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Effects of Lithium on Basal and Modulated Activities of the Particulate and Soluble Guanylate Cyclases in Retinal Rod Outer Segments†

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ABSTRACT: A large amount of information regarding the kinetics of biochemical reactions involved in visual transduction was derived from electrophysiological studies on dark-adapted rod outer segments. Hodgkin et al. [(1985) *J. Physiol. 358*, 447–468] observed that when Na was replaced with Li in the perfusion solution bathing the rod outer segment, the dark current slowly declined to zero. This decline was thought to result from a rise in intracellular calcium which was hypothesized to inhibit guanylate cyclase activity and reduce the cyclic GMP concentration. Rod outer segments contain membrane and soluble guanylate cyclase activities, and we show here that Li directly inhibits both types of activities very strongly. Both the basal (at high calcium) and the stimulated (at low calcium) activities of the membrane enzyme were inhibited by Li. Half-maximal inhibition of the stimulated enzyme was at 30 mM Li while for the basal activity it was at 100 mM. Over 80% of the activated enzyme was inhibited at 110 mM Li. The soluble guanylate cyclase activity was stimulated by nitroprusside. One hundred millimolar Li inhibited the basal activity by 20–30%, but the inhibition of the nitroprusside-stimulated (soluble) enzyme was much stronger, resembling that of the activated membrane enzyme. Half-maximal inhibition occurred at 30 mM, and about 80% inhibition was found at 100 mM Li. Stimulation of the soluble enzyme by nitroprusside was independent of calcium in the physiological range. The inhibition of the stimulated enzyme by Li was similarly independent of calcium, except at unphysiologically high concentrations. These results demonstrate that, irrespective of calcium concentrations, Li inhibits both membrane and soluble forms of guanylate cyclases, thereby reducing the cyclic GMP synthesis in rod outer segments. These observations could explain the decline in dark current reported when Li replaced Na in the perfusion medium in electrophysiological experiments.

It is generally accepted that cyclic GMP is the internal messenger in vertebrate visual transduction (Pugh & Cobbs, 1986). Light-sensitive channels conducting cations into the dark-adapted photoreceptor rod outer segments (ROS)[1] are gated by cyclic GMP (Fesenko et al., 1985). The amount of dark current flowing into the ROS is therefore thought to vary with the concentration of cyclic GMP. The free cyclic GMP concentration is maintained at a constant level by a balance between the basal activities of cyclic GMP phosphodiesterase (PDE) which hydrolyzes the nucleotide and guanylate cyclase (GC) which synthesizes it from GTP (Pugh & Cobbs, 1986; Hodgkin & Nunn, 1988). A light flash on rod outer segments increases cyclic GMP hydrolysis, thereby decreasing the dark current (Cote et al., 1984; Cohen & Blazysnki, 1988). Recovery of the dark current is thought to result from a reduction in hydrolysis of cyclic GMP to the basal level, coupled with the restoration of cyclic GMP concentration by cyclase.

ROS guanylate cyclase is hypothesized to be under feedback regulation by calcium (Pugh & Cobbs, 1986; Yau & Nakatani, 1985). In the dark-adapted ROS, calcium enters through the cyclic GMP-gated channel and is extruded through the Na/Ca exchanger (Yau & Nakatani, 1985). When a light flash closes the channels and the exchanger remains unaffected, internal free calcium concentration decreases (McNaughton et al., 1986; Ratto et al., 1988). It was shown that GC is activated at the lower concentration of calcium (Lolley & Racz, 1982), leading to an increase in cyclic GMP concentration, which in turn increases the dark current and thus the internal free calcium concentration. Restoration of the internal free calcium concentration to the dark-adapted level reduces GC activity to its basal level (Pugh & Cobbs, 1986).

Hodgkin et al. (1985) noted that replacing Na with Li in the solution bathing the rod outer segment leads to a small but rapid increase in the dark current, followed by a slower decline to zero. From this, and other observations, the authors suggested that Li permeates the channel somewhat better than Na and that Li does not replace Na in the Na/Ca exchange, thereby leading to an increase in internal calcium. The decline of the current to zero is thought to be due to inhibition of GC by the increased calcium concentration.

