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cGMP Inhibits L-Type Ca^{2+} Channel Currents Through Protein Phosphorylation in Rat Pinealocytes

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In this study, the effect of cGMP on the dihydropyridine-sensitive (L-type) Ca^{2+} current was investigated using the whole cell version of the patch-clamp technique in rat pinealocytes. Dibutyryl-cGMP (1×10^{-4} M) induced a pronounced inhibition of the L-type Ca^{2+} channel current. The dibutyryl-cGMP effect was concentration dependent. Elevation of cGMP by nitroprusside had a similar inhibitory action on the L-type Ca^{2+} channel current. Norepinephrine, which increased cGMP in rat pinealocytes, also inhibited this current. The action of cGMP was independent of cAMP elevation since the cAMP antagonist, Rp-cAMPs, had no effect on the inhibitory action of dibutyryl-cGMP. The involvement of cyclic GMP-dependent protein kinase was suggested by the blocking action of two protein kinase inhibitors, (1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide (HA1004), on the dibutyryl-cGMP effect on the L-type Ca^{2+} channel current. Taken together, these results suggest that (1) cGMP modulates L-type Ca^{2+} channel currents in rat pinealocytes, causing inhibition of this current; (2) the action of cGMP appears to be independent of cAMP elevation; and (3) phosphorylation by cGMP-dependent protein kinase may be involved.

[Key words: cGMP, cAMP, L-type Ca^{2+} current, nitroprusside, cGMP-dependent protein kinase, pineal]

In the rat pinealocyte, norepinephrine is released at night from the sympathetic nerve terminal to stimulate a large increase in cAMP and cGMP accumulation (Klein, 1985). cAMP controls the activity of arylalkyalamine *N*-acetyltransferase, the enzyme regulating the nocturnal increase in the synthesis of the pineal hormone melatonin (Klein and Weller, 1975). The role of cGMP is unknown. The spectrum of cGMP-regulated events in other tissues, however, include photo- and olfactory reception, cardiac and smooth muscle contractility and steroidogenesis (Schmidt et al., 1993).

In heart, cGMP regulates contractility partly through its action on the dihydropyridine (DHP)-sensitive, L-type Ca^{2+} current. The predominant action of cGMP on this Ca^{2+} current is inhibitory and several signal transduction mechanisms are involved (Lohmann et al., 1991). For instance, in isolated frog myocytes cGMP inhibits this current by stimulation of cAMP-phosphodiesterase, causing a reduction in cAMP (Hartzell and Fischmeister, 1986), whereas in purified rat ventricular myocytes, cGMP predominantly inhibits this current via a mechanism involving cGMP-dependent protein kinase (Mery et al., 1991). However, under certain conditions, a stimulatory effect of cGMP on the Ca^{2+} current through inhibition of cAMP-phosphodiesterase has also been reported in frog myocytes (Mery et al., 1993).

The L-type Ca^{2+} channels are composed of several transmembrane subunits and the α_1 subunit of this channel binds organic Ca^{2+} channel blockers (Catterall et al., 1988). Recently, multiple forms of the α_1 subunit of this channel have been isolated from rat brains (Vaghy et al., 1987). One of these isoforms has primary structure that is significantly different from its skeletal and cardiac muscle counterparts (Hui et al., 1991). This novel brain isoform of the α_1 subunit is highly expressed in rat pineal gland (Chin et al., 1992).

In contrast to the numerous reports on the effects of cGMP on the L-type Ca^{2+} current in heart, little is known about the action of cGMP on the L-type Ca^{2+} current in mammalian neural tissue. In rat sympathetic neurons and rabbit pelvic ganglia, cGMP increases this current (Nishimura et al., 1992; Chen and Schofield, 1993). However, cGMP inhibits this current in guinea pig hippocampal neurons through activation of phosphodiesterase (Doerner and Alger, 1988).

The dihydropyridine-sensitive L-type Ca^{2+} channel current has recently been characterized in the rat pinealocyte (Aguayo and Weight, 1988; Chik et al., 1992) and the high abundance of the novel brain isoform of the α_1 subunit of this channel in the rat pineal (Chin et al., 1992) offers us an opportunity to investigate the relationship between cGMP and the brain L-type Ca^{2+} channel in mammalian neural tissue. The purpose of this study is therefore to determine (1) the effect of dibutyryl-cGMP and other cGMP elevating agent on this current using the whole cell version of the patch-clamp technique, and (2) whether cGMP-dependent protein kinase or phosphodiesterase is involved in the intracellular mechanism of action of cGMP in this tissue. Our results indicate that cGMP inhibits the L-type Ca^{2+} channel current in rat pinealocytes and unlike its action on other mammalian brain tissue, this effect of cGMP appears to be mediated through a mechanism that involves cGMP-dependent protein kinase.

