Inhibition of Voltage-Gated K Channels in Synaptosomes by sn-1,2-Dioctanoylglycerol, an Activator of Protein Kinase C

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Tracer efflux studies were used to determine the effect of activation of protein kinase C on K channel function in rat brain synaptosomes. Hippocampal synaptosomes were treated with sn-1,2-dioctanoylglycerol (diC8), a synthetic diacylglycerol (DG) analog that activates protein kinase C. DiC8 inhibited depolarization-induced ^{86}Rb efflux through voltage-gated K channels but did not affect the component of efflux corresponding to Ca-activated K channels. In time-course experiments, diC8 inhibited two components of ^{86}Rb efflux: efflux through a rapidly inactivating, voltage-gated K channel (responsible for the "A" current) and that through a slowly inactivating, voltage-gated K channel (believed to be the "delayed rectifier"). Experiments with specific blockers of these voltage-gated K channels supported this observation. Inhibition of K-stimulated ^{86}Rb efflux by diC8 was time dependent: at least 15 sec of preincubation was required before the effect could be observed. The effect of diC8 was concentration dependent: 50 μM diC8 produced a half-maximal inhibition of K-stimulated ^{86}Rb efflux.

The idea that the inhibition of synaptosome K channels by diC8 resulted from activation of C kinase was supported by pharmacological evidence. The action of diC8 was mimicked by 1-oleoyl-2-acetylglycerol, another DG analog that activates protein kinase C, but not by deoxy-diC8, a DG analog that does not activate C kinase. Inhibition of C kinase by sphingosine or H-7 prevented the diC8 effect.

These studies demonstrate that synaptosomes are a good model in which to study modulation of mammalian CNS K channels. The data suggest that protein kinase C plays an important role in the regulation of the activity of some types of K channels. Modulation of K channel activity may, in turn, regulate the release of neurotransmitter from the presynaptic terminal.

Potassium (K) channels located in the presynaptic terminal control excitability and neurotransmitter release and thus have the potential to be key regulators of synaptic transmission. Previous work from our laboratory has shown that isolated nerve terminals (synaptosomes) contain a variety of K channels that can be studied with ^{86}Rb efflux methods. The various K channels can be distinguished on the basis of their kinetic and pharmacological characteristics (Bartschat and Blaustein, 1985a, b; Benishin et al., 1987; Blaustein et al., 1988) and correspond to known K conductances determined by electrophysiological techniques. Synaptosomes are a convenient mammalian system in which to study modulation of K channel activity; use of synaptosomes allows a parallel examination of the physiology, pharmacology, and biochemistry of presynaptic K channels.

Much evidence suggests that protein kinase C—calcium/phospholipid-dependent protein kinase, "C kinase" (Takai et al., 1979)—plays an important role in neuronal function. Activators of C kinase can modulate neurotransmitter release in hippocampal slices (Malenka et al., 1987), in cultured embryonic brain neurons (Zurgil et al., 1986), and in synaptosomes prepared from striatum, hippocampus, and other forebrain structures (Lynch and Bliss, 1986; Nichols et al., 1987; Shu and Selmanoff, 1988). Protein kinase C may be involved in long-term potentiation (LTP), a mammalian model of learning (Akers et al., 1986; Malenka et al., 1986; Hu et al., 1987; Lovinger et al., 1987). The mechanisms that underlie C kinase modulation of neurotransmitter release or its involvement in LTP have not yet been elucidated. Either of these effects could result from modulation of K channel activity because electrophysiological evidence indicates that activators of C kinase inhibit K conductances in hippocampal slices (Baraban et al., 1985), in cultured or acutely dissociated hippocampal neurons (Doerner et al., 1987), and in cultured dorsal root ganglion neurons (Werz and Macdonald, 1987). Furthermore, C kinase has been implicated in a simple form of invertebrate learning which results, in part, from modulation of K conductances (Farley and Auerbach, 1986).

With the aforementioned observations in mind, we investigated the modulation of rat brain synaptosome K channels by sn-1,2-dioctanoylglycerol (diC8), an analog of diacylglycerol (DG) that activates protein kinase C. A preliminary report of some of our findings has been published (Colby and Blaustein, 1987).

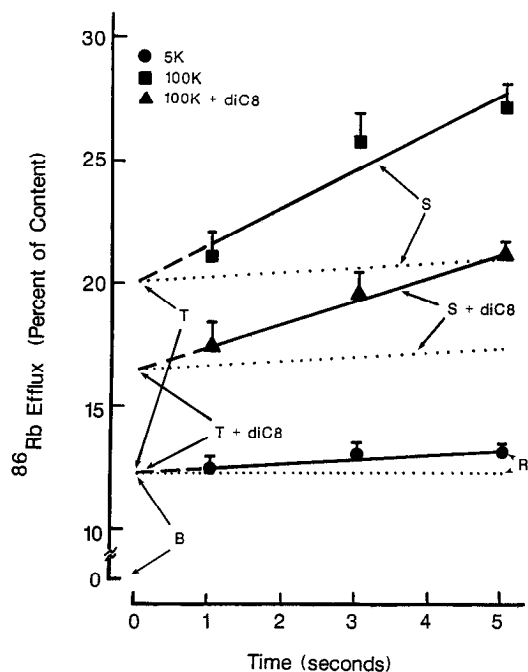## Materials and Methods

Materials

DG analogs (diC8 and OAG) in chloroform were obtained from Avanti Polar Lipids (Birmingham, AL). Deoxy-diC8 was generously donated by Dr. R. M. Bell, Duke University. Immediately before use, aliquots of these compounds were dried under a stream of nitrogen and resuspended in dimethylsulfoxide (DMSO). Dilutions were made with a buffered salt solution (5K) described below. Final DMSO concentration did not exceed 0.1% and had no effect on ^{86}Rb efflux. DG analogs were sonicated before use. Sphingosine-SO$_4$ (Sigma Chemical Co., St. Louis) was dissolved in ethanol and diluted with 5K buffer. Final ethanol concentration did not exceed 0.1%, a concentration that had no effect on ^{86}Rb efflux (not shown). 1-(5-Isoquinolinesulfonyl)-2-

