The Journal of Neuroscience, August 1995, 15(8): 5486–5501

Thalamocortical Projections Have a K^+ Channel That Is Phosphorylated and Modulated by cAMP-Dependent Protein Kinase

Herman Moreno,[1] Clifford Kentros,[1] Earl Bueno,[1] Michael Weiser,[1] Arturo Hernandez,[2] Eleazar Vega-Saenz de Miera,[1] Arturo Ponce,[1] William Thornhill,[3] and Bernardo Rudy[1]

[1]Department of Physiology and Neuroscience, New York University Medical Center, New York, New York 10016, [2]Instituto de Fisiologia Celular, UNAM, Mexico City, Mexico, and [3]Department of Physiology and Biophysics, Mount Sinai Medical Center, New York

The finding that some K^+ channel mRNAs are restricted to certain populations of neurons in the CNS suggests that there are K^+ channels tailored to certain neuronal circuits. One such example are the transcripts from the KV3.2 gene, the majority of which are expressed in thalamic relay neurons. To gain insights into the specific roles of KV3.2 subunits, site specific antibodies were raised to determine their localization in thalamic relay neurons. Immunohistochemical and focal lesioning studies demonstrate that KV3.2 proteins are localized to the terminal fields of thalamocortical projections. It is also shown that KV3.2 channels expressed *in vitro* are strongly inhibited through phosphorylation by cAMP-dependent protein kinase (PKA). Channels containing KV3.1 subunits, which otherwise exhibit nearly identical electrophysiological properties in heterologous expression systems but have a different and less restricted pattern of expression in the CNS, are not affected by PKA. Therefore, this modulation might be associated with the specific roles of KV3.2 subunits. Furthermore, we demonstrate that KV3.2 proteins can be phosphorylated *in situ* by intrinsic PKA. KV3.2 subunits display properties and have a localization consistent with a role in the regulation of the efficacy of the thalamocortical synapse, and could thereby participate in the neurotransmitter-mediated control of functional states of the thalamocortical system associated with global states of awareness.

[Key words: thalamus, cAMP, protein kinase A (PKA), synaptic efficacy, channel modulation, KV3.2, Shaw, phosphorylation, immunohistochemistry]

K^+ channels exhibit a striking diversity, endowing neurons with a wide repertoire of excitable properties, including variations in membrane resting potential, electrical resistance, and action potential threshold, waveform, and frequency. Therefore this diversity contributes to the ability of individual neurons to respond in a specific fashion to a given input (Llinas, 1988; Rudy, 1988; Latorre et al., 1989; McCormick, 1990; Baxter and Byrne, 1991; Hille, 1992). K^+ channels are also one of the most frequent targets of second messenger cascades activated by neurotransmitters and other stimuli (Kaczmareck and Levitan, 1987; Caterall, 1988; Levitan, 1988; Rudy, 1988; Hille, 1992). The ensuing changes (such as the phosphorylation of K^+ channel proteins) result in the stimulus-dependent modulation of the electrical properties of neurons. The diversity of K^+ channels allows for a large variation in the responses to these modulators. An example of the potential physiological significance of the modulation of K^+ channels by kinases is found in *Aplysia* sensory neurons, where a K^+ channel known as the S channel is inhibited by phosphorylation by cAMP-dependent protein kinase. This results in an increase of the duration of the action potential, which in turn produces an increase in neurotransmitter release. This modulation is thought to play a role in short term learning processes in *Aplysia* (Klein et al., 1982; Siegelbaum et al., 1982).

A large number of subunits of mammalian K^+ channels expressed in the CNS have been identified in recent years by molecular cloning techniques (Jan and Jan, 1990; Perney and Kaczmareck, 1991; Rudy et al., 1991; Pongs, 1992; Salkoff et al., 1992; Butler et al., 1993; Kubo et al., 1993a,b; Warmke and Ganetzky, 1994). Along with providing a wealth of structural information (MacKinnon, 1991; Miller, 1991; Jan and Jan, 1992), the availability of molecular probes provides tools for understanding the contribution of K^+ channels to the electrical properties of CNS neurons, which are especially useful in areas inaccessible to direct electrophysiological analysis. The Sh family, the most extensively studied family of subunits of voltage-dependent K^+ channels, consists of over 20 members divided into four subfamilies based on sequence similarities: Shaker-related, Shab-related, Shaw-related, and Shal-related (reviewed by Jan and Jan, 1990; Perney and Kaczmareck, 1991; Rudy et al., 1991; Pongs, 1992; Salkoff et al., 1993).

The mRNAs of some Sh family members are widely distributed in the CNS, while others have a more restricted pattern of expression, suggesting their involvement in more specific roles (e.g. Beckh and Pongs, 1990; Drewe et al., 1992; Hwang et al., 1992; Perney et al., 1992; Rudy et al., 1992; Sheng et al., 1992, 1994; Tsaur et al., 1992; Wang et al., 1994; Weiser et al., 1994). The transcripts of the KV3.2 gene (originally called RKShIIIA or KShIIIA; McCormack et al., 1990; Rudy et al., 1991a; the nomenclature of Chandy et al., 1991, is used in this article), members of the Shaw-related subfamily, are among those K^+ channel transcripts showing highly localized expression. *In situ*

Received Jan. 3, 1995; revised Mar. 23, 1995; accepted Mar. 29, 1995.

This research was supported by NIH Grant NS30989 and NSF Grant IBN 9209523 to B. R; and NIH Grant NS29633, to W. T. Arturo Ponce is a fellow of the Human Frontier Science Program Organization. We thank Dr. Dean Hillman for his assistance with stereotaxic injection procedures. Herman Moreno and Cliff Kentros contributed equally to this work.

Correspondence should be addressed to Bernardo Rudy, Department of Physiology and Neuroscience, New York University Medical Center, 550 First Avenue, New York, NY 10016.

hybridization histochemistry with KV3.2 probes in the rat CNS shows that KV3.2 mRNAs are predominantly expressed in thalamic relay neurons (Rudy et al., 1992; Weiser et al., 1994). Furthermore, based on quantitation of Northern blots, we estimate that close to 90% of all KV3.2 mRNAs in the rat CNS are found in the dorsal thalamus, and that, among those tested, KV3.2 mRNAs are one of the most abundant K^+ channel mRNAs in thalamic poly (A) RNA (Rudy et al., 1992; Weiser et al., 1994). KV3.2 mRNAs have so far not been detected outside of the CNS (Weiser et al., 1994; Vega-Saenz de Miera et al., 1994). Cloned originally from rat (McCormack et al., 1990), the KV3.2 gene has also been identified in mouse and human (Vega-Saenz de Miera et al., 1994) and chicken (unpublished data). The sequence of KV3.2 proteins has been strongly conserved throughout vertebrate evolution (≈95% amino acid identity between rat and chicken in the membrane portion of the protein). These data suggest that channels containing KV3.2 subunits (KV3.2 channels) may play an important role in the function of thalamic relay neurons. Moreover, high levels of KV3.2 mRNAs are found throughout the dorsal thalamus, including nuclei involved in auditory, visual and somatosensory processing, suggesting that the roles of KV3.2 channels are general to thalamic function.

The dorsal thalamus acts as a gate between neurons receiving sensory information and the cerebral cortex. To reach the neocortex, sensory information must first, with few exceptions, be processed by thalamic relay neurons (Jones, 1985; Steriade and Llinas, 1988; Sherman and Koch, 1990; Steriade et al., 1990). Given the role of the dorsal thalamus in the processing of sensory information, thalamic relay neurons have been the subject of extensive electrophysiological study (McCormick and Prince, 1985; McCormick and Prince, 1986; McCormick and Prince, 1987b; Steriade and Llinas, 1988; Steriade et al., 1990; Sherman and Koch, 1990; Agmon and Connors, 1991; Huguenard and Prince, 1991; Huguenard et al., 1991; Agmon and Connors, 1992; McCormick, 1992a,b; McCormick and Huguenard, 1992). To understand the possible relationship between the channels identified in these electrophysiological studies and KV3.2 subunits, it is necessary to know which compartments of the neuronal membrane contain these proteins. For this purpose we raised site-specific antibodies and utilized immunohistochemical techniques to determine the localization of the subunits at the subcellular level.

Many studies have demonstrated that neurotransmitter-induced activation of second messenger cascades has a dramatic effect upon the function of thalamic relay neurons, often via the regulation of K^+ currents (McCormick and Prince, 1985; McCormick and Prince, 1986; McCormick and Prince, 1987a,b; Steriade et al., 1990; Huguenard and McCormick, 1992; McCormick, 1992a,b; McCormick and Huguenard, 1992). These modulations are believed to underlie changes in the thalamocortical circuit associated with global states of awareness such as sleep, wakefulness, arousal, coma, or changes in attention (Steriade and Llinas, 1988; Sherman and Koch, 1990; Steriade et al., 1990; McCormick, 1992a,b). The finding that the excitability of thalamic relay neurons is modulated by neurotransmitters has contributed to the now widely held view of the dorsal thalamus as an active structure capable of regulating sensory input to the cortex rather than a passive relay station.

Given the significance of ion channel modulation in thalamocortical function and to gain further insight into the roles of KV3.2 proteins in thalamic relay neurons, we investigated the possibility that KV3.2 channels are targets of regulation by receptor-mediated activation of second messenger cascades. Among several possible cascades we have focused on the cAMP system because it is one of the key pathways modulated by neurotransmitters and neuropeptides acting on the thalamocortical circuit. Moreover, a putative PKA phosphorylation site is a key distinction between KV3.2 subunits and the products of KV3.1, a closely related gene. In *Xenopus* oocytes, KV3.1 and KV3.2 transcripts express currents with nearly identical kinetics and voltage dependence (Vega-Saenz de Miera et al., 1994). However, KV3.1 transcripts have an entirely different, and sometimes complementary, pattern of expression in the rat CNS (Weiser et al., 1994). Modulation of KV3.2 channels by cAMP may then be closely associated with its specific roles in the mammalian CNS.

Materials and Methods

Antibody production. To raise antibodies against KV3.2 proteins, rabbits were injected with the peptide DLGGKRLGIEDAAGLGGPDGKC coupled via the cysteine to keyhole limpet hemocyanin (KLH) (Harlow and Lane, 1988). The peptide corresponds to a sequence present in the constant region of KV3.2 proteins (residues 184–204 plus a C-terminal cysteine added to facilitate coupling) prior to the first membrane spanning domain (McCormack et al., 1990, 1991), in an area where K^+ channel proteins are less similar (Vega-Saenz de Miera et al., 1994). The KLH-linked KV3.2 peptide was injected into rabbits using standard procedures for antiserum production by Berkeley Antibody Co., Richmond, CA. For affinity purification, the KV3.2 peptide was coupled via the cysteine to Sulfolink Sepharose resin (Pierce, Rockford, IL) and antibodies purified following supplier's protocols. The final volume of purified antibodies was three times that of the original volume of serum applied to the column and had a protein concentration of 150 μg/ml.

Several tests were carried out to characterize the KV3.2 antibodies and assess their specificity. The antibodies immunoprecipitate a protein of ~80 kDa from membranes derived from CHO cells permanently transfected with a KV3.2a cDNA but not from control CHO cells (see Fig. 8*A*, lanes 3, 4). The size of this band is larger than the predicted (~67 kDa) size of the KV3.2a polypeptide, or the observed size of in vitro synthesized protein (also ~67 kDa, data not shown) probably due to glycosylation or other posttranslational modifications. A somewhat broader band is seen in immunoprecipitates from rat brain membranes (see Fig. 8*A*, lane 1) probably reflecting the heterogeneity in brain of splice variants and perhaps also posttranslational modifications. The immunoprecipitation of KV3.2 proteins from transfected CHO cells (data not shown) or from brain (Fig. 8*A*, lane 2) can be blocked by preincubating the antibodies with an excess of KV3.2 peptide. Moreover, the KV3.2 antibodies immunostained CHO cells transfected with a KV3.2 cDNA, but not with a KV3.1 or KV3.3 cDNAs, in contrast to antibodies against KV3.1, which immunostained only the KV3.1 transfected cells (Fig. 1). Taken together these tests show that the KV3.2 antibodies react specifically with KV3.2 proteins. Furthermore, the pattern of immunostaining in the brain is consistent with the pattern of expression of KV3.2 mRNAs (Rudy et al., 1992; Weiser et al., 1994). Of particular significance in terms of specificity being the immunostaining of a small subset of neurons in the cortex and the hippocampus (Figs. 2–4) with a similar distribution to neurons expressing KV3.2 mRNAs (Rudy et al., 1992; Weiser et al., 1994).

Kainic acid lesions. Adult male rats (275–300 gm) were deeply anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine and placed in a stereotaxic apparatus. Upon removal of a small piece of the overlying skull, 100 nl of 10 mg/ml kainic acid was injected stereotaxically with a glass micropipette into the ventromedial/ventroposterior thalamic area (AP +6.7 mm, ML +1.8 mm, DV +6.8 mm; according to the atlas of Paxinos and Watson, 1986) over a period of 10 min using a Drummond 10 μl microdispenser. The micropipette was kept in place an additional 10 min to minimize diffusion of the neurotoxin into the injection shaft, following which the wound was packed with Gelfoam and sutured, and the animals allowed to recover from the anesthetic.