Materials and Methods

Materials. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H7), *N*-(2-guanidinoethyl)-5-isoquinoline-sulfonamide (HA1004), and nitroprusside were obtained from Sigma Chemical Corp. (St. Louis, MO). Dibutyryl-cGMP and Bay K 8644 were obtained from Calbiochem Corp (San Diego, CA) and Rp-cAMPs was obtained from BioLog Life Sci. Inst. (La Jolla, CA). Cs_2-aspartate was prepared by Dr. H. J. Liu (Department of Chemistry, University of Alberta). All other chemicals were of the purest grades available and were obtained commercially. ^{125}I-

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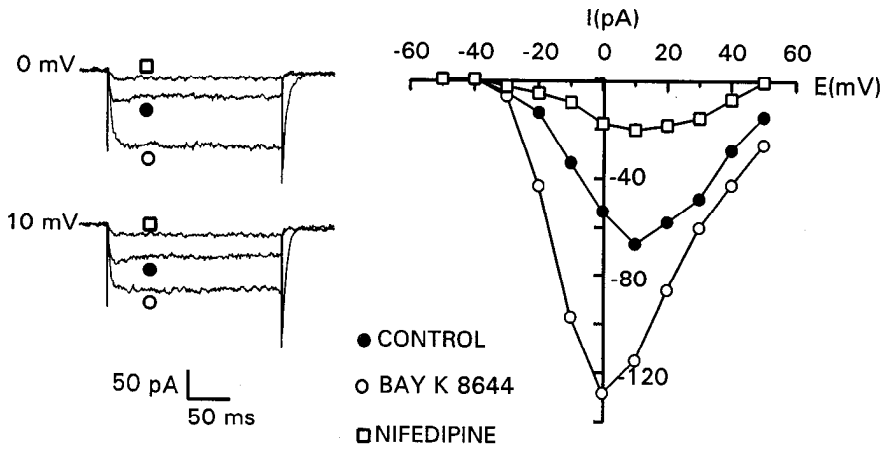

Figure 1. Effect of Bay K 8644 and nifedipine on the L-type Ca^{2+} channel current in a single rat pinealocyte. *A*, L-type Ca^{2+} channel currents (20 mM Ba^{2+} charge carrier) after the sequential additions of Bay K 8644 (1×10^{-6} M) and nifedipine (1×10^{-6} M) are shown. The currents were activated by depolarizing the pinealocyte from a holding potential of −50 mV to the test potentials indicated next to the currents. The leakage was corrected on line. The *I–V* relationships after the sequential additions of Bay K 8644 and nifedipine are shown in *B*. The currents shown in *A* were used to construct the *I–V* relationships in *B*. This inward current was increased 100% by Bay K 8644 and the subsequent addition of nifedipine reduced the total current by 85%.

CGMP was obtained from ICN ImmunoBiologicals (Lisle, IL) and antibody for the radioimmunoassay of cGMP was a gift from Dr. A. Baukal (National Institute of Child Health and Human Development, NIH, Bethesda, MD).

Cell preparations. Male Sprague–Dawley rats (150 gm) were decapitated after cervical dislocation and no anesthetic was used. Pinealocytes were then prepared by trypsinization as described previously (Buda and Klein, 1978). The cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and maintained at 37°C in a mixture of 95% air and 5% CO_2 overnight before the experiments.

