Dendrotoxin-Binding Brain Membrane Protein Displays a K^+ Channel Activity That Is Stimulated by both cAMP-Dependent and Endogenous Phosphorylations†

Hubert Rehm,‡ Siegried Pelzer,§ Claude Cochet,∥ Edmond Chambaz,∥ Bruce L. Tempel,⊥ Wolfgang Trautwein,§ Dieter Pelzer,*,§ and Michel Lazdunski*,‡

Centre de Biochimie du Centre National de la Recherche Scientifique, Parc Valrose, F-06034 Nice Cedex, France, Physiologisches Institut II, Universität des Saarlandes, 6650 Homburg/Saar, FRG, LBIO/DRF/CENG-85X, Unité 244 INSERM, 38041 Grenoble, France, and GRECC, 182-B, Medical Center, Veterans Administration, 1660 South Columbian Way, Seattle, Washington 98108

Received February 23, 1989; Revised Manuscript Received April 26, 1989

ABSTRACT: The purified protein that binds the K^+ channel ligands dendrotoxin I and mast cell degranulating peptide can be phosphorylated by cAMP-dependent protein kinase and by an endogenous protein kinase, which may be a specific K^+ channel kinase. Phosphorylations take place on the toxin-binding subunit, a polypeptide of 76–80 kDa. Phosphorylation by both kinases leads to activation of the reconstituted dendrotoxin-sensitive K^+ channel.

Dendrotoxin I (DTX_I)[1] from snake venom and mast cell degranulating peptide (MCD) from bee venom are epileptogenic peptides which facilitate neurotransmitter release (Harvey & Karlsson, 1982; Harvey & Anderson, 1985; Bidard et al., 1987a). When injected at low concentrations, they generate hippocampal θ rhythm (Bidard et al., 1987a) and, in the case of MCD, long-term potentiation (Cherubini et al., 1987). Both peptides are blockers of a unique class of K^+ channels (Benoit & Dubois, 1986; Halliwell et al., 1986; Penner et al., 1986; Stansfeld et al., 1987; Stansfeld & Feltz, 1988). Specific, high-affinity binding sites exist for DTX_I and MCD in brain membranes (Taylor et al., 1984; Black & Dolly, 1986) which are located on the same protein complex, the DMB protein (D for DTX_I, M for MCD, and B for binding) (Bidard et al., 1987b; Rehm et al., 1988; Rehm & Lazdunski, 1988a,b; Schmidt et al., 1988). The DMB protein from rat brain has recently been purified to homogeneity (Rehm & Lazdunski, 1988b). It consists of two types of subunits of 76–80 kDa (α-subunit) and 38 kDa (β-subunit). The toxin-binding component of the DMB protein was shown by affinity cross-linking to be the α-subunit peptide of 76–80 kDa (Rehm et al., 1988). The purified protein is in fact a mixture of structurally closely related proteins which differ in their affinity for β-bungarotoxin (β-BTX) (Rehm & Lazdunski, 1988a), another snake venom toxin with K^+ channel blocking activity (Dreyer & Penner, 1987).

This paper shows that the α-subunit of the DMB protein is phosphorylated by two different kinases and that this phosphorylation enhances its K^+ channel activity.

Experimental Procedures

Materials. The DMB protein was purified from rat brain as described in Rehm and Lazdunski (1988b). DTX_I and MCD were purified and iodinated as described in Rehm et al. (1988b). The catalytic subunit of cAMP kinase, cholesterol, and soybean phosphatidylcholine were from Sigma. The phosphatidylcholine (bovine brain), phosphatidylethanolamine (bovine heart), and phosphatidylserine (bovine brain) that were used for the reconstitution and electrophysiological experiments were from Avanti Polar Lipids.

Preparation of Antiserum and Western Blotting. An amino-terminal peptide (amino acids 1–27, N-terminal end) predicted by a mouse K^+ channel cDNA clone (MBK1) (Tempel et al., 1988) was chemically synthesized and purified by HPLC. The peptide was conjugated to keyhole limpet hemocyanin (Liu et al., 1979), and polyclonal antibodies against this conjugate were raised in rabbits by Berkeley Antibody Co., Richmond, CA. The DMB protein was electrotransferred from SDS–PAGE onto nitrocellulose (Towbin et al., 1979). After staining for protein with Ponceau S, the blots were blocked and incubated with the anti-MBK1 antibodies (dilution 1:500). Blots were developed with a goat anti-rabbit IgG coupled to alkaline phosphatase (Sigma).

Phosphorylation of the DMB Protein. The purified DMB protein (40–140 ng) was incubated with cAMP kinase (37 ng) and [γ-^{32}P]ATP (75 μM, 3–5 cpm/fmol) in 14 mM Na-Hepes, 10 mM Tris-HCl, pH 7.2, 10 mM $MgCl_2$, 0.7 mM EDTA, 80 mM KCl, 0.1% (w/v) Triton X-100, 7% (v/v) glycerol, and 0.02% (w/v) soybean phosphatidylcholine at 30 °C in a total volume of 10–16 μL. The reaction was stopped with Lämmli sample buffer and analyzed by SDS–PAGE. The amount of phosphate incorporated into the individual subunits of DMB protein was determined by counting the Coomassie-stained bands of the dried gel in a toluene-based scintillator. For the phosphorylation of DMB protein by the endogenous protein kinase, similar conditions were used, but cAMP kinase was omitted.

Phosphorylation of the reconstituted DMB protein was carried out with 4–8 ng of reconstituted DMB protein and 100 μM [γ-^{32}P]ATP (5–10 cpm/fmol) in 14 mM Na-Hepes, pH 7.2, 80 mM KCl, 10 mM $MgCl_2$, and 0.7 mM EDTA for 20 min at 30 °C. The amount of phosphate incorporated was determined by a TCA-precipitation method (Sandoval & Cuatrecasas, 1976).