Preparation of CHO cells expressing channel proteins. For transient expression of channel proteins, CHO (Chinese Hamster Ovary) K1 cells (ATCC, Bethesda, MD) were plated at 40% confluence and microin-

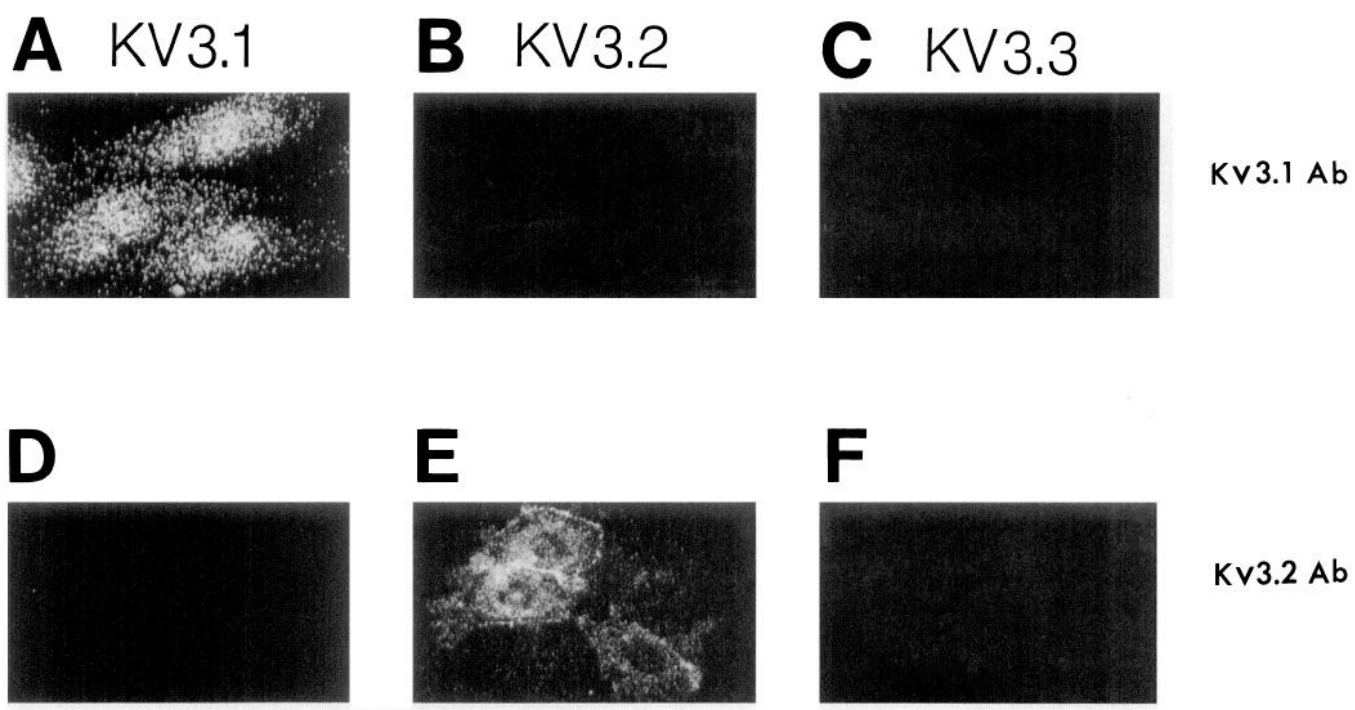

Figure 1. KV3.2 antibodies immunostain CHO cells transfected with KV3.2 cDNAs but not other Shaw-related genes. Immunofluorescent images of CHO cells permanently transfected with KV3.1b (*A* and *D*), KV3.2a (*B* and *E*), or KV3.3a (*C* and *F*) cDNAs were immunostained with antibodies against the KV3.1b protein (*A–C*) or KV3.2 proteins (*D–F*).

jected with in vitro transcripts (cRNA) (McCormack et al., 1990) of the appropriate cDNA. Cells, (usually of rounded shape and about 15 μm diameter) were injected in the cytoplasm utilizing an Eppendorf microinjector 5242 (Eppendorf, Hinz, Germany) with a mixture of cRNA (1 μg/μl) and 0.01% dextran fluorescein (Molecular Probes Inc.) centrifuged immediately before use. Injection micro-needles were made of 1BBL Glass (WPI) in a 720 Kopf puller (Kopf, Tujunga, CA). Fluorescent cells were recorded 12–36 hr after injection.

For permanent expression of KV3.2 proteins, the KV3.2a cDNA (McCormack et al., 1990) was subcloned into a mammalian expression vector containing an SV40 promoter and a neomycin resistance gene (a kind gift of Dr. R. Kris, Dept. of Pharmacology, NYU). Permanent CHO-K1 cell lines expressing KV3.2a proteins were prepared by stable transfection utilizing DOTAP (Boehringer Mannheim) to introduce the recombinant vector by lipofection according to the manufacturer's protocols. Following selection for neomycin resistance, cell lines were isolated and electrophysiologically tested, selected, and grown in DMEM with 10% (vol) fetal bovine serum (GIBCO) in the presence of neomycin.

In situ *hybridization and immunohistochemistry.* Adult male Sprague–Dawley rats were anesthetized with sodium pentobarbital (Nembutal, 120 mg/kg, i.p.) and perfused transcardially with cold saline containing 0.5% $NaNO_2$ and 10 U/ml heparin followed by 400 ml of cold 0.1 M phosphate buffer containing 4% formaldehyde. The brains were removed, cut into appropriate blocks and postfixed for 2 hr, following which they were placed in 30% sucrose at 4°C overnight. Tissue sections (30-40 μm, unless otherwise indicated) were cut on a freezing sliding microtome and processed for *in situ* hybridization histochemistry (Rudy et al., 1992; Weiser et al., 1994) or immunohistochemistry. For immunohistochemistry, the sections were preincubated for 30 min with a solution containing 1% bovine serum albumin (BSA, Sigma, fraction V), 0.2% Triton X-100 in 0.1 M phosphate-buffered saline (PBS). After washing, the sections were incubated overnight in 0.5% BSA in PBS (PBS-BSA) containing KV3.2 antibodies at a 1:75 to 1:100 dilution. After several washes in PBS-BSA, the sections were incubated with biotinylated anti-rabbit secondary antibodies (Vector, Burlingame, CA) at a 1:200 dilution for 1 hr and then washed. Bound antibodies were detected using the avidin–biotin–complex following the instructions in the Vectastain Elite ABC kit (Vector), using 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB, Aldrich Milwaukee WI) as a chromogen. Tissue sections were mounted on gelatin coated glass slides, dried, dehydrated, counterstained with cresyl violet when necessary, and coverslipped.

To prepare tangential sections of primary somatosensory cortex, following transcardial perfusion and craniotomy of adult (175 gm) male rats, the hemispheres were separated by a midsagittal cut and postfixed and cryoprotected as above. Prior to sectioning, a tangential cut was made, grossly separating cortex from underlying structures. The cortices were sectioned serially, parallel to the pial surface of the primary somatosensory cortex, in 40 μm thick sections (Welker and Woolsey, 1974).

For immunofluorescence on CHO cells, the cells were grown on glass coverslips. After two washes with PBS the cells were fixed with 4% formaldehyde, washed with PBS, and permeabilized for 15 min in PBS containing 0.1% Triton X-100 (PBS-Triton). The cells were then incubated for 1 hr with a blocking solution containing 10% fetal bovine serum, 2% goat serum, 0.2% biotin, and 1% BSA in PBS-Triton and then incubated overnight with KV3.2 antibodies at a 1:50 dilution or KV3.1b antibodies (Weiser et al., 1995) at 1:1000. After several 15 min rinses with PBS-Triton the cells were treated with biotinylated anti-rabbit IgG (Amersham) followed by fluorescein labeled streptavidin (Amersham). The cells were then rinsed with PBS. Following a rinse in H_2O the coverslips were mounted to a microscope slide with Vectashield (Vector Laboratories) and sealed with nail polish.

Electrophysiological analysis. Whole-cell currents were obtained at room temperature by the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) with an Axopatch 1A amplifier (Axon Instruments, Foster City, CA). The patch pipettes contained a solution of 130 mM KCl, 10 mM EGTA, 1 mM $MgCl_2$, 5 mM MgATP, and 10 mM HEPES (pH adjusted to 7.4 with KOH). The extracellular solution contained 135 mM NaCl, 3.5 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM glucose, and 10 mM HEPES (pH adjusted to 7.4 with NaOH); 1 μM tetrodotoxin (TTX) was added when intrinsic Na^+ currents were present. Seal resistance was typically 10 GΩ. Recordings were obtained with partial series resistance compensation (50%–70%) and most of the cell capacitance canceled. Experiments with cAMP or IBMX were only performed in recordings that were stable. The currents were low-pass filtered at 2 kHz using an eight pole Bessel filter (Frequency Devices) and digitized at 2.5 kHz. Subtraction of leak and remaining capacitance was obtained using a P/4 protocol. To generate voltage clamp protocols, and for data acquisition and analysis we used the pCLAMP software (Axon Instruments).

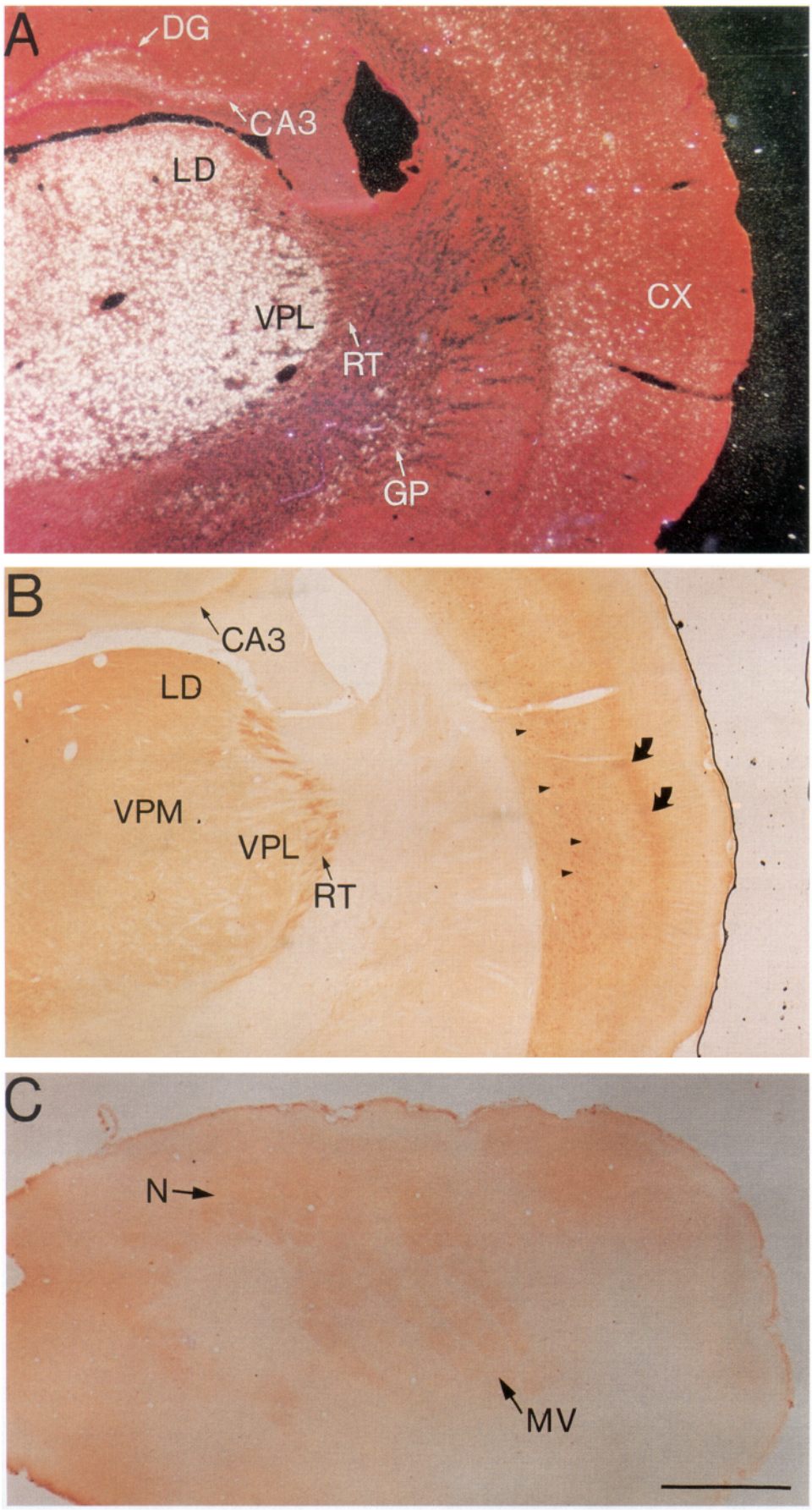

Figure 2. KV3.2 proteins are present in thalamocortical projections. *A*, Dark-field photomicrograph of a rat brain coronal section at the level of the ventroposterolateral thalamic nucleus hybridized with a ^{35}S-labeled probe specific to KV3.2 mRNAs. Note the strong hybridization signals throughout the dorsal thalamus and the punctate staining in deep cortical layers and the hippocampus. Observation under bright field of counterstained sections shows that in the dorsal thalamus the hybridization grains are concentrated over the somata of thalamic relay neurons (Rudy et al., 1992; Weiser et al., 1994). *B*, Immunohistochemistry of a parallel section with affinity purified KV3.2 antibodies. Note the strong fiber-like staining of the RT. The staining of the dorsal thalamus is comparatively weaker, and does not appear to be associated to neuronal somas. This pattern is the converse of that seen by *in situ* hybridization (*A*). Note also the presence of a diffuse band of immunostaining in the cortex approximately at the level of layer IV (*curved arrows*), and the somatic staining of a subset of neurons in deep layers of the cortex (*arrowheads*) and in the hippocampus which have a distribution similar to the subsets of neurons seen expressing KV3.2 mRNAs in these regions (*A*). *C*, Tangential section of primary somatosensory cortex immunostained with KV3.2 antibodies. Note the labeling of the layer IV barrel field. Uniform background staining was seen in control sections treated with peptide depleted antibodies (data not shown). Abbreviations: *VPL*, ventroposterolateral thalamic nucleus; *VPM*, ventroposteromedial thalamic nucleus; *LD*, laterodorsal thalamic nucleus; *CX*, cortex; *RT*, reticular thalamic nucleus; *CA3*, CA3 field of Ammon's horn; *DG*, dentate gyrus; *GP*, globus pallidus; *N*, barrels of the rostral face projections; *MV*, barrels of the projections corresponding to the mystacial pad vibrissae. Scale bars: *A* and *B*, 1.2 mm; *C*, 2 mm.