There are, however, two observations inconsistent with the above hypothesis. First, free calcium concentration in dark-adapted ROS was reported to be 200–500 nM and decreases rapidly after a light flash (McNaughton et al., 1986; Ratto et al., 1988). Biochemical measurements on cyclase activity show that while the enzyme is activated at free calcium concentrations below 200–500 nM, the basal activity of the enzyme is not inhibited when free calcium concentration is

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[1] Abbreviations: ROS, rod outer segments; GC, guanylate cyclase; PDE, cyclic GMP phosphodiesterase; DTT, dithiothreitol; NO, nitric oxide.

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increased beyond the 500 nM level up to 100 μM (Koch & Stryer, 1988; Lambrecht & Koch, 1991; Dizhoor et al., 1991). Second, Fain et al. (1989) observed that replacing Na with Li in the bathing solution that contained nominally zero calcium concentration also resulted in a rapid increase in current followed by a slow decline, much like the observation Hodgkin et al. (1985) made using 1 mM calcium. The decline in circulating dark current could not therefore be due to inhibition of cyclase by the elevation of internal calcium. Fain et al. (1989) speculated that Li may directly interfere with the mechanisms controlling the dark current. Since cyclic GMP controls the dark current, Li could have increased its hydrolysis or decreased its synthesis. We have recently shown that Li, at concentrations up to 100 mM, does not influence the PDE activity, or its activation by light (Sitaramayya & Margulis, 1992). We have since found that, in addition to the calcium-modulated membrane cyclase, ROS also have a soluble guanylate cyclase activated by nitroprusside (Margulis et al., 1992). In this report we show that Li directly inhibits both the membrane and soluble GCs, as well as the mechanisms of their activation. Since Li effects on dark current were observed in physiological experiments at both high and low external calcium concentrations (Hodgkin et al., 1985; Fain et al., 1989), we also tested the effects of Li on cyclase activity at different calcium concentrations. The effects of Na and K are compared to those of Li.

MATERIALS AND METHODS

Isolation of Intact Rod Outer Segments. Intact ROS were isolated from dark-adapted bovine retinas using the protocol of Schnetkamp and Daemen (1982), except that calcium was excluded from all solutions. All operations were done under infrared light with the aid of an image converter. On the basis of the amount of protein released from ROS when homogenized in hypotonic buffer [10 mM Tris, 1 mM dithiothreitol (DTT), pH 7.5] and centrifuged, 80–90% of the outer segments were intact (Godchaux & Zimmerman, 1979).

Soluble and Membrane Fractions. Intact ROS were homogenized in hypotonic buffer and frozen at −50 °C. A measured aliquot of the thawed suspension was centrifuged for 30 min at 100000*g*. The supernatant was respun to remove any residual membranes. The pellet was washed once in hypotonic buffer, suspended in the same buffer, and made up to a desired volume. The soluble and membrane fractions usually contained 3–5 mg of protein/mL.

Guanylate Cyclase Assays. The assays on homogenates and membranes were done under infrared light, and those on soluble fraction were done in room light. The assay volume was 40 μL and contained 25 mM Tris, pH 7.5, 1.5 mM GTP, 2 mM cyclic GMP, 5 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM DTT, about 10 μCi of [α-^{32}P]GTP, and about 0.1 μCi of [^{3}H]cyclic GMP. In some experiments nitroprusside, $CaCl_2$, NaCl, or KCl was added to the assays at the desired concentrations. Blank samples contained buffer instead of the enzyme preparations. The assays were done for 10 min at 25 °C. The reactions were terminated with the addition of an equal volume of 150 mM EDTA containing cyclic GMP, GMP, and guanosine each at 2 mM, and the tubes were held at 80 °C for 5 min. The tubes were cooled and centrifuged, and aliquots were analyzed for [^{32}P]cyclic GMP by thin-layer chromatography, as described earlier (Hakki & Sitaramayya, 1990).