Ca^{2+} current recordings. The whole-cell version of the patch-clamp technique was used to measure the Ca^{2+} channel current (Hamill et al., 1981). Patch electrodes were pulled from borosilicate glass capillary tubes (OF = 1.2 mm, ID = 0.9 mm, FSH, Brunswick, ME) and heat polished. They were filled with a solution containing (in mM) 70 Cs_2-aspartate, 20 HEPES, 11 EGTA, 1 $CaCl_2$, 5 $MgCl_2 \cdot 6\ H_2O$, 5 glucose, 5 ATP-Na_2, and 5 K-succinate. Creatine phosphokinase (50 U/ml) and 20 mM phosphocreatine-Na_2 were added to the pipette solution to prevent current run-down (10). The bath solution contained (in mM) 105 Tris Cl, 0.8 $MgCl_2$, 5.4 KCl, 20 $BaCl_2$, 0.02 tetrodotoxin, and 10 HEPES. In all experiments, Ba^{2+} (20 mM) was used as the charge carrier. All solutions were filtered (0.22 μm) before use. The osmolarity was adjusted to 320 mOsm and the pH to 7.4. The membrane currents were measured using an Axon (Axopatch, β=1) patch-clamp amplifier (Axon Instruments, Foster City, CA). The data were sampled using pCLAMP software (pCLAMP 5.5) and a Labmaster analog-to-digital interface (Axon Instrument). Analysis was also performed using the pCLAMP software. The effects of the drugs were monitored continuously using depolarizing pulses at a frequency of 0.03 Hz except when generating current–voltage relationships. To generate current–voltage relationships, 250 msec depolarizing test pulses of increasing amplitude were applied at a frequency of 0.3 Hz. On-line leakage subtraction was implemented using the P/N protocol in pCLAMP software. The drugs were added into the bath solution after the inward current reached a steady-state. The experiments were performed at room temperature (20–22°C).

cGMP determination. Aliquots of cells (50,000 cells/500 μl) were treated with drugs which had been prepared in 100× concentrated solutions in water. The duration of the drug treatment period was 10 min for cGMP accumulation. At the end of the treatment period, cells were collected by centrifugation (2 min, 10,000 × *g*), the supernatant was aspirated, and the tube was placed on solid CO_2. The frozen cell pellets were lysed by the addition of 5 mM acetic acid (100 μl) and boiling (5 min). The lysates were stored frozen at −20°C until analysis.

The lysates were centrifuged (12,000 × *g*, 10 min) and samples of the supernatant were used to determine cellular cGMP contents, using a radioimmunoassay procedure in which samples were acetylated prior to analysis (Harper and Brooker, 1975). Since there was a small batch-to-batch variation of cGMP response between cell preparations, all comparisons were performed within the same batch of cells.

Statistical analysis. Data are presented as the means ± SEM of the amount of cGMP in four aliquots of cells. The amount of cyclic nucleotide in each cell pellet was based on duplicate determination. Data were analyzed by Duncan's multiple range test (1955). For the patch-clamp studies, data are presented as the means ± SEM percentages of control values. The control current–voltage relationship was plotted and used as a control. The peak inward current occurred at 0 or 10 mV and hence this current was used for the determination of inhibition of cGMP elevating agents. Group comparisons were analyzed by Student's *t* tests, with statistical significance set at $P < 0.05$ (*, significantly different from control).

Results

Characterization of L-type Ca^{2+} channel currents in rat pinealocytes

As described previously, dissociated pinealocytes were found to express a DHP-sensitive Ca^{2+} channel current (L-channel current) (Aguayo and Weight, 1988; Chik et al., 1992). This current was activated by depolarizing the cells from a holding potential of −50 mV. Current through this channel showed very little inactivation during the depolarizing pulse and was increased by Bay K 8644 (1×10^{-6} M) and inhibited by nifedipine (1×10^{-6} M) (Fig. 1). Bay K 8644 not only increased the current by 100%, but also shifted the peak inward current towards more negative potentials (Fig. 1), which is a common characteristic of this channel (Bean et al., 1986). The effects of nifedipine and Bay K 8644 were seen 1–2 min after addition of the drugs to the bath. Nifedipine applied after Bay K 8644 inhibited the total current by 85% (Fig. 1).

Effect of a cGMP analog and a cGMP elevating agent on L-type Ca^{2+} channel currents in rat pinealocytes

The effect of dibutyryl-cGMP on the L-type Ca^{2+} channel current is shown in Figure 2, *A* and *B*. Dibutyryl-cGMP (1×10^{-4} M) decreased the amplitude of the peak L-type Ca^{2+} channel current by 50.2%. The onset of the inhibition occurred within 1–2 min and maximal inhibition was observed within 4 min. The inhibition of the inward L-type Ca^{2+} channel current by dibutyryl-cGMP was reproducible and reversible. There was no change in the voltage at which the current was activated, nor was the peak current shifted along the voltage axis by dibutyryl-cGMP (Fig. 2*B*). The inhibition by dibutyryl-cGMP was concentration dependent (Fig. 2*C*). No inhibition was seen with dibutyryl-cGMP (1×10^{-9} M) and a 26.9% inhibition was seen with dibutyryl-cGMP (1×10^{-6} M). These observations suggest that similar to cardiac and neural tissues, cGMP inhibits the L-type Ca^{2+} channel current in the rat pineal gland.