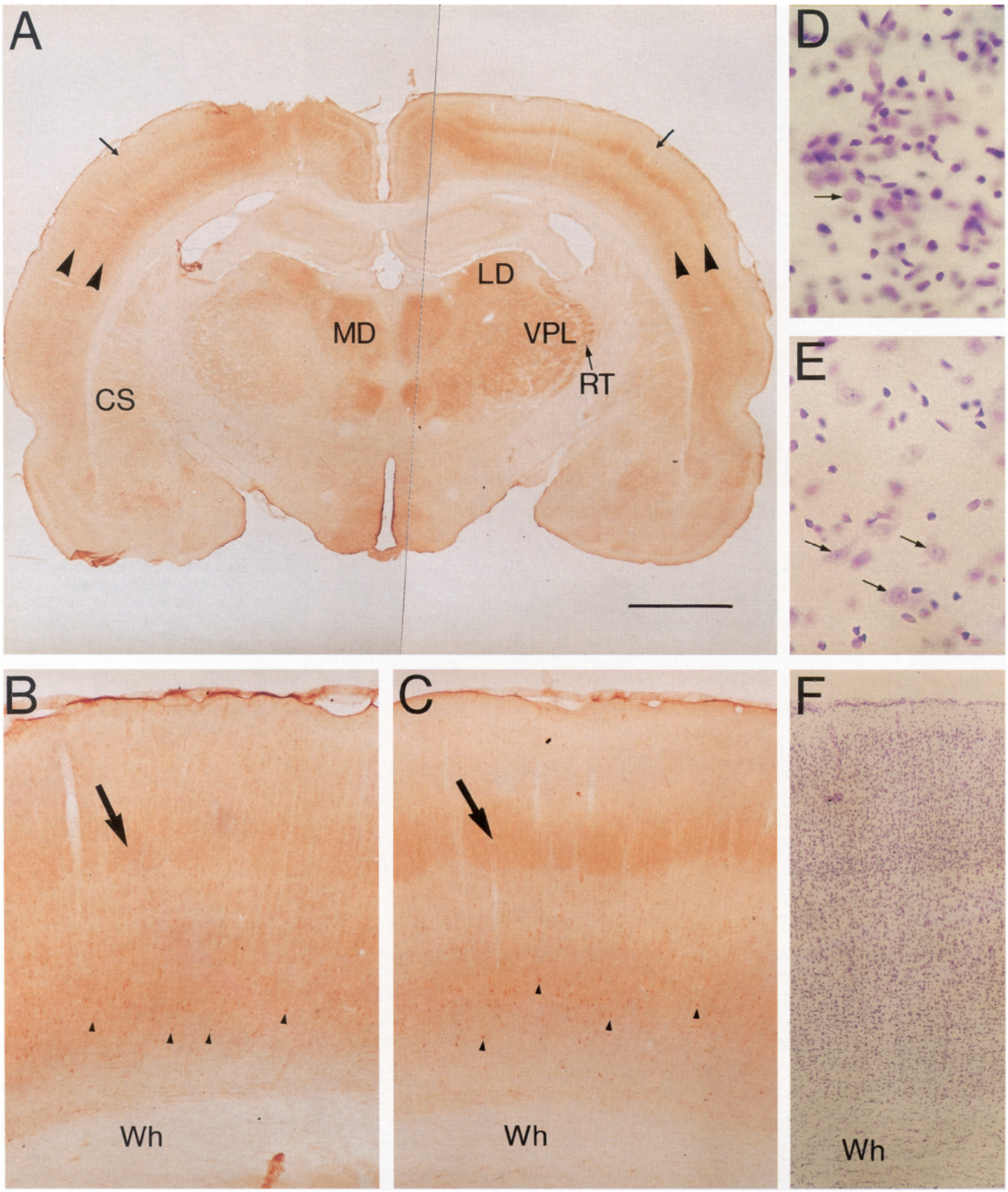

Figure 3. Unilateral thalamic lesions eliminate KV3.2 antibody staining in layer IV neocortex. *A*, Immunohistochemistry with KV3.2 antibodies of a coronal section from the brain of a rat unilaterally injected with kainic acid. The extent of the primary lesion (left side) in the ventral posterior thalamus is associated with a loss of staining. Note that cortical layer IV on the side ipsilateral to the lesion shows a marked reduction in stain relative to that of the contralateral cortex, while the staining in deep cortical layers is largely unchanged. *Arrows* indicate blood vessels that can be used as landmarks to identify the areas amplified in *B* and *C*. *B* and *C*, High power micrographs of primary somatosensory cortex ipsilateral (*B*) and contralateral (*C*) to the lesion site stained with KV3.2 antibodies showing the marked reduction in label in layer IV (*arrow*) but not in the neuronal somata in deep layers (*arrowheads*) on the lesioned side. Note in the normal side (*C*) that at this magnification it is possible to see that the band of immunostaining on layer IV is not homogeneous but consists of stained cylinders (representing the cortical barrels in cross-section)

Single channel recordings utilized the cell-attached configuration of the patch-clamp technique (Hamill et al., 1981) with an Axopatch 200-A amplifier (Axon Instruments). The extracellular solution utilized for whole cell recordings was used for both the bath and the pipette. The currents were low pass filtered at 2 kHz and digitized at 2 kHz. Leak subtraction was digitally performed using a template generated by averaging sweeps with no openings and this average subtracted from sweeps containing openings. The data was plotted utilizing an additional software-generated 0.5 kHz Gaussian filter. Data analysis utilized the FETCHAN and PSTAT programs of the PCLAMP software.

Preparation of membrane extracts. Rat brain membrane extracts were prepared from a P3 fraction of tissue homogenate (Hartshorne and Catterall, 1984) solubilized for 1 hr in a 2% Triton X-100 solution containing 50 mM potassium phosphate buffer pH 7.4, 50 mM KCl, 2 mM EDTA, and 1 μM pepstatin A, 1 mM 1,10 phenanthroline, 0.2 mM PMSF, and 1 mM iodoacetamide to inhibit proteases. The suspension was spun at 100,000 × *g* to remove nonsolubilized material, and the top 2/3 of the supernatant used for further experiments. Membranes from confluent CHO cell cultures were prepared by homogenizing the cells in a 25 mM potassium phosphate (pH 7.4), 2 mM EDTA ice cold solution containing the aforementioned protease inhibitors. Membranes were pelleted at 35,000 × *g* for 1 hr, resuspended in the same solution at 2–3 mg/ml, and stored at −70°C. Before use the membranes were thawed and solubilized as above.

Immunoprecipitation. Before immunoprecipitation, 300 μl of solubilized membranes (~400 μg protein) were precleared for 30 min at 4°C with protein A–sepharose beads (Pharmacia). After removing the beads, the extracts were incubated for 4 hr at 4°C with KV3.2 antibodies at a 1:10 dilution. At the end of the incubation period fresh protein A–sepharose beads were added and the suspension incubated for 2–3 hours at 4°C. The complexed beads were collected and washed two times in 1% Triton X-100 in 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.4. Proteins were then extracted with a sample buffer [10% (vol) glycerol, 5% (vol) β-mercaptoethanol; 60 mM Tris-HCl pH 6.8; 0.001% (wt) bromphenol blue and 3% SDS], heated for 3 min at 80°C and electrophoresed in an 9% (for the experiment shown in Fig. 8*A*) or 10% (for the experiment shown in Fig. 8*B,C*) SDS polyacrylamide gel (Harlow and Lane, 1988). For the experiments shown in Figures 8, *B* and *C*, the gels were dried and exposed to Kodak XAR x-ray film in the presence of intensifying screens for 2 d at −80°C. For immunoblotting (as in the experiment shown in Fig. 8*A*), the electrophoresed proteins were transferred onto a nitrocellulose filter (Bio-Rad). The Western blot was incubated with KV3.2 antibodies at a 1:100 dilution, followed by incubation with horseradish peroxidase-linked anti-rabbit secondary antibodies. Bound antibodies were detected using chemiluminescence with an ECL detection kit (Amersham).

Phosphorylation by exogenous protein kinase A. Solubilized membrane proteins (200 μl containing ~400 μg of protein) were preincubated for 2 min at 36°C prior to the addition of 4 units of catalytic subunit of PKA (New England Biolabs) and 25 μCi of γ^{32}-ATP (3000 Ci/mmol; New England Nuclear). The reaction was incubated for 30 sec at 36°C, stopped with 200 mM EDTA and the tubes immediately placed on ice and processed for immunoprecipitation. Immunoprecipitated proteins were analyzed by SDS gel electrophoresis. To quantify the bands of immunoprecipitated protein the autoradiograms of the gel were read in an Ambis 4000 radioanalytic imaging detector (Ambis, San Diego, CA).

Back-phosphorylation. Purified cortical synaptosomes were prepared from freshly dissected rat cortices by the method of Gray and Whittaker following the protocols described by Krueger et al. (1979). Synaptosomes (2.5 mg protein/200 μl sample) in 145 mM NaCl, 2.6 mM KCl, 1.3 mM $MgSO_4$, 10 mM HEPES pH 7.4, 1 mM $CaCl_2$, 10 mM glucose were incubated with various concentrations of CPT-cAMP for 10 min at 36°C, and the reactions stopped with 0.1% SDS on ice. The synaptosomes were immediately diluted threefold and solubilized for 30 min at 4°C in (final concentrations) 1% Triton X-100, 5 mM $MgCl_2$, 25 mM Hepes-Tris (pH 7.4), 5 mM EGTA, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM $NaVO_4$, 1 μM pepstatin A, 1 mM 1,10 phenanthroline, 0.2 mM PMSF, and 1 mM iodoacetamide. Nonsolubilized membranes were removed by centrifugation for 30 min in an Eppendorf centrifuge at 4°C. The supernatants were then phosphorylated with exogenous catalytic subunit of PKA and then immunoprecipitated as described above.

Results

Immunolocalization of KV3.2 proteins to thalamocortical projections

The KV3.2 gene (McCormack et al., 1990, 1991) is one of four known Shaw-related genes (Vega-Saenz de Miera et al., 1992, 1994). It generates four different proteins by alternative usage of 3′ exons resulting in proteins with divergent carboxyl domains. All KV3.2 transcripts express indistinguishable currents in *Xenopus* oocytes and appear to be coexpressed in similar neuronal populations in the CNS (McCormack et al., 1990, 1991; Luneau et al., 1991; Rudy et al., 1992; Moreno and Kentros, unpublished observations). Antibodies that recognize all KV3.2 isoforms (see Materials and Methods) were used to study the localization of KV3.2 proteins in the rat brain.

Surprisingly, strong immunostaining was seen (Fig. 2*B*) in the reticular thalamus (RT), a nucleus of the embryologically and functionally distinct "ventral thalamus" (Rose, 1942; Jones, 1985; Steriade, et al., 1990) which expresses little KV3.2 mRNAs (see Fig. 2*A* and Rudy et al., 1992; Weiser et al., 1994), while the dorsal thalamus, or thalamus proper, showed relatively weaker and diffuse immunostaining (Fig. 2*B*). In particular, thalamic relay neurons, the cells expressing the highest levels of KV3.2 mRNAs (Rudy et al., 1992; Weiser et al., 1994), displayed weak somatic labeling. The immunostaining in the RT was not in neurons, but in fiber-like structures that cross the RT transversely (Fig. 2*B*).

In addition, KV3.2 antibodies immunostained a diffuse, fiber-like band in layer IV of the cerebral cortex (indicated with curved arrows in Fig. 2*B*), another area which does not show appreciable mRNA expression (see Fig. 2*A* and Rudy et al., 1992; Weiser et al., 1994), as well as staining the somata of a subset of neurons in deep cortical layers (indicated by arrowheads in Fig. 2*B*) with a similar distribution to a subset of neurons expressing KV3.2 mRNAs (see Fig. 2*A* and Rudy et al., 1992; Weiser et al., 1994).

Layer IV of the neocortex is the main area of termination of thalamocortical projections, the axons of thalamic relay neurons (Jones and Burton, 1976; Herkenham, 1980; Jones, 1985; Steriade, et al., 1990). Together with the results of mRNA localization we hypothesized that the band of immunostaining seen in the neocortex is produced by the presence of KV3.2 proteins in the terminals of thalamocortical projections. Thalamocortical axons pass through the RT on their way to the cortex and provide collateral inputs to reticular thalamic neurons (Jones, 1985; Steriade, et al., 1990), thus the pattern of immunostaining seen in

←

separated by less stained septa. *D* and *E*, High power micrographs of the VPL in Nissl-stained sections ipsilateral (*D*) and contralateral (*E*) to the injection site. Note the marked reduction in thalamic relay neurons (indicated by *arrows* in *E*) and the increase in glial cells in the ipsilateral side (*D*). *F*, Photomicrograph of a Nissl stained section from the same brain showing somatosensory cortex ipsilateral to the lesion site. Note the lack of apparent cytotoxic effects as judged by the normal cytoarchitecture of the six cortical layers. Essentially the same results were obtained in two additional lesioning experiments, one with a more extensive lesion (Fig. 4*A–C*) and one with a smaller lesion (not shown). Abbreviations: *VPL*, ventroposterolateral thalamic nucleus; *LD*, laterodorsal thalamic nuclei; *MD*, mediodorsal thalamic nuclei; *RT*, reticular thalamic nucleus; *CS*, corpus striatum; *Wh*, cortical white matter. Scale bars: *A*, 2.1 mm; *B* and *C*, 525 μm; *D* and *E*, 60 μm; *F*, 525 μm.

