 kDa form, indicating that the two size forms differ at their COOH-terminal ends (Westenbroek *et al.*, 1992; Hell *et al.*, 1994b). Similar results were obtained when calcium channels were solubilized and isolated from hippocampal slices by immunoprecipitation with anti-CNB1 followed by immunoblotting with the same antibody. Two bands with apparent molecular masses of 220 and 240 kDa were labeled (Figure 1, lane 1). Corresponding bands were also observed when anti-CNB1 was used in a double immunoprecipitation procedure in which CNB1 precipitates were phosphorylated with PKA and [γ-^{32}P]ATP, dissolved and re-immunoprecipitated with anti-CP(1382–1400) (Figure 2, lane1). The anti-peptide antibody

anti-CP(1382–1400) was originally produced against a sequence of the skeletal muscle L-type channel α1 subunit (Striessnig *et al.*, 1990). This sequence is highly conserved in neuronal calcium channels, and anti-CP(1382–1400) recognizes all calcium channel α1 subunits described to date. As expected, anti-CNB3 immunoprecipitated only the longer size form of the class B α1 subunit from hippocampus when employed as second antibody during double immunoprecipitation experiments (Figure 2, lane 3). These results identified both size forms of the class B N-type channel α1 subunit in the hippocampus and showed that the class B α1 subunit can be detected by our double immunoprecipitation method after labeling with PKA. Specificity of the double immunoprecipitation method was demonstrated by the absence of any signal if non-specific control antibody was used during the immunoprecipitations (Figure 2, lanes 2, 5 and 8).

Anti-CNC1 was raised against a peptide corresponding to a sequence in the loop between domains II and III of the class C α1 subunit, and anti-CNC2 against a peptide derived from the COOH-terminus of the full-length form of this subunit. Two size forms of the class C α1 subunit with apparent molecular masses of ~200 and 220 kDa have been detected in total rat brain with anti-CNC1 by immunoblotting (Hell *et al.*, 1993a), as well as by double immunoprecipitation in combination with phosphorylation by PKC (Hell *et al.*, 1993b). Anti-CNC2 bound only to the longer size form of the class C α1 subunit, suggesting that the two isoforms of the class C α1 subunit also differ at their COOH-termini (Hell *et al.*, 1993b). When calcium channels were solubilized from hippocampal slices, two protein bands were detectable after immunoprecipitation and subsequent immunoblotting using anti-CNC1 for both steps (Figure 1, lane 2). This result indicates the presence of both size forms of the class C L-type α1 subunit in the hippocampus. However, if class C L-type channels were solubilized from hippocampus, isolated by double immunoprecipitation with anti-CNC1 as the first antibody and anti-CP(1382–1400) as the second antibody, and phosphorylated with PKA, only the upper band was detectable (Figure 2, lane 4), even though both bands were present in the immunoprecipitates as observed when PKC was used for phosphorylation rather than PKA (data not shown). The upper band was also detectable when the second immunoprecipitation was performed with anti-CNC2 instead of anti-CP(1382–1400). These observations are in agreement with earlier findings analyzing class C L-type channels from total rat brain. These results suggest that only the full-length form of the class C α1 subunit is a substrate for PKA and that the phosphorylation site(s) for PKA may be located in the COOH-terminal part of the full-length form which is missing in the short form (Hell *et al.*, 1993b).

Anti-CNE2 was raised against a peptide derived from a sequence found between domains II and III of the class E α1 subunit. This antibody is specific for the class E α1 subunit and does not recognize any of the other calcium channels. A rather broad band just above the 240 kDa marker was detectable after immunoblotting of calcium channels from whole rat brain and probing the blot with anti-CNE2 (Yokoyama, Westenbroek, Hell, Snutch and Catterall, submitted). This broad immunoreactive band can be resolved into two separate, closely spaced bands. Calcium channels were solubilized from hippocampal slices, immunoisolated with anti-CNE2 as precipitating antibody and immunoblotted with anti-CNE2 as probing antibody. These experiments revealed the existence of two size forms of the class E α1 subunit which migrated close together just above the 240 kDa standard (Figure 1, lane 3). Corresponding bands were detectable when solubilized hippocampal calcium channels were isolated by double immunoprecipitation with anti-CNE2 utilized for the first precipitation and either anti-CP(1382–1400) or anti-CNE2 for the second precipitation, and labeled by phosphorylation with PKA (Figure 2, lanes 7 and 9).