To determine whether other cGMP elevating agents also have a similar effect on the L-type Ca^{2+} channel current, cGMP was

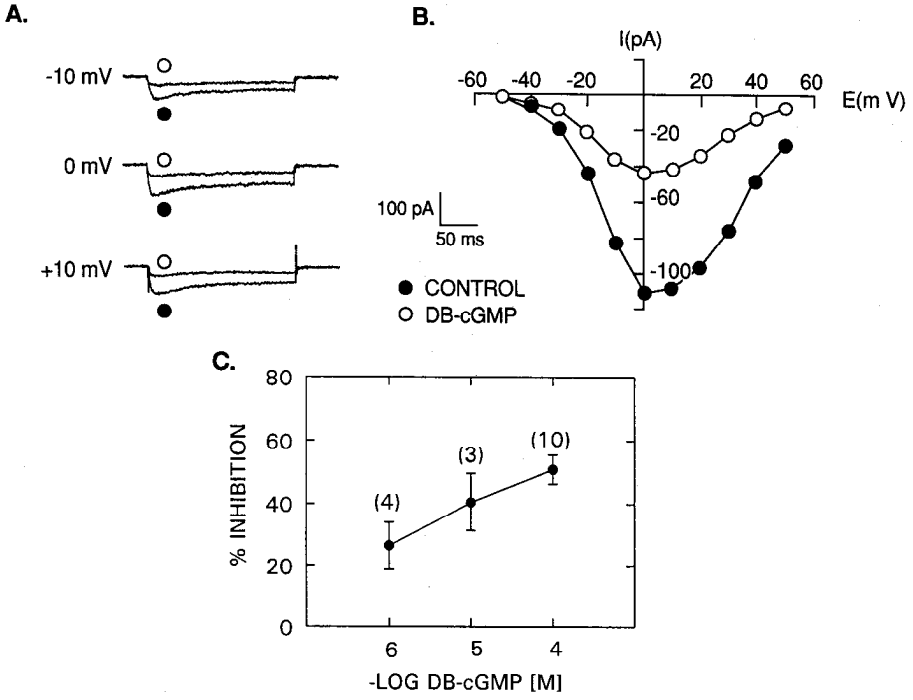

Figure 2. Effect of dibutyryl-cGMP on the L-type Ca^{2+} channel current in rat pinealocytes. *A*, L-type Ca^{2+} channel currents at two test potentials (indicated next to the currents) before (●) and after (○) dibutyryl-cGMP (DB-cGMP, 1×10^{-4} M) are shown. The *I–V* relationship before and after DB-cGMP is shown in *B*. The currents shown in *A* were used to construct the *I–V* relationship in *B*. *C*, The effect of DB-cGMP as a function of concentration on L-type Ca^{2+} channel currents. The peak inward L-channel current amplitude was recorded after the current reached the steady state. Cells were then exposed to different concentrations of DB-cGMP for 10 min. The peak inward currents were measured and expressed as a percentage of the reduction of the control current (% inhibition). The points are plotted as the means ± SEM and the *n* values are shown in parenthesis above each data point.

pharmacologically increased with nitroprusside. Treatment with nitroprusside (1×10^{-4} M) reduced the amplitude of the L-type Ca^{2+} channel current by 35.7% without shifting the current–voltage relationship (Fig. 3*A,B*). The onset of the nitroprusside effect appeared within 3 min and reached its peak after 5–6 min. The inhibitory action of nitroprusside on the L-channel current was concentration dependent (Fig. 3*C*) and paralleled the stimulatory action of nitroprusside on cGMP accumulation (Fig. 3*D*).

The effect of norepinephrine, a known stimulator of cGMP in rat pinealocytes (Klein, 1985), on the L-type Ca^{2+} channel current was also determined. Treatment with norepinephrine (1×10^{-7} M), which increased cGMP sixfold (from 9.1 ± 1.5 to 55.5 ± 5.0 pmol/10^5 cells, $n = 4$), reduced the amplitude of the L-type Ca^{2+} channel current by 20.1 ± 5.9% ($n = 4$) (Fig. 4*A,B*). The onset of the norepinephrine effect appeared within 3 min and reached its peak after 5–6 min.