Figure 1. Effect of diC8 on time course of ^{86}Rb efflux from hippocampal synaptosomes. A representative time course is shown; components of Rb efflux illustrated here (R, B, S, T) are fully described in Materials and Methods. Each point represents the mean of 3–5 individual determinations ± SE. Similar results were observed in 2 additional experiments. The *solid lines* were determined by linear-regression analysis. The *bottom dotted line* is parallel to the abscissa; the 2 *upper dotted lines* are parallel to the 5K line and illustrate the increase in slope caused by depolarizing efflux solution. ΔK, the K-stimulated ^{86}Rb efflux, includes 2 distinguishable Rb efflux components, T (ordinate-intercept) and S_v (part of the increase in slope, "S"), that correspond to 2 different voltage-gated K channels (see Materials and Methods and Bartschat and Blaustein, 1985a). Incubation of hippocampal synaptosomes with 150 μM diC8 for 6 min inhibited both T and S, suggesting that the activity of both types of voltage-gated K channels can be modulated by C kinase. DiC8 had no effect on efflux in 5K (not shown).

methylpiperazine (H-7) was purchased from Seikagaku America, Inc. (St. Petersburg, FL). Crude venom from the scorpion *Centruroides sculpturatus* was purchased from Sigma; dendrotoxin ("α-DaTX"; see Harvey and Karlsson, 1980) was purified from crude *Dendroaspis angusticeps* venom (Sigma) as described (Blaustein et al., 1988). ^{86}RbCl was obtained from New England Nuclear (Boston). All other reagents were obtained from commercial sources in the highest grade available.

Methods

Preparation of synaptosomes. Hippocampal or striatal P_2 synaptosomes were prepared from female Sprague-Dawley rats, 100–125 gm, as described (Krueger et al., 1979). The loosely packed portion of the pellet from the second high-speed (10,000 × g) centrifugation (P_2 pellet), enriched with nerve terminals, was resuspended in 0.32 M sucrose and saved; the dense (bottom) part of the pellet (containing primarily mitochondria) was discarded. K channel function in P_2 synaptosomes is identical to that seen in synaptosomes that have been purified on a 1-step sucrose density gradient (K. A. Colby and M. P. Blaustein, unpublished observations). The synaptosomes were slowly equilibrated with a physiological salt solution (5K), containing (in mM) NaCl, 145; KCl, 5; RbCl, 0.1; MgCl$_2$, 2; glucose, 10; NaH$_2$PO$_4$, 0.5; and HEPES, 10, adjusted to pH 7.4. After equilibration the synaptosomes were centrifuged, and the resulting pellet was resuspended in a small volume of 5K buffer (composition as above) containing 100 μM Ca. The final protein concentration of the resuspended synaptosomes was 3–4 mg/ml.

Synaptosome ^{86}Rb efflux assay. Efflux of ^{86}Rb from the synaptosomes was measured as previously described (Bartschat and Blaustein, 1985a, b). Briefly, the resuspended synaptosomes were loaded with tracer (to a steady-state ^{86}Rb level) by incubation with 10–20 μCi ^{86}Rb/ml syn-

aptosomes (3–4 mg protein/ml) for 30 min at 30°C. Aliquots of the ^{86}Rb-loaded synaptosomes were added, at timed intervals, to tubes containing diC8 (or other DG analogs) or an appropriate vehicle; these suspensions were preincubated at 37°C for a fixed amount of time, usually 6–9 min (see Results). When sphingosine was used, it was included along with diC8 during this preincubation. In the experiments with H-7, the synaptosomes were first preincubated for 9 min with or without H-7, before incubation with diC8.

After the preincubation period, an aliquot of each sample was transferred to a glass fiber filter (Schleicher and Schuell #25). The synaptosomes trapped on the filter were rinsed with 5K buffer (containing 0.1% BSA) and subjected to gentle vacuum filtration to remove extrasynaptosomal ^{86}Rb. Rb efflux was stimulated by addition of an efflux solution at a constant time (no later than 10 sec) after the final 5K wash. Efflux solutions contained either 5 mM KCl (control) or 50–100 mM KCl (to depolarize the synaptosomes) with or without 1 mM CaCl$_2$. The composition of the depolarizing solutions (50 or 100K) was similar to that of the 5K solution, except that KCl replaced NaCl mole-for-mole; when 1 mM CaCl$_2$ was added (50K/Ca or 100K/Ca), the concentration of MgCl$_2$ was decreased to 1 mM. Efflux was measured for 1–5 sec (timed with a metronome) and was terminated by addition of an isotonic buffered stop solution containing (in mM) tetraethylammonium chloride, 145; tetrabutylammonium chloride, 5; RbCl, 0.1; NiCl$_2$, 10; MgCl$_2$, 10 and HEPES, 10, titrated to pH 7.4 with NaOH. Vacuum filtration was used to collect the efflux and stop solutions. The radioactivity in the filtrate (representing ^{86}Rb efflux) and that on the filter (containing the immobilized synaptosomes) were quantified by liquid scintillation spectrometry. The percentage of total radioactivity released during the efflux period was calculated using the following formula:

$$^{86}\text{Rb efflux (\% of total)} = \frac{(\text{cpm in filtrate})}{(\text{cpm in filtrate}) + (\text{cpm in filter})} \times 100$$

Components of ^{86}Rb efflux. Hippocampal synaptosomes (Fig. 1), like those prepared from rat forebrain (Bartschat and Blaustein, 1985a, b) and from rat corpus striatum (not shown), exhibit several kinetically and pharmacologically distinct components of Rb efflux: a resting efflux ("R"), a K-stimulated, voltage-gated component ("ΔK"), and a Ca-dependent component ("ΔCa"). The ^{86}Rb efflux into 5K (nondepolarizing) solution includes a small, time-dependent "resting efflux" ("R," 0.3–0.5%/sec) and a larger, time-independent "background" ("B," 8–12% of the "zero-time" Rb efflux; Bartschat and Blaustein, 1985a; see also Fig. 1). The "background" includes tracer that was incompletely removed during the preceding washes.