Phosphorylation of calcium channels by PKA in intact hippocampal neurons

We used the method of back-phosphorylation in combination with double immunoprecipitation to determine the state of phosphorylation of calcium channels in acutely isolated hippocampal slices. This approach enabled us to investigate whether calcium channels were phosphorylated in intact neurons during incubation of hippocampal slices with different drugs which activate PKA. After the first immunoprecipitation, samples were incubated with PKA in the presence of [γ-^{32}P]ATP. During this step, [^{32}P]phosphate can only be incorporated into those phosphorylation sites which are not already occupied. Quantification of [^{32}P]phosphate in the channel protein reflects the phosphorylation status of the channel before the incubation with purified kinase (Forn and Greengard, 1978). The amount of [^{32}P]phosphate would be decreased if the channel had been phosphorylated in intact neurons during treatment of the hippocampal slices. No [^{32}P]phosphate was incorporated into calcium channels if PKA was omitted from the incubation medium or if PKA was blocked by its specific inhibitor PKI (Hell, Murphy and Catterall, unpublished observations). These findings indicate that only PKA, and no other protein kinases which may have been associated with immunopurified calcium channels or co-purified with PKA from bovine heart, were phosphorylating the channels during the *in vitro* incubation.

The class-specific antibodies anti-CNB1, -CNC1 and -CNE2 were utilized to isolate the corresponding calcium channels during the first immunoprecipitation. The class-specific antibodies anti-CNB3, -CNC2 and -CNE2 were employed for the second immunoprecipitation. Utilizing COOH-terminal antibodies against the class B and class C channels for immunoprecipitation focused our analysis on the full-length size forms of these two α1 subunits. Additional information, however, could not have been gained by including the short forms of the class B and class C α1 subunits in our studies because the shorter form of the class C α1 subunit is not a substrate for PKA during back-phosphorylation (see above) and the two size forms of the class B α1 subunit show identical phosphorylation patterns, as determined by two-dimensional phosphopeptide mapping (Hell and Catterall, unpublished results).

8-(4-Chlorophenylthio)adenosine-3′,5′-cyclic monophosphate (8-CPT-cAMP) is a membrane-permeable analog of cAMP and activates PKA with high efficacy. Application of 8-CPT-cAMP to hippocampal slices reduced back-phosphorylation of the class C α1 subunit with PKA by

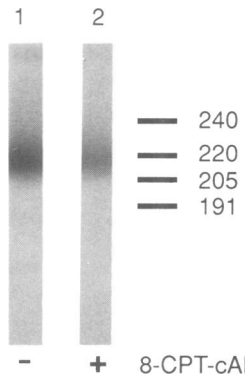

Fig. 3. Treatment of hippocampal slices with the cAMP analog 8-CPT-cAMP reduces back-phosphorylation of the class C α1 subunit with PKA. Six hippocampal slices were incubated for 20 min under control conditions or with 500 μM 8-CPT-cAMP and solubilized. The phosphorylation status of class C α1 subunits was determined by double immunoprecipitation in combination with back-phosphorylation using purified PKA. Autoradiography after SDS–PAGE revealed a strong decrease of incorporation of [^{32}P]phosphate during back-phosphorylation (here 50% as determined by Cerenkov counting of the excised bands). This result indicates that class C α1 subunits have been phosphorylated during the treatment of hippocampal slices with 8-CPT-cAMP where no radioactive phosphate was present for incorporation.