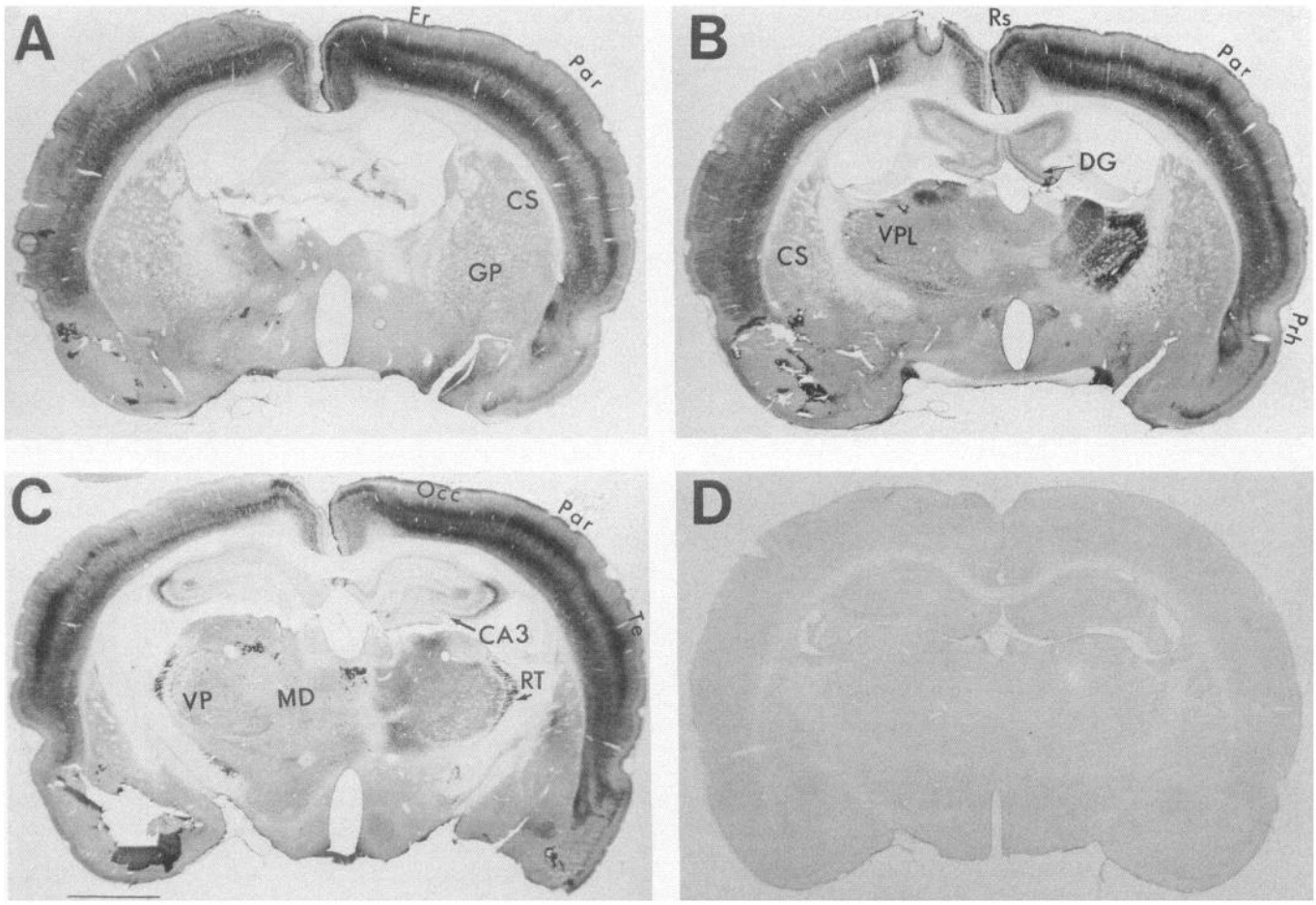

Figure 4. The staining in layer IV of the cortex after unilateral thalamic lesions is not affected in areas receiving projections from undisturbed sections of the thalamus. *A–C*, Immunohistochemistry with KV3.2 antibodies of coronal sections at three different anteroposterior levels of the brain from a rat unilaterally injected with kainic acid. Note in *B* and *C* the loss of staining in the left thalamus, the site of the primary lesion. The site of entry of the injection needle is apparent on the section shown in *B*. Note the marked reduction in staining of cortical layer IV on the parietal and occipital areas of the cortex on the side ipsilateral to the lesion, but not in the ipsilateral temporal cortex. The staining in deep cortical layers is largely unchanged. *D*, Immunohistochemistry of a section from a normal rat at a level similar to that for the section shown in C, treated with affinity purified KV3.2 antibodies preincubated with an excess of KV3.2 peptide. Note that all areas labeled by KV3.2 antibodies in *A–C* and in Figures 2*B* and 3*A–C* show close-to-background signals in this experiment. Abbreviations: *CA3*, CA3 field of the hippocampus; *CS*, corpus striatum; *DG*, dentate gyrus; *Fr*, Frontal area of the cortex; *GP*, globus pallidus; *MD*, mediodorsal thalamic nuclei; *Occ*, occipital area of the cortex; *Par*, parietal area of the cortex; *Prh*, perirhinal cortex; *Rs*, retrosplenial cortex; *RT*, reticular thalamic nucleus; *Te*, temporal cortex; *VP*, ventroposterior thalamic complex; *VPL*, ventroposterolateral thalamic nucleus. Scale bar, 2.05 mm.

the RT supports this interpretation. Furthermore, the KV3.2 antibodies immunostained the barrels of the primary somatosensory cortex (discrete multicellular units representing the cortical fields of sensory units; Woolsey, and Van der Loos, 1970; Welker, 1971; Welker and Woolsey, 1974) in tangential sections of layer IV (Fig. 2*C*). Fink-Heimer staining of degenerating thalamocortical terminals reveals the segregation of these terminals into discrete clusters which fill the cortical barrels (Killackey, 1973). The images produced by KV3.2 antibodies in cortical layer IV (Figs. 2*C*, 3*C*) are very similar to those produced by degenerating thalamocortical terminals.

To confirm that the band of immunostaining with KV3.2 antibodies in layer IV of the cortex is due to the presence of KV3.2 proteins in the terminal fields of thalamocortical axons, unilateral kainic acid lesions of the thalamus were performed. Kainic acid is an excitotoxic neurotoxin which specifically destroys cells (such as thalamic relay neurons) expressing cell surface kainate receptors. Thus, upon injection into the thalamus, thalamic relay neurons are destroyed while afferent fibers and fibers of passage, as well as glial cells are spared (Coyle et al., 1978). The method has been applied extensively to selectively destroy thalamic-relay neurons in rat (Marty et al., 1991; Vukelic et al., 1991). In the experiment illustrated in Figure 3, 5 d after the lesion the brains were dissected out and processed for histology and KV3.2 immunohistochemistry. The treatment destroyed most thalamic relay neurons on the lesioned side of the thalamus (compare Fig. 3*D,E*). Consistent with the hypothesis laid out here, the immunostaining of the layer IV band is much weaker in the cortex ipsilateral to the lesion, while the contralateral side was unaffected (compare Fig. 3*B,C*). The cortex on the lesioned side appeared undamaged as judged by Nissl stain (Fig. 3*F*) and immunostaining with antibodies against KV3.1b proteins which are expressed somatodendritically by cortical layer II–VI cells (data not shown). Moreover, the KV3.2-positive neurons in deep layers of the cortex were unaffected by the lesion (arrowheads on Fig. 3*B*; see also Fig. 4*A–C*). The effect of thalamic kainic

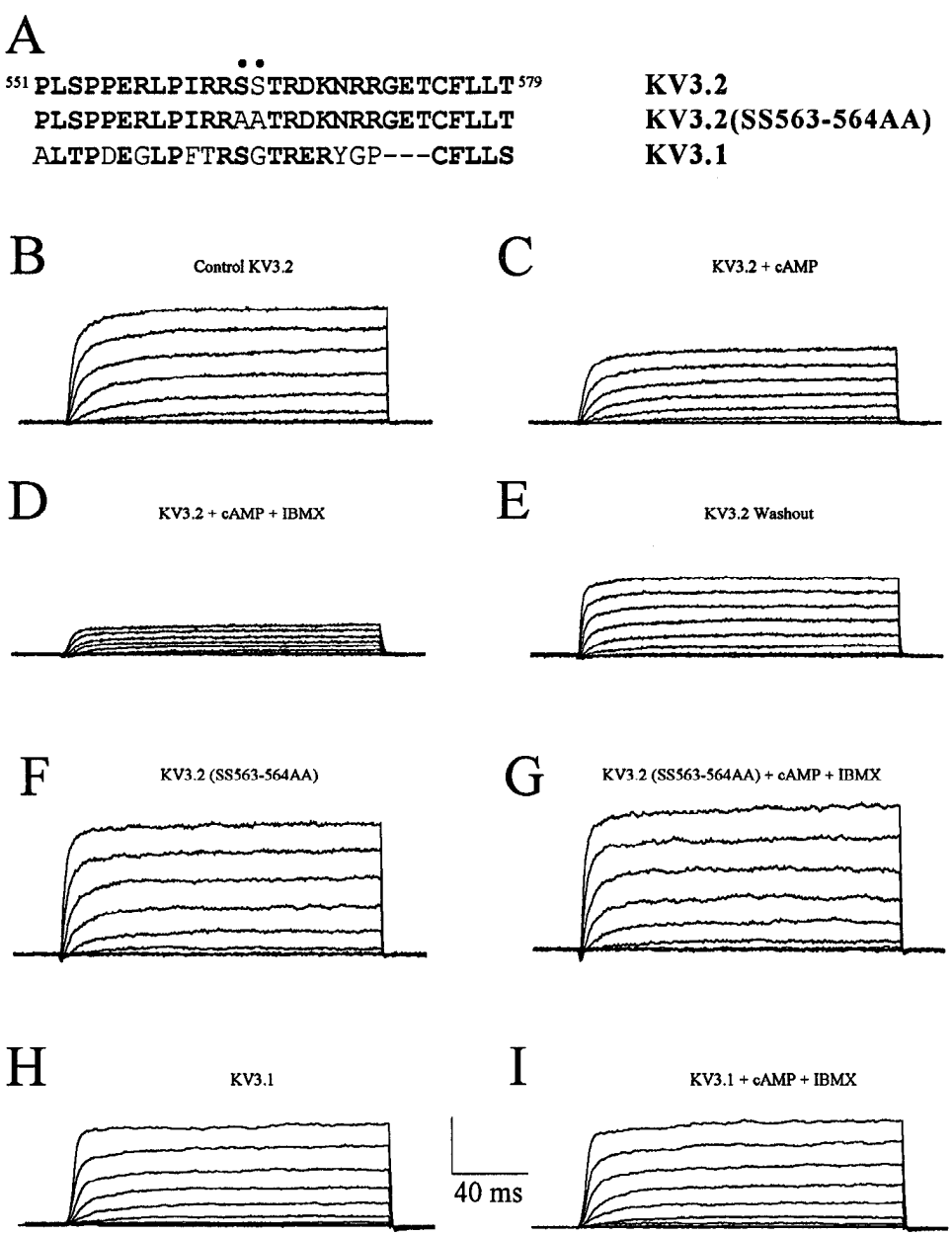

Figure 5. Effects of PKA induction on KV3.2 channels expressed in CHO cells. *A*, The amino acid sequence of residues 551–579 of KV3.2 proteins (McCormack et al., 1991; Vega-Saenz de Miera et al., 1994) containing putative PKA phosphorylation sites (●) (Kemp and Pearson, 1990; Kennelly and Krebs, 1991) is compared to the amino acid sequence of the equivalent region of KV3.1b (see Vega-Saenz de Miera et al., 1994, for details on the genomic structure of Shaw-related genes). Also shown is the sequence of the KV3.2 (SS563-564AA) mutant where the two serines in the putative PKA phosphorylation site have been changed to alanine. The sequence of KV3.1b and KV3.2 proteins in this region is very similar (conserved residues are shown in *boldface*); however, KV3.1b proteins lack a PKA phosphorylation site. In the KV3.2 gene, alternative splicing takes place downstream from the sequence shown here, and therefore this sequence is present in all KV3.2 isoforms. In these proteins the sequence RRSS conforms to the strongest consensus sequence for PKA phosphorylation (R-[R/K]-X-[S/T]). In the KV3.1b protein there are two substitutions (T for R and G for S) such that the resulting sequence (TRSG) does not conform even with the weaker consensus sequences for PKA phosphorylation (R-X-[S/T]) or (R-X-X-[S/T]). The threonine immediately following the glycine in KV3.1b is not a good substrate for phosphorylation because of the arginine on the C-terminal side (Kennelly and Krebs, 1991; and literature cited there). *B*, KV3.2 currents from a KV3.2 transfected CHO cell elicited in response to depolarizing steps from −50 to +40 mV in 10 mV increments from a holding potential of −90 mV. *C*, KV3.2 currents in the same cell 8 min after bath application of 1 mM 8-(4 chlorophenylthio)-cAMP (CPT cAMP). *D*, KV3.2 currents after bath application of 1 mM CPT cAMP and 1 mM IBMX following the recording shown in *C*. *E*, Currents obtained from the same cell following a 15 min washout of both cAMP and IBMX. *F*, Currents obtained from a CHO cell microinjected with mutant KV3.2 (SS563-564AA) cRNA. *G*, Currents in the same cell as in *F* 16 min after bath application of 1 mM CPT cAMP and 1 mM IBMX. *H*, KV3.1b currents from a CHO cell transfected with KV3.1b cDNA. *I*, KV3.1b currents in the same cell as in *H* 15 min after bath application of 1 mM CPT cAMP and 1 mM IBMX. All recordings utilized the same pulse protocol as that described in *B*. Effects of cAMP or IBMX were seen about 1 min after the application of the reagent, but reached maximal effects within 5–7 min. Calibration: *B–E*, 600 pA; *F* and *G*, 200 pA; *H* and *I*, 500 pA.

acid lesions on the staining in layer IV of the neocortex was specific to those cortical areas innervated by the lesioned thalamus. For example, in an animal in which the medial geniculate body remained intact, the staining in layer IV was affected in parietal and occipital cortex but not in temporal cortex (Fig. 4*A–C*).