The K-stimulated efflux (ΔK) is the efflux into 50K or 100K minus the efflux into 5K; ΔK can be divided into 2 components, component "T" and component "S," on the basis of time-course experiments in which ^{86}Rb efflux is measured for intervals of 1–5 sec (see Fig. 1). Component T is the increment in the ordinate-intercept of the extrapolated time-course line caused by depolarization. This component represents efflux through a rapidly inactivating (within 1 sec), voltage-gated K channel that is blocked by relatively low concentrations of 4-aminopyridine (IC$_{50}$ ≈ 0.2 mM; Bartschat and Blaustein, 1985a) and by α-DaTX (Benishin et al., 1988; Blaustein et al., 1988), and thus probably corresponds to the "A current" (Rogawski, 1985).

Component S is the increase in the slope of the line through the various time points measuring Rb efflux when 5K is replaced by depolarizing solutions (see Fig. 1); S can be further divided into 2 components: "S_R" and "S_V." Component S_R represents efflux through the ohmic "resting K channels" that results from the increase in electrical driving force during depolarization (Bartschat and Blaustein, 1985a). At the K concentrations used in our experiments, approximately one-half to two-thirds of component S is attributable to component S_R. The remaining one-third to one-half (component S_V) cannot be accounted for by electrodiffusion and appears to represent efflux through a voltage-gated, noninactivating K channel that may correspond to the "delayed rectifier" (Hille, 1984). Component S_V is sensitive to low concentrations of phencyclidine (IC$_{50}$ ≈ 1–2 μM; Bartschat and Blaustein, 1986); it is also selectively blocked by crude venoms from various scorpions (Schneider et al., 1988) and by cetain newly identified polypeptide toxins from the snake, *D. angusticeps* (Benishin et al., 1988).

The Ca-dependent efflux (ΔCa; Fig. 2) equals the efflux into 50K/Ca or 100K/Ca minus the efflux into 50K or 100K, respectively. This corresponds to efflux through Ca-activated, voltage-gated K channels (Bartschat and Blaustein, 1985b; Blaustein et al., 1988).
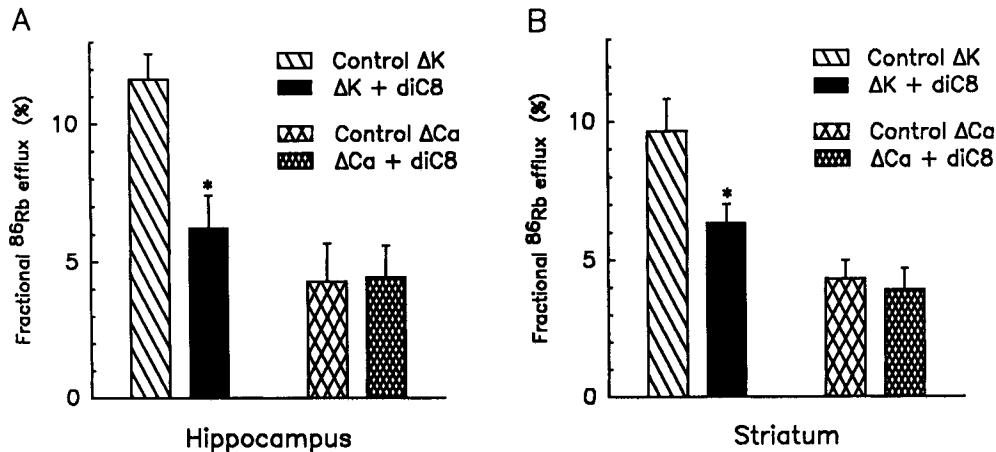
Figure 2. Effect of diC8 on ^{86}Rb efflux from hippocampal or striatal synaptosomes. ΔK (K-stimulated Rb efflux) = (efflux into 50K or 100K) − (efflux into 5K), ΔCa (Ca-dependent, K-stimulated Rb efflux) = (efflux into 50K/Ca or 100K/Ca) − (efflux into 50K or 100K). *A,* Hippocampal synaptosomes were incubated with 150 μM diC8 for 6 min at 37°C before ^{86}Rb efflux was measured for 5 sec. DiC8 markedly reduced ΔK but had no effect on ΔCa. Results from one experiment are shown (values are means of 4 individual determinations ± SE). Similar results were seen in 2 additional experiments. The mean decrease in ΔK with diC8 in 3 experiments was 44% (range, 41–46% inhibition). There was no change in ΔCa by diC8; mean ΔCa + diC8 = 101% control ΔCa (range, 83–118% control). *, significantly different from control ΔK, $p < 0.05$. *B,* Striatal synaptosomes were exposed to 150 μM diC8 as described above. Results from a representative experiment are illustrated (values are the means of 4–5 individual determinations ± SE). DiC8 exposure decreased ΔK (mean inhibition seen in 3 experiments was 25%; range, 19–34%) but had no effect on ΔCa (mean ΔCa + diC8 = 102% control ΔCa; range, 91–112% control; measured in 2 separate experiments). *, significantly different from control ΔK, $p < 0.05$.

Protein determination. Protein concentration was determined as described by Smith et al. (1985) using BSA as the standard.