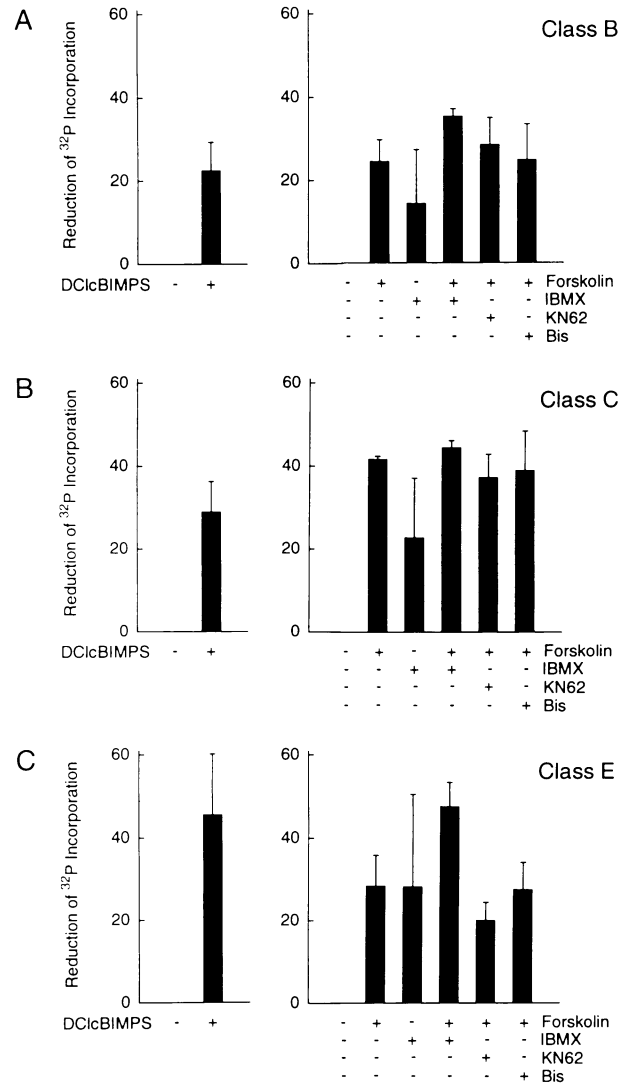

Fig. 4. Activation of PKA in hippocampal slices decreases back-phosphorylation of class B, C and E α1 subunits with PKA. Four hippocampal slices were treated with or without 500 μM DCl-cBIMPS (left side) or with 50 μM forskolin or 1,9-dideoxyforskolin for control in the presence or absence of 50 μM IBMX, 10 μM KN-62 or 2 μM bisindolylmaleimide (Bis; right side). After solubilization, class B (panel **A**), C (panel **B**) and E (panel **C**) α1 subunits were subsequently double immunoprecipitated and their phosphorylation status was analyzed by back-phosphorylation, SDS–PAGE and Cerenkov counting of excised bands. For each experiment, values were normalized with respect to control (no additions) which was set as 100%. Shown is the amount of reduction of back-phosphorylation which corresponds to the difference of the normalized data and 100%. Accordingly, control values are equivalent to 0% reduction. DCl-cBIMPS, as well as forskolin (applied alone or in any combination), decreased back-phosphorylation significantly based on *t*-test analysis with a 99% confidence interval. The forskolin effect was increased by IBMX and not affected by KN-62 or bisindolylmaleimide. Bars indicate the average of 3–8 experiments ± SEM.

~50 % (Figure 3). Further studies were performed with Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-cyclic monophosphorothioate (DCl-cBIMPS), another membrane-permeable cAMP analog which is a more specific PKA activator. DCl-cBIMPS reduced back-phosphorylation of the class B, C and E α1 subunits by >25, 30 and 50%, respectively (Figure 4). The effect of DCl-cBIMPS on back-phosphorylation with PKA was significant for each channel, demonstrating that all three channels were phosphorylated by PKA during the incubation of hippocampal slices with this drug. These findings demonstrate that phosphorylation sites on the class B, C and E α1 subunits are available for phosphorylation by PKA in intact neurons.

To study the activation of PKA by endogenously generated cAMP, forskolin, an activator of a variety of adenylyl cyclases, and 3-isobutyl-1-methylxanthine (IBMX), a general inhibitor of phosphodiesterases, were used. Since non-specific effects of forskolin on ion channels have been reported (reviewed by Laurenza *et al.*, 1989), 1,9-dideoxyforskolin was used in control incubations. This forskolin derivative mimics side effects of forskolin without stimulating adenylyl cyclases. Similar to DCl-cBIMPS, forskolin reduced back-phosphorylation of class B, C and E calcium channels significantly when compared with control conditions, confirming that all three calcium channels are phosphorylated by PKA in intact hippocampal neurons after activation of adenylyl cyclase (Figure 4). The phosphodiesterase inhibitor IBMX should increase the concentration of cAMP by prolonging its half-life. IBMX by itself appeared to reduce back-phosphorylation of all three calcium channels (Figure 4), but this effect was statistically insignificant because of the large variability of the measurements. This deviation may be due to the indirect action of IBMX which depends on basal production of cAMP in the slices. However, IBMX enhances the effect of forskolin on phosphorylation of the class B and E α1 subunits (Figure 4). Pre-treatment with either KN-62, a specific inhibitor of CaMKII, or the bisindolylmaleimide GF109203X, a specific inhibitor of PKC, had no effect on the forskolin-induced phosphorylation of class B, C or E α1 subunits at concentrations which were at least three times higher than those shown to be effective in hippocampal slices (e.g. Wyllie *et al.*, 1994; ffrench-Mullen, 1995; Figure 4). These results demonstrate that

Table I. Activation of PKA does not affect the amount of calcium channel α1 subunits as detected by immunoblotting

	Long form	Short form
Class B	100.1 ± 4.2%	107.1 ± 4.2%
Class C	98.8 ± 8.1%	100.4 ± 11.3%
Class E	104.5 ± 8.2%	106.1 ± 30.5%

50 μM forskolin or 1,9-dideoxyforskolin as control were applied to hippocampal slices. After solubilization and immunoprecipitation of calcium channels with the affinity-purified antibodies anti-CNB1 (class B), -CNC1 (class C) or -CNE2 (class E), proteins were separated by SDS–PAGE and transferred onto nitrocellulose which was probed with the same antibody used for immunoprecipitation (see Materials and methods for details). The blots were developed with the ECL reagent and protein bands corresponding to the long and short forms of the class B, C and E calcium channels were quantified by densitometry. Given are the relative amounts of the two different size forms of each channel after forskolin application in comparision with the control treatments which correspond to 100%.

neither CaMKII nor PKC activity contribute to the observed phosphorylation at PKA sites.

