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Sulfhydryl G Proteins and Phospholipase A_2-Associated G Proteins Are Involved in Adrenergic Signal Transduction in the Rat Pineal Gland

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The rat pineal gland with its circadian noradrenaline-regulated melatonin rhythm is an excellent model for studying adrenergic signal transduction with respect to cAMP and cGMP formation. The stimulatory G_s proteins play a well-established role in this process. In contrast, the potential roles of the inhibitory G_i proteins, the functionally unclear other G_o proteins, and a number of G protein subtypes are not known. The present study examines the effects on β_1- and β_1-plus-α_1-stimulated cAMP and cGMP formation of a number of G protein modulators in rat pinealocyte suspension cultures. The effects of the nitric oxide donor sodium nitroprusside on cGMP were also examined. The results showed that drugs that activate G proteins of the G_i/G_o family, i.e., pertussis toxin, mastoparan, and compound 48/80, had no effect on unstimulated, isoproterenol (β_1)-stimulated, or combined isoproterenol/phenylephrine (β_1-plus-α_1)-stimulated cAMP and cGMP accumulation. However, in this experimental paradigm, the inhibitors of sulfhydryl G proteins (*N*-ethylmaleimide) and those of phospholipase A2-related G proteins (isotetrandrine) exerted a clear inhibitory effect. Sodium-nitroprusside-stimulated cGMP accumulation was also inhibited. These results confirm a previous report that members of the G_i/G_o family, which are present in the rat pineal gland, do not play a major role in adrenergic signal transduction. The new finding that sulfhydryl G proteins and phospholipase A2-associated G proteins exert a clear stimulatory effect on adrenergic signal transduction suggests that they are subtypes of G_s proteins. © 2001 Academic Press

Key Words: **rat pineal gland; G proteins; cAMP; cGMP; arylalkylamine *N*-acetylransferase; NO.**

INTRODUCTION

GTP-binding proteins (G proteins) play an important role in hormonal signal transduction, acting as regulatory links between cell-membrane-bound receptors and second messengers such as cAMP, cGMP, and certain phospholipid derivatives (see Helmreich and Hofmann, 1996). According to their function, G proteins are classified as stimulatory (G_s), inhibitory (G_i), or other (G_o) (see Offermann and Schultz, 1994). Moreover, a distinction can be made as to whether the G proteins contain sulfhydryl groups (Gudermann *et al.,* 1997) or which enzymes are affected (see Offermann and Schultz, 1994), e.g., phospholipase A2 (PLA2) (Burch and Axelrod, 1987).

The rat pineal gland is an excellent *in vitro* model for studying adrenergic signal transduction involving G_s proteins. *In vivo,* the gland exhibits a pronounced day–night rhythm of melatonin synthesis, depending on the circadian release of noradrenaline from intrapineal postganglionic sympathetic fibers regulated by the hypothalamic suprachiasmatic nucleus (see Korf *et al.,* 1998). *In vitro* studies have shown that adrenergic stimulation involves β_1-adrenoceptors, α_1-adrenore-

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ceptors, and G_s proteins, which enhance cAMP and cGMP accumulation by activating membrane-bound adenylyl cyclase and soluble guanylyl cyclase (sGC), respectively (Sugden and Klein, 1987; Babila and Klein, 1992, 1994; White and Klein, 1995; Korf *et al.,* 1998). β_1-Adrenergic stimulation alone, e.g., by isoproterenol (ISO), leads to a moderate increase of cAMP and cGMP levels (Vanecek *et al.,* 1985). α_1-Adrenergic agonists, e.g., phenylephrine (PHE), given alone have no effects (Vanecek *et al.,* 1985). However, conjoint administration of β_1- and α_1-adrenoceptor agonists greatly increase cAMP and cGMP levels, pointing to a potentiation effect of α_1-adrenergic mechanisms (Vanecek *et al.,* 1985). This effect is the result of increased intracellular Ca^{2+} levels (Sugden *et al.,* 1986, 1987). With respect to cGMP formation, but not to cAMP synthesis, it is relevant that the α_1-mediated Ca^{2+} influx activates NO synthase type I (NOS I) (Lin *et al.,* 1994), which in turn increases sGC activity and cGMP accumulation (Spessert *et al.,* 1993). Whereas the enhanced cAMP levels stimulate the penultimate and rate-limiting enzyme of melatonin synthesis, arylalkylamine *N*-acetylransferase (AA-NAT), and hence melatonin synthesis (see Korf *et al.,* 1998), the function of cGMP in the pineal gland is largely unknown.

In contrast to the G_s proteins, G_i, G_o and subtypes of G proteins have been little studied in the rat pineal gland. There is clear evidence that G_i and G_o are present (Sugden, 1990; Babila *et al.,* 1992), without showing significant day/night differences in their levels (Babila *et al.,* 1992). The G_i levels are constant throughout life, but the G_o protein exhibits a developmental pattern similar to that of the 42-kDa form of G_s, increasing fivefold between day 7 and day 40 (Babila *et al.,* 1992). With respect to the functions of the G_i and G_o proteins in the rat pineal gland, only one study exists. Following exposure of pineal tissue to the G_i/G_o protein antagonist pertussis toxin (PTX) for 18 h, there was no demonstrable effect on α-adrenergic potentiation of β-adrenergic stimulation of cAMP and cGMP formation (Sugden, 1990), leaving open the question of whether short-term treatment with PTX or other antagonists such as mastoparan and compound 48/80 is also without effect.