Mechanism through which cGMP inhibited L-type Ca^{2+} channel currents

To test the possible involvement of the cAMP pathway, Rp-cAMPs was added to the bath solution prior to treatment with dibutyryl-cGMP. Treatment with Rp-cAMPs had no effect (50.2% ± 3.9% vs 47.0 ± 7.1% in the presence or absence of Rp-cAMPs) on the inhibition of the L-type Ca^{2+} channel current by dibutyryl-cGMP (Fig. 5*A,B*), suggesting that the observed

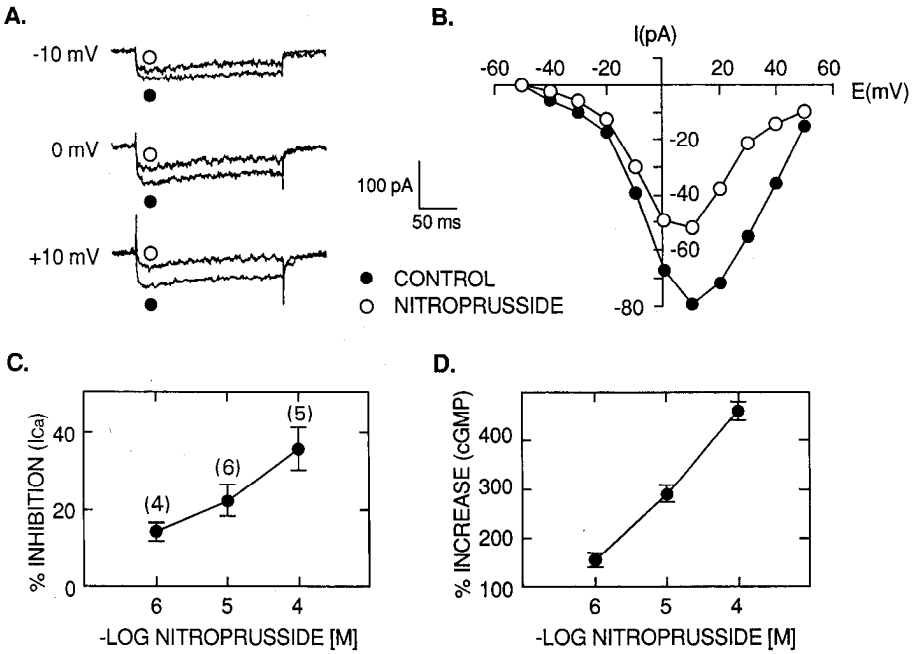

Figure 3. Effect of nitroprusside on the L-type Ca^{2+} channel current and cGMP accumulation in rat pinealocytes. *A*, L-type Ca^{2+} channel currents at two test potentials (indicated next to the currents) before (●) and after (○) nitroprusside (1×10^{-4} M) are shown. The *I–V* relationship before and after nitroprusside is shown in *B*. The currents shown in *A* were used to construct the *I–V* relationship in *B*. *C*, The effect of nitroprusside as a function of concentration on L-type Ca^{2+} channel currents (I_{Ca}). For additional methods on Ca^{2+} current recordings, see Material and Methods. *D*, The effect of nitroprusside as a function of concentration on cGMP accumulation. The values plotted represent the means ± SEM, $n = 4$. Data shown are representative of one of three experiments.