All areas labeled by KV3.2 antibodies, including the band on cortical layer IV, display little staining above background in control sections treated with antibodies preincubated with KV3.2 peptide (Fig. 4*D*).

Modulation of KV3.2 channels by cAMP-dependent phosphorylation

To obtain further clues as to the functions of KV3.2 channels in thalamocortical projections we studied their modulation by second-messenger systems. KV3.2 proteins have two consecutive serines present in a sequence matching the consensus sequence for phosphorylation by cAMP-dependent protein kinase (PKA) (Fig. 5*A*). Given the results of localization of KV3.2 proteins suggesting that this K^+ channel may influence action potential duration in thalamocortical projections and transmitter release in thalamocortical synapses, as well as the role played by PKA phosphorylation in synaptic function in other systems (Klein et al., 1982; Siegelbaum et al., 1982; Nestler and Greengard, 1984; Kaczmareck and Levitan, 1987; Caterall, 1988; Hille, 1992; Chavez-Noriega and Stevens, 1994) we explored the effects of cAMP upon the electrophysiological properties of KV3.2 channels expressed in Chinese hamster ovary (CHO) cells.

CHO cells permanently transfected with a KV3.2 cDNA expressed voltage-dependent K^+ currents (Fig. 5*B*) absent in nontransfected cells, closely resembling the currents seen in *Xenopus* oocytes injected with *in vitro* KV3.2 transcripts (McCormack et al., 1990; Vega-Saenz de Miera et al., 1994). These currents were about 40% inhibited by bath application of a membrane permeable cAMP analog (Fig. 5*C*, Table 1). This inhibition was reversed upon removal of the cAMP (data not shown) and potentiated by the further addition of 3-isobutyl methyl xanthine (IBMX), an inhibitor of phosphodiesterase (Fig. 5*D*, Table 1). Most of the current recovered several minutes after washing out the cAMP and IBMX (Fig. 5*E*). Similar effects were seen on CHO cells microinjected with KV3.2a cRNA (Table 1).

In contrast to the observations on KV3.2 currents, no effects of cAMP or IBMX were seen on the currents expressed in CHO cells by the related KV3.1b cDNA (Fig. 5*H,I*; Table 1), which expresses currents with very similar voltage-dependence and kinetics, but encodes proteins lacking the PKA-phosphorylation site present in KV3.2 proteins (Fig. 5*A*; see also Vega-Saenz de Miera et al., 1994).

The effects of the elevation of cAMP concentrations on KV3.2 currents in CHO cells are due to the activation of PKA and not to other possible actions of cyclic nucleotides since they are suppressed by H8, an inhibitor of cyclic nucleotide dependent protein kinases, at concentrations which do not produce a significant block of protein kinase C (PKC), and prevented by KT5720, a potent and selective inhibitor of PKA, a purified microbial metabolite of *Nocardiopsis* sp (Gadbois et al., 1992) (Table 1).

Furthermore, the effects of PKA are most likely the result of direct phosphorylation of KV3.2 channel proteins since no effects are seen on KV3.2 currents expressed in CHO cells microinjected with *in vitro* transcribed RNA (cRNA) from a KV3.2 cDNA in which the serines in the consensus PKA site have been mutated (Fig. 5*F,G*; Table 1). In addition, as shown in Figure 8*B*, KV3.2 proteins from brain tissue can be phosphorylated *in vitro* by the catalytic subunit of PKA.

The inhibition of KV3.2 channels upon activation of PKA activation is probably not the result of a positive shift in voltage dependence, since the currents remaining after the addition of cAMP (Fig. 6) or IBMX (data not shown) have similar kinetics and voltage dependence than the currents seen prior to the addition of PKA modulators. Apparently, the inhibition of KV3.2 channels is also not due to a reduction of single channel conductance (Fig. 7).

In situ *phosphorylation of KV3.2 proteins by intrinsic cAMP-dependent protein kinases*

If the KV3.2 channels present in the terminals of thalamocortical projections were modulated in a similar fashion *in vivo*, this could affect transmission across thalamocortical synapses. For this to occur the thalamocortical presynaptic terminal must have all the molecular machinery necessary for phosphorylation properly located in relation to the channel (Scott, 1991). To test whether KV3.2 proteins can be phosphorylated in situ, we explored their phosphorylation in intact cortical synaptosomes by endogenous cAMP-dependent protein kinases. Synaptosomes are resealed presynaptic terminals that maintain many functions, including Ca^{2+} transport, ATP synthesis, and neurotransmitter release (Girod et al., 1992; Whittaker, 1993). To study the phosphorylation of KV3.2 proteins *in situ* we utilized the technique developed by Costa and Catterall to study the phosphorylation of Na^+ channels in intact synaptosomes (Costa and Catterall, 1984; Rossie and Catterall, 1987). The method, based on the technique developed by Forn and Greengard (1978) to study phosphorylation in rat brain slices, consists of indirectly estimating the extent of endogenous phosphorylation by measuring the amount of protein made unavailable for phosphorylation by exogenous catalytic subunit of PKA (in the presence of γ-^{32}P-ATP) after solubilizing the synaptosomes. Thus, a reagent increasing the phosphorylation of the channel in intact synaptosomes decreases the incorporation of labelled phosphate following rupture of the synaptosomes. This method, known as back-phosphorylation, is useful to study the phosphorylation of rare proteins, such as channels, when it is not possible to radiolabel the intracellular ATP pool to high specific activities (Costa and Catterall, 1984; Rossie and Catterall, 1987).

Incubation of intact cortical synaptosomes with permeable analogs of cAMP produced a marked dose-dependent decrease in the incorporation of ^{32}P into KV3.2 proteins by added PKA (Fig. 8*C,D*). Nearly all the available PKA phosphorylation sites in KV3.2 proteins appear to be phosphorylated in synaptosomes incubated for 10 min with $\geq$1 mM cAMP analog (Fig. 8*C,D*) or $\geq$0.5 mM IBMX (data not shown). The dose–response found here is very similar to that reported by Costa and Catterall (1984) for the *in situ* phosphorylation of Na^+ channels. As in their study, relatively high concentrations of cAMP analogs are required, probably due to their low permeability across the membrane (Adams et al., 1991). These experiments provide strong evidence that the molecular components necessary to phosphorylate KV3.2 proteins via the cAMP cascade are present *in vivo*.

Discussion

In situ hybridization studies had previously shown that mRNAs encoding some K^+ channel subunits have a wide distribution in the mammalian CNS, while others are restricted to specific sub-

Table 1. Inhibition of KV3.2 currents in CHO cells by cAMP and IBMX

Cell	Condition	Fraction blocked	*n*
KV3.2 CHO K1	+cAMP	0.37 ± 0.15	15
KV3.2 CHO K1	+cAMP, IBMX	0.73 ± 0.12	8
KV3.2 CHO K1	cAMP + H8	0.1 ± 0.05	4
KV3.2 CHO K1	cAMP, IBMX + H8	0.25 ± 0.17	4
KV3.2 CHO K1	cAMP + KT5720	0.05 ± 0.08	3
KV3.2 CHO K1	cAMP, IBMX + KT5720	0.07 ± 0.07	3
KV3.1 CHO K1	+cAMP, IBMX	0.06 ± 0.03	3
CHO K1 + KV3.1	+cAMP	0	2
CHO K1 + KV3.1	+cAMP, IBMX	0	2
CHO K1 + WT	+cAMP	0.3	2
CHO K1 + WT	+cAMP, IBMX	0.7	2
CHO K1 + SS(563,564)AA	+cAMP	0.02 ± 0.07	7
CHO K1 + SS(563,564)AA	+cAMP, IBMX	0.01 ± 0.06	4

Shown is the fraction of current blocked at +20 mV (mean ± SD) by the extracellular addition of the indicated reagents to CHO cells transfected with KV3.2 cDNA (KV3.2 CHO K1), CHO cells transfected with KV3.1b cDNA (KV3.1 CHO K1), or nontransfected CHO K1 cells injected either with KV3.1b cRNA (CHO K1 + KV3.1), wild-type KV3.2 cRNA (CHO K1 + WT), or a KV3.2 mutant where serine 563 and serine 564 have been replaced by alanines [CHO K1 + SS(563,564)AA]. Reagents added were 1 mM CPT-cAMP, 1 mM IBMX, 20 μM *N*-[2-(methylamino)ethyl]-5 isoquinoline-sulfonamide hydrochloride (H8; RBI, Natick, MA), or 200 nM KT5720 (Calbiochem-Novabiochem Corp.).

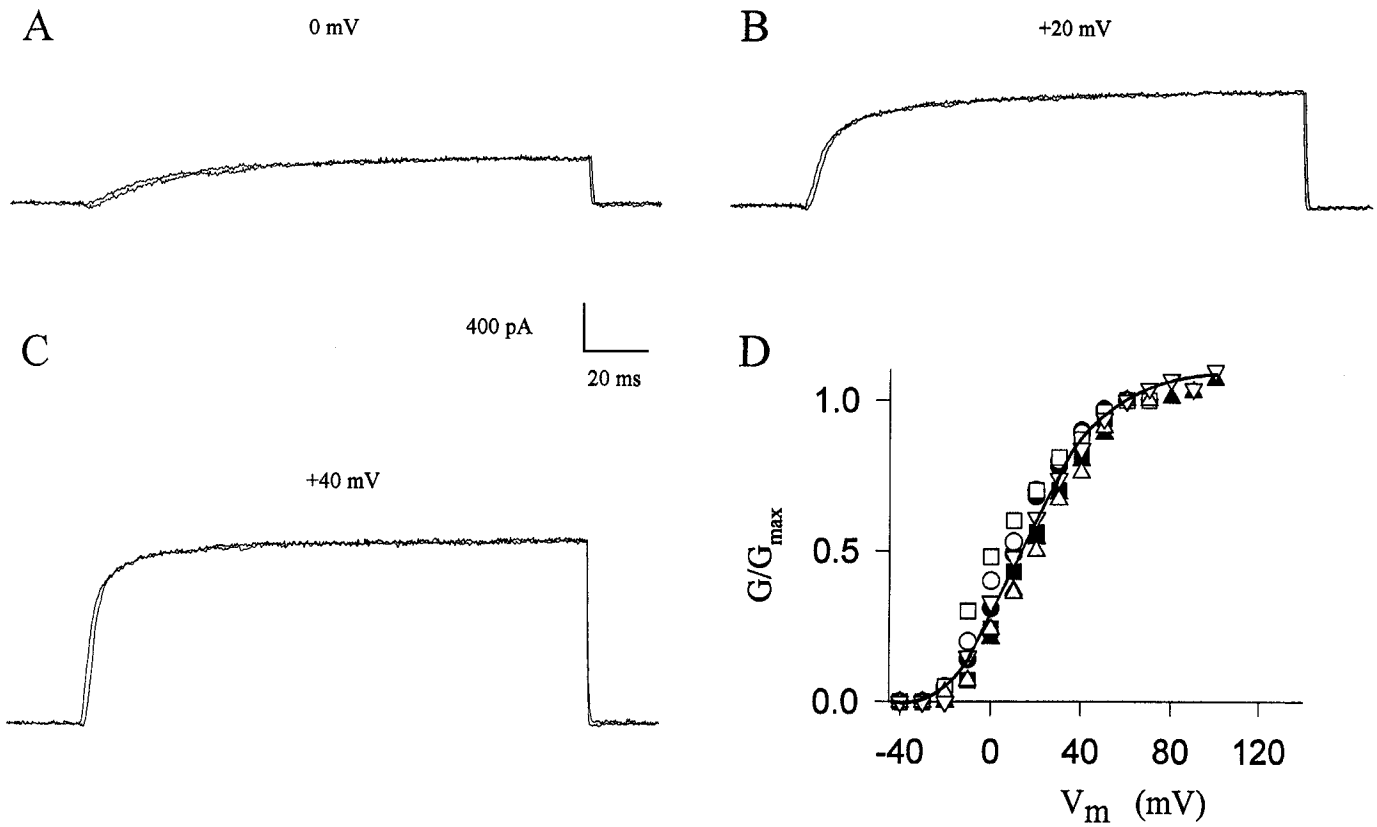

Figure 6. The inhibition of KV3.2 currents by cAMP is not produced by a positive shift in voltage-dependence of channel activation. *A–C*, The currents after addition of 1 mM CPT-cAMP recorded during depolarizing potentials to the indicated voltage were scaled up and superimposed on the currents recorded during the same voltage steps before the addition of cAMP analog. Similar results were obtained in the presence of IBMX (data not shown). *D*, Curves of normalized conductance (G/G_{max}) as a function of membrane potential calculated from the currents elicited in three cells before the addition of cAMP or IBMX (*solid symbols*), after the addition of 1 mM CPT-cAMP (*open circles* and *upright triangles*), and two of these cells after the addition of IBMX (*open squares* and *inverted triangles*). Conductance was calculated using a reversal potential of −80 mV.