Statistical analysis. Significance was determined by a 2-tailed Student's *t* test. A *p* value of less than 0.05 was considered to be significant. Where representative experiments are illustrated in the figures, similar results were obtained in at least 2 separate experiments with an *N* of 4–6 for each condition during each experiment. The standard errors (SE) of the ΔK and ΔCa values were calculated as follows (see Nachshen and Blaustein, 1979):

$$SE(\Delta K) = \sqrt{\{[SE(5K)]^2 + [SE(50K)]^2\}},$$
$$SE(\Delta Ca) = \sqrt{\{[SE(50K)]^2 + [SE(50K + Ca)]^2\}}.$$

Results

Effects of diC8 on K-stimulated Rb efflux (ΔK) and Ca-dependent Rb efflux (ΔCa)

Figure 2*A* shows data from a representative experiment in which the effect of diC8 was tested on Rb efflux from hippocampal synaptosomes. Exposure to diC8 significantly inhibited ΔK, but did not affect ΔCa. Although not illustrated here, diC8 had no significant effect on the resting efflux component. Virtually identical results were obtained with striatal synaptosomes (Fig. 2*B*), except that the inhibition of ΔK was somewhat smaller in magnitude. In subsequent experiments we focused exclusively on the effects of diC8 on hippocampal synaptosomes.

DiC8 treatment inhibits 2 types of voltage-gated K channels

The K-stimulated Rb efflux is due to efflux through 2 voltage-gated K channels (corresponding to components T and S$_{\text{v}}$), as well as to an increase in electrodiffusion through resting K channels (component S$_{\text{R}}$; see Methods and Blaustein et al., 1988).

Time-course experiments demonstrated that diC8 inhibited both voltage-gated components of ΔK (Fig. 1): the ordinate-intercept (component T) and the slope (part of which is component S$_{\text{v}}$) of the 100K + diC8 line were both decreased with respect to the control 100K line. This observation suggests that the activity of both the noninactivating K channel and the inactivating K channel can be modulated by C kinase.

Pharmacological evidence supports this hypothesis (Fig. 3*A*): diC8 still decreased ^{86}Rb efflux in the presence of α-DaTX, a polypeptide from *D. angusticeps* venom that specifically blocks component T (Benishin et al., 1988; Blaustein et al., 1988). Furthermore, diC8 inhibition was still apparent in the presence of crude venom from the scorpion *C. sculpturatus* that specifically blocks component S$_{\text{v}}$ (Blaustein et al., 1988; Schneider et al., 1988) through activity that appears identical to that of noxiustoxin (Carbone et al., 1987). However, when components T and S$_{\text{v}}$ were both completely inhibited by a high concentration (10 mM) of 4-aminopyridine (4-AP; Bartschat and Blaustein, 1985a), diC8 had no further effect on the residual ΔK, component S$_{\text{R}}$ (see Materials and Methods).

It is possible to determine the relative contributions of the various components of Rb efflux using inhibitors, such as *C. sculpturatus* venom and α-DaTX, that selectively block, respectively, components S$_{\text{v}}$ (Schneider et al., 1988) and T (Benishin et al., 1988; Blaustein et al., 1988). Component S$_{\text{R}}$, the toxin- and 4-AP-insensitive increase in efflux due to electrodiffusion in depolarizing solutions, constitutes a significant portion (usually 50–60%) of ΔK (right-hand bar in Fig. 3*B*). If one subtracts component S$_{\text{R}}$ from the total ΔK, the remaining fraction of ΔK represents the efflux through the 2 classes of voltage-gated K channels. In the experiment illustrated in Figure 3, the total ΔK was 12.9 units, 6.3 units of which was contributed by component S$_{\text{R}}$. The fraction of ΔK corresponding to efflux through voltage-gated channels (i.e., components S$_{\text{v}}$ + T in Fig. 3*B*) was therefore 6.6 units. Addition of diC8 reduced this fraction of ΔK to 2.2 units (total ΔK + diC8 equals 8.5 units minus 6.3 units from component S$_{\text{R}}$), representing a 70% inhibition of ^{86}Rb efflux through the voltage-gated K channels (black bar in Fig. 3*A*). Analysis of this sort revealed that 150 μM diC8 rou-