When these experiments were initiated, cyclase assays were done at 37 and 25 °C. It was noted that the activation of membrane cyclase at low calcium concentrations was much lower at 37 °C than at 25 °C. Since at the time our emphasis was on the membrane enzyme, all further assays were done at 25 °C. However, we recently found that ROS also have a soluble enzyme and that it is activated by nitric oxide (NO), generating substances such as nitroprusside (Margulis et al., 1992). The activation of the soluble enzyme was higher at 37 °C than at 25 °C primarily because the liberation of NO from nitroprusside is temperature dependent (Feelisch & Noack, 1987). Therefore, it would have been better to assay the soluble enzyme at 37 °C, but for the sake of uniformity, all the assays, on both membrane and soluble fractions, were done at 25 °C.

Cyclic GMP Phosphodiesterase Assays. The [^{3}H]cyclic GMP added to the cyclase assays provided a measure of hydrolysis of cyclic GMP under the assay conditions. Under the conditions employed in this series of experiments, less than 10% of the cyclic GMP was hydrolyzed during the 10-min assays. Nitroprusside, calcium, lithium, or the other cations tested had no influence on the cyclic GMP hydrolysis.

Free Calcium Concentrations. Free calcium concentrations were calculated using the software Max Chelator, version 4.12, from Chris Patton, Stanford University, Stanford, CA. All the cyclase assays contained 1 mM EGTA unless stated otherwise. When no $CaCl_2$ was added, the free calcium concentration in the assays was assumed to be zero and was referred to as the "low calcium concentrations". To obtain the desired free calcium concentrations shown in parentheses, calcium was added to the assays to the following final concentrations: 0.7 mM (104 nM), 0.8 mM (178 nM), 0.85 mM (252 nM), 0.9 mM (399 nM) and 1.0 mM (5.65 μM). Assays with "high calcium concentration" were those to which no EGTA was added but which contained calcium chloride at a final concentration of 100 μM. The free calcium concentration in these assays was calculated to be 77 μM.

Protein. Protein was measured by the method of Sedmak and Grossberg (1977) using bovine serum albumin as standard.

Materials. Fresh bovine eyes were purchased from MO-PAC, Souderton, PA. [^{3}H]Cyclic GMP and [α-^{32}P]GTP were from ICN. All other chemicals were from Sigma Chemical Co.

Data Analysis. All assays were done in triplicate, and the mean ± SD values are shown in all the tables and figures. The experiments shown here were done with different batches of ROS with similar results.

RESULTS

Effect of Lithium on Rod Outer Segment Membrane Cyclase. To test the effects of Li on membrane cyclase, we chose to use homogenates rather than washed membranes because the soluble enzyme did not interfere with the study: First, the basal activity of soluble cyclase constitutes only 2–4% to the total activity of the homogenate (Margulis et al., 1992). Second, the membrane enzyme needed a soluble protein for activation at low calcium concentrations (Koch & Stryer, 1988). Under conditions when the membrane enzyme was activated by about 650% (Table I), the activity of the soluble enzyme increased by only about 30% (data not shown). Therefore, at low calcium concentration and in the absence of nitroprusside, the soluble enzyme activity was an insignificant fraction of the total cyclase activity. Studies on the membrane enzyme could therefore be carried out using the homogenate with little or no interference from the soluble enzyme.

Table I: Effect of Li, Na, and K on Basal and Stimulated Guanylate Cyclase Activities in ROS Homogenates[a]

additions	cyclase activity in ROS homogenate [nmol min^{-1} (mg of protein)$^{-1}$]	
	EGTA (no added calcium)	100 μM $CaCl_2$
none (control)	5.56 ● 0.04	0.86 ● 0.04
lithium		
25 mM	2.84 ± 0.16	0.51 ± 0.06
100 mM	0.98 ± 0.12	0.32 ± 0.02
sodium		
25 mM	4.89 ± 0.22	0.76 ± 0.02
100 mM	3.36 ± 0.21	0.74 ● 0
potassium		
25 mM	5.02 ± 0.17	0.86 ± 0.01
100 mM	3.27 ● 0.07	0.91 ± 0.04

[a] The guanylate cyclase activity in ROS homogenate was measured at low (1 mM EGTA, no added calcium) and high (100 μM $CaCl_2$ added; free Ca calculated to be 77 μM) calcium concentrations as described under Materials and Methods.