The observed reduction of back-phosphorylation could be due to a loss of substrate, possibly by proteolysis induced by drug treatment. We measured the amount of the class B, C and E α1 subunits present during the back-phosphorylation reaction. After the usual immunoprecipitation with anti-CNB1, -CNC1 or -CNE2, immunoblotting was performed using the same antibodies to probe the corresponding precipitates, and the amount of immunoprecipitated α1 subunits was quantitated by densitometric analysis. As shown in Table I, incubation with forskolin does not decrease the amount of either of the two size forms of the class B, C or E α1 subunits in comparison with control treatments with 1,9-dideoxyforskolin. Evidently, stimulation of PKA by drug treatment of hippocampal slices did not reduce the amount of the class B, C or E α1 subunit available for back-phosphorylation. These results also exclude the possibility that the longer size form of the class C α1 subunit was converted into the shorter form by proteolytic processing stimulated by elevated cAMP. Such a reaction would not decrease the total number of class C L-type channels, but it would reduce the amount of substrate for back-phosphorylation because the short form is not phosphorylated *in vitro* by PKA.

TEA induces phosphorylation of hippocampal calcium channels

Tetraethylammonium ion (TEA) depolarizes neurons and promotes repetitive action potentials and the release of different neurotransmitters by blocking potassium channels. We applied TEA to test if endogenous electrical activity in the hippocampal slice is sufficient to induce phosphorylation of calcium channels. TEA treatment clearly diminished back-phosphorylation of the class B, C and E α1 subunits by ~40 % (Figure 5). The same TEA-induced reduction of back-phosphorylation was also observed when the slices were pre-incubated with either KN-62 or GF109203-X to block CaMKII or PKC, respectively. These experiments show that TEA increases the phosphorylation of class B, C and E α1 subunits without requiring direct or indirect action of the calcium-dependent enzymes CaMKII or PKC. TEA did not affect

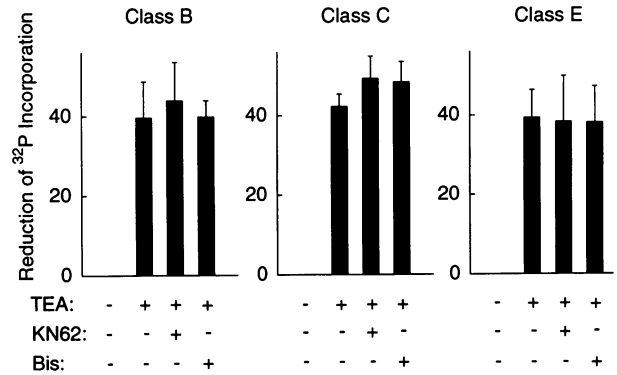

Fig. 5. TEA treatment of hippocampal slices reduces back-phosphorylation of class B, C and E α1 subunits with PKA. Four hippocampal slices were incubated for 20 min with standard incubation buffer or incubation buffer in which 25 mM NaCl was replaced by 25 mM TEA-Cl. The effect of 10 μM KN-62 or 2 μM bisindolylmaleimide (Bis) on TEA-induced phosphorylation was also tested. Class C, B and E α1 subunits were subsequently immunoisolated and their phosphorylation status was analyzed by back-phosphorylation. Values are given as in Figure 4. TEA decreased back-phosphorylation of all three channels in the absence or presence of KN-62 or bisindolylmaleimide significantly, based on *t*-test analysis with a 99% confidence interval. Bars indicate the average of 3–7 experiments ± SEM.

Table II. TEA application does not reduce the amount of calcium channel α1 subunits as detected by immunoblotting

	Long form	Short form
Class B	113.2 ± 7.8%	115.6 ± 25.5%
Class C	103.5 ± 14.6%	104.3 ± 11.6%
Class E	99.4 ± 10.4%	93.2 ± 11.4%

Hippocampal slices were treated under control conditions or with 25 mM TEA and solubilized. Calcium channels were extracted with the affinity-purified antibodies anti-CNB1 (class B), -CNC1 (class C) or -CNE2 (class E) on protein A–Sepharose. After SDS–PAGE, proteins were transferred onto nitrocellulose which was probed with the same antibody used for immunoprecipitation (see Materials and methods for details). The blots were developed with the ECL reagent and protein bands corresponding to the long and short forms of the class B, C and E calcium channels were quantified by densitometry. Given are the relative amounts of the two different size forms of each channel after TEA application in comparision with the control treatments which correspond to 100%.

the amount of α1 subunits available during back-phosphorylation, as tested by immunoblotting with subsequent densitometry (Table II). Taken together, our findings indicate that elevation of either cellular cAMP levels or electrical activity can increase phosphorylation of the α1 subunits of all three calcium channels investigated.