† This work was supported by the Association Française contre la Myopathie, the Centre National de la Recherche Scientifique, the Deutsche Forschungsgemeinschaft (SBF 246 Projekt A1), and the Ministère de la Défense Nationale (Grant DRET 85/138). H.R. is a stipend recipient of the Deutsche Forschungsgemeinschaft.

* To whom correspondence should be addressed.

‡ Centre de Biochimie du Centre National de la Recherche Scientifique.

§ Universität des Saarlandes.

∥ Unité 244 INSERM.

⊥ Veterans Administration.

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTX_I, dendrotoxin I; MCD, mast cell degranulating peptide; β-BTX, β-bungarotoxin; DMB protein, dendrotoxin and mast cell degranulating peptide binding protein.

0006-2960/89/0428-6455$01.50/0 © 1989 American Chemical Society

Reconstitution of the DMB Protein. The purified DMB protein (4–10 μg/mL) was diluted 10-fold with 20 mM Na-Hepes, pH 7.2, containing 120 mM KCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (w/v) octyl glucoside, 1.3 mg/mL phosphatidylcholine, 0.6 mg/mL phosphatidylethanolamine, and 0.1 mg/mL phosphatidylserine. The mixture was dialyzed for 48 h at 4 °C against three changes of 20 mM Na-Hepes, pH 7.2, 1 mM EDTA, and 120 mM KCl. The resulting liposomes with the reconstituted DMB protein were stored in liquid nitrogen. The reconstituted and the soluble DMB proteins had similar affinity (K_D = 60–80 pM) for ^{125}I-DTX$_I$.

Determination of the Electrophysiological Activity of the Purified and Reconstituted DMB Protein. Solvent-free lipid bilayers were formed from monolayers of 70% bovine heart phosphatidylethanolamine, 15% bovine brain phosphatidylserine (Avanti Polar Lipids), and 15% cholesterol (Sigma), dissolved in *n*-hexane (1 mg of lipid/mL), at the tip of patch pipets (5–10 MΩ) by the contact method described in detail elsewhere (Hanke et al., 1984). Glass–bilayer seal resistances were in the order of 2–40 GΩ. The bath and pipet filling solutions contained 100 or 150 mM KCl, 20 mM $CaCl_2$, and 5 mM K^+-Hepes (pH 7.4, room temperature). For reasons given elsewhere (Pelzer et al., 1989), only those bilayers with stable artifact-free current base lines for 15–30 min at V_p of −100 or +100 mV were selected for reconstitution experiments with the DMB protein containing liposomes. Incorporation of DMB protein containing liposomes into the performed artifact-free phospholipid bilayers occurred spontaneously within 1–10 min after addition of ≈10 μL of the liposome suspension to the bath side (bath volume 2.0 mL) underneath the bilayer membrane. Voltage clamp was applied with a conventional patch-clamp amplifier (L/M-EPC 7, List Medical Electronic, Darmstadt, FRG). Elementary currents through reconstituted channels were recorded at constant voltages by clamping the pipet potential (*Vp*) with respect to the bath. Single-channel currents were recorded continuously on an FM tape recorder at $7^1/_2$ or 15 ips, and then played back through a four-pole Bessel filter (−3 dB, low pass) at one-third to one-fourth of the digitizing rate (e.g., Figure 3) for computer analysis as described elsewhere (Cavalié et al., 1986). At digitizing rates <500 Hz (e.g., Figure 4), elementary currents were sampled without filtering. Leak currents were subtracted electronically. Channel openness (*Np*) was assessed in continuous 10-s analysis segments over a period of 3 min in bilayer recordings with predominant 21-pS single-channel activity. These segments were integrated, and the time-average current was divided by the predominant open channel current amplitude (see text for explanation).

The responsiveness of channel activity to phosphorylating agents was tested by their addition to the bath side of the lipid bilayer. No comparable effects were observed within usual recording times when ATPγS or ATPγS and cAMP kinase were included in the pipet. This indicates that the DMB protein is likely to be in a fixed orientation in our bilayers with its cytoplasmic side facing the bath, resulting in an inside-out configuration of single-channel recording. In this recording configuration, positive current pulses (upward deflections = channel openings) at the current monitor output reflect current flowing from the pipet into the bath at positive pipet potentials (positive *Vp*). Positive *Vp* are displayed as positive values on the pipet voltage and correspond to negative membrane potentials.

DTX$_I$-containing liposomes were prepared from a 0.1 mM DTX$_I$ stock solution, and 10–50 μL of the DTX$_I$–liposome suspension was added to the bath side underneath the bilayer.