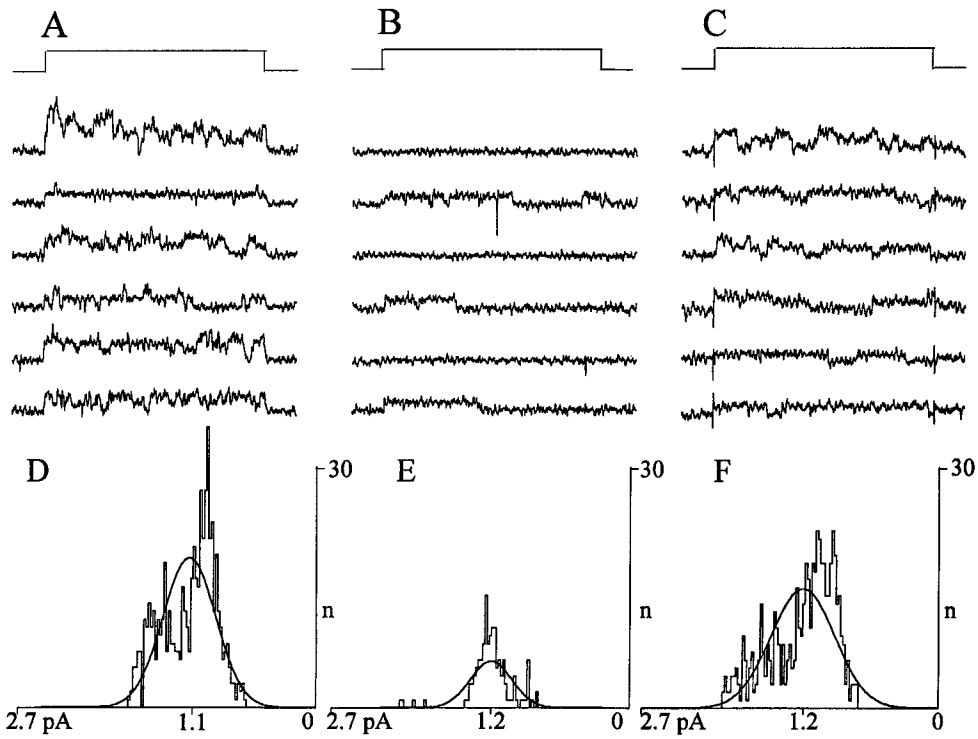

Figure 7. Inhibition of KV3.2 single channels in cell-attached patches by cAMP application. *A–C*, Single channels in a cell-attached patch from a KV3.2-transfected CHO cell during 40 mV depolarizations of 800 msec delivered at a rate of 1/10 sec. *A*, Representative sweeps recorded before the application of cAMP. *B*, Sweeps following local continuous application of 1 mM CPT cAMP. Note the reduction in channel openings in the presence of cAMP. *C*, Sweeps recorded following a 15 min washout of the CPT cAMP. Note that by this stage the leak of the patch had increased, making leak subtractions more inaccurate, but the reversal of the inhibition of channel openings is nevertheless clear. *D–F*, Histograms of single channel amplitude distributions before cAMP application (*D*), in the presence of cAMP analog (*E*) and after washout of the cAMP (*F*). The data was obtained from 16 sweeps (for each condition) from the experiment depicted in (*A–C*). The data were fitted to a Gaussian curve. Note that the mean single-channel current is not reduced in the presence of cAMP. In the three conditions studied a single channel conductance of 14–15 pS is obtained assuming a resting potential of −40 mV (based on average measurements from several transfected cells) and using a reversal potential of −80 mV. Similarly, a slope single channel conductance of ≈15 pS was calculated from the currents obtained during ramp voltage steps in all conditions (data not shown).

populations of neurons, leading to the idea that there are K^+ channels tailored to certain neuronal circuits, and providing hints as to the roles of K^+ channel diversity. The experiments presented here were aimed at obtaining further clues as to the specific roles of KV3.2 subunits, one intriguing example of a K^+ channel protein with a highly restricted pattern of expression in the rat brain. It was previously known that KV3.2 transcripts are predominantly expressed in thalamic relay neurons throughout the dorsal thalamus. In the present studies, immunohistochemistry with site specific antibodies was utilized to investigate the localization of KV3.2 subunits in thalamic relay neurons. The experiments provide compelling evidence that in these neurons the subunits are present mainly in thalamocortical axons and their terminal fields in the cortex and the reticular thalamus, implying that these are key structures where KV3.2 subunits play specific roles.

Additional clues as to the possible roles of KV3.2 proteins were obtained from the studies of phosphorylation and modulation of these proteins by cAMP-dependent protein kinase. We have shown that KV3.2 subunits are phosphorylated by PKA and that this phosphorylation results in a marked inhibition of KV3.2 channels in CHO cells. Moreover, we have obtained evidence that KV3.2 proteins are phosphorylated in cortical synaptosomes, demonstrating that the machinery to phosphorylate the channels is present *in vivo*.

The potential significance of the modulation of KV3.2 subunits by the cAMP pathway in the specific roles played by these proteins in the CNS is underscored by the fact that the motif in KV3.2 proteins responsible for this modulation is absent in KV3.1 proteins, which otherwise express very similar currents, but have a different distribution in the mammalian CNS (Weiser et al., 1994). Consistent with the lack of the strong consensus motif for PKA phosphorylation in KV3.1 proteins, the kinase had no effect on KV3.1 channels in CHO cells.

The idea that KV3.1 and KV3.2 subunits have specialized functions is supported by the fact that most of the differences between KV3.1 and KV3.2 proteins have been conserved throughout vertebrate evolution (Vega-Saenz de Miera et al., 1994) implying that areas with gene-specific sequence encode functionally distinct domains; however, no significant differences between KV3.1 and KV3.2 currents had previously been observed by electrophysiological analysis in heterologous expression systems. Here we showed that KV3.2 but not KV3.1 currents are modulated by PKA-dependent phosphorylation. Moreover, experiments with phorbol esters reveal the opposite situation in the case of protein kinase C, which has effects on KV3.1b but not KV3.2a currents (Moreno et al., 1994). It is thus possible that the various KV3.1 and KV3.2 channels are different versions of the same channel types adapted for modulation by specific stimuli in distinct neuronal circuits.

The results of the studies described in this article allow us to consider testable hypotheses about the roles of voltage-gated K^+

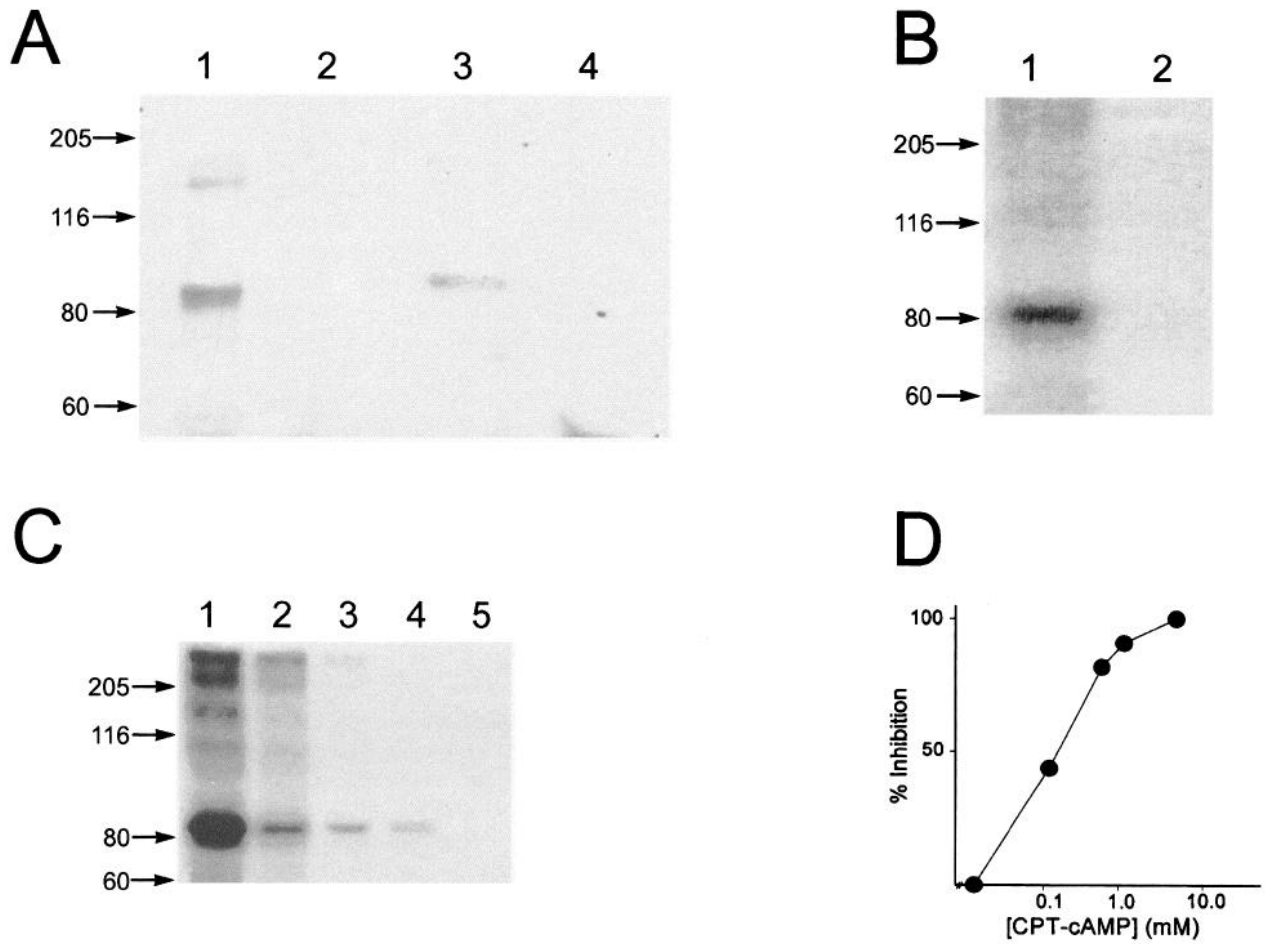

Figure 8. Immunoprecipitation and *in situ* phosphorylation of KV3.2 proteins. *A*, Immunoblots of proteins immunoprecipitated from Triton X-100 membrane extracts. *Lane 1*, Immunoprecipitation of a rat brain membrane extract with KV3.2 antibodies; *lane 2*, immunoprecipitation of a rat brain membrane extract with KV3.2 antibodies preincubated with an excess of KV3.2 peptide; *lane 3*, immunoprecipitation of an extract of membranes derived from CHO cells permanently transfected with KV3.2 cDNA; the immunoprecipitation of the band shown here is blocked by preincubating the antibodies with an excess of KV3.2 peptide (data not shown); *lane 4*, Immunoprecipitation of an extract of membranes derived from control CHO-K1 cells. *B*, Autoradiograms of immunoprecipitates of solubilized rat brain membranes phosphorylated by catalytic subunit of PKA in the presence of γ-^{32}P-ATP. *Lane 1*, Immunoprecipitation with KV3.2 antibodies; *lane 2*, immunoprecipitation with KV3.2 antibodies preincubated with an excess of KV3.2 peptide. *C*, Back-phosphorylation of solubilized KV3.2 proteins derived from cortical synaptosomes incubated in the presence of varying concentrations of cAMP prior to solubilization. *Lane 1*, 0 CPT-cAMP; *lane 2*, 0.1 mM CPT-cAMP; *lane 3*, 0.5 mM CPT-cAMP; *lane 4*, 1.0 mM CPT-cAMP; *lane 5*, 5.0 mM CPT-cAMP. *D*, Effect of incubation of intact synaptosomes with increasing concentrations of CPT-cAMP on the rephosphorylation of KV3.2 proteins by added catalytic subunit of PKA. Shown is the percentage of the signal seen when the synaptosomes are incubated in the absence of cAMP (100%) that remains when the synaptosomes are incubated with the indicated concentration of cAMP.

channels containing KV3.2 proteins in the function of the thalamocortical circuit.

Modulation of KV3.2 channels by PKA phosphorylation

We have shown here that cAMP-dependent phosphorylation inhibits KV3.2 channels expressed in CHO cells. Inhibition of voltage-gated K^+ channels by the activation of the cAMP cascade has been reported in native K^+ channels *in situ* (Deterre, 1981; Klein et al., 1982; Siegelbaum et al., 1982; Kaczmareck et al., 1984; Strong, 1984; Soliven and Nelson, 1990) as well as in one of the products of the *Drosophila* Shaker gene expressed in heterologous expression systems (Drain et al., 1994), but to our knowledge not yet in other cloned mammalian voltage-gated K^+ channels. It is important to point out however that cAMP-dependent phosphorylation can have the opposite effect in other voltage-gated K^+ channels (Giles et al., 1989; Yazawa and Kameyama, 1990; Frace and Hartzell, 1993; Huang et al., 1994). Therefore, a given neurotransmitter receptor activating the same second messenger cascades can produce opposite changes in the electrical properties of a cell, depending on the channel types that are the target of the cascade.