Discussion

Biochemical properties of class B, C and E α1 subunits from hippocampal neurons

The biochemical characterization of class B N-type, class C L-type and class E calcium channel α1 subunits from total rat brain is described elsewhere (Dubel *et al.*, 1992; Westenbroek *et al.*, 1992; Hell *et al.*, 1993a,b, 1994b; Yokoyama, Westenbroek, Hell, Snutch and Catterall, submitted). Class B, C and E α1 subunits isolated from hippocampal slices showed biochemical properties virtually identical to those of calcium channels from whole

brain. All three α1 subunits exist in two size forms with apparent molecular masses very similar to the corresponding isoforms isolated from total brain, as detected with the class-specific antibodies anti-CNB1, -CNC1 and -CNE2 against the central loop. Furthermore, COOH-terminal antibodies specific for either the class B or the class C α1 subunit recognize only the larger size form of the corresponding channel. No antibody against the COOH-terminal sequence of the class E calcium channel is currently available. Therefore, we could not test if the two size forms of the class E α1 subunit also vary at their COOH-termini. However, cloning and sequencing of cDNA from rabbit brain indicated that differential splicing occurs near the COOH-terminus of the class E α1 subunit which probably gives rise to two different isoforms (Niidome *et al.*, 1992). The difference of the predicted molecular masses of these two size forms is ~10 kDa, in good agreement with the difference of the apparent molecular masses of the two class E bands immunoreactive for anti-CNE2. These findings are analogous to those for the class B α1 subunit which shows both alternative splicing at its COOH-terminal end and the existence of two size forms which differ at their COOH-terminal ends.

Phosphorylation of class B, C and E α1 subunits in hippocampal slices

We employed our class-specific antibodies in a double immunoprecipitation procedure to isolate analytical amounts of the class B, C or E α1 subunits rapidly from hippocampal slices. Multiple phosphatase inhibitors were present throughout the fast isolation procedure to preserve the phosphorylation state of the calcium channels. Their phosphorylation status was determined after different drug treatments of the slices by back-phosphorylation with PKA. Activation of PKA by treatment with membrane-permeant cAMP derivatives or by activation of cellular adenylyl cyclase diminished back-phosphorylation of class B, C and E α1 subunits substantially. The amount of α1 subunits present during the back-phosphorylation step was not reduced for any of these channels by stimulation of PKA in hippocampal slices. Therefore, the observed reduction of back-phosphorylation reflects phosphorylation of all three classes of calcium channels in intact hippocampal neurons. An increase of cytosolic cAMP concentrations and stimulation of PKA may stimulate calcium influx. We tested, therefore, if the calcium-dependent protein kinases CaMKII or PKC may have contributed directly or indirectly to the phosphorylation induced by PKA activation by using inhibitors that have been shown previously to inhibit the actions of these kinases effectively in hippocampal slices. Neither CaMKII nor PKC are necessary or responsible for the observed phosphorylation at PKA sites, since pre-treatment of hippocampal slices with the corresponding inhibitors did not change the reduction of back-phosphorylation as detected upon forskolin application.

Our results suggest that all three of these α1 subunits may be regulated by PKA. PKA must be bound to A kinase anchoring proteins (AKAPs) in close proximity to some target proteins, including glutamate receptors of the AMPA type and class C L-type channels, to be able to modify their activity upon stimulation (Johnson *et al.*, 1994; Rosenmund *et al.*, 1994). Therefore, our results also suggest that PKA may be anchored near class B, C and E calcium channels in nerve terminals, cell bodies and dendrites of hippocampal neurons.