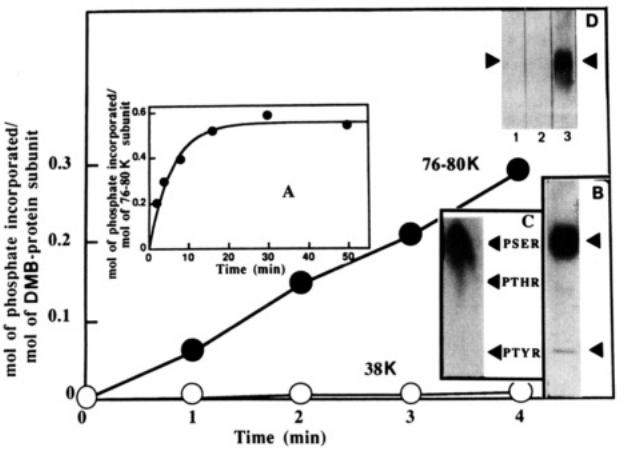

FIGURE 1: Phosphorylation of DMB protein by cAMP kinase. Purified DMB protein (140 ng) was phosphorylated with the catalytic subunit of cAMP kinase (37 ng) as described under Experimental Procedures. After the times indicated, the phosphate incorporated into the subunits of DMB protein was determined. (Inset A) Saturation kinetics of phosphate incorporation into the toxin-binding subunit of DMB protein (60 ng) by cAMP kinase (37 ng). (Inset B) Autoradiogram of an SDS–PAGE of purified DMB protein (140 ng) which was phosphorylated by cAMP kinase (37 ng) for 4 min. Arrowheads indicate the position of the 76–80-kDa (upper) and the 38-kDa (lower) subunits. (Inset C) DMB protein was phosphorylated by cAMP kinase, and the phosphoamino acids were analyzed by thin-layer chromatography. An autoradiogram of the thin-layer plates is shown in which migration positions of marker phosphoamino acids are indicated. (Inset D) Western blot of purified DMB protein (200 ng per lane). The blots were immunostained with antibodies against a peptide derived from the amino-terminal end of the mouse K^+ channel (see Experimental Procedures). In lanes 1 and 2 the antibodies were preincubated with 6 or 0.6 nM amino-terminal peptide, respectively. Arrowheads indicate the position of the toxin-binding α-subunit of the DMB protein.

Miscellaneous. SDS–PAGE was in 8% separating gels (Laemmli, 1970) except for the analysis of the proteinase K digestion products of phosphorylated 76–80-kDa subunits where 14% separating gels were used. Protein was determined with the BCA assay (Pierce) or by scanning Coomassie-stained gels, with bovine serum albumin as a standard.

Results and Discussion

The sequence of the K^+ channel involved in the *Shaker* mutation in *Drosophila* has recently been established (Tempel et al., 1987). *Drosophila* probes have been subsequently used to identify protein sequences for a class of voltage-dependent K^+ channels in mouse and rat brain (Baumann et al., 1988; Tempel et al., 1988). Injection into *Xenopus* oocytes of RNA synthesized in vitro with rat brain cDNA as a template or nuclear injection of cDNA results in the expression of functional K^+ channels (Stuehmer et al., 1988), which are blocked by dendrotoxin, MCD, and 4-aminopyridine and contain one consensus sequence for cAMP kinase phosphorylation. The K^+ channel protein identified by cloning experiments is probably identical with or very similar to the α-subunit identified by purification and affinity labeling since polyclonal antibodies prepared against the N-terminal end (1–27) of the sequence predicted by the molecular biology approach cross-react with the purified α-subunit (Figure 1, inset D).

Incubation of the purified DMB protein with the catalytic subunit of cAMP kinase in the presence of [γ-^{32}P]ATP and Mg^{2+} led to the rapid phosphorylation of the 76–80-kDa toxin-binding subunit of DMB protein (Figure 1) up to a maximal incorporation of 0.45–0.60 mol of phosphate/mol of α-subunit (Figure 1, inset A). It may be that a portion of the α-subunits corresponding to subtypes of DTX$_I$-sensitive K^+ channel cannot be phosphorylated. Phosphorylation of the

38-kDa subunit of DMB protein was negligible (Figure 1). Phosphorylation of DMB protein with cAMP kinase had no influence on the binding of its ligands MCD and DTX_I and vice versa (not shown). The cAMP kinase phosphorylated serine residues (Figure 1, inset C). The rate of phosphorylation of the DMB protein was similar to rates of phosphorylation by cAMP kinase of the dihydropyridine-sensitive Ca^{2+} channel (Callahan et al., 1988).

The 76–80-kDa subunit of DMB protein was still phosphorylated when the purified DMB protein was incubated with [γ-^{32}P]ATP and Mg^{2+} without any added protein kinase (Figure 2). Within 1 h, 0.13–0.25 mol of phosphate/mol of α-subunit was incorporated. Again, phosphorylation of the 38-kDa subunit was negligible. These results indicate the presence of an endogenous kinase activity. The endogenous kinase also phosphorylated serine residues (Figure 2A, upper-left inset) and had a K_m for ATP of 15 μM (not shown). Peptide maps indicated that the serine residues that are phosphorylated by the endogenous kinase and by cAMP kinase respectively are different (Figure 2C). The cDNA-derived amino acid sequence of the voltage-dependent rat brain K^+ channel referred to above contains, aside from the cAMP kinase consensus sequence, another potential serine phosphorylation site (Baumann et al., 1988).

Phosphorylation of the DMB protein by the endogenous protein kinase was not influenced by cAMP kinase inhibitor, by a Ca^{2+}/calmodulin mixture, by cAMP, by the phorbol ester 4β-phorbol 12-myristate 13-acetate (4 μM), or by the toxins DTX_I, MCD, or β-BTX (Figure 2B and data not shown). The phosphorylation of the DMB protein by the endogenous kinase was however inhibited by GTP [IC_{50} = 500 μM (not shown)]. An exchange of Mg^{2+} for Mn^{2+} in the reaction mixture decreased but did not eliminate (as for cAMP kinase) the endogenous kinase activity (Figure 2B).

The endogenous kinase also phosphorylated other substrates such as protamine, histones, and casein (not shown). However when the DMB protein with its endogenous kinase activity was reconstituted into liposomes, the activity of the enzyme for the α-subunit of the DMB protein was increased by a factor of 4–5 (0.6–0.7 mol of phosphate/mol of α-subunit) (Figure 2D) while its activity against protamine was decreased by a factor of 30 (not shown).