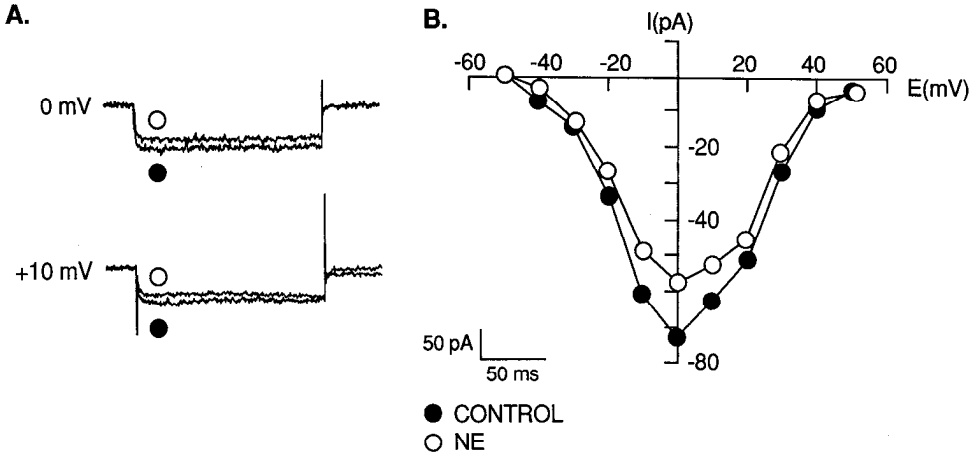

Figure 4. Effect of norepinephrine on the L-type Ca^{2+} channel current in rat pinealocytes. *A*, L-type Ca^{2+} channel currents at two test potentials (indicated next to the currents) before (●) and after (○) norepinephrine (1×10^{-7} M) are shown. The *I–V* relationship before and after norepinephrine is shown in *B*. The currents shown in *A* were used to construct the *I–V* relationship in *B*. For additional methods on Ca^{2+} current recordings, see Material and Methods.

effect of cGMP on the current is independent of elevation of cAMP and possibly dependent on cGMP-dependent protein kinase, a known mechanism of cGMP on L-type Ca^{2+} channel currents in ventricular myocytes (Mery et al., 1991).

To examine the involvement of protein kinases, two protein kinase inhibitors, H7 and HA1004 (Hidaka et al., 1984) were used to examine their actions on the inhibitory effect of dibutyryl-cGMP. Treatment with H7 (1×10^{-4} M), which had no effect on the L-channel current, reduced the inhibitory action of dibutyryl-cGMP on this current from 50.2% to 18.2% (Fig. 6*A,B*), suggesting the involvement of cGMP-dependent protein kinase in the inhibitory action of dibutyryl-cGMP. Treatment with HA1004 (1×10^{-4} M), which caused a small reduction of the L-channel current, was also effective in reducing the inhibitory action of dibutyryl-cGMP on this current (Fig. 7*A,B*).

Discussion

Although the signal transduction mechanisms involved in the regulation of pineal function are well established, very little is known about the relationship between second messengers and the electrophysiology of the rat pineal gland. The dihydropyridine-sensitive Ca^{2+} channel current has recently been characterized in the rat pinealocyte (Aguayo and Weight 1988; Chik et al., 1992) and the brain form of this channel is highly expressed in the rat pineal gland (Chin et al., 1992). In this study, it was found that cGMP inhibits the L-type Ca^{2+} channel current probably through a mechanism that involves cGMP-dependent protein kinase.

As indicated in the introductory section, norepinephrine is released at night from the sympathetic nerve terminal to stimulate a large increase in pineal cAMP and cGMP accumulation (Klein, 1985). The role of cAMP on the activity of arylalkyalamine

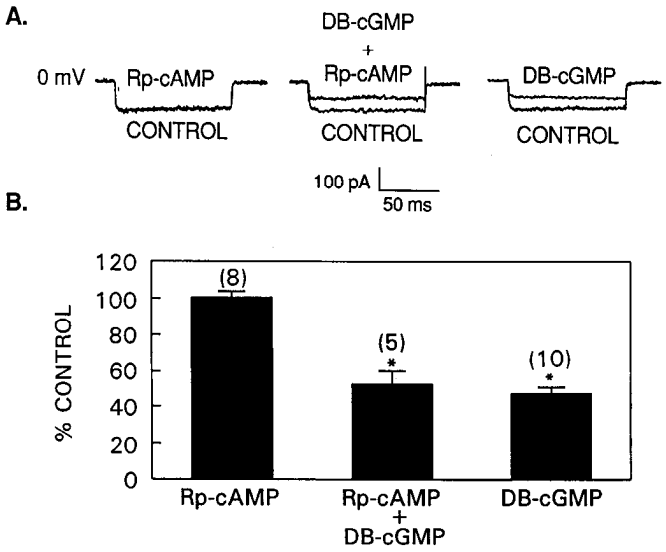

Figure 5. Effect of Rp-cAMPs on the dibutyryl-cGMP inhibition of the L-type Ca^{2+} channel current in rat pinealocytes. *A*, The effect of dibutyryl-cGMP (*DB-cGMP*, 1×10^{-4} M) on the peak inward L-channel current in the presence of Rp-cAMPs (1×10^{-4} M). Rp-cAMPs had no effect on the DB-cGMP effect on L-type Ca^{2+} channel currents. *B*, The results from a group of cells are shown (*n* values are indicated in parentheses above the histograms). Rp-cAMPs had no effect on L-type Ca^{2+} channel currents. Treatment with Rp-cAMPs had no effect on the inhibitory effect of DB-cGMP on the peak inward Ca^{2+} currents. For additional methods on Ca^{2+} current recordings, see Material and Methods.