A

B
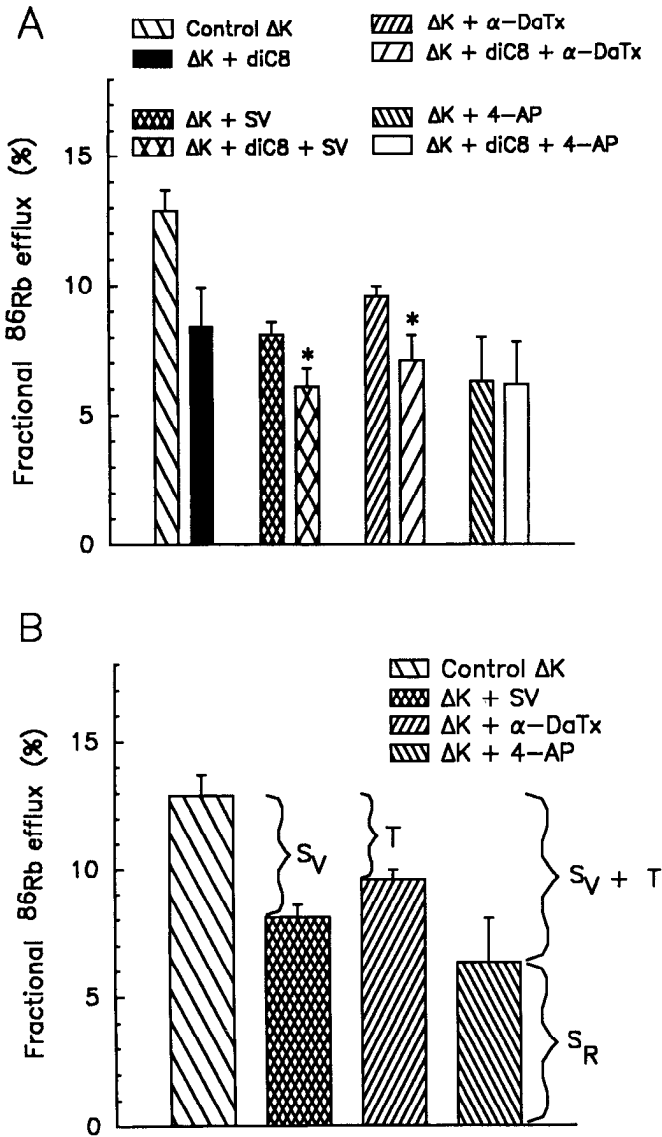
Figure 3. Effect of K channel blockers on diC8 inhibition of ΔK. *A,* Hippocampal synaptosomes were incubated for 9 min with 150 μM diC8 ± 5 μg/ml crude scorpion (*C. sculpturatus*) venom (*SV*) to block component S_V, 100 nM α-DaTX to block component T, or 10 mM 4-AP to block both components T and S_V. DiC8 further inhibited ^{86}Rb efflux in the presence of either SV or α-DaTX, supporting the conclusion from the time-course experiment (Fig. 1) that both a rapidly inactivating, voltage-gated K channel and a noninactivating, voltage-gated K channel were inhibited by diC8. DiC8 had no further effect when components T and S_V were completely blocked with 4-AP. A representative experiment is shown; data are expressed as means of 4–6 individual measurements ± SE. All values are significantly different from control ΔK, $p < 0.05$. *, significantly different from SV or α-DaTX alone, $p < 0.05$. *B,* The relative contributions of components T (α-DaTX-sensitive), S_V (scorpion venom-sensitive), and S_R (α-DaTX-, SV-, and 4-AP-insensitive) to the total ΔK value are shown. The α-DaTX-, SV-, and 4-AP-insensitive portion of ΔK (S_R = 50–60% of total ΔK) is the increased efflux through resting K channels as a result of depolarization (see Materials and Methods). DiC8 therefore blocked a major portion (70%) of the part of ΔK that represents efflux through voltage-gated K channels (components S_V and T).
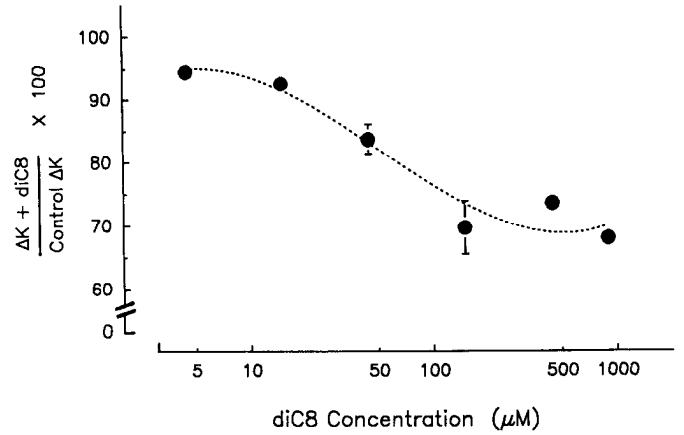
Figure 4. Concentration dependence of diC8 inhibition of ΔK. Hippocampal synaptosomes were incubated for 7 min at 37°C with concentrations of diC8 from 4.5 to 900 μM. ^{86}Rb efflux was then measured for 5 sec. Inhibition of ΔK could be seen at concentrations as low as 15 μM; 50 μM diC8 produced half-maximal inhibition (an approximately 20–25% reduction in total ΔK); there was little additional inhibition at concentrations greater than 150 μM. The data points represent the mean inhibition seen in 2–3 separate experiments, except for the 150 μM point (measured in 6 experiments) and the 900 μM point (measured in only 1 experiment); N for each experiment was 4–6. Error bars represent the SE of the mean inhibition and are shown only where the SE exceeds the size of the symbol. The *dashed line* is a computer-fitted curve of the data points.

tinely blocked 60–100% of the non-S_R components of ΔK that represent efflux through the 2 types of voltage-gated (inactivating and noninactivating) K channels.

Concentration and time dependence of the diC8 effect

The magnitude of inhibition of K-stimulated Rb efflux by diC8 depended upon both the concentration of diC8 (Fig. 4) and the length of the preincubation (Fig. 5). The maximal inhibition observed in any experiment was approximately a 45–50% reduction in total ΔK; inhibition of this magnitude was produced by high concentrations of, or long incubations with, diC8. DiC8, 50 μM, inhibited the 4-AP-sensitive fraction of ΔK (corresponding to components S_V and T) by about 50%; the inhibition plateaued above 150 μM diC8. A similar dose–response relationship with diC8 has been observed in other systems (Conn et al., 1985).

There were day-to-day variations in the absolute amount of inhibition produced by 150 μM diC8, the concentration used for most of our experiments. In 12 separate experiments in which hippocampal synaptosomes were preincubated with 150 μM diC8 for 6 min, inhibition ranged from 21 to 47% of total control ΔK; the average inhibition was 36%.