Phosphorylation of the class B, C and E channels could also be induced by TEA application to hippocampal slices. This membrane-impermeable cation cannot activate PKA directly. TEA exerts its effects by blocking potassium channels, which causes membrane depolarization and calcium influx and stimulates repetitive action potentials and exocytosis of neurotransmitters. It is, therefore, likely that some of the released neurotransmitters activate G protein-coupled receptors which are linked to cAMP production and ultimately activate PKA. In addition, depolarization of the plasma membrane may make certain PKA phosphorylation sites on these calcium channels accessible to PKA. For example, depolarization-dependent phosphorylation by PKA is thought to enhance activation of L-type calcium channels in chromaffin cells (Artalejo *et al.*, 1992), skeletal muscle cells (Sculptoreanu *et al.*, 1993a), mammalian cells transfected with class C α1 subunit (Sculptoreanu *et al.*, 1993b) and neuronal class C L-type channels expressed in *Xenopus* oocytes (Bourinet *et al.*, 1994). Depolarization-induced calcium influx could increase the activity of CaMKII or PKC as well. Pre-incubation with CaMKII- or PKC-specific inhibitors did not affect the TEA-induced reduction of back-phosphorylation with PKA, suggesting that activation of CaMKII or PKC is neither responsible nor required for the TEA effects. Calcium influx may, however, contribute to PKA activation by stimulating the activity of the calcium/calmodulin-sensitive type I adenylyl cyclase. Nevertheless, the results with TEA show that increased electrical activity in hippocampal neurons leads to phosphorylation of three distinct classes of calcium channels in different neuronal compartments by PKA.

Physiological relevance of phosphorylation of hippocampal calcium channels

Electrophysiological experiments indicate that neurotransmitters regulate the activity of neuronal calcium channels through phosphorylation by second messenger-activated serine/threonine protein kinases (for review, see Dolphin, 1990; Miller, 1990). The activity of L-type calcium channels was increased by PKA in GH3 pituitary cells (Armstrong and Eckert, 1987), guinea-pig hippocampal neurons (Gray and Johnston, 1987), rat nodose ganglion neurons (Gross *et al.*, 1990), chromaffin cells (Artalejo *et al.*, 1990) and rat neostriatal neurons (Surmeier *et al.*, 1995). PKA augmented the activity of N-type channels in nodose ganglion cells (Gross *et al.*, 1990), but decreased it in dorsal root ganglion neurons (Gross and Macdonald, 1988, 1989) and rat neostriatal neurons (Surmeier *et al.*, 1995). PKA also increased the activity of unidentified neuronal calcium channels in snail neurons (Doroshenko *et al.*, 1984; Chad and Eckert, 1986). Our present results indicate that direct phosphorylation of N- and L-type calcium channels is a likely mechanism responsible for regulation of these channels in hippocampal neurons.

Immunocytochemical analysis revealed that the majority of class B N-type channels are found on dendrites and nerve terminals in the hippocampus and other areas of the brain (Westenbroek *et al.*, 1992; Dado *et al.*, 1993).

Furthermore, functional studies indicated that N-type calcium channels control calcium-dependent release of glutamate, acetylcholine, monoamines and other neurotransmitters (Miller, 1987; Takahashi and Momiyama, 1993; Turner *et al.*, 1993; Wheeler *et al.*, 1994). Therefore, regulation of the activity of presynaptic N-type channels by PKA may influence neurotransmitter release and may be involved in modulation of synaptic transmission.

Several forms of sustained potentiation and depression can be induced by different electrical stimulation paradigms in the hippocampus (reviewed recently by Bliss and Collingridge, 1993; Malenka and Nicoll, 1993; Bear and Malenka, 1994). Short-term potentiation (STP) and long-term potentiation which lasts for several hours (LTP) or, with extended stimulation, for days (L-LTP) in the CA1 area of the hippocampus depend on calcium influx through postsynaptic NMDA receptors and may involve modulation of both pre- and postsynaptic properties. In contrast, LTP at the mossy fiber terminal is NMDA receptor independent and based on increased neurotransmitter release from the presynaptic side. Activation of PKA is both necessary and sufficient for the induction of mossy fiber LTP (Huang *et al.*, 1994; Weisskopf *et al.*, 1994). Since N-type channels are present in mossy fiber terminals (Westenbroek *et al.*, 1992) and, along with P-type channels, are involved in the control of synaptic transmission at these synapses (Castillo *et al.*, 1994), phosphorylation of these calcium channels by PKA may contribute to the induction and maintenance of mossy fiber LTP.

PKA activity is also necessary to establish L-LTP at CA1 synapses (Frey *et al.*, 1993). In contrast to mossy fiber LTP, L-LTP in CA1 neurons may involve an increase of the postsynaptic response to neurotransmitter stimulation. Class C L-type channels are clustered in patches in the membrane of postsynaptic cells in the hippocampus (Hell *et al.*,1993a; Westenbroek and Catterall, in preparation) and their activity is significantly increased by PKA (Sculptoreanu *et al.*, 1993a). Furthermore, activation of NMDA receptors in CA1 pyramidal neurons by tetanic stimulation increased both cAMP levels and calcium channel activity (Chetkovich *et al.*, 1991). Elevated calcium influx through class C L-type channels may, therefore, contribute to the increase of synaptic transmission during L-LTP. In fact, TEA which evokes the phosphorylation of class C L-type channels (see above), can induce LTP in hippocampal slices under certain conditions. This induction depends on calcium influx through postsynaptic L-type channels rather than through NMDA receptors (Aniksztejn and Ben-Ari, 1991; Huang and Malenka, 1993).