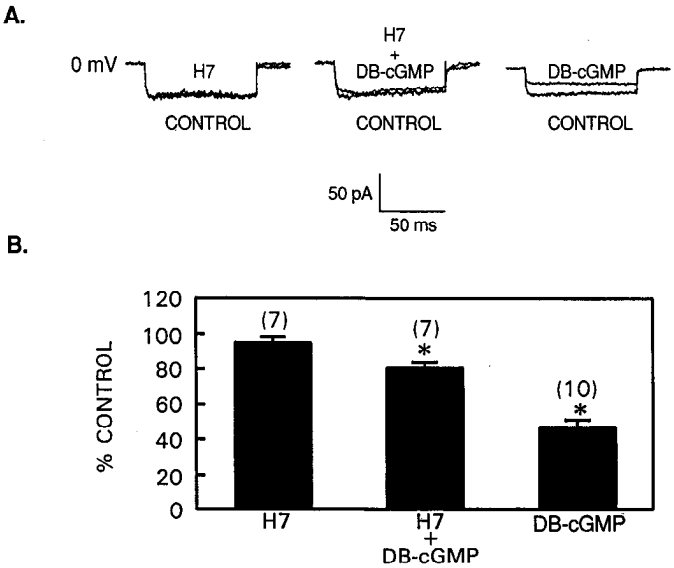

Figure 6. Effect of H7 on the dibutyryl-cGMP inhibition of the L-type Ca^{2+} channel current in rat pinealocytes. *A*, The effect of dibutyryl-cGMP (*DB-cGMP*, 1×10^{-4} M) on the peak inward L-channel current in the presence of H7 (1×10^{-4} M). *B*, The results from a group of cells are shown (n values are indicated in parentheses above the histograms). *H7*, Which had no effect on the current, was effective in reducing the inhibitory effect of DB-cGMP on the peak inward Ca^{2+} current from 50.2% to 18.2%. For additional methods on Ca^{2+} current recordings, see Material and Methods.

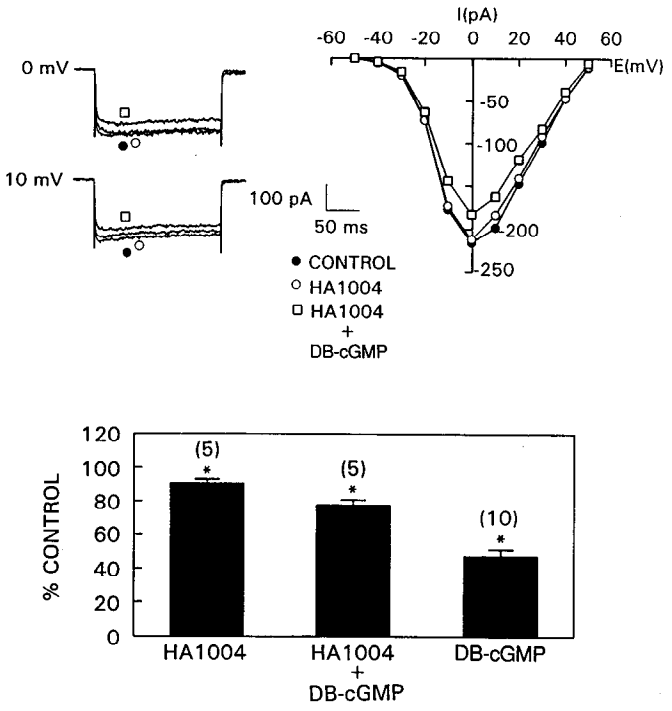

Figure 7. Effect of HA1004 on the dibutyryl-cGMP inhibition of the L-type Ca^{2+} channel current in rat pinealocytes. *A*, The effect of dibutyryl-cGMP (*DB-cGMP*, 1×10^{-4} M) on the peak inward L-channel current in the presence of HA1004 (1×10^{-4} M). *B*, The results from a group of cells (the *n* values are shown in parentheses above the histograms) are shown. HA1004, which caused a small reduction in the current, was effective in reducing the inhibitory effect of DB-cGMP on the peak inward Ca^{2+} current from 50.2% to 22.3%. For additional methods on Ca^{2+} current recordings, see Material and Methods.