Inhibition of ΔK by diC8 was observed only when the synaptosomes were preincubated with the lipid for at least 15 sec before efflux was initiated (Fig. 5). The inhibitory effect was not observed when diC8 was present only in the efflux solution (no preincubation), suggesting that diC8 was not acting as a direct blocker of synaptosomal K channels (see Discussion). Inhibition of ΔK increased with increasing length of preincubation with diC8; half-maximal inhibition was observed after 15–30 sec with 150 μM diC8. The inhibition plateaued after approximately 2 min of preincubation. The effect of diC8 was partially reversed by centrifuging the diC8-treated synaptosomes and resuspending them in lipid-free media for 20 min before efflux was measured (not shown).
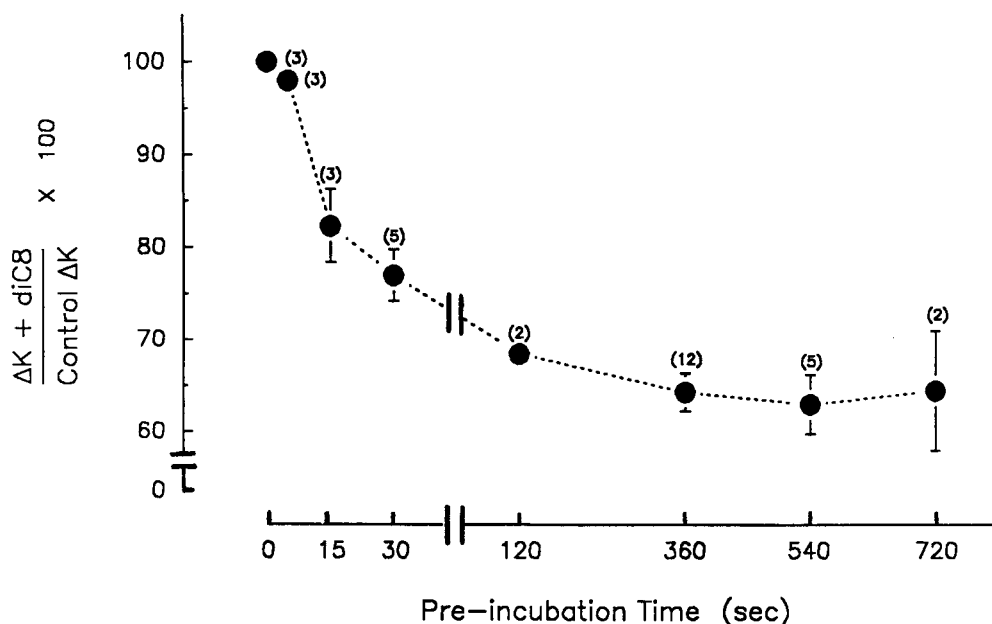
Figure 5. Time dependence of diC8 inhibition of ΔK. Hippocampal synaptosomes were preincubated with 150 μM diC8 for 5 sec to 12 min before ^{86}Rb efflux was measured for 5 sec. Inhibition of ΔK was apparent following preincubations of at least 15 sec and increased rapidly as the length of preincubation increased up to approximately 2 min, at which point the inhibition began to plateau. Addition of diC8 to the efflux solution alone (no preincubation) or preincubations of less than 15 sec had no effect on control ΔK. Points shown are the mean inhibition observed in 2–12 separate experiments (number of experiments for each point shown in parentheses); 4–6 individual determinations were made in each experiment. Bars represent the SE of the mean inhibition and are shown only where the SE exceeds the size of the symbol. Note the change in scale after the 30 sec time point.

Structure-activity relationship supports C kinase involvement

There is a well-defined relationship between the structure of DG analogs and their ability to activate C kinase; for example, DG analogs that lack the 3'OH group do not stimulate C kinase (Conn et al., 1985). To determine if the diC8-induced inhibition of ΔK was due to activation of protein kinase C, we compared the effect of diC8 with that of 1-oleoyl-2-acetylglycerol (OAG), another C-kinase activating DG analog, and with deoxy-diC8, which does not activate C kinase. As illustrated in Figure 6, OAG mimicked the inhibitory effect of diC8 on ΔK but appeared to be less potent; in contrast, deoxy-diC8 had no effect on ΔK.

Two C kinase-activating phorbol esters (Castagna et al., 1982), TPA (12-O-tetradecanoylphorbol 13-acetate) and PDBu (phorbol 12,13-dibutyrate), in concentrations from 10^{-9} to 10^{-5} M, had no effect on any of the components of Rb efflux from hippocampal or striatal synaptosomes (not shown). Furthermore, neither PDA (phorbol 12,13-diacetate) nor DPB (12-deoxyphorbol 13-isobutyrate), at a concentration of 100 nM, affected the K-stimulated Rb efflux, ΔK (not shown).

Inhibitors of C kinase prevent the diC8 effect

A further test of the hypothesis that the action of diC8 on the K-stimulated Rb efflux is due to C kinase activation is to determine if inhibitors of C kinase can prevent the effect. We examined 2 inhibitors of C kinase, sphingosine (Hannun et al., 1986) and H-7 (Kawamoto and Hidaka, 1984), for their effects on the inhibition of ΔK caused by diC8. Sphingosine inhibits C kinase activity by interfering with the formation of an active lipid–enzyme complex. It prevented the diC8-induced inhibition of ΔK when it was added to synaptosomes concurrently with diC8 (Fig. 7). H-7 inhibits C kinase through a direct interaction with the catalytic site of the kinase. When synaptosomes were incubated with H-7 before exposure to diC8, the inhibition caused by diC8 was significantly reduced (Fig. 7). Neither sphingosine nor H-7, alone, had any effect on resting or K-stimulated efflux (not shown).

Discussion

Modulation of ion channel activity is an important cellular regulatory mechanism (Kaczmarek, 1987; Reuter, 1987). K channels play a key role in neuronal excitability, repolarization and neurotransmitter release and thus are obvious targets for regulation. Evidence currently available demonstrates that the activity of K channels in a variety of systems can be influenced by neurotransmitters, second messengers, and protein kinase activators (Siegelbaum and Tsien, 1983; Levitan, 1985). K channel modulation may underlie fundamental, but poorly understood, processes such as learning and memory (Kandel and Schwartz, 1982; Alkon, 1984). There is thus a great incentive to uncover the mechanisms responsible for the regulation of K channel activity.