The endogenous kinase activity reproducibly copurified (n = 4) with the DMB protein in a protocol involving affinity chromatography on both a DTX_I column and a wheat germ agglutinin column (Rehm & Lazdunski, 1988b). Thus, the endogenous protein kinase is probably closely associated with the DMB protein.

Protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase, and casein kinase II did not phosphorylate the DMB protein, even in 10-fold excess of enzyme over substrate (not shown).

Reconstitution of the DMB protein containing liposomes (three different protein preparations) into preformed artifact-free lipid bilayers at the tip of glass patch pipets was associated with the reconstitution of multiple conductance levels of elementary current (Figure 3) in 35 out of 48 trials. Figure 3A shows single-channel current pulses of different amplitude in symmetrical 150 mM K^+ solution at a constant Vp of +80 mV sampled at high temporal resolution and spline-interpolated for better visual representation (see legend). As indicated by this example record, openings and closings of different size were mostly complete without resolvable intermediate transitions into subconductance states. Average open-channel current amplitudes in this experiment were 0.54,

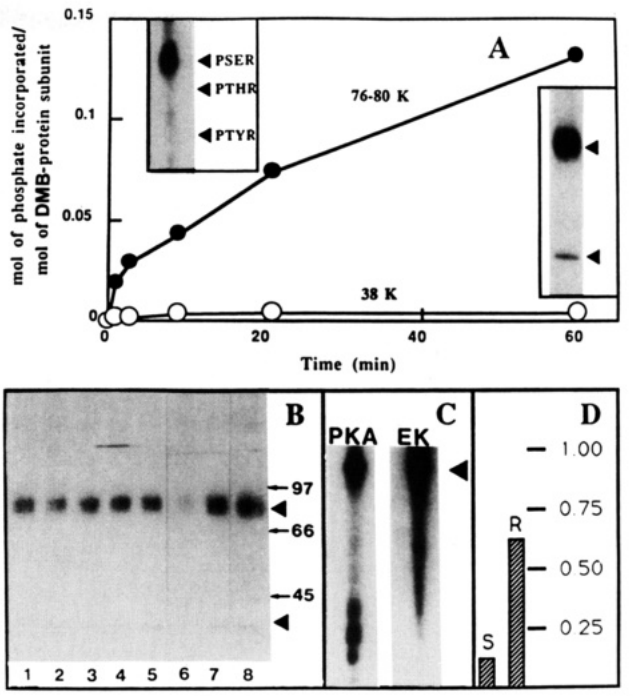

FIGURE 2: Phosphorylation of DMB protein by an endogenous protein kinase. (A) Purified DMB protein was incubated with [γ-^{32}P]ATP and Mg^{2+} as described under Experimental Procedures. After the times indicated the amount of phosphate incorporated into the subunits of DMB protein was determined. Inset (upper left): DMB protein was phosphorylated by the endogenous kinase, and the phosphoamino acids were analyzed by thin-layer chromatography. The migration positions of the marker amino acids are indicated. Inset (lower right): Autoradiogram of an SDS–PAGE of DMB protein phosphorylated by the endogenous kinase for 60 min. Arrowheads indicate the position of the 76–80-kDa (upper) and of the 38-kDa (lower) subunits. (B) Purified DMB protein was incubated for 4 min with [γ^{32}P]ATP and Mg^{2+} in the presence of the indicated agents and the reaction mixture then analyzed on SDS–PAGE. The autoradiogram shows the extent of phosphorylation of DMB protein in the presence of cAMP (16 μM) (lane 1), Ca^{2+}/calmodulin (240 μM/2.4 μM) (lane 2), DTX_I (0.5 μM) (lane 3), MCD (0.5 μM) (lane 4), β-BTX (0.1 μM) (lane 5), Mn^{2+} (instead of Mg^{2+}) (lane 6), EGTA (0.5 mM) (lane 7), and no agent added (control) (lane 8). Molecular mass markers (arrows) and the position of the 76–80- and 38-kDa subunits of the DMB protein (arrowheads) are indicated. (C) Purified DMB protein was phosphorylated by cAMP kinase or the endogenous kinase, and the subunits of DMB protein were separated on SDS–PAGE. The gel pieces containing the 76–80-kDa subunit of the DMB protein were cut out and digested with 500 ng of proteinase K in the stacking gel of another SDS–PAGE (Cleveland et al., 1977). The autoradiogram shows the pattern of phosphorylated peptides obtained from 76–80-kDa subunits which were phosphorylated by cAMP kinase (PKA) or the endogenous protein kinase (EK). The arrowhead points to the position of the undigested 76–80-kDa subunit. (D) Phosphorylation of the reconstituted DMB protein (R) by the endogenous protein kinase. Reconstitution of DMB protein and its phosphorylation were carried out as described under Experimental Procedures. For comparison, the extent of phosphorylation of soluble DMB protein is also shown (S). The scale is in moles of phosphate incorporated per mole of 76–80-kDa subunit.