N-acetyltransferase and melatonin synthesis is well established (Klein and Weller, 1975). In contrast, the role of cGMP is unknown. The observed inhibitory action of cGMP on L-type Ca^{2+} currents represents the first demonstration of a cGMP-mediated event in the rat pineal gland.

From previous electrophysiological studies, it has been established that pinealocytes have resting potentials which are approximately −30 mV and that they generate action potentials which do not involve fast Na^+ channels (Parfitt et al., 1975; Freschi and Parfitt, 1986). When the gland is activated by norepinephrine, the cells are hyperpolarized and this hyperpolarization is related to K^+ efflux (Cena et al., 1991). The hyperpolarization together with the inhibitory action on the L-type Ca^{2+} channel current produced by the increase of cGMP accumulation by norepinephrine (Vanecek et al., 1985) would favor stabilization of the membrane and reduced electrical activity. This would be conducive for the large increase in the synthetic activity for melatonin production at night (Klein, 1985). This is an attractive hypothesis and it is of interest to note that in the chicken pineal gland, there is a close correlation between the activity of the L-channel and melatonin production (Harrison and Zatz, 1989; Zatz and Mullen, 1988).

In other cells, activation of the L-type Ca^{2+} channel current is one mechanism whereby excitation and secretion are coupled and modulation of these channels provides a precise control of secretion (Hosey and Lazdunski, 1988). In the rat pineal gland, since the secretion of melatonin is governed by synthesis rather than release, it is possible that activation and modulation of the L-type Ca^{2+} channel current may regulate other Ca^{2+}-mediated events (Ho et al., 1987, 1988, 1989). These other events may be directly related to the induction of *N*-acetyltransferase and melatonin synthesis as suggested by other studies in the chicken pineal gland (Zatz and Mullen, 1987; Harrison and Zatz, 1988).

The observation that the inhibitory action of dibutyryl-cGMP on the L-type Ca^{2+} channel current is mimicked by nitroprusside suggest that the soluble form of guanylyl cyclase may be involved in the action of cGMP on this current. Recent studies showed that cGMP formation in the rat pineal gland is linked with the nitric oxide–dependent activation of cytosolic guanylyl cyclase (Spessert, 1993; Spessert et al., 1993). It is possible that nitric oxide is involved in the inhibitory action of cGMP on the L-type Ca^{2+} channel current as demonstrated in bullfrog cardiac tissue (Mery et al., 1993).

Since the brain L-type Ca^{2+} channel is highly expressed in the rat pineal gland (Chin et al., 1992), it is important to compare the regulation of this channel with that of other neural tissue. There are major similarities and differences in the modulating action of cGMP on this current. Unlike the action of cGMP in peripheral neurons which is stimulatory (Nishimura et al., 1992; Chen and Schofield, 1993), the effect of cGMP on this current in the rat pineal gland is inhibitory, similar to that observed in the guinea pig hippocampal neurons (Doerner and Alger, 1988). However, the mechanism through which cGMP modulates this current differs in the two tissues. While the inhibitory action in the guinea pig hippocampal neuron is through activation of cyclic nucleotide phosphodiesterase, the cGMP effect on the rat pineal gland is through activation of cGMP-dependent protein kinase. This suggestion is based on the observation that two kinase inhibitors, H7 and HA 1004, inhibit the effect of GMP on the L-type Ca^{2+} channel current. Since neither cGMP elevating agents has an effect on basal cAMP accumulation in the pineal (our unpublished observation), it would appear that cyclic nucleotide phosphodiesterase is not involved in the action of cGMP on this current. These results suggest that the mechanism of action of cGMP in the rat pineal gland appears to be similar to that of rat ventricular myocytes (Mery et al., 1991) even though these two tissues presumably express different isoforms of the α_1 subunit of L-type Ca^{2+} channels. Furthermore, the brain L-type Ca^{2+} channels appear to be regulated by cGMP through two separate mechanisms: cGMP-dependent protein kinase as in the pineal or cyclic nucleotide phosphodiesterase as in the hippocampus. Perhaps the controlling factor that determines which mechanism is operating in a particular tissue reflects the relative abundance of the two enzymes present.

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