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FURTHER EVIDENCE AGAINST THE COUPLING OF DOPAMINE RECEPTORS TO PHOSPHOINOSITIDE HYDROLYSIS IN RAT STRIATUM

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Abstract—The effects of D_1 and D_2 dopamine receptor agonists on phosphoinositide hydrolysis were studied by measuring the accumulation of radioactive inositol phosphates in slices of rat corpus striatum prelabelled with [^{3}H]inositol. All assays were performed in the presence of lithium. Neither the D_1 receptor agonist SKF 38393 nor the D_2 receptor agonist quinpirole, alone or in combination, had an effect on basal accumulation of inositol phosphates. The muscarinic receptor agonist carbachol produced a robust increase in the accumulation of inositol monophosphate and a smaller increase in the accumulation of inositol bisphosphate. These effects were not altered by the presence of quinpirole. Additionally, quinpirole also had no effect when assays were conducted in the presence of the muscarinic receptor antagonist scopolamine, the glutamic acid receptor antagonist kynurenic acid, and the antioxidant glutathione. These results are discussed in relation to recent contradictory reports and lend support to the position that D_2 dopamine receptors are not coupled to phosphoinositide hydrolysis in rat striatum.

Dopamine (DA§), acting at the D_1 DA receptor, has long been known to stimulate the production of cyclic AMP by adenylate cyclase [1]. The D_2 receptor has now been shown to be linked to inhibition of adenylate cyclase in many tissues [2] but since the efficacy of this response is limited [3], and not universal [4, 5], the search for other possible actions of this receptor has continued. It has been proposed recently that D_2 receptors inhibit the hydrolysis of phosphoinositides (PI) by phospholipase C (PLC). Working with rat or bovine anterior pituitary cells in primary culture, two groups have reported that DA, acting through a D_2 receptor, inhibits stimulation of PI hydrolysis induced by angiotensin II or thyrotropin-releasing hormone [6–8]. Other workers, however [9–13], did not find acute DA treatment to affect either basal or antiotensin II, thyrotropin-releasing hormone or neurotensin-stimulated PI hydrolysis (although some chronic effects [10, 11] as well as an acute decrease in incorporation of $^{32}P_i$ into inositol phospholipids [11, 12, 14] were noted). Additional negative results have also been reported recently in mouse embryonic striatal neurons in primary culture [15] and in the transformed neural-related clonal cell line NCB-20 [16, 17]. Studies using slice preparations of rat corpus striatum have also yielded discrepant findings. Pizzi *et al.* [18, 19] have reported that DA (as well as a number of selective D_2 agonists) inhibits basal PI hydrolysis. In contrast, Kelly *et al.* [20] were unable to find any effect by DA agonists on basal or either muscarinic- or KCl-stimulated accumulation of inositol phosphates (IPs). Additionally, Petcoff and Cooper [21] have reported that DA has no effect on histamine-stimulated production of IPs.

Given the disagreement in the literature to date and the significance of understanding the function of the D_2 receptor, we felt it important to describe our own findings on the effects of DA agonists on PI hydrolysis in striatum. We report here that DA agonists did not affect basal or carbachol-stimulated accumulation of IPs in rat striatal slices. In addition to confirming the conclusion of Kelly *et al.* [20], we also replicated certain experimental conditions employed by Pizzi *et al.* [18, 19] and show that these methodological variations did not account for the differences in results.

EXPERIMENTAL PROCEDURES

For each experiment, the corpus striata from five to six male Sprague–Dawley rats (200–250 g) were pooled and cross-chopped at 350 μm using a McIlwain tissue chopper. Slices were placed in 10 mL of ice-cold Krebs–Ringer–bicarbonate buffer (KRB) of the following composition (in mM): NaCl, 122; KCl, 3; $NaHCO_3$, 25; KH_2PO_4, 0.4; $MgSO_4$, 1.2; $CaCl_2$, 1.3; D-glucose, 10; pH 7.4 after gassing for 30 min with 95% O_2/5% CO_2. Slices were rinsed twice, incubated for 60 min, and rinsed twice again. The tissue was then labeled for 2 hr with 0.7 μM *myo*-[2-^{3}H]inositol (Amersham, sp. act. = 22.8 Ci/mmol) in a final volume of 1 mL/100 mg wet tissue weight. Extra-slice label was removed with five washes of fresh KRB. Fifty microliters of labeled, gravity-packed slices (approx. 1.5 mg protein) was