Class C L-type channels and class E channels are found on cell bodies of hippocampal pyramidal cells (Hell *et al.*, 1993a; Yokoyama, Westenbroek, Hell, Snutch and Catterall, submitted). L-type channels are involved in the regulation of gene expression in hippocampal neurons, and calcium entering through L-type calcium channels is more effective than calcium entering through glutamate receptors in the regulation of transcription (Murphy *et al.*, 1991; Bading *et al.*, 1993). Phosphorylation of class C and, possibly class E, channels by PKA may therefore indirectly affect the expression of neuronal genes. Thus, our results suggest that phosphorylation of calcium channels in different cellular compartments may be involved in the control of multiple neuronal functions including presynaptic neurotransmitter release, postsynaptic excitability and induction of gene expression. With the methods described here, it is now possible to identify signaling pathways which lead to calcium channel phosphorylation in intact hippocampal cells.

Materials and methods

Materials

[γ-^{32}P]ATP (111 TBq/mmol) was obtained from New England Nuclear-DuPont (Boston, MA), the ECL detection kit for immunoblotting from Amersham (Arlington Heights, IL), 8-CPT-cAMP, protein A–Sepharose (PAS) and bovine serum albumin (BSA; IgG-free) from Sigma (St. Louis, MO), DCl-cBIMPS from BioLog (La Jolla, CA), microcystin-LR, KN-62 and GF109203X from Calbiochem (San Diego, CA) and control antibodies (rabbit IgG) from Zymed (South San Francisco, CA). Three-week-old Sprague–Dawley rats were purchased from Bantin and Kingman (Bellevue, WA). PKA was isolated by established procedures (Kaczmarek *et al.*, 1980) and generously provided by Dr E.I.Rotman, Department of Pharmacology, University of Washington. All other reagents purchased from commercial sources were of standard biochemical quality.

Production and purification of peptides and antibodies

The production of the anti-peptide antibodies anti-CP(1382–1400), -CNB1, -CNC1, -CNC2, -CNB3 and -CNE2 has been described elsewhere (Striessnig *et al.*, 1990; Westenbroek *et al.*, 1992; Dubel *et al.*, 1992; Hell *et al.*, 1993b,1994b; Yokoyama, Westenbroek, Hell, Snutch and Catterall, submitted, respectively). The methods for the synthesis and the purification of peptides, the immunization of the rabbits and the affinity purification of the specific antibodies from serum on peptide-affinity columns are described in Westenbroek *et al.* (1992).

Incubation of hippocampal slices

Three-week-old rats were killed by cervical dislocation and hippocampi were quickly collected in ice-cold dissection buffer (230 mM sucrose, 10 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$, 25.6 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES, equilibrated with 95% O_2/5% CO_2 and adjusted with NaOH to pH 7.0). Slices (0.5 mm) were prepared with a McIlwain Tissue Chopper and incubated for 10–20 min in 24-well cell culture plates at room temperature under 95% O_2/5% CO_2. The dissection buffer was replaced by incubation buffer (126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.4 mM $CaCl_2$, 25.6 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES, equilibrated with 95% O_2/5% CO_2 and adjusted with NaOH to pH 7.0) and the slices were kept for 90 min at 32°C. Drugs were added and usually four slices per sample were collected and solubilized in 400 µl ice-cold 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 20 mM ethylenediaminetetraacetic acid (EDTA), 10 mM [ethylenebis(oxyethylenenitrilo)] tetraacetic acid (EGTA), 10 mM Tris–Cl, pH 7.4 containing the phosphatase inhibitors β-glycerol phosphate (20 mM), NaF (50 mM), sodium pyrophosphate (50 mM), microcystin-LR (2 µM) and *p*-nitrophenyl phosphate (1 mM) and the protease inhibitors pepstatin A (1 µg/ml), leupeptin (10 µg/ml), aprotinin (20 µg/ml), benzamidine (100 µg/ml), calpain inhibitor I and II (8 µg/ml each) and phenylmethanesulfonyl fluoride (PMSF, 200 µM) by triturating with an insulin syringe. Nonsolubilized material was removed by high-speed centrifugation (70.1 Ti rotor from Beckman, 40 000 r.p.m., 30 min, 4°C).