1.12, 1.8, 2.52, and 3.24 pA, corresponding to conductance levels of 6.75, 14, 22.5, 31.5, and 40.5 pS, respectively. In another experiment in 150 mM symmetrical K^+ solution at a Vp of +80 mV, the histogram of open-channel current amplitudes revealed six well-separated conductance peaks (Figure 3B, main graph). The majority of openings (927 out of 1352) clustered around 1.92 pA = 24 pS. The number of openings belonging to the other conductance levels of 7, 15, 33, 42.75, and 50.75 pS was 65, 153, 118, 65, and 24, respectively. In summary, up to seven conductance peaks with average values of 7.3 ± 1.3, 12.4 ± 1.6, 20.7 ± 2.3, 30.1 ± 1.9, 40.3 ± 2.6,

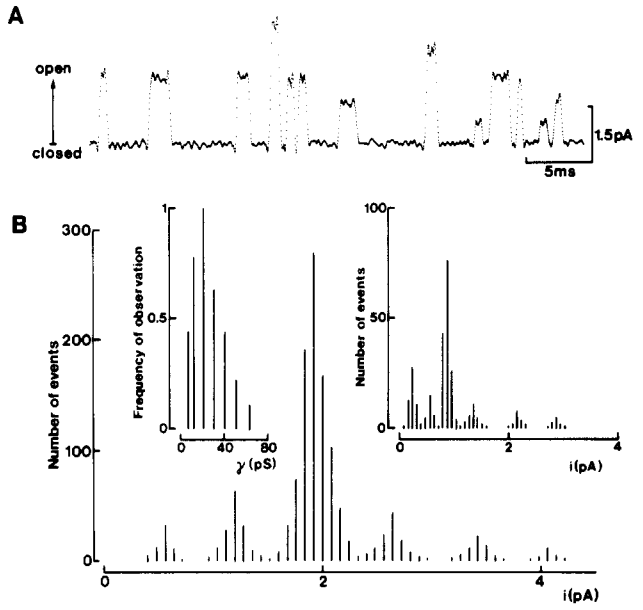

FIGURE 3: Electrophysiological activity and K^+ dependence of purified DMB channels. (A) Recording segment of single-channel activity in symmetrical 150 mM K^+ solution at *Vp* of +80 mV. The current record was digitized at 7 kHz, filtered (low pass, −3 dB) at 1.75 kHz, and spline interpolated for improved visual representation. The cubic spline interpolation technique (Colquhoun & Sigworth, 1983) was used, in which a cubic polynomial spans the interval between each pair of data points. A different polynomial is used for each interval, with coefficients chosen to match the function values as well as the first and second derivatives at the sample points. (B) (Main graph) Histogram of open-channel current amplitudes in symmetrical 150 mM K^+ solution at *Vp* of +80 mV. Distinct well-separated peaks occurred at 0.56, 1.2, 1.92, 2.64, 3.42, and 4.06 pA. (Left inset) Plot of the open-channel conductances (γ) at constant positive *Vp* against their frequency of observation. The positions of the bars correspond to the average conductance values. (Right inset) Histogram of open-channel current amplitudes in symmetrical 100 mM K^+ solution at *Vp* of +80 mV. Distinct well-separated peaks occurred at 0.24, 0.56, 0.88, 1.36, 2.16, and 2.88 pA. For open-channel current histograms, elementary current records were sampled at 3.5 kHz and filtered between 0.875 and 1.15 kHz (low pass, −3 dB).

51.0 ± 2.3, and 63.4 ± 2.9 pS were detected in histograms of open-channel conductance levels from 28 experiments in symmetrical 150 mM K^+ solution. The ≈21-pS single-channel activity was always observed (Figure 3B, left inset), and in 25 experiments, >65% of all openings in the histograms of elementary current amplitudes belonged to the ≈21-pS open-channel conductance level (e.g., Figure 3B, main graph). In symmetrical 100 mM K^+ solution, a representative elementary current amplitude histogram at *Vp* of +80 mV gave peak conductance levels of open-channel current of 3, 7, 11, 17, 27, and 36 pS (Figure 3B, right inset). On average, conductance peaks in symmetrical 100 mM K^+ solution were 3.5 ± 0.7, 6.4 ± 0.4, 9.0 ± 1.1, 15.5 ± 2.3, 28.0 ± 1.2, and 36 pS; the frequency of observation (number of observations/number of experiments) was 7/7, 7/7, 7/7, 5/7, 3/7, and 1/7, respectively. Under symmetrical ionic conditions (100 or 150 mM K^+), elementary current amplitude–*Vp* relations were linear at positive *Vp* with chord conductances within the standard deviations of the conductance values given above. Pronounced rectification was not observed in occasional recordings of elementary current at negative *Vp*. 4-Aminopyridine (4-AP, 1 mM) appeared to reduce the frequency of channel openings without markedly affecting the respective single-channel current amplitude. The dependence of the size of single-channel current pulses on K^+ concentrations as well as their sensitivity to 4-AP suggests that reconstitution of the DMB protein was associated with the reconstitution of multiple functional K^+ channels. The observed heterogeneity of elementary current conductance levels is probably related to the fact that the purified DMB protein is a mixture of closely related K^+ channel structures with similar but not identical pharmacological properties (Rehm & Lazdunski, 1988a). The pharmacological sensitivities as well as the selectivity properties of each putative channel in the DMB protein mixture remain yet to be determined electrophysiologically.