There are several biophysical mechanisms by which modulation by second messenger cascades could affect ion channel activity, including changes in single-channel conductance and inactivation kinetics, voltage-dependence shifts, and recruitment (or elimination) of channels in the membrane. Of these potential mechanisms, shifts in the voltage dependence of activation are well documented in some cases, including both voltage-gated and Ca^{2+}-activated K^+ channels and N-type Ca^{2+} channels (Bean, 1989; Giles et al., 1989; Perozo and Bezanilla, 1990; Yazawa and Kameyama, 1990; Mathie et al., 1992; Delcour and Tsien, 1993; Esguerra et al., 1994). Changes in the number of active channels in the membrane have also been proposed in some systems (Strong et al., 1987; Blumenthal and Kaczmarek, 1992). In the case of *Drosophila* Shaker channels, it has been demonstrated that phosphorylation modulates the rate of inacti-

vation (Drain et al., 1994). However, in most cases that have been analyzed, channel modulation is produced by changes in the probability of channel opening due to as of yet unexplained biophysical mechanisms, which could include transitions of affected channels into (or out of) nonconductive states and are difficult to distinguish experimentally from changes in the number of channel proteins in the membrane. This appears to be also the case for the inhibition of KV3.2 currents by cAMP-dependent phosphorylation. The inhibition was not the result of a positive shift in voltage dependence. The voltage range tested (Fig. 6*D*) excludes a shift in voltage dependence of up to ~100 mV. Furthermore, no relevant change in kinetics (Fig. 6) or single channel conductance (Fig. 7) was observed after stimulation of PKA.

Experiments with cloned subunits have the advantage that one can manipulate the protein and determine whether the effects of a kinase are due to the direct phosphorylation of the channel protein or are indirect, resulting for example, from the phosphorylation of proteins associated with the channel or the activation of other second messenger pathways. The data with KV3.2 proteins with mutations on the serines in the PKA site (Fig. 5, Table 1) indicate that in the case of KV3.2 proteins the effects of PKA are due to phosphorylation of the channel subunit at a site located in the carboxyl end region of the protein. One may now design experiments to help understand the molecular mechanism by which phosphorylation of channel proteins translates into functional changes. For example in one study with cloned channel proteins (Drain et al., 1994), it was shown that the molecular mechanism of modulation may not be the simple result of introducing negative charges.

Possible properties of native channels containing KV3.2 subunits

Before considering the role that KV3.2 channels may have on thalamocortical circuits it is necessary to discuss what the properties of these channels might be, based on studies in heterologous expression systems. Like all Sh proteins, KV3.2 subunits can form functional tetrameric channels by themselves. In *Xenopus* oocytes (McCormack et al., 1990; Luneau et al., 1991; Vega-Saenz de Miera et al., 1994) and CHO cells (this article) these channels produce delayed rectifier-type currents that are first seen when the membrane is depolarized beyond −20 mV and are very sensitive to TEA and 4-AP. However, as in the case of other Sh subunits, KV3.2 proteins can also combine with other members of the same subfamily (but not with proteins of other subfamilies) leading to the formation of heteromultimeric channels (Weiser et al., 1994). Although KV3.2 mRNAs overlap with transcripts of the other three Shaw-related genes in some neuronal populations in the CNS, for the most part they are expressed in different areas, suggesting that in many neuronal populations heteromultimer formation might not be a factor determining the properties of native KV3.2 channels (Weiser et al., 1994). Specifically, in the dorsal thalamus there is negligible expression of KV3.4 mRNAs and low levels (much lower than KV3.2) of expression of KV3.1 and KV3.3 transcripts in some, but not all thalamic nuclei. In those nuclei where there is coexpression of KV3.2, KV3.1 and/or KV3.3 mRNAs (e.g., the ventral posterior complex and the dorsal lateral geniculate; see Weiser et al., 1994, for details) KV3.2 proteins may indeed form heteromultimers with these subunits. It is thus possible that KV3.2 channels vary in subunit composition in different thalamocortical axons. In *Xenopus* oocytes, the presence of KV3.1 subunits does not alter the electrophysiological properties of KV3.2 channels. Coexpression of KV3.2 and KV3.3 proteins (which by themselves express inactivating currents) results in channels that inactivate relatively slowly ($\tau > 20$ msec at +20 mV, at 37°C, depending on the number of KV3.3 proteins in the channel complex). In all cases the channels are activated when the membrane is depolarized beyond −20 mV and are very sensitive to TEA and 4-AP (Vega-Saenz de Miera et al., 1994; Weiser et al., 1994). Heteromultimer formation may thus cause some kinetic differences in the behavior of channels containing KV3.2 subunits in the projections of different thalamic nuclei.

In addition, native channels containing KV3.2 subunits could have properties different than those of homomultimeric or heteromultimeric KV3.2 channels *in vitro* due to the effects of β-subunits or other factors that could influence channel function (Rudy et al., 1991). For example, the β_1-subunit induces fast inactivation of delayed rectifier channels of the Shaker subfamily (Rettig et al., 1994). These factors may also affect the physiological effects of phosphorylation. Whether these factors are important in the case of KV3.2 or other Shaw-related proteins remains to be discovered.

The role of K^+ channels containing KV3.2 proteins in thalamocortical function

Although native channels containing KV3.2 proteins may have properties somewhat different from those of KV3.2 channels in heterologous expression systems as explained in the previous section, we will assume for the purposes of this discussion that as *in vitro* these channels are not activated until the membrane is depolarized beyond −20 mV. If this were the case, we expect that the channels will be activated mainly during action potentials. They may be activated also by receptor-mediated depolarizations that depolarize the membrane to potentials more positive than −20 mV. KV3.2 channels could be effective in repolarizing action potentials or postsynaptic depolarizations if they exist in sufficient numbers such that the current generated by the small fraction of channels that will open (given the membrane potential and/or time available for channel opening) can overcome depolarizing currents. The predominantly axonal localization of KV3.2 subunits in thalamic relay neurons suggests that their primary role here is in modulating action potentials.

By accelerating the repolarization of action potentials the activation of KV3.2 channels would decrease action potential duration. The expression of KV3.2 subunits in thalamocortical axons may help to faithfully transmit the high firing rates of bursting thalamic relay neurons through the axon and terminals (Steriade and Llinas, 1988; Sherman and Koch, 1990; Steriade et al., 1990; McCormick, 1992a,b). The presence of KV3.2 channels in terminals, and their modulation by cAMP-dependent phosphorylation may allow for the modulation of action potential duration at the presynaptic terminal and thereby affect neurotransmitter release.

Although K^+ channels that activate at more negative voltages (≤ -40 mV) could also repolarize spikes and control action potential duration, the effects of high voltage-activating K^+ channels on the action potential would be specific to these functions by virtue of the fact that they are not opened until the spike has been generated. In a cell containing both currents, their modulation by independent second messenger systems would allow the cell to regulate action potential duration separately from action potential initiation and the amount of transmitter release per spike separately from firing frequency.

Modulation of excitability of the thalamocortical system by neurotransmitters and neuropeptides is thought to play a fundamental role in controlling the level of cortical activation during behavioral states such as sleep, wakefulness, arousal, coma, or changes in attention (Steriade and Llinas, 1988; Steriade et al., 1990; Sherman and Koch, 1990; McCormick, 1992a,b). Some of the actions of neurotransmitters on the thalamocortical circuit have been demonstrated to involve the regulation of K^+ channels by receptor-activated second messenger cascades (McCormick, 1992a,b). We hypothesize that modulation of KV3.2 channels in thalamocortical terminals by the cAMP cascade may be also a mechanism of control of the functional state of the thalamocortical circuit. Known actions of neurotransmitters in this system include effects at postsynaptic sites in thalamic and cortical neurons (McCormick, 1992a,b). While presynaptic modulation of the thalamocortical synapse has yet to be directly demonstrated, there are numerous examples of presynaptic modulation in other systems (Klein et al., 1982; Chesselet, 1984; Hevron et al., 1986; Chavez-Noriega and Stevens, 1994). Moreover, facilitation of thalamocortical synapses has been demonstrated (Ashe and Metherate, 1993; Lee and Ebner, 1992), although the relative contribution of pre- and postsynaptic mechanisms to this facilitation have not been determined. In addition, several neurotransmitter receptors have been localized to the presynaptic aspect of the thalamocortical synapse (Sahin et al., 1992; Vogt et al., 1992), although it is also possible that somatic changes in cAMP concentration could diffuse and activate PKA at the terminal (Bacskai et al., 1993).

Recently developed experimental paradigms, such as the thalamocortical slice preparation of Agmon and Connors (1991, 1992), allowing the recording of synaptically evoked responses from functionally identified cortical neurons should provide the opportunity to test predictions emanating from our hypothesis. Further studies *in vitro*, including finding specific–inhibitors for KV3.2 channels and discovering neurotransmitters or other modulators that regulate the cAMP cascade in thalamocortical synaptosomes will provide further clues for *in vivo* experiments. The potential significance of modulation of KV3.2 subunits by the cAMP pathway in other neuronal circuits expressing KV3.2 proteins has also yet to be explored.

References

Adams SR, Harootunian AT, Buechler YJ, Taylor SS, Tsien RY (1991) Fluorescence ratio imaging of cyclic AMP in single cells. Nature 349: 695–697.

Agmon A, Connors BW (1991) Thalamocortical responses of mouse somatosensory (barrel) cortex *in vitro*. Neuroscience 41:365–380.

Agmon A, Connors BW (1992) Correlation between intrinsic firing patterns and thalamocortical synaptic responses of neurons in mouse barrel cortex. J Neurosci 12:319–329.

Ashe JH, Metherate R (1993) Nucleus basalis stimulation facilitates thalamocortical synaptic transmission in rat auditory cortex. Synapse 14:132–143.

Bacskai BJ, Hochner B, Mahaut-Smith M, Adams SR, Kaang BK, Kandel ER, Tsien RY (1993) Spatially resolved dynamics of cAMP and protein kinase A subunits in *Aplysia* sensory neurons. Science 260: 222–226.

Baxter DA, Byrne JH (1991) Ionic conductance mechanisms contributing to the electrophysiological properties of neurons. Curr Opin Neurobiol 1:105–112.

Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. Nature 340:153–156.

Beckh S, Pongs O (1990) Members of the RCK potassium channel family are differentially expressed in the rat nervous system. EMBO J 9:777–782.

Blumenthal EM, Kaczmarek LK (1992) Modulation by cAMP of a slowly activating potassium channel expressed in *Xenopus* oocytes. J Neurosci 12:290–296.

Butler A, Tsunoda S, McCobb D, Wei A, Salkoff L (1993) mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels. Science 261:221–224.

Catterall WA (1988) Structure and function of voltage-sensitive ion channels. Science 242:50–61.

Chandy KG, Douglas J, Gutman GA, Jan L, Joho R, Kaczmarek L, McKinnon D, North RA, Numa S, Philipson L, Rivera AB, Rudy B, Salkoff L, Swanson R, Steiner D, Tanouye M, Tempel BL (1991) Simplified gene nomenclature. Nature 352:26.

Chavez-Noriega LE, Stevens CF (1994) Increased transmitter release at excitatory synapses produced by direct activation of adenylate cyclase in rat hippocampal slices. J Neurosci 14:310–317.

Chesselet MF (1984) Presynaptic regulation of neurotransmitter release in the brain: fact and hypothesis. Neuroscience 12:327–375.

Costa M, Catterall WA (1984) Cyclic AMP-dependent phosphorylation of the α subunit of the sodium channel in synaptic nerve ending particles. J Biol Chem 259:8210–8218.

Coyle JT, Molliver ME, Kuhar MJ (1978) *In situ* injection of kainic acid: a new method for selectively lesioning neural cell bodies while sparing axons of passage. J Comp Neurol 180:301–324.

Delcour AH, Tsien RW (1993) Altered prevalence of gating modes in neurotransmitter inhibition of N-type calcium channels. Science 259: 980–984.

Deterre P, Paupardin-Tritsch D, Bockaert J, Gerschenfeld HM (1981) Role of cyclic AMP in a serotonin-evoked slow inward current in snail neurones. Nature 290:783–785.

Drain P, Dublin AE, Aldrich RW (1994) Regulation of Shaker K^+ channel inactivation gating by the cAMP-dependent protein kinase. Neuron 12:1097–1109.

Drewe JA, Verma S, Frech G, Joho RH (1992) Distinct spatial and temporal expression patterns of K^+ channel mRNAs from different subfamilies. J Neurosci 12:538–548.

Dubinsky JM, Oxford GS (1985) Dual modulation of K channels by thyrotropin-releasing hormone in clonal pituitary cells. Proc Natl Acad Sci USA 82:4282–4286.

Esguerra M, Wang J, Foster CD, Adelman JP, North RA, Levitan IB (1994) Cloned Ca^{2+}-dependent K^+ channel modulated by a functionally associated protein kinase. Nature 369:563–565.

Forn J, Greengard P (1978) Depolarizing agents and cyclic nucleotides regulate phosphorylation of specific neuronal proteins in rat cerebral cortex slices. Proc Natl Acad Sci USA 75:5195–5199.

Frace AM, Hartzell HC (1993) Opposite effects of phosphatase inhibitors on L-type calcium and delayed rectifier currents in frog cardiac myocytes. J Physiol (Lond) 472:305–326.

Gadbois DM, Crissman HA, Tobey RA, Bradburry EM (1992) Multiple kinase arrest points in the G1 phase of nontransformed mammalian cells are absent in transformed cells. Proc Natl Acad Sci USA 89:8626–8630.

Giles W, Nakajima T, Ono K, Shibata EF (1989) Modulation of a delayed rectifier K^+ current by isoprenaline in bull-frog atrial myocytes. J Physiol (Lond) 415:233–249.