DiC8 blocks ^{86}Rb efflux components corresponding to "A" and "delayed rectifier" channels

The data described in this report indicate that certain activators of protein kinase C modulate the activity of voltage-gated K channels in synaptosomes from mammalian brain. Incubation of hippocampal synaptosomes with diC8 markedly inhibited K-stimulated Rb efflux corresponding to 2 types of K channels — a rapidly inactivating, voltage-gated K channel and a noninactivating, voltage-gated K channel. The effect was both time and concentration dependent. Experiments with α-DaTX, which blocks T, and *C. sculpturatus* venom, which blocks S_V, demonstrated that diC8 blocked both inactivating and noninactivating K channels; diC8 had no further effect when components T and S_V were both completely blocked by 10 mM 4-AP.

The maximum inhibition of ΔK observed in our experiments was approximately 50% of the total control ΔK. About half of the K-stimulated ^{86}Rb efflux is attributable to electrodiffusion through the "resting" K conductance (see Materials and Methods, Fig. 3, and Bartschat and Blaustein, 1985a); thus, a 50% reduction in ΔK represents virtually complete shut-down of the voltage-gated K channels. Furthermore, diC8 specifically inhib-
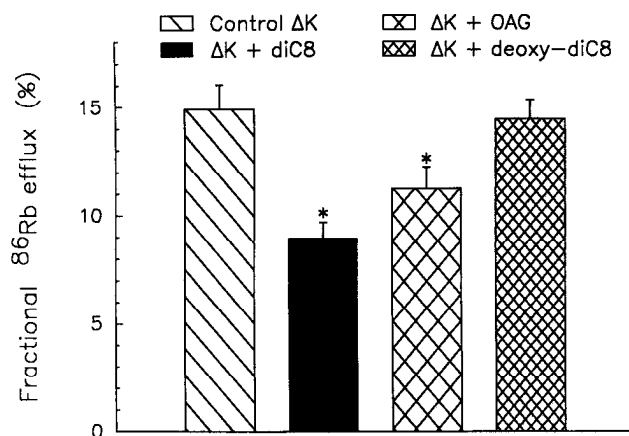
Figure 6. Effect of DG analogs on ^{86}Rb efflux from hippocampal synaptosomes. Hippocampal synaptosomes were incubated with 150 μM diC8, deoxy-diC8, or OAG for 7–9 min before ^{86}Rb efflux was measured for 5 sec. OAG inhibited ΔK, but was slightly less effective than diC8; deoxy-diC8 had no effect on ΔK. Data shown are from 2 representative experiments (one with each analog) and are expressed as the means $\pm$ SE. $N = 10$–12 for control ΔK and ΔK + diC8; $N = 4$–6 for each analog. Similar results were obtained in at least 2 separate experiments. *, significantly different from control ΔK, $p < 0.05$.

Figure 7. Effect of C kinase inhibitors on diC8 inhibition of ΔK. Hippocampal synaptosomes were incubated with 150 μM diC8 $\pm$ 100 μM sphingosine (*SPH*) for 9 min or with 50 μM H-7 for 9 min followed by incubation with diC8 for 9 min, before ^{86}Rb efflux was measured for 5 sec. SPH completely prevented the inhibition of ΔK by diC8; H-7 significantly reduced the diC8-induced inhibition of ΔK. Neither SPH nor H-7 alone had any effect on ΔK (not shown). Data from 2 representative experiments (one with each inhibitor) are illustrated as the means $\pm$ SE. $N = 8$–10 for control ΔK and ΔK + diC8; $N = 4$–5 for each inhibitor. Similar results were obtained in at least 2 separate experiments. *, significantly different from control ΔK, $p < 0.05$; **, significantly different from ΔK + diC8 (alone), $p < 0.05$.
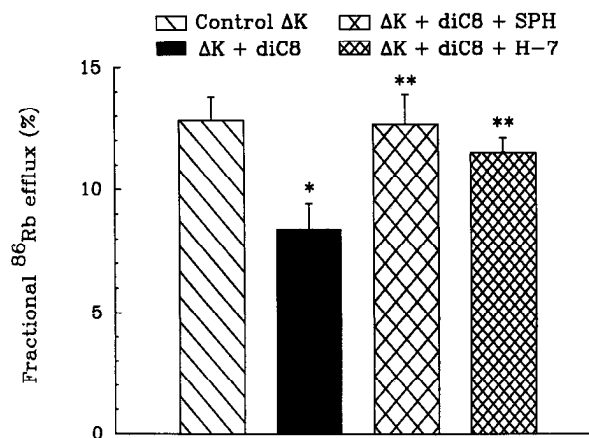
ited the K-stimulated component of Rb efflux; it had no effect on ΔCa, the efflux through Ca-activated K channels, or on resting efflux.

DiC8 blocks K channels by activating C kinase

Pharmacological evidence supports the idea that the diC8 inhibition of the ΔK results from activation of protein kinase C. Structure–activity data showed that inhibition of ΔK was only produced by DG analogs capable of activating C kinase; deoxy-diC8, an inactive analog, had no effect on K-stimulated Rb efflux. Our data indicated that OAG was less potent at inhibiting K-stimulated Rb efflux. OAG may be less effective at traversing membranes; other studies have shown that, although diC8 and OAG have similar potencies in activating C kinase *in vitro*, diC8 is more effective in stimulating luteinizing hormone release from pituitary cells (Conn et al., 1985).

Two inhibitors of C kinase, sphingosine and H-7, blocked the diC8 effect. Neither of these inhibitors is completely specific for C kinase; however, they inhibit C kinase by different mechanisms. The fact that both were able to prevent the diC8 effect strongly suggests that C kinase is involved in the diC8-induced inhibition of K-stimulated Rb efflux.