The DMB protein induced 21-pS K^+ channel activity (symmetrical 150 mM K^+, constant *Vp* of +80 mV) was highly sensitive to augmentation by phosphorylation. Phosphorylation was carried out with either ATP or ATPγS, the two kinase substrates giving quantitatively similar results. However, ATPγS was preferred to ATP for electrophysiological experiments because thiophosphorylated proteins are more resistant to hydrolysis than phosphorylated proteins and cAMP kinase can use ATPγS as substrate as well as ATP (Gratecos & Fischer, 1974). In 21 bilayer recordings with predominant 21-pS single-channel activity, 21-pS channel openness (*Np*) was 0.0626 ± 0.0361 under control conditions (Figure 4Aa–c, left panels; Figure 4Ad). In the absence of added cAMP kinase, 0.4 mM ATPγS primarily increased 21-pS channel openness to 0.5361 after about 10 min of bath application (Figure 4Aa). The ATPγS-induced augmentation of 21-pS channel activity was stable over the next 20 min of continous recording (*Np* = 0.6580 at 30 min) (Figure 4Aa). By contrast, 0.1 mM ATPγS in the absence of added cAMP kinase did not enhance 21-pS channel openness prior to about 30 min after bath addition (Figure 4Ab). Thereafter, *Np* of the 21-pS channel activity was increased to 0.5989 (Figure 4Ab). If, however, 0.1 mM ATPγS was applied to the bath together with 0.1 μM catalytic subunit of cAMP kinase (C of PKA), augmentation of 21-pS channel openness to 0.5611 occurred earlier, at about 10 min, and was stable over the next 20 min (*Np* = 0.4763 at 30 min) (Figure 4Ac). Thus at a fixed time, for example, 15 min after bath application of the phosphorylating agents, *Np* of the 21-pS channel activity was unchanged by 0.1 mM ATPγS alone (0.0626 ± 0.0361 versus 0.0642 ± 0.0467), increased to 0.5199 ± 0.0724 by 0.1 mM ATPγS in the presence of 0.1 μM C of PKA, and also increased to 0.5229 ± 0.0992 by a higher ATPγS concentration of 0.4 mM (Figure 4Ad). The basis for this apparent concentration dependence of ATPγS alone and the additional effect of cAMP kinase at a given ATPγS concentration was the shortening of latencies to stimulation from 27.75 ± 2.5 to 10.125 ± 1.3 min upon increasing ATPγS from 0.1 to 0.4 mM, and to 9.875 ± 2 min in the presence of cAMP kinase at 0.1 mM ATPγS (Figure 4Ae).

The ATPγS-induced augmentation of 21-pS channel openness depends on ATP hydrolysis. In three experiments, 2 mM AMP-PNP, a poorly hydrolyzable ATP analogue, did not increase 21-pS channel activity within about 30 min (Figure 4B). Throughout the example experiment, *Np* clustered around 0.1324.

The activity of the phosphorylated 21-pS channel was reduced from 0.4488 to 0.0652 by DTX_I (Figure 4Ca), which was applied to the pipet side of the channel by fusion of DTX_I-containing liposomes with the bilayer (*n* = 4). When DTX_I was applied prior to ATPγS, it also reduced basal 21-pS elementary current from 0.0496 to 0.0034, and subsequent application of 0.4 mM ATPγS was then unable to increase DTX_I-suppressed 21-pS channel activity within the normal latency to 0.4 mM ATPγS stimulation (*n* = 2) (Figure 4Cb).

The activation of the mammalian DMB K^+ channels demonstrated herein is to our knowledge a newly described type