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§ Abbreviations: AF, ammonium formate; DA, dopamine; FA, formic acid; GSH, glutathione; IPs, inositol phosphates; IP_1 inositol monophosphate; IP_2 inositol bisphosphate; IP_3 inositol trisphosphate; IP_4 inositol tetrakisphosphate; KYN, kynurenic acid; PI, phosphoinositides; PLC, phospholipase C; and SCO, scopolamine.

added to 450 μL KRB containing 10 mM LiCl for incubation with test drugs. All labeling and incubations were carried out at 37°, in capped vessels under a continuous stream of 95% O_2/5% CO_2. Incubations were terminated by the addition of 1.5 mL chloroform/methanol/conc HCl (100:200:1) and vigorous vortexing.

The phases were separated by the addition of 0.5 mL chloroform and 0.5 mL water followed by centrifugation (1000 g × 15 min). A 1-mL sample of the aqueous phase was added to 4 mL H_2O and applied to columns packed with 2 mL anion exchange resin (Biorad AG1-X8, 100–200 mesh, formate form). After washing the columns with 50 mL of H_2O and then 50 mL of 60 mM ammonium formate (AF)/5 mM sodium tetraborate, IPs were sequentially eluted as follows: Fraction 1, 2 × 10 mL: 200 mM AF/100 mM formic acid (FA); fraction 2, 3 × 10 mL: 400 mM AF/100 mM FA; fraction 3: 1 × 10 mL 1 M AF/100 mM FA. Modified from the protocol of Berridge *et al.* [22], these fractions represent inositol monophosphate (IP_1), inositol bisphosphate (IP_2), and inositol trisphosphate (IP_3) plus inositol tetrakisphosphate (IP_4) respectively. For ease, we refer to the third fraction simply as IP_3.

The fractionated IPs were quantified by liquid scintillation counting at approximately 32% efficiency. A 100-μL sample of the organic phase containing a mixture of labeled PI was dried and counted at approximately 50% efficiency. All counts were converted to dpm using an external standard. Results were calculated as dpm IPs per 10,000 dpm PI. To determine background levels of [^{3}H]IPs, "blank" samples were inactivated by addition of the $CHCl_3$/MeOH/HCl mixture to labeled slices prior to the incubation with test drugs. As discussed later, these values, however, were not subtracted from the experimental samples for purposes of data analysis.

Each experiment was performed in triplicate on three to five separate occasions. Statistical analysis was performed using repeated measures and post-hoc paired t-tests.

Materials. Quinpirole was the gift of Eli Lilly & Co. (Indianapolis, IN). (+)-SKF 38393 was purchased from Research Biochemicals Inc. (Natick, MA). All other drugs were from the Sigma Chemical Co. (St. Louis, MO).

RESULTS

In one series of five experiments, striatal slices were incubated for 30 min in the presence or absence of the selective D_2 agonist quinpirole. A 10 mM concentration of LiCl was present during the incubation but not prior to the addition of quinpirole. As shown in Table 1, quinpirole in concentrations ranging from 0.1 to 10 μM had no effect on the basal production of IP_1, IP_2 or IP_3. In the same experiments, the muscarinic agonist carbachol, at a concentration of 0.5 mM, stimulated IP_1 accumulation to 450% of control (Fig. 1). The presence of 1 μM quinpirole together with carbachol did not affect significantly this stimulation. Carbachol produced a more modest, but statistically significant, stimulation of IP_2 accumulation but had no significant effect on IP_3. Quinpirole again had no effect on these levels. In preliminary experiments (data not shown), neither lower (0.01 μM) nor higher (100 μM) concentrations of quinpirole had any effect.

Table 1. Lack of effect of quinpirole on basal accumulation of inositol phosphates

Drug concentration	N	IP_1	IP_2	IP_3
		(% of control)		
Quinpirole, 