When evaluating the mode of action of any compound that inhibits ion channel activity, one must consider the possibility that the agent in question simply blocks the channel directly. While it is conceivable that diC8 acts externally to block synaptosome K channels rather than internally through activation of a second-messenger system, the following evidence makes this unlikely. First, diC8 had no effect on ^{86}Rb efflux when it was present only in the efflux solution and had not been preincubated with the synaptosomes. In contrast, compounds that inhibit ^{86}Rb efflux by a direct block of K channels (e.g., dendrotoxins, 4-AP, phencyclidine, tetraethylammonium) do not require a preincubation period and are effective when present only in the efflux solution (Bartschat and Blaustein, 1985a, b, 1986; Blaustein et al., 1988). Second, deoxy-diC8, a structural analog of diC8 that differs only in the absence of the 3'OH group,

had no effect on ^{86}Rb efflux. If diC8 were simply blocking the K channels externally, it is likely that a closely related analog would have a similar (or at least a partial) effect. Finally, the fact that inhibitors of C kinase blocked the inhibition of ^{86}Rb efflux by diC8 also suggests that diC8 acts internally; it seems unlikely that the effects of an external channel blocker could be antagonized without affecting the properties of the channel itself. In combination with the pharmacological data discussed above, these observations support our hypothesis that diC8 acts within the synaptosome to inhibit ^{86}Rb efflux.

Presynaptic K channels may be modulated by only certain types of C kinase

Because diC8 appeared to block synaptosome K channels via activation of protein kinase C, it was somewhat surprising that phorbol esters, which are known to activate C kinase *in vitro*, had no effect on ΔK. There are several possible explanations for this finding.

DG analogs and phorbol esters are often assumed to have identical effects; however, this is not always the case (e.g., Brockenbrough and Korc, 1987; Kolesnick and Paley, 1987; Ways et al., 1987). In fact, Nishizuka (1986) mentions that differences in the actions of phorbols and DG analogs should not be surprising. An important distinction between phorbol esters and DG, the physiological messenger they imitate, is their duration of action (Nishizuka, 1986). The intracellular concentration of DG increases transiently in response to receptor activation and quickly returns to baseline as the lipid is metabolized. In contrast, phorbol esters are not readily degraded within the cell. This difference may underlie seeming incongruities in the effects of DG analogs and phorbol esters and suggests that DG analogs more closely replicate normal physiological responses.

Several groups have isolated C kinase clones that encode structurally distinct forms of C kinase (Coussens et al., 1986; Knopf et al., 1986). The physiological significance of these iso-

zymes has not yet been established. These isozymes may have distinct physiochemical properties that could include differing sensitivity to activation by phorbol esters; for example, Robinson and coworkers (1987) have identified an isozyme of C kinase that is not activated by phorbol esters. It is possible that presynaptic K channels are modulated only by certain subclasses (perhaps phorbol-insensitive) of C kinase. While previous studies have demonstrated modulation of postsynaptic K conductances by phorbol esters (Baraban et al., 1985; Doerner et al., 1987; Werz and Macdonald, 1987), our results are the first to address their effect on presynaptic K channels. The difference in the response to phorbol esters suggests that the regulation of presynaptic K channels may not be identical to that of analogous channels located postsynaptically on the soma. Moreover, the situation is complicated by observations suggesting that the relative density of various K and Ca channels, a characteristic that would be expected to vary between the terminal and the soma, may determine the response to phorbol esters (Werz and Macdonald, 1987).

Finally, it is likely that activation of C kinase within the synaptosome has multiple effects. Indeed, C kinase activation *in vivo* may promote neurotransmitter release through a dual mechanism, by increasing both the influx of calcium into the presynaptic terminal (our results and Zurgil et al., 1986) and the sensitivity of the exocytotic process to calcium (Knight and Baker, 1983; Nichols et al., 1987). Conceivably, certain isozymes of C kinase might differentially modulate K channel function, and influx of calcium, while others preferentially control the Ca sensitivity of processes distal to Ca entry. Such redundant regulation of the effects of calcium would allow the neuron to maintain fine control over the essential process of transmitter release.

Implications of C kinase modulation of presynaptic K channels

Activation of protein kinase C by DG analogs (Lynch and Bliss, 1986) or phorbol esters (Zurgil et al., 1986; Malenka et al., 1987; Nichols et al., 1987) produces an increase in neurotransmitter release in a number of different experimental preparations from the mammalian CNS. Our results suggest that this increase in transmitter release may result from a block of the K channels that are responsible for the repolarization phase of the action potential, in a manner analogous to that previously demonstrated in invertebrates (Kandel and Schwartz, 1982). Inhibition of these channels would slow neuronal repolarization, thereby allowing more calcium to enter the presynaptic terminal and, in turn, promoting increased neurotransmitter release. This scenario is supported by observations that K channel blockers such as α-DaTX ("dendrotoxin"; Harvey and Karlsson, 1980), 4-AP, and tetraethylammonium (Glavinovic, 1987) increase neurotransmitter release. Indeed, inhibition of K channels underlies the increased neurotransmitter release caused by C kinase activators in the squid giant synapse (Augustine et al., 1986).

In conclusion, our data directly demonstrate that C kinase activators can modulate K channel function in the mammalian presynaptic terminal. These results have interesting implications in light of the participation of K channel modulation in simple forms of invertebrate learning and memory (e.g., Kandel and Schwartz, 1982; Farley and Auerbach, 1986) and the role of protein kinase C in LTP, a mammalian model of synaptic plasticity (e.g., Malenka et al., 1986). Our data strengthen the hypothesis that modulation of voltage-gated K channels in the

presynaptic terminal may be involved in the C kinase-induced increase in neurotransmitter release that is thought to be at least one component of LTP (Malenka et al., 1986).

Studies are currently underway to determine if the inhibition of K-stimulated Rb efflux by diC8 is accompanied by a change in the phosphorylation of synaptic proteins. Of particular interest is whether changes in phosphorylation can be detected in peptides, labeled by K channel blockers from *D. angusticeps* venom, that are thought to be components of neuronal K channels (Sorensen and Blaustein, 1987).

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