Immunoisolation, back-phosphorylation and immunoblotting of calcium channels from hippocampal slices

All purification steps were performed on ice using pre-cooled solutions in the presence of protease inhibitors and phosphatase inhibitors. Proteins which bind nonspecifically to Sepharose or protein A were removed by pre-incubation of each sample with 75 µl Sepharose CL-4B and 2 mg PAS for 30 min and subsequent centrifugation (Heraeus Biofuge 13, 13000 r.p.m., 1 min). Supernatants were incubated with 5 µg of affinity-purified anti-CNC1 antibody for 90 min on ice. PAS (3 mg), pre-blocked with 0.5% BSA in TBS (150 mM NaCl, 10 mM Tris–Cl, pH 7.4) and 1% Triton X-100 were added, samples were mixed on a tilting mixer for 150 min and centrifuged as before. The PAS/antibody/calcium

channel complex was washed three times with TBS, 1% Triton X-100 and processed as described below. Supernatants were collected and class B N-type channels were immediately precipitated with 5 μg anti-CNB1 and PAS as detailed for class C. After precipitating the immunocomplex, supernatants were collected again, frozen and stored at −80°C and thawed later for immunoprecipitating class E calcium channels with 10 μg anti-CNE2 following the procedure outlined above. Class B and E calcium channels were isolated from solubilized hippocampal samples without previous precipitation of other calcium channels where indicated.

For immunoblotting, immunocomplexes were washed once with 10 mM Tris–Cl, pH 7.4 and extracted with SDS sample buffer (20 μl 125 mM Tris–HCl, pH 6.8, 6% SDS, 2 mM EGTA, 10% sucrose, 20 mM dithiothreitol, 1 μM pepstatin A, 2 μg/ml leupeptin, 4 μg/ml aprotinin, 100 μg/ml benzamidine) at 50–60°C for 30 min. The samples were cleared by a short spin in a microfuge and analyzed by electrophoresis in standard SDS–polyacrylamide gels (SDS–PAGE) made from 5% acrylamide plus 0.13% bisacrylamide (separating gel) and 3% acrylamide plus 0.08% bisacrylamide (top gel). Minigels were run at 50 mA/gel until all pre-stained protein markers had entered the separating gel, when the current was reduced to 20 mA/gel. Electrophoresis was continued until the pre-stained 117 kDa protein marker reached the bottom of the gel. This protocol resulted in optimal recovery and resolution of calcium channel α1 subunits. The immunoblotting procedure is given in Westenbroek *et al.* (1992). ECL signals were detected by film exposure and quantified by densitometry.

For *in vitro* back-phosphorylation, immunocomplexes were washed once with basic phosphorylation buffer (50 mM HEPES–NaOH, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA). The complexes were phosphorylated with 0.5–1 μg PKA in 50 μl of basic phosphorylation buffer containing 1 mM dithiothreitol, 1 μM pepstatin A, 2 μg/ml leupeptin, 4 μg/ml aprotinin and 0.2 μM [γ-^{32}P]ATP. Samples were incubated at 32–34°C for 20 min with careful mixing every 2–3 min, washed three times with 1% Triton X-100 in RIA buffer (25 mM Tris–HCl, pH 7.4, 20 mM EDTA, 10 mM EGTA, 75 mM NaCl, 20 mM Na pyrophosphate, 20 mM β-glycerolphosphate, 50 mM NaF, 1 mM *p*-nitrophenyl phosphate) and once in 10 mM Tris–HCl, pH 7.4. Pellets were extracted with 20 μl of 50 mM Tris–Cl, pH 8.0, 1.5% SDS, 5 mM dithiothreitol, 20 mM β-glycerolphosphate, 1 μM *p*-nitrophenyl phosphate, 1 μM pepstatin A, 2 μg/ml leupeptin and 4 μg/ml aprotinin. Samples were spun as described above after dilution with 240 μl of 1% Triton X-100, 0.1% BSA, 20 mM β-glycerolphosphate, 1 μM *p*-nitrophenyl phosphate, 1 μM pepstatin A, 2 μg/ml leupeptin, 4 μg/ml aprotinin, 100 μg/ml benzamidine and 200 μM PMSF in RIA buffer. The supernatants were collected and incubated with 25 μg affinity-purified anti-CP(1382–1400), 20 μg anti-CNC2 or -CNB3 or 10 μg anti-CNE2 for 90 min on ice. Calcium channel α1 subunits were precipitated with 2 mg PAS as detailed above, washed three times with 1% Triton X-100 in RIA, once with 10 mM Tris–HCl, pH 7.4, incubated with SDS sample buffer and separated by SDS–PAGE as indicated above. Radiolabeled protein bands were localized by autoradiography, excised and quantified by Cerenkov counting. ^{32}P in control areas with no obvious phosphoprotein band was also determined for each gel lane and these values were subtracted from the phosphoprotein counts to correct for differences in background amounts of ^{32}P in each lane.

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