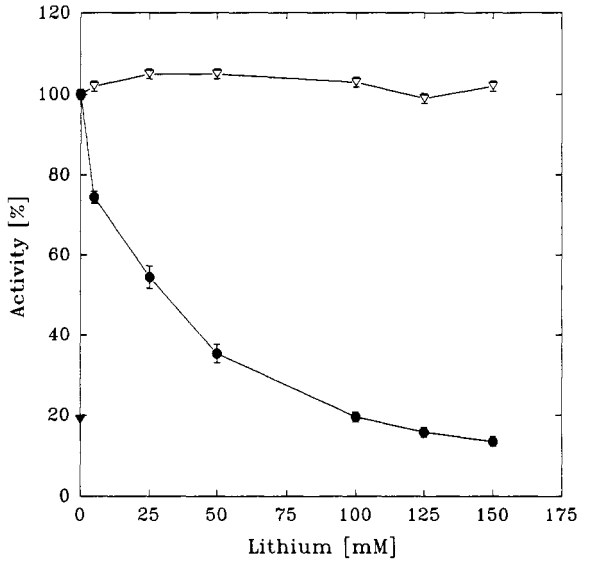

FIGURE 1: Influence of Li on ROS guanylate cyclase activity. The cyclase (●) and phosphodiesterase (▽) activities were measured in ROS homogenate at low calcium concentration (1 mM EGTA, no added calcium) in the presence of the specified concentrations of Li. The GC activity measured at 77 μM free Ca in the absence of Li is marked by (▼).

Figure 1 shows the effects of various concentrations of Li on GC and PDE activities in ROS homogenate at low calcium concentration. Cyclase activity was inhibited by Li in a dose-dependent manner. Half-maximal inhibition was observed at about 30 mM Li. At 110 mM Li, the extracellular concentration used in several electrophysiological studies, the inhibition was more than 80%. PDE activity was unaffected at all concentrations of Li tested.

Lithium Inhibits the Activity as Well as the Activation of Membrane Cyclase. The cyclase activity in the ROS membranes is reported to be activated by a soluble protein at free calcium concentrations of about 100 nM and lower (Koch & Stryer, 1988). The activation was abolished when free calcium concentration is increased to 120–600 nM (Koch & Stryer, 1988; Lambrecht & Koch, 1991; Dizhoor et al., 1991). At concentrations of calcium above 1 μM, cyclase activity remained invariant (Lambrecht & Koch, 1991) and therefore was considered basal activity. The effects of Li shown in Figure 1 were therefore on the activated GC. In order to test the effects of Li on the basal cyclase activity, Li was added to homogenates at high calcium or to membrane fractions separated by high-speed centrifugation and washed in hypotonic buffer. Table I shows that Li inhibited the stimulated activity more strongly than the basal activity: 100 mM Li inhibited basal activity by 40–60% and the activated enzyme by about 80%. Effects of Li on cyclase activity in washed membranes were identical to those on basal activity in homogenates (data not shown).