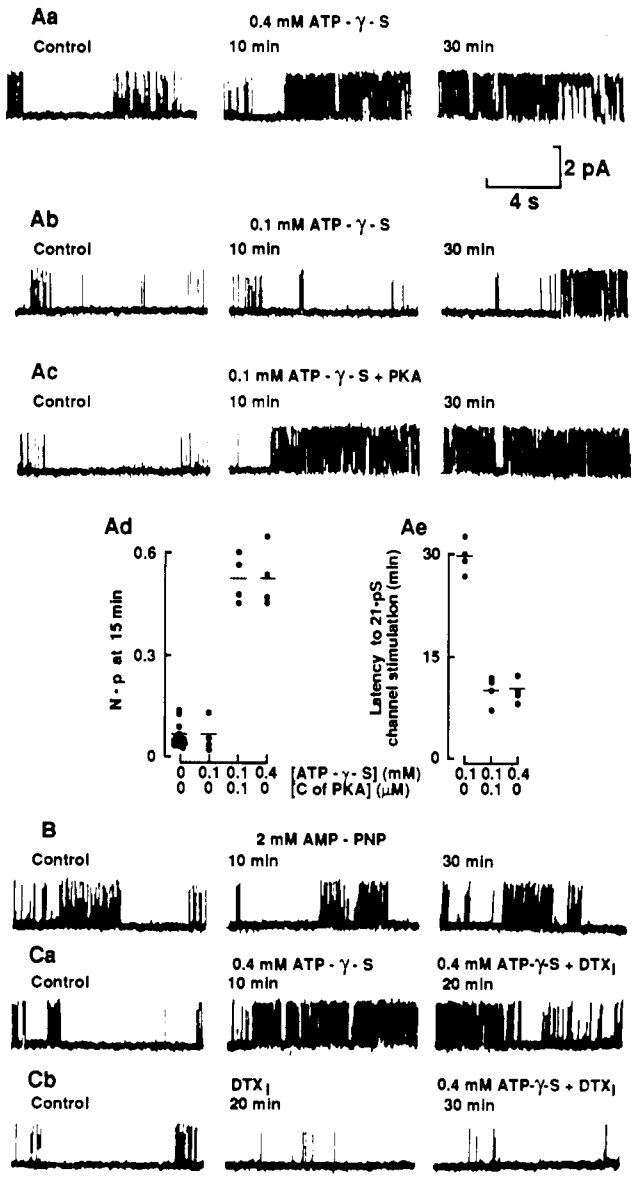

FIGURE 4: Regulation of the 21-pS channel activity reconstituted from the purified DMB protein. (Aa) Control (left panel) and 10 min (middle panel) and 30 min (right panel) after bath addition of 0.4 mM ATPγS. No kinase was present throughout the experiment. (Ab) Control (left panel) and 10 min (middle panel) and 30 min (right panel) after bath addition of 0.1 mM ATPγS. No kinase was present throughout the experiment. (Ac) Control (left panel) and 10 min (middle panel) and 30 min (right panel) after bath addition of 0.1 mM ATPγS in the presence of 0.1 μM catalytic subunit of cAMP kinase. (Ad) 21-pS channel openness (*Np*) after 15 min of successful reconstitution of 21-pS channel activity and 15 min after bath addition of 0.1 mM ATPγS, 0.1 mM ATPγS in the presence of 0.1 μM catalytic subunit of cAMP kinase, and 0.4 mM ATPγS, respectively. (Ae) Latencies to 21-pS channel stimulation after bath addition of 0.1 mM ATPγS, 0.1 mM ATPγS in the presence of 0.1 μM catalytic subunit of cAMP kinase, and 0.4 mM ATPγS, respectively. (B) Control (left panel) and 10 min (middle panel) and 30 min (right panel) after bath addition of 2 mM AMP-PNP. (Ca) Control (left panel) and 10 min after bath addition of 0.4 mM ATPγS (middle panel) and 20 min after subsequent addition of 30 μL of the DTX_I–liposome suspension to the bath side of the bilayer (right panel). (Cb) Control (left panel) and 20 min after bath addition of 30 μL of the DTX_I–liposome suspension (middle panel) and 30 min after subsequent bath addition of 0.4 mM ATPγS (right panel). Continous recordings from the same bilayers in A–C, respectively. All recordings were made at a constant pipet potential (*Vp*) of +80 mV, and examples were digitized at 307 Hz. No filter.

of regulation for K^+ channels. Phosphorylation is also essential for the activation of L-type Ca^{2+} channels (Klee et al., 1988; Reuter, 1983).

A key property of the dendrotoxins is their facilitating effect on neuromuscular transmission (Harvey & Anderson, 1985). The demonstration made in this paper that the DMB protein channel is activated by phosphorylation may have important consequences for the control of neurosecretion. Factors that activate the endogenous kinase or elevate cAMP with a subsequent activation of cAMP kinase will lead to K^+ channel opening, increase of membrane polarization, and decrease of neurosecretion. Conversely, events leading to decreased cAMP levels via cyclase inhibition or phosphodiesterase activation or to a decrease of the endogenous kinase activity will lead to depolarization, activation of Ca^{2+} channels, and increased neurosecretion. It will be particularly important in the future to see how the regulation described here alters the set of K^+ channels, which are the target of MCD in hippocampus and which are involved in the control of long-term potentiation (Cherubini et al., 1987).

ACKNOWLEDGMENTS

We thank J. Barhanin for fruitful discussions. We also thank F. Aguila, R. A. Newitt, P. Hammes, C. Roulinat-Bettelheim, and D. F. Treichel for skillful technical assistance.

Registry No. K, 7440-09-7; ATP, 56-65-5; endogenous kinase, 9026-43-1.

REFERENCES

Baumann, A., Grupe, A., Ackermannn, A., & Pongs, O. (1988) *EMBO J. 8*, 2457–2463.

Benoit, E., & Dubois, J.-M. (1986) *Brain Res. 377*, 374–377.

Bidard, J.-N., Gandolfo, G., Mourre, C., Gottesmann, C., & Lazdunski, M. (1987a) *Brain Res. 418*, 235–244.

Bidard, J.-N., Mourre, C., & Lazdunski, M. (1987b) *Biochem. Biophys. Res. Commun. 143*, 383–389.

Black, A., & Dolly, J. O. (1986) *Eur. J. Biochem. 156*, 609–617.

Callahan, C., Ptasienski, J., & Hosey, M. M. (1988) *J. Biol. Chem. 263*, 17342–17349.

Cavalié, A., Pelzer, D., & Trautwein, W. (1986) *Pfluegers Arch. 406*, 241–258.

Cherubini, E., Ben Ari, Y., Gho, M., Bidard, J.-N., & Lazdunski, M. (1987) *Nature 328*, 70–73.

Cleveland, D., Fischer, S., Kirschner, M., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Colquhoun, D., & Sigworth, F. J. (1983) in *Single-Channel Recording* (Sakmann, B., & Neher, E., Eds.) pp 191–263, Plenum Press, New York and London.

Dreyer, F., & Penner, R. (1987) *J. Physiol. 386*, 455–463.

Gratecos, D., & Fischer, E. (1974) *Biochem. Biophys. Res. Commun. 58*, 960–967.

Halliwell, J., Othman, I., Pelchen, A., & Dolly, J. O. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 493–497.

Hanke, W., Methfessel, C., Wilmsen, U., & Boheim, G. (1984) *Bioelectrochem. Bioenerg. 12*, 329–339.

Harvey, A., & Karlsson, E. (1982) *Br. J. Pharmacol. 77*, 153–161.

Harvey, A., & Anderson, A. (1985) *Pharmacol. Ther. 31*, 33–55.

Klee, C. B., Draetta, G. F., & Hubbard, M. J. (1988) *Adv. Enzymol. Relat. Press Mol. Biol. 61*, 149–200.

Laemmli, U. K., (1970) *Nature 227*, 680–685.

Liu, F., Zinnecker, M., Hamaoka, T., & Katz, D. (1979) *Biochemistry 18*, 690–697.

Pelzer, D., Grant, A. D., Cavalié, A., Pelzer, S., Sieber, M., Hofmann, F., & Trautwein, W. (1989) *Ann. N.Y. Acad. Sci.* (in press).