Moreover, it is of interest to study whether there are hints for the presence of PLA2-associated G proteins in the rat pineal gland. This search seems warranted as mepacrine, an inhibitor of the arachidonic-acid-generating enzyme PLA2, blocks the pinealocyte cGMP response to a combined treatment with ISO and PHE (Vanecek *et al.,* 1986) and PLA2 activity is stimulated by α_1-adrenergic mechanisms (Ho and Klein, 1987). Also, cyclic nucleotide formation is enhanced by arachidonic acid metabolites and decreases following the inhibition of lipoxygenase activity (Chik *et al.,* 1991).

These observations taken together prompted the following four questions concerning G protein-mediated adrenergic signal transduction affecting cAMP and cGMP levels in the rat pineal gland. Is the evidence firm that G_i/G_o proteins play no role? Are there hints that PLA2-associated G proteins are involved? Do sulfhydryl G proteins play a role? Finally, how is NO-stimulated cGMP accumulation affected by G protein inhibitors? To answer these questions, suspension cultures of rat pinealocytes and organ-cultured rat pineal glands were stimulated adrenergically or with NO in the presence and absence of the G protein modulators PTX, mastoparan, and compound 48/80, which preferentially affect the G_i/G_o family (Weingarten *et al.,* 1990; Mukai *et al.,* 1992; Higashijima *et al.,* 1990, 1988), *N*-ethylmaleimide (NEM), which inhibits sulfhydryl G proteins (Jin and Akaike, 1998; Joshi *et al.,* 1996), and isotetrandrine (IT), which is an inhibitor of PLA2-associated G proteins (Hashizume *et al.,* 1991; Akiba *et al.,* 1992). The endpoints studied were cAMP levels, cGMP levels, and AA-NAT activity.

MATERIALS AND METHODS

Chemicals

BME basal medium (Eagle), fetal calf serum (FCS), and BGJb medium Fitton–Jackson modified were obtained from Gibco, Scotland. Collagenase, ISO, PHE, sodium nitroprusside (SNP), isobutylmethylxanthine (IBMX), PTX, and NEM were obtained from Sigma, Germany. Mastoparan, compound 48/80, and IT were purchased from Calbiochem, Germany. Radioimmunoassay (RIA) kits for measuring cAMP and cGMP were obtained from Amersham Buchler, Germany.

Animals

Male Sprague–Dawley rats (body weight: 120–150 g) were purchased from a commercial supplier and

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were maintained under standard laboratory conditions (light: 12 h, dark: 12 h; lights on at 5:00 a.m.; light intensity: 200 Lux at cage level; room temperature: 22 ± 2°; water and food *ad libitum*) for 2 weeks prior to experimental study.

Suspension Culture of Pinealocytes

Rats were killed between 9:00 and 10:00 a.m. under open ether anaesthesia. Pineals were removed quickly under sterile conditions and pooled. Pinealocytes were prepared following the method described earlier (Buda and Klein, 1978) with slight modification (Spessert and Vollrath, 1993). In brief, pinealocytes were obtained by incubation of the pineals in 0.1% collagenase in BME medium at 37° under an atmosphere of 95% oxygen and 5% carbon dioxide in a gas incubator (Cytoperm, Heraeus, Germany) for 1 h. The pinealocytes were then washed, aliquoted, and further incubated in BME containing 10% FCS under similar conditions of atmosphere and temperature for 6 h. Aliquots of pinealocytes (10^6 cells/ml) were treated with the relevant drugs for 15 min in the presence of 1 mM theophylline and 0.1 mM IBMX (Spessert *et al.,* 1995). After treatment with drugs, aliquots were centrifuged at 10,000*g* for 1 min. The supernatant was discarded and cells were stored in liquid nitrogen. Cells were sonicated in 0.15 ml of a mixture of 0.05 M Tris–HCl buffer (pH 8), 0.1 mM IBMX, 1 mM theophylline, and sodium acetate buffer (pH 4), heated at 95° for 3 min, and centrifuged; the supernatants were used for RIA of cGMP and cAMP. In each experimental series, suspension cultures without the drugs to be tested served as controls.

Organ Culture

Rats were killed between 10:00 and 11:00 a.m. Pineal glands were quickly removed under sterile conditions and placed on plastic meshes in culture dishes containing 1.5 ml of BGJ_b medium Fitton–Jackson modified with 1 mg/ml bovine serum albumin fraction V, 2 mM glutamine, 0.125 mg/ml $CaCO_3$, 0.1 mg/ml ascorbic acid, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The pineal glands were preincubated for 24 h at 37° under an atmosphere of 95% O_2 and 5% CO_2, to allow the intrapineal noradrenaline-containing nerve fibers to degenerate, reducing the noradrenaline lcvels of the gland to zero (Cardinali *et al.,* 1987). The pineals were subsequently treated with the drugs indicated for 6 h. To determine AA-NAT activity, pineals were stored in liquid N_2 at the end of incubation. In each experimental series, pineals cultured without the drugs to be tested served as controls.

RIA of cGMP and cAMP

cGMP and cAMP were assayed following acetylation. In brief, 0.025 ml acetylation reagent (two volumes of triethylamine and one volume of acetic anhydride) was added to a mixture of 0.1 ml of the supernatant and 0.4 ml of 0.05 M sodium acetate buffer (pH 5.8). Aliquots (in duplicate) of 0.1 ml of the acetylated mixtures were incubated with 0.1 ml of the primary antibodies against cGMP and cAMP at room temperature for 1 h. After addition of 0.1 ml of [^{125}I]cGMP and [^{125}I]cAMP, the aliquots were further incubated at 4–8° for 15 to 18 h. The aliquots were then incubated with 0.5 ml of the respective second antibodies at room temperature for 10 min. The bound fraction was isolated by a magnetic separation method. The radioactivity present in the bound fraction was measured in terms of counts per minute (CPM) by a Gamma Scintillation Counter (Packard, Crystal II: Multidetector System). The concentrations of cGMP and cAMP were calculated by interpolation of %B/Bo from a standard curve and expressed as fmol/mg protein.