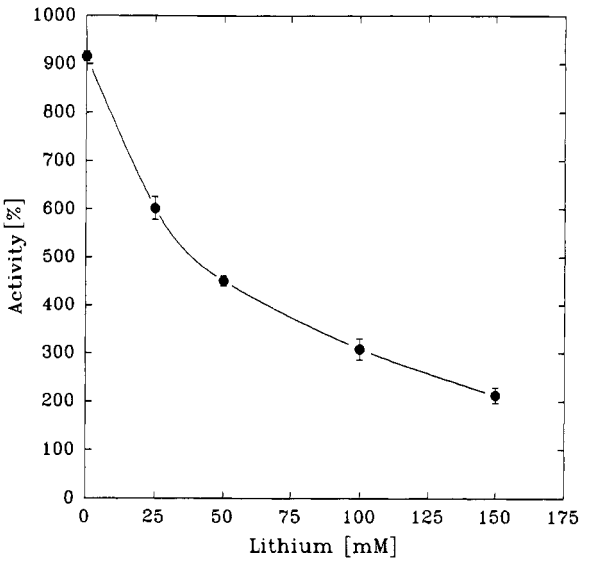

FIGURE 2: Effect of Li on nitroprusside-activated soluble guanylate cyclase. The soluble guanylate cyclase was assayed in the presence of 100 μM nitroprusside and 1 mM EGTA at the specified concentrations of Li in the assay. The basal activity (100%) was measured in the absence of nitroprusside and Li.

Na and K did not have a significant effect on the basal cyclase activity, but they inhibited the activated enzyme, though to a much lesser extent than Li (Table I).

Effect of Lithium on Soluble Cyclase. ROS also have a soluble cyclase whose basal activity constitutes 2–4% of the total activity (Margulis et al., 1992). This enzyme also showed a small, but consistent, increase in activity (about 30% in most preparations) at low calcium concentration compared to that at high calcium. At both lower and higher calcium concentrations, 100 mM Li inhibited the cyclase activity by 20–30%. Na and K had no measurable effect (data not shown).

Effect of Lithium and Calcium on the Activation of Soluble Cyclase by Nitroprusside. Like the soluble GCs in other tissues, the soluble enzyme in ROS is highly activated by nitric oxide-generating substances such as nitroprusside (Waldman & Murad, 1987; Margulis et al., 1992). It was shown recently that the maximal activation was found at 100 μM nitroprusside (Margulis et al., 1992). Figure 2 shows that, in the absence of Li, 100 μM nitroprusside activated the enzyme more than 9-fold. Lithium reduced the activation drastically. Half-maximal reduction was observed at about 30 mM Li. At 110 mM Li, the inhibition was about 80%. Na and K at 100 mM also reduced the activation, but only by 20–30% (Table II).

Because production of nitric oxide in ROS in vivo by NO synthase depends on calcium (Venturini et al., 1991), we studied the level of nitroprusside activation of cyclase at various free calcium concentrations (Table III). At all submicromolar concentrations of calcium, nitroprusside activated cyclase by about 10-fold. At higher concentrations the activation was reduced to about 7-fold at 5.6 μM and to 2-fold at 77 μM. Knowles et al. (1989) also reported that calcium reduced the activation of rat brain soluble cyclase by nitric oxide, though the effects were observed at lower calcium concentrations than in our experiments. We also tested the effect of Li on

Table II: Effect of Li, Na, and K on Nitroprusside-Activated Soluble Guanylate Cyclase in ROS[a]

additions	soluble cyclase activity (%)
none (control)	100
100 μM nitroprusside	868 ± 26
100 μM nitroprusside + 100 mM LiCl	295 ± 20
100 μM nitroprusside + 100 mM NaCl	710 ± 26
100 μM nitroprusside + 100 mM KCl	623 ● 23

[a] Guanylate cyclase activity in 100000*g* supernatant of ROS homogenate was measured at low calcium concentration (1 mM EGTA, no added calcium) as described under Materials and Methods.