Penner, R., Petersen, M., Pierau, F. K., & Dreyer, F. (1986) *Pfluegers Arch. 407*, 365-369.
Rehm, H., & Lazdunski, M. (1988a) *Biochem. Biophys. Res. Commun. 153*, 231-240.
Rehm, H., & Lazdunski, M. (1988b) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4919-4923.
Rehm, H., Bidard, J.-N., Schweitz, H., & Lazdunski, M. (1988) *Biochemistry 27*, 1827-1832.
Reuter, H. (1983) *Nature 301*, 569-574.
Sandoval, I. V., & Cuatrecasas, P. (1976) *Biochemistry 15*, 3424-3432.
Schmidt, R., Betz, H., & Rehm, H. (1988) *Biochemistry 27*, 963-967.
Stansfeld, C., & Feltz, A. (1988) *Neurosci. Lett. 93*, 49-55.
Stansfeld, C., Marsh, S., Parcej, D., Dolly, J. O., & Brown, D. (1987) *Neuroscience 23*, 893-902.
Stuehmer, W., Stocker, M., Sakmann, B., Seeburg, P., Baumann, A., Grupe, A., & Pongs, O. (1988) *FEBS Lett. 242*, 199-206.
Taylor, J. W., Bidard, J.-N., & Lazdunski, M. (1984) *J. Biol. Chem. 259*, 13957-13967.
Tempel, B., Jan, Y., & Jan, L. (1988) *Nature 332*, 837-839.
Tempel, B., Papazian, D., Schwartz, T., Jan, Y., & Jan, L. (1987) *Science 237*, 770-775.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. U.S.A. 76*, 4350-4354.

Mechanism of Isomerization of Rhodopsin Studied by Use of 11-Cis-Locked Rhodopsin Analogues Excited with a Picosecond Laser Pulse†

Hideki Kandori,‡ Sinzi Matuoka,‡,§ Yoshinori Shichida,‡ Tôru Yoshizawa,*,‡ Masayoshi Ito,∥ Kiyoshi Tsukida,∥ Valeria Balogh-Nair,⊥,# and Koji Nakanishi⊥

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan, Kobe Women's College of Pharmacy, Kobe 658, Japan, and Department of Chemistry, Columbia University, New York, New York 10027

Received November 2, 1988; Revised Manuscript Received March 7, 1989

ABSTRACT: Primary photochemical behaviors of cattle rhodopsin analogues (Rh5 and Rh7) having cyclopenta- and cycloheptatrienylidene 11-cis-locked retinals (Ret5 and Ret7, respectively) were studied by excitation with a picosecond laser pulse (wavelength 532 nm; duration 21 ps). Picosecond absorption and fluorescence measurements of Rh5 showed formation of only a long-lived excited singlet state ($\tau_{1/e}$ = 85 ps). The excited state of the retinal analogue having a five-membered ring was stabilized in protein (Rh5) more than in solvent (protonated Schiff base of Ret5; PSB5). Excitation of Rh7 produced two ground-state photoproducts, Rh7(580) and Rh7(630). According to the analysis of photon density dependency, Rh7(580) was a single-photon product of Rh7, while Rh7(630) was the photoproduct of Rh7(580). Fluorescence emitted from a seven-membered ring system like Rh7 or a protonated Schiff base of Ret7 (PSB7) was weaker than that in a corresponding five-membered ring system, especially in protein (Rh7). The difference in photoreaction between Rh5 and Rh7 may originate from the difference in fixation of the 11-cis form. On the basis of the spectral and kinetic similarities between Rh7(580) and photorhodopsin, a precursor of bathorhodopsin, it was proposed that both have twisted all-trans chromophores in the way of the isomerization. The protein moiety of rhodopsin which fixes the chromophore at both ends seems to accelerate the rotation of the C_{11}–C_{12} double bond and to prevent it from going through relaxation processes other than the isomerization. This may be a plausible reason why rhodopsin has a large quantum yield (0.67).

Absorption of a photon by rhodopsin isomerizes its 11-*cis*-retinylidene chromophore to all-trans form. The photoisomerization takes place at extremely high speed so that rhodopsin has a high photosensitivity. Until recently, it was believed that bathorhodopsin would be the earliest intermediate of photobleaching of rhodopsin. Since bathorhodopsin has a twisted *all-trans*-retinal as its chromophore (Yoshizawa & Wald, 1963; Yoshizawa & Horiuchi, 1972; Fukada et al., 1984; Eyring et al., 1982), it was recognized that the isomerization of the chromophore began with a Franck–Condon state and was completed at the ground state via an excited common state of rhodopson and bathorhodopsin.

Several years ago, Shichida et al. (1984) detected an earlier intermediate than bathorhodopsin, called photorhodopsin. This intermediate has its absorption maximum at a longer wavelength than that of bathorhodopsin. What is the structure of the chromophore in photorhodopsin? Is the chromophore of photorhodopsin an intermediate on the way to the isomerization? A powerful tool to solve this question is to use some retinal analogues in which the isomerization of the C_{11}–C_{12} double bond is locked. Two kinds of analogues have been synthesized; one is locked by a five-membered ring (Ret5; Ito

† This work was supported in part by a Special Coordination Fund of the Science and Technology Agency of the Japanese Government, by Grants-in-Aid for Specially Promoted Research to T.Y. (63065002), for Scientific Research on Priority Areas to Y.S. (63621003), and for Encouragement of Young Scientists to H.K. (63790474) from the Japanese Ministry of Education, Culture and Science, and by a grant from the National Institutes of Health to K.N. (GM 36564).
* To whom correspondence should be addressed.
‡ Kyoto University.
§ Present address: Department of Applied Physics, Faculty of Technology, Nagoya University, Nagoya 464, Japan.
∥ Kobe Women's College of Pharmacy.
⊥ Columbia University.
Present address: Department of Chemistry, City University of New York, City College, New York, NY 10031.

0006-2960/89/0428-6460$01.50/0 © 1989 American Chemical Society