Measurement of AA-NAT Activity

The cultured pineals were sonicated in 0.075 ml ice-cold 0.05 M phosphate buffer (pH 6.5), and AA-NAT activity was determined following the method described by Deguchi and Axelrod (1972). The detection limit of the assay was 3.4 nmol/h × mg protein.

Protein Estimation

Protein concentrations of all the samples were determined following the method described by Lowry *et al.* (1951) with bovine serum albumin as a standard.

Statistical Analysis

Nucleotide accumulation and AA-NAT activities were expressed as mean ± SE. The data were statisti-

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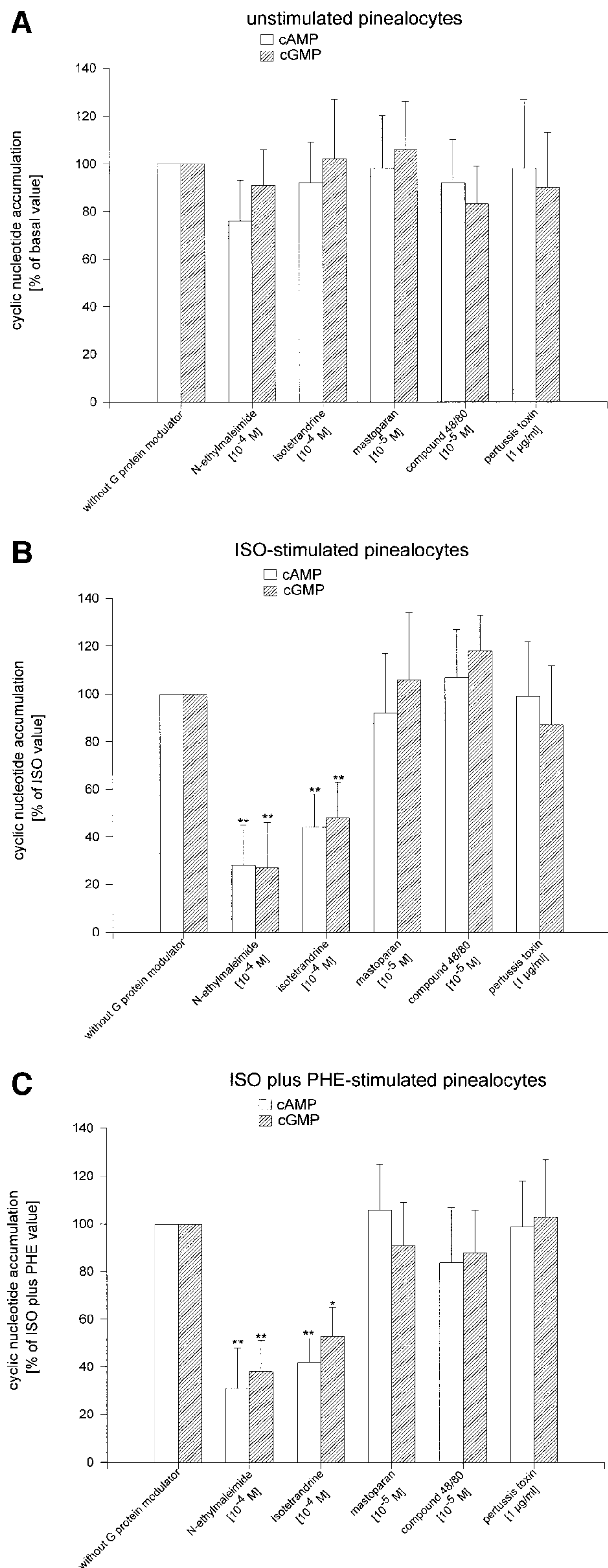

cally analyzed by means of Student's *t* test (nucleotide accumulation) or Mann–Whitney's *U* test (AA-NAT activity); $P < 0.05$ was considered significant.

RESULTS

Figure 1A shows that none of the G protein modulators altered the basal levels of the cyclic nucleotides significantly compared with those of the untreated controls.

To assess the nature of G proteins involved in the adrenergic stimulation of cAMP and cGMP accumulation, the effects of G protein inhibitors on β_1- (Fig. 1B) and β_1-plus-α_1-adrenergically (Fig. 1C) stimulated pinealocytes were investigated. As expected, β_1-adrenergic stimulation by ISO moderately increased both the cyclic nucleotides, whereas β_1-plus-α_1-adrenergic stimulation by ISO plus PHE strongly enhanced the accumulation of the nucleotides (not shown). Although NEM and IT significantly inhibited ISO-induced accumulation of both cAMP and cGMP, mastoparan, compound 48/80, and PTX had no significant effects on the accumulation of the cyclic nucleotides (Fig. 1B). Similarly, NEM and IT, but not mastoparan, PTX, and compound 48/80 attenuated ISO-plus-PHE-induced accumulation of both cyclic nucleotides (Fig. 1C).

FIG. 1. Effects of G protein modulators on unstimulated (A), isoproterenol (ISO, 10^{-4} M)-stimulated (B), and ISO (10^{-4} M) plus phenylephrine (PHE, 10^{-4} M)-stimulated (C) cAMP and cGMP accumulation in rat pinealocyte suspension culture, as outlined under Materials and Methods. Note that the G protein inhibitors *N*-ethylmaleimide (NEM) and isotetrandrine (IT) significantly decreased ISO-induced and ISO-plus-PHE-induced accumulation of both cAMP and cGMP. For comparison of cAMP and cGMP formation, accumulation of nucleotides was expressed as percentage of the value without G protein modulators. The absolute values for 100% cGMP and cAMP accumulation were as follows: (A) 180 fmol/mg protein (cAMP), 68 fmol/mg protein (cGMP); (B) 1887 fmol/mg protein (cAMP), 312 fmol/mg protein (cGMP); (C) 7341 fmol/mg protein (cAMP), 814 fmol/mg protein (cGMP). Data are means ± SE (bars) for three or more samples. * $P < 0.05$ compared with pinealocytes stimulated by ISO or ISO-plus-PHE; ** $P < 0.01$ compared with pinealocytes stimulated by ISO (B) or ISO-plus-PHE (C), in the absence of G protein modulators.