Table III: Influence of Ca on the Activation of Soluble Cyclase by Nitroprusside and Effect of Li on the Activated Enzyme

	soluble cyclase activity in the presence of 100 μM nitroprusside[a] [nmol min^{-1} (mg of protein)$^{-1}$]	
free Ca concn[b]	control	+100 mM LiCl
0 (1 mM EGTA, no $CaCl_2$)	0.31 ± 0	0.11 ● 0.01
104 nM	0.31 ± 0.01	0.10 ± 0.02
252 nM	0.31 ± 0.03	0.09 ± 0.01
5.65 μM	0.23 ± 0.02	0.07 ± 0.01
77 μM	0.06 ● 0	0.05 ± 0

[a] The basal activity of the soluble enzyme (in the absence of nitroprusside) was 0.03 nmol min^{-1} (mg of protein)$^{-1}$. [b] The various free calcium concentrations were obtained by mixing EGTA and $CaCl_2$ at the concentrations described under Materials and Methods.

nitroprusside-activated cyclase at various calcium concentrations. At all submicromolar calcium concentrations, Li reduced the activation by nitroprusside by 65–70%. At high concentrations of calcium (77 μM), where the enzyme activation was already minimal, Li did not have a significant effect on the activation.

DISCUSSION

The aim of this investigation was to test if Li directly influenced the activity of guanylate cyclase, and, if it did, whether the inhibition was independent of calcium at the submicromolar physiological concentrations. Since ROS have two GCs, the effects of Li on both enzymes were investigated.

Li inhibited the basal activity of membrane GC. The inhibition was relatively specific in that K and Na had little or no effect. The enzyme activated at low calcium concentration was also inhibited by Li but more strongly than the basal enzyme. Na and K also had the inhibitory effects, though to a much smaller extent than Li. These results show that the decline in dark current observed by Hodgkin et al. (1985) and Fain et al. (1989) when Li replaced Na in the perfusion solution could at least in part be due to direct inhibition of membrane cyclase activity.

The basal activity of soluble cyclase constitutes only about 2–4% of the total cyclase activity in ROS (Margulis et al., 1992). However, it can potentially be activated by 30–100-fold (Margulis et al., 1992; Craven & DeRubertis, 1978), limited only by the availability of its activator, nitric oxide. NO is produced by nitric oxide synthase, a calcium/calmodulin-dependent enzyme present in ROS (Venturini et al., 1991). At calcium concentrations of hundreds of nanomolar as in the dark-adapted ROS, NO synthase is active, and therefore soluble cyclase should be in an activated state. We have recently shown that, under optimally activated conditions, the cyclic GMP synthesis due to the soluble enzyme is comparable to that of the membrane enzyme at high calcium (Margulis et al., 1992). The soluble cyclase is therefore an essential element in the balance between synthesis and hydrolysis of cyclic GMP in the dark-adapted ROS. The electrophysiological observations of Schmidt et al. (1992) that nitroprusside increased the dark current as well as accelerated the recovery phase of the light response support this hypothesis.

Li inhibited the soluble enzyme also. Here again the inhibition of basal activity was less severe (about 30% at 100 mM Li) than that of the nitroprusside-activated activity (about 70%). The inhibition of the soluble enzyme by Li was independent of calcium in the submicromolar range. Therefore, it is possible that Li directly inhibited the soluble enzyme at low or high external calcium concentrations as in the experiments of Fain et al. (1989) and Hodgkin et al. (1985), respectively. However, whether the eventual decline in the dark current in Li-perfused ROS was due to inhibition of cyclases depends upon how much Li entered the cell. From the results shown in Figures 1 and 2 it is apparent that as little as 1–5 mM Li in the cell could cause a 5–20% decrease in the cyclase activities. PDE activity was unaffected at Li concentrations of 1–100 mM (Figure 1 and Sitaramayya & Margulis, 1992). This raises the possibility that any reduction in the activity of cyclases without a change in that of PDE would slowly decrease the cyclic GMP concentration in the cell and cause an eventual decline in the dark current.

The mechanism of Li inhibition of cyclases remains unclear. Since both basal and activated forms of both cyclases are inhibited, and the dose–response curves look similar for the two enzymes, Li may be binding at a site common to both enzymes, such as the catalytic site. Inhibitory effects of Li on cyclases with such widely different properties suggest that Li may inhibit cyclases from other tissues also. Further studies are needed to test this possibility and to determine the mechanism of Li inhibition.

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