Girod R, Eder-Colli L, Medilanski J, Dunant Y, Tabti N, Poo MM (1992) Pulsatile release of acetylcholine by nerve terminal (synaptosomes) isolated from *Torpedo* electric organs. J Physiol (Lond) 450: 325–340.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cell and cell-free membrane patches. Pfluegers Arch 391:85–100.

Harlow E, Lane D (1988) Antibodies: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratories.

Hartshorne RP, Catterall WA (1984) The sodium channel from rat brain: purification and subunit composition. J Biol Chem 259:1667–1675.

Herkenham M (1980) Laminar organization of thalamic projections to the rat neocortex. Science 207:532–534.

Hevron E, David G, Arnon A, Yaori Y (1986) Acetylcholine modulates two types of presynaptic potassium channels in vertebrate motor nerve terminals. Neurosci Lett 72:87–92.

Hille B (1992) Ionic channels of excitable membranes, 2nd ed. Sunderland, MA: Sinauer.

Huang XY, Morielli AD, Parlta EG (1994) Molecular basis of cardiac

potassium channel stimulation by protein kinase A. Proc Natl Acad Sci USA 91:624–628.
Huguenard JR, McCormick DA (1992) Simulation of the currents involved in rhythmic oscillations in thalamic relay neurons. J Neurophysiol 68:1373–1383.
Huguenard JR, Prince DA (1991) Slow inactivation of TEA-sensitive K current in acutely isolated rat thalamic relay neurons. J Neurophysiol 66:1316–1328.
Huguenard JR, Coulter DA, Prince DA (1991) A fast transient potassium current in thalamic relay neurons: kinetics of activation and inactivation. J Neurophysiol 66:1304–1315.
Hwang PM, Glatt CE, Bredt DS, Yellen G, Snyder SH (1992) A novel K^+ channel with unique localizations in mammalian brain: molecular cloning and characterization. Neuron 8:473–482.
Jan LY, Jan YN (1990) How might the diversity of potassium channels be generated? Trends Neurosci 13:415–419.
Jan LY, Jan YH (1992) Structural elements involved in specific K^+ channel functions. Annu Rev Physiol 54:537–555.
Jones EG (1985) The thalamus. New York: Plenum.
Jones EG, Burton H (1976) Areal differences in the laminar distribution of thalamic afferents in cortical fields of the insular, parietal and temporal regions of primates. J Comp Neurol 168:197–248.
Kaczmareck L, Levitan IB (1987) Neuromodulation: the biochemical control of neuronal excitability. New York: Oxford UP.
Kaczmarek KL, Kaczmarek LK, Strumwasser R (1994) A voltage clamp analysis of current underlying cAMP-induced membrane modulation in isolated peptidergic neurons of *Aplysia*. J Neurophysiol 52: 340–349.
Kemp B, Pearson RB (1990) Protein kinase recognition sequence motifs. Trends in Biochem Sci 15:342–346.
Kennelly PJ, Krebs EG (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J Biol Chem 266:15555–15558.
Killackey HP (1973) Anatomical evidence for cortical subdivision based on vertically discrete thalamic projections from the ventral posterior nucleus to cortical barrels in the rat. Brain Res 51:326–331.
Klein M, Camardo J, Kandel ER (1982) Serotonin modulates a specific potassium current in the sensory neurons that show presynaptic facilitation in *Aplysia*. Proc Natl Acad Sci USA 79:5713–5717.
Krueger BK, Ratzlaff RW, Strichartz GR, Blaustein MB (1979) J Membrane Biol 50:287–310.
Kubo Y, Balwin TJ, Jan YN, Jan LY (1993a) Primary structure and functional expression of mouse inward rectifier potassium channel. Nature 362:127–133.
Kubo Y, Reuveny E, Slesinger PA, Jan YN, Jan LY (1993b) Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. Nature 364:802–806.
Latorre R, Aberhauser A, Labarca P, Alvarez O (1989) Varieties of calcium-activated potassium channels. Annu Rev Physiol 51:385–399.
Lee KM, Ebner FF (1992) Induction of high frequency activity in the somatosensory thalamus of rats *in vivo* results in long-term potentiation of responses in SI cortex. Exp Brain Res 90:253–261.
Levitan IB (1988) Modulation of ion channels in neurons and other cells. Annu Rev Neurosci 11:119–136.
Llinas R (1988) The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function. Science 242:1654–1664.
Luneau CJ, Wiedmann R, Smith JS, Williams JB (1991) Shaw-like rat brain potassium channel cDNAs with divergent 3′ ends. FEBS Lett 288:163–167.
MacKinnon R (1991) New insights into the structure and function of potassium channel. Curr Opin Neurobiol 1:14–19.
Marty S, Dusart I, Peschanski M (1991) Glial changes following an excitotoxic lesion in the CNS. I. microglia/macrophages. Neuroscience 45:529–530.
Mathie A, Bernheim L, Hille B (1992) Inhibition of N- and L-type calcium channels by muscarinic receptor activation in rat sympathetic neurons. Neuron 8:907–914.
McCormack T, Vega-Saenz de Miera E, Rudy B (1990) Molecular cloning of a member of a third class of Shaker-family K^+ channel genes in mammals. Proc Natl Acad Sci USA 87:5227–5231.
McCormack T, Vega-Saenz de Miera E, Rudy B (1991) Molecular cloning of a member of a third class of Shaker-family K^+ channel genes in mammals. Correction. Proc Natl Acad Sci USA 88:4060.
McCormick DA (1990) Membrane properties and neurotransmitter actions. In: The synaptic organization of the brain, 3d ed (Shepherd, GM, ed), pp 32–66. New York: Oxford UP.
McCormick DA (1992a) Neurotransmitter actions in the thalamus and cerebral cortex. J Clin Neurophysiol 9:212–223.
McCormick DA (1992b) Neurotransmitter actions in the thalamus and cerebral cortex and their role in neuromodulation of thalamocortical activity. Prog Neurobiol 39:337–388.
McCormick DA, Huguenard JR (1992) A model of the electrophysiological properties of thalamocortical relay neurons. J Neurophysiol 68:1384–1400.
McCormick DA, Prince DA (1985) Two types of muscarinic response to acetylcholine in mammalian cortical neurons. Proc Natl Acad Sci USA 82:6344–6348.
McCormick DA, Prince DA (1986) Acetylcholine induces burst firing in thalamic reticular neurons by activating a potassium conductance. Nature 319:402–405.
McCormick DA, Prince DA (1987a) Neurotransmitter modulation of thalamic neuronal firing pattern. J Mind Behav 8:573–590.
McCormick DA, Prince DA (1987b) Actions of acetylcholine in the guinea-pig and cat medial and lateral geniculate nuclei, *in vitro*. J Physiol (Lond) 392:147–165.
Miller C (1991) Annus mirabilis of K channels. Science 252:1092–1096.
Nestler EJ, Greengard, P (1984) Protein phosphorylation in the nervous system. New York: Wiley.
Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates, 2d ed. Boca Raton: Academic.
Perney TM, Kaczmarek LK (1991) The molecular biology of K channels. Curr Opin Cell Biol 3:663–670.
Perney TM, Marshall J, Martin KA, Hockfield S, Kaczmarek LK (1992) Expression of the mRNAs for the Kv3.1 potassium channel gene in the adult and developing rat brain. J Neurophysiol 68:756–766.
Perozo E, Bezanilla F (1991) Phosphorylation affects voltage gating of the delayed rectifier K^+ channel. Neuron 5:685–690.
Pongs O (1992) Molecular biology of voltage-dependent potassium channels. Physiol Rev 72:S79–S88.
Rettig J, Heinemann SH, Wunder F, Lorra C, Parcej DN, Dolly JO, Pongs O (1994) Inactivation properties of voltage-gated K^+ channels altered by presence of β-subunit. Nature 369:289–294.
Rose JE (1942) The ontogenetic development of the rabbit's diencephalon. J Comp Neurol 77:61–129.
Rossie S, Catterall W (1987) Cyclic-AMP-dependent phosphorylation of voltage-sensitive sodium channels in primary cultures of rat brain neurons. J Biol Chem 262:12735–12744.
Rudy B (1988) Diversity and ubiquity of K channels. Neuroscience 25:729–750.
Rudy B, Kentros C, Vega-Saenz de Miera E (1991) Families of K channel genes in mammals: toward an understanding of the molecular basis of K channel diversity. Mol Cell Neurosci 2:89–102.
Rudy B, Kentros C, Weiser M, Fruhling D, Serodio P, Vega-Saenz de Miera E, Ellisman MH, Pollock JA, Baker H (1992) Region-specific expression of a K^+ channel gene in brain. Proc Natl Acad Sci USA 89:4603–4607.
Sahin M, Bowen WD, Donoghue JP (1992) Location of nicotinic and muscarinic cholinergic and μ-opiate receptors in rat cerebral neocortex: evidence from thalamic and cortical lesions. Brain Res 579:135–147.
Salkoff L, Baker K, Butler A, Covarrubias M, Pak MD, Wei A (1992) An essential "set" of K^+ channels conserved in flies, mice and humans. Trends Neurosci 15:161–166.
Scott JD (1991) Cyclic nucleotide-dependent protein kinases. Pharmacol Ther 50:123–145.
Sheng M, Tsaur M-L, Jan YN, Jan LY (1992) Subcellular segregation of two A-type K^+ channel proteins in rat central neurons. Neuron 9:271–284.
Sheng M, Tsaur M-L, Jan YN, Jan LY (1994) Contrasting subcellular localization of the KV1.2 K^+ channel subunit in different neurons of rat brain. J Neurosci 14:2408–2417.
Sherman SM, Koch C (1990) Thalamus. In: The synaptic organization of the brain, 3d ed (Shepherd GM, ed), pp 246–278. New York: Oxford UP.
Siegelbaum SA, Camardo J, Kandel ER (1982) Serotonin and cyclic AMP closes single K^+ channels in *Aplysia* sensory neurones. Nature 299:413–417.

Soliven B, Nelson DJ (1990) Beta adrenergic modulation of K^+ current in human T lymphocytes. J Membr Biol 117:263–274.

Steriade M, Llinas RR (1988) The functional states of the thalamus and the associated neuronal interplay. Physiol Rev 68:649–742.

Steriade M, Jones EG, Llinas RR (1990) Thalamic oscillations and signaling. New York: Wiley.

Strong JA (1984) Modulation potassium current kinetics in bag cell neurons of *Aplysia* by an activator of adenylate cyclase. J Neurosci 4:2772–2783.

Strong JA, Fox AP, Tsien RW, Kaczmarek LK (1987) Stimulation of protein kinase C recruits covert calcium channels in *Aplysia* bag cell neurones. Nature 325:714–717.

Tsaur M-L, Sheng, Lowenstein DH, Jan YN, Jan LY (1992) Differential expression of K^+ channel mRNAs in the rat brain and down regulation in the hippocampus following seizures. Neuron 8:1055–1067.

Vega-Saenz de Miera E, Moreno H, Fruhling D, Kentros C, Rudy B (1992) Cloning of ShIII (Shaw-like) cDNAs encoding a novel high-voltage-activating, TEA-sensitive, type-A K^+ channel. Proc R Soc Lond [Biol] 248:9–18.

Vega-Saenz de Miera E, Weiser E, Kentros C, Lau D, Moreno H, Serodio P, Rudy B (1994) Shaw-related K^+ channels in mammals. In: Handbook of membrane channels, pp 41–78. New York: Academic.

Vogt BA, Crino PB, Jensen EL (1992) Multiple heteroreceptors on limbic thalamic axons: M_2 acetylcholine, serotonin$_{1B}$, β_2-adrenoceptors, μ-opioid, and neurotensin. Synapse 10:44–53.

Vukelic JI, Yamamoto T, Hertzberg EL, Nagy JI (1991) Depletion of connexin43-immunoreactivity in astrocytes after kainic acid-induced lesions in rat brain. Neurosci Lett 130:120–124.

Wang H, Kunkel DDM, Schwartzkroin PA, Tempel BL (1994) Localization of Kv1.1 and Kv1.2, two K channel proteins, to synaptic terminals, somata and dendrites in the mouse brain. J Neurosci 14: 4588–4599.

Warmke JW, Ganetsky B (1994) A family of potassium channel genes related to eag in *Drosophila* and mammals. Proc Natl Acad Sci USA 91:3438–3442.

Weiser M, Vega-Saenz de Miera E, Kentros C, Moreno H, Franzen L, Hillman D, Baker H, Rudy B (1994) Differential expression of Shaw-related K^+ channels in the rat central nervous system. J Neurosci 14:949–972.

Weiser M, Bueno E, Sekirnjak C, Martone ME, Baker H, Hillman D, Chen S, Thornhill W, Ellisman M, and Rudy B (1995) The potassium channel subunit KV3.1b is localized to somatic and axonal membranes of specific populations of CNS neurons. J Neurosci 15: in press.

Welker C (1971) Microelectrode delineation of the fine grain somatotopic organization of SmI cerebral neocortex in albino rat. Brain Res 26:259–275.

Welker C, Woolsey TA (1974) Structure of layer 14 in the somatosensory neocortex of the rat: description and comparison with the mouse. J Comp Neurol 158:437–454.

Whittaker VP (1993) Thirty years of synaptosome research. J Neurocytol 22:735–742.

Woolsey TA, Van der Loos H (1970) The structural organization of layer IV in the somatosensory region of the mouse cerebral cortex: the description of a cortical field composed of discrete cytoarchitectonic units. Brain Res 17:205–242.

Yazawa K, Maski K (1990) Mechanism of receptor-mediated modulation of the delayed outward potassium current in guinea-pig ventricular myocytes. J Physiol (Lond) 421:135–150.