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To determine whether NEM affected cAMP and cGMP responses to β_1-adrenergic stimulation to similar extents, the effects of NEM on β_1-adrenergic cAMP and cGMP responses as a function of concentration were compared (Fig. 2A). NEM inhibited cAMP and cGMP formation in a dose-dependent manner and was equally effective in blocking both the nucleotides.

In the rat pineal, cAMP reportedly induces the rate-limiting enzyme in melatonin synthesis, AA-NAT. The possibility that NEM-sensitive G proteins play a role in AA-NAT induction was studied in organ culture. As illustrated in Fig. 2B, NEM depressed AA-NAT activity under v_{max} conditions in a dose-dependent manner.

The adrenergic cGMP response requires NO-dependent activation of sGC. To investigate whether G protein modulators affect adrenergic cGMP transduction, the effect of G protein modulators on NO-stimulated cGMP formation was tested. The NO donor SNP strongly stimulated cGMP accumulation (Fig. 3). The presence of NEM and IT significantly inhibited SNP-induced accumulation of cGMP in the pinealocytes. Compared with IT, NEM was more effective in blocking the SNP-induced cGMP accumulation. Mastoparan, compound 48/80, and PTX had no detectable effects on SNP-induced cGMP accumulation (Fig. 3).

DISCUSSION

It is well established that G_s proteins are abundantly present in the rat pineal gland (Babila *et al.,* 1992) and play important roles in adrenergic dual-signal transduction, resulting in increased levels of cAMP and cGMP (Sugden and Klein, 1987; Babila and Klein, 1992, 1994; White and Klein, 1995). The G_s protein comes in two forms, i.e., the 42- and the 45-kDa species (Babila *et al.,* 1992). The 45-kDa form is present in the fetal and adult rat pineal at nearly identical levels, with a significant increase at postnatal day 3, whereas the amount of the 42-kDa form is relatively low at birth, increasing sevenfold between day 7 and day 40 (Babila *et al.,* 1992). The late appearance of the small form of G_s coincides with the developmental occurrence of the cGMP response to adrenergic stimulation and the occurrence of the melatonin-forming enzyme hydroxyindole-*O*-methyltransferase.

There is also evidence that the G_i and the G_o proteins are present in the rat pineal gland (Babila *et al.,* 1992). Whereas the G_i levels are constant throughout life, the G_o protein exhibits a developmental pattern similar to that of the 42-kDa form of G_s, increasing fivefold between day 7 and day 40 (Babila *et al.,* 1992). In view of the prominent cAMP-dependent day–night variation of pineal melatonin synthesis (see Foulkes *et al.,* 1997), it is interesting to note that there are no significant day/night differences in the levels of G_s, G_i, and G_o (Babila *et al.,* 1992). Phylogenetically, the mammalian pinealocytes are photoreceptor-related cells. It is therefore relevant to note that the retinal G protein transducin $G_t\alpha$ is not demonstrable in the rat pineal gland (Babila *et al.,* 1992).

The results of the present study show that, although G_i/G_o proteins are demonstrable in the rat pineal gland (Babila *et al.,* 1992), neither the basal nor the adrenergically stimulated cAMP and cGMP accumulation are affected by a number of G_i/G_o protein activators, applied in sufficient concentrations (Akiba *et al.,* 1992; Igarashi *et al.,* 1993; Klinker *et al.,* 1996). This observation confirms that the G_i/G_o proteins do not play an important role in the adrenergic dual-signaling pathway in the rat pineal gland. This conclusion agrees with that of Sugden (1990), who has found that 18-h PTX treatment of rat pinealocytes is without effect on α_1-adrenergic potentiation of β-adrenergic stimulation of cAMP and cGMP accumulation.

In the present study, significant effects were obtained with the sulfhydryl G protein blocker NEM and the PLA2-related antagonist IT, both of which inhibited adrenergically induced cAMP and cGMP accumulation. This is apparently the first report showing that sulfhydryl G proteins participate in adrenergic signal transduction in the mammalian pineal gland. The role of sulfhydryl G proteins in adrenergic signal transduction has, however, been established in organs other than the pineal, e.g., NEM inhibits the contractile response to noradrenaline in rat aorta and mesenteric artery (Abebe *et al.,* 1995). In the aorta, an NEM-sensitive pathway has also been shown to play a role in NO-mediated vasorelaxation produced by acetylcholine (Andriambeloson *et al.,* 1999). This latter ob-

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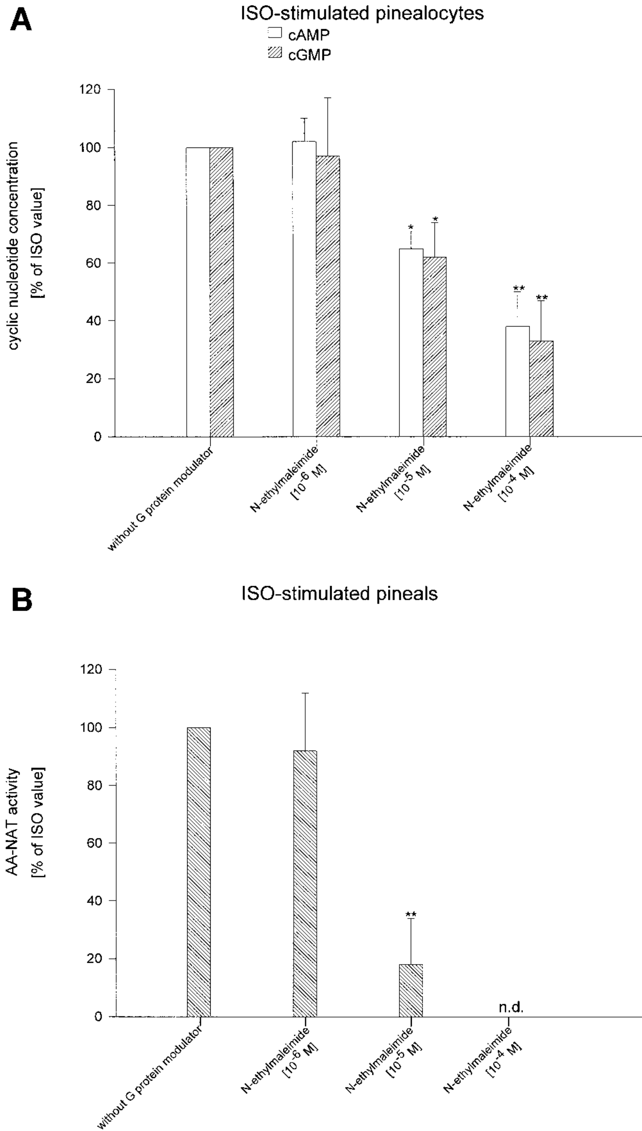

FIG. 2. Effects of various concentrations of *N*-ethylmaleimide (NEM) on isoproterenol (ISO, 10^{-4} M)–stimulated nucleotide formation in pinealocyte suspension cultures (A) and on AA-NAT activity in pineal organ cultures (B). For details see Materials and Methods. Note the dose-dependent decrease of all the parameters. For ease of comparison, the data are expressed as percentage of the value without G protein modulators. Determination was performed in duplicate on at least three (cyclic nucleotide accumulation) or six (AA-NAT activity) samples. The absolute values for 100% are as follows: 1976 fmol/mg protein (cAMP), 361 fmol/mg protein (cGMP), and 30,8 nmol/h × mg protein (AA-NAT). Data are means ± SE (bars) for three or more samples. * $P < 0.05$; ** $P < 0.01$ compared with pinealocytes stimulated by ISO in the absence of G protein modulators.

servation is of interest as NO plays an important mediatory role in cGMP accumulation in the rat pineal gland (see below).

The presence of a G-protein-coupled PLA2 in the rat pineal gland has been anticipated by observations that the activation of α_1-adrenoceptors increases pineal PLA2 activity (Ho and Klein, 1987) and that α_1-adrenergic mechanisms are generally mediated by G proteins (see Gudermann *et al.,* 1997; Summers and McMartin, 1993). Similarly, IT (an inhibitor of G protein-coupled PLA2) diminishes adrenergically induced cAMP and cGMP accumulation (this study). G protein-mediated regulation of PLA2 activity has also been found in tissues other than pineal (Tong *et al.,* 1998; Qian *et al.,* 1997; Gebicke-Haerter *et al.,* 1991) but appears to be less common, or at least less well investigated, than the G-protein-dependent regulation of phospholipase C (PLC) (see Helmreich and Hofmann, 1996).

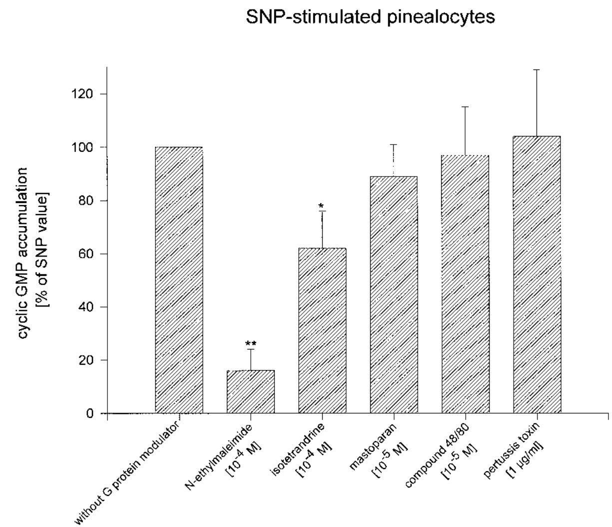

FIG. 3. Effects of G protein modulators on sodium nitroprusside (SNP; 10^{-4} M)-stimulated cGMP accumulation in rat pinealocyte suspension cultures. Note that the G protein inhibitors *N*-ethylmaleimide and isotetrandrine significantly decreased SNP-induced accumulation of cGMP. For comparison of individual experiments, cGMP accumulation is expressed as percentage of the value without G protein modulators and represented as means ± SE (bars) for three or more samples. The absolute value for 100% cGMP accumulation was 818 fmol/mg protein. Data are means ± SE (bars) for three or more samples. * $P < 0.05$; ** $P < 0.01$ compared with pinealocytes stimulated by SNP in the absence of G protein modulators.

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The effects of NEM and IT observed in the present study raise the question as to whether NEM- and IT-sensitive G proteins represent distinct groups of G proteins or whether they belong to the G_s and/or the G_i/G_o families. Both NEM and IT exerted clear inhibitory effects on adrenergically stimulated cAMP and cGMP accumulation. As in the rat pineal, the adrenergically induced cAMP and cGMP stimulation are G_s dependent (Sugden and Klein, 1987; Babila and Klein, 1992, 1994; White and Klein, 1995) and, since there were no demonstrable effects of G_i/G_o modulators (see also Sugden, 1990), it may be assumed that the NEM- and IT-sensitive G proteins belong to the G_s family. However, data from the literature indicate that NEM- and IT-sensitive G proteins belong more to the G_i/G_o group than to the G_s family (Morishita *et al.,* 1997; Yan and Surmeier, 1996; Choi and Lovinger, 1996; Tsunoda and Owyang, 1995). Interestingly, in astrocytes, the IT-sensitive PLA2-associated G protein seems to be a G_s (Gebicke-Haerter *et al.,* 1991). It thus seems that, in the rat pineal gland, which is characterized by the simultaneous presence of pinealocytes and astrocytes (Vollrath, 1981), G_s involvement predominates over G_i/G_o-mediated processes.

A frequently discussed problem is whether dual adrenergic signaling, i.e., the concomitant regulation of two separate second messengers by noradrenaline, is regulated by a common pool of G proteins or by different types of G proteins. The present results favor the conclusion that, in the rat pineal gland, a common pool is involved. This conclusion is based on the present findings that both cAMP and cGMP respond uniformly when G protein modulators are applied. Thus, NEM and IT, but not mastoparan, COM, and PTX, inhibit adrenergically stimulated cAMP and cGMP formation in a dose-dependent manner (see also Sugden, 1990).

An interesting new finding in the present study is that NEM and IT inhibit SNP-stimulated cGMP formation. It is generally accepted that SNP is an NO donor and that NO activates sGC followed by an increase of cyclic GMP accumulation. This concept is also valid for the rat pineal gland (Spessert *et al.,* 1993, 1995). The observations that SNP-stimulated cGMP formation is affected by the G_s activator cholera toxin (White and Klein, 1995) and by NEM and IT suggest that NO-stimulated sGC activity is modulated by NEM- and IT-sensitive G_s proteins. The way in which G proteins modulate the NO responsiveness of sGC activity is unclear. The possibility of a direct interaction between G protein and sGC is unlikely, because G proteins are cell membrane bound, whereas in the rat pineal, NO-sensitive sGC is located in the cytosolic fraction (Spessert *et al.,* 1992). Because of the effectiveness of IT in the inhibition of NO-stimulated cGMP synthesis (this study), a possible mechanism that does not require such a direct interaction involves G-protein-associated PLA2 and the sensitization of sGC by arachidonic acid metabolites. This suggestion is in line with the findings that (1) PLA2 inhibition depresses the pineal cGMP response (Vanecek *et al.,* 1986), (2) arachidonic acid metabolites enhance the pineal cGMP response (Chik *et al.,* 1991), and (3) arachidonic acid metabolites have been demonstrated to stimulate sGC in tissues other than the pineal (Glass *et al.,* 1977; Snider *et al.,* 1984). However, NO may also have direct effects on G proteins (Christopoulos and El-Fakahany, 1999). Lander *et al.* (1993) have found that NO increases GTPase activity of isolated α subunits of G_s and G_i in human blood mononuclear cells. Moreover, Miyamoto *et al.* (1997) have reported that NO can directly inhibit G_i and G_o proteins coupled to the bradykinin BK2 receptor in bovine aortic endothelial cells. In view of these findings, it remains possible that, in the rat pineal, NO also stimulates cGMP formation via G proteins.

It is of interest to consider the implications of the present findings for melatonin synthesis in the rat pineal gland. There is ample evidence that the rate-limiting enzyme of melatonin, AA-NAT, is adrenergically induced via G_s and the second messenger cAMP (Roseboom *et al.,* 1996). In the present study, NEM has been shown not only to depress adrenergically stimulated cAMP levels, but also to inhibit adrenergically stimulated AA-NAT activity. Recently, NEM has been reported to have a direct inhibitory effect on recombinant AA-NAT activity (Zhan-Poe and Craft, 1999). Although a direct effect on AA-NAT by NEM in the present study is possible, it seems much more likely that, for the reasons given above, NEM acts via G_s proteins. If this is the case, it is concluded that the G_s proteins involved in adenylyl cyclase and melatonin formation in the rat pineal gland contain a functionally important sulfhydryl component.

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REFERENCES

Abebe, W., Edwards, J. D., and Agrawal, D. K. (1995). G-proteins in blood vessels II. Assessment of functional involvement. *Gen. Pharmacol.* **26,** 75–83.

Akiba, S., Kato, E., Sato, T., and Fujii, T. (1992). Biscoclaurine alkaloids inhibit receptor-mediated phospholipase A_2 activation probably through uncoupling of a GTP-binding protein from the enzyme in rat peritoneal mast cells. *Biochem. Pharmacol.* **44,** 45–50.

Andriambeloson, E., Stoclet, J. C., and Andriantsitohaina, R. (1999). Mechanism of endothelial nitric oxide-dependent vasorelaxation induced by wine polyphenols in rat thoracic aorta. *J. Cardiovasc. Pharmacol.* **33,** 248–254.

Babila, T., and Klein, D. C. (1992). Stimulus deprivation increases pineal $G_{s\alpha}$ and G_{β}. *J. Neurochem.* **59,** 1356–1362.

Babila, T., and Klein, D. C. (1994). Cholera toxin-induced $G_s\alpha$ down regulation in neural tissue: Studies on the pineal gland. *Brain Res.* **638,** 151–156.

Babila, T., Schaad, N. C., and Klein, D. C. (1992). Rat pineal Gsa, Gia and Goa: Relative abundance and development. *Brain Res.* **572,** 232–235.

Buda, M., and Klein, D. C. (1978). A suspension culture of pinealocytes: Regulation of *N*-acetyltransferase activity. *Endocrinology* **103,** 1483–1493.

Burch, R. M., and Axelrod, J. (1987). Dissociation of bradykinin-induced prostaglandin formation from phosphatidylinositol turnover in Swiss 3T3 fibroblasts: Evidence for G protein regulation of phospholipase A_2. *Proc. Natl. Acad. Sci. USA* **84,** 6374–6378.

Cardinali, D. P., Vacas, M. I., Keller Sarmiento, M. I., Etchegoyen, G. S., Pereyra, E. N., and Chuluyan, H. E. (1987) Neuroendocrine integrative mechanisms in mammalian pineal gland: Effects of steroid and adenohypophysial hormones on melatonin synthesis in vitro. *J. Steroid Biochem.* **27,** 565–571.

Chik, C. L., Young, I., and Ho, A. K. (1991). Differential involvement of the arachidonic acid cascade on the α_1-adrenergic potentiation of vasoactive intestinal peptide- versus β-adrenergic-stimulated cyclic AMP and cyclic GMP accumulation in rat pinealocytes. *J. Neurochem.* **57,** 1534–1539.

Choi, S., and Lovinger, D. M. (1996). Metabotropic glutamate receptor modulation of voltage-gated Ca^{2+} channels involves multiple receptor subtypes in cortical neurons. *J. Neurosci.* **16,** 36–45.

Christopoulos, A., and El-Fakahany, E. E. (1999). The generation of nitric oxide by G protein-coupled receptors. *Life Sci.* **64,** 1–15.

Deguchi, T., and Axelrod, J. (1972). Sensitive assay of serotonin *N*-acetyltransferase activity in rat pineal. *Anal. Biochem.* **50,** 174–179.

Foulkes, N. S., Whitmore, D., and Sassone-Corsi, P. (1997). Rhythmic transcription: The molecular basis of circadian melatonin synthesis. *Biol. Cell* **89,** 487–494.

Gebicke-Haerter, P. J., Schober, A., and Hertting, G. (1991). Pertussis and cholera toxins inhibit prostaglandin synthesis in rat astrocyte cultures at distinct metabolic steps. *Biochem. Pharmacol.* **42,** 1267–1271.

Glass, D. B., Frey, W., Carr, D. W., and Goldberg, N. D. (1977). Stimulation of human platelet guanylate cyclase by fatty acids. *J. Biol. Chem.* **252,** 1279–1285.

Gudermann, T., Schoneberg, T., and Schultz, G. (1997). Functional and structural complexity of signal transduction via G-protein-coupled receptors. *Annu. Rev. Neurosci.* **20,** 399–427.

Hashizume, T., Yamaguchi, H., Sato, T., and Fujii, T. (1991). Suppressive effect of biscoclaurine alkaloids on agonist-induced activation of phospholipase A_2 in rabbit platelets. *Biochem. Pharmacol.* **42,** 419–423.

Helmreich, E. J. M., and Hofmann, K. P. (1996). Structure and function of proteins in G-protein-coupled signal transduction. *Biochim. Biophys. Acta* **1286,** 285–322.

Higashijima, T., Burnier, J., and Ross, E. M. (1990). Regulation of Gi and Go by mastoparan, related amphiphilic peptides and hydrophobic amines. *J. Biol. Chem.* **265,** 14176–14186.

Higashijima, T., Uzu, S., Nakajima, T., and Ross, E. M. (1988) Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G-proteins). *J. Biol. Chem.* **263,** 6491–6494.

Ho, A. K., and Klein, D. C. (1987). Activation of α_1-adrenoceptors, protein kinase C, or treatment with intracellular free Ca^{2+} elevating agents increases pineal phospholipase A_2 activity. *J. Biol. Chem.* **262,** 11764–11770.

Igarashi, M., Strittmatter, S. M., Vartanian, T., and Fishman, M. C. (1993). Mediation by G proteins of signals that cause collapse of growth cones. *Science* **259,** 77–79.

Jin, Y. H., and Akaike, N. (1998). Tandospirone-induced K^+ current in acutely dissociated rat dorsal raphe neurones. *Br. J. Pharmacol.* **124,** 897–904.

Joshi, S., Abebe, W., and Agrawal, D. K. (1996). G-proteins in guinea pig airway smooth muscle: Identification and functional involvement. *Pharmacol. Res.* **33,** 195–202.

Klinker, J. F., Laugwitz, K.-L., Hagelüken, A., and Seifert, R. (1996) Activation of GTP formation and high-affinity GTP hydrolysis by mastoparan in various cell membranes. G-protein activation via nucleoside diphosphate kinase, a possible general mechanism of mastoparan action. *Biochem. Pharmacol.* **51,** 217–223.

Korf, H.-W., Schomerus, C., and Stehle, J. H. (1998). The pineal organ, its hormone melatonin, and the photoneuroendocrine system. *Adv. Anat. Embryol. Cell Biol.* **146,** 1–100.

Lander, H. M., Sehajpal, P. K., and Novogrodsky, A. (1993). Nitric oxide signaling: A possible role for G proteins. *J. Immunol.* **151,** 7182–7187.

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Lin, A. M. Y., Schaad, N. C., Schulz, P. E., Coon, S. L., and Klein, D. C. (1994). Pineal nitric oxide synthase: Characteristics, adrenergic regulation and function. *Brain Res.* **651,** 160–168.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–271.

Miyamoto, A., Laufs, U., Pardo, C., and Liao, J. K. (1997). Modulation of bradykinin receptor ligand binding affinity and its coupled G-proteins by nitric oxide. *J. Biol. Chem.* **272,** 19601–19608.

Morishita, W., Kirov, S. A., Pitler, T. A., Martin, L. A., Lenz, R. A., and Alger, B. E. (1997). *N*-ethylmaleimide blocks depolarization-induced suppression of inhibition and enhances GABA release in the rat hippocampal slice in vitro. *J. Neurosci.* **17,** 941–950.

Mukai, H., Munekata, E., and Higashijima, T. (1992). G protein antagonists. *J. Biol. Chem.* **267,** 16237–16243.

Offermann, S., and Schultz, G. (1994). Complex information processing by the transmembrane signaling system involving G proteins. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **350,** 329–338.

Qian, Y., Essenberg, R. C., Dillwith, J. W., Bowman, A. S., and Sauer, J. R. (1997). A specific prostaglandin E2 receptor and its role in modulating salivary secretion in the female tick, *Anblyomma americanum* (L.). *Insect Biochem. Mol. Biol.* **27,** 387–395.

Roseboom, P. H., Coon, S. L., Baler, R., McCune, S. K., Weller, J. L., and Klein, D. C. (1996). Melatonin synthesis: Analysis of the more than 150-fold nocturnal increase in serotonin *N*-acetyltransferase messenger ribonucleic acid in the rat pineal gland. *Endocrinology* **137,** 3033–3044.

Snider, R. M., McKinney, M., Forray, C., and Richelson, E. (1984). Neurotransmitter receptors mediate cGMP formation by involvement of arachidonic acid and lipoxygenase. *Proc. Natl. Acad. Sci. USA* **81,** 3905–3909.

Spessert, R., Gupta, B. B. P., Seidel, A., Maitra, S. K., and Vollrath, L. (1992). Involvement of cyclic guanosine monophosphate (cGMP) and cytosolic guanylate cyclase in the regulation of synaptic ribbon numbers in rat pineal gland. *Brain Res.* **570,** 231–236.

Spessert, R., Hill, G., and Vollrath, L. (1995). In rat pinealocytes the cyclic GMP response to NO is regulated by Ca^{2+} and protein kinase C. *Brain Res.* **694,** 207–212.

Spessert, R., Layes, E., and Vollrath, L. (1993). Adrenergic stimulation of cyclic GMP formation requires NO-dependent activation of cytosolic guanylate cyclase in rat pinealocytes. *J. Neurochem.* **61,** 138–143.

Spessert, R., and Vollrath, L. (1993). Muscarinic agonists have no measurable effect on cGMP formation of rat pinealocytes. *Brain Res. Bull.* **32,** 589–592.

Sugden, D. (1990). Pertussis toxin does not inhibit α_1-adrenergic potentiation of β-adrenergic stimulation of cAMP accumulation in rat pinealocytes. *Biochem. Biophys. Res. Commun.* **168,** 871–877.

Sugden, A. L., and Klein, D. C. (1987). A cholera toxin substrate regulates cytosolic cyclic GMP content of rat pinealocytes. *J. Biol. Chem.* **262,** 7447–7450.

Sugden, A. L., Sugden, D., and Klein, D. C. (1986). Essential role of calcium influx in the adrenergic regulation of cAMP and cGMP in rat pinealocytes. *J. Biol. Chem.* **261,** 11608–11612.

Sugden, A. L., Sugden, D., and Klein, D. C. (1987). α_1-Adrenoceptor activation elevates cytosolic calcium in rat pinealocytes by increasing net influx. *J. Biol. Chem.* **262,** 741–745.

Summers, R. J., and McMartin, L. R. (1993). Adrenoceptors and their second messenger systems. *J. Neurochem.* **60,** 10–23.

Tong, L. J., Dong, L. W., and Liu, M. S. (1998). GTP-binding protein mediated phospholipase activation in rat liver during the progression of sepsis. *Mol. Cell Biochem.* **189,** 55–61.

Tsunoda, Y., and Owyang, C. (1995). The regulatory site of functional GTP binding protein coupled to the high affinity cholecystokinin receptor and phospholipase A_2 pathway is on the G_β subunit of G_q protein in pancreatic acini. *Biochem. Biophys. Res. Commun.* **211,** 648–655.

Vanecek, J., Sugden, D., Weller, J. L., and Klein, D. C. (1985). Atypical synergistic α_1- and β-adrenergic regulation of adenosine 3′5′-monophosphate and guanosine 3′5′-monophosphate in rat pinealocytes. *Endocrinology* **116,** 2167–2173.

Vanecek, J., Sugden, D., Weller, J. L., and Klein, D. C. (1986). See-saw signal processing in pinealocytes involves reciprocal changes in the α_1-adrenergic component of the cyclic GMP response and the β-adrenergic component of the cAMP responses. *J. Neurochem.* **47,** 678–686.

Vollrath, L. (1981). The pineal organ. *In* "Handbuch der Mikroskopischen Anatomie des Menschen" (A. Oksche and L. Vollrath, Eds.), Vol VI/7, pp. 1–665. Springer-Verlag, Berlin.

Weingarten, R., Ransnas, L., Mueller, H., Sklar, L. A., and Bokoch, G. M. (1990). Mastoparan interacts with the carboxyl terminus of the α-subunit of Gi. *J. Biol. Chem.* **265,** 11044–11049.

White, B. H., and Klein, D. C., (1995). Stimulation of cGMP accumulation by sodium nitroprusside is potentiated via a Gs mechanism in intact pinealocytes. *J. Neurochem.* **64,** 711–717.

Yan, Z., and Surmeier, D. J. (1996). Muscarinic (m2/m4) receptors reduce N- and P-type Ca^{2+} currents in rat neostriatal cholinergic interneurons through a fast, membrane-delimited, G-protein pathway. *J. Neurosci.* **16,** 2592–2602.

Zhan-Poe, X., and Craft, C. M. (1999). Biochemical characterization of recombinant serotonin N-acetyltransferase. *J. Pineal Res.* **27,** 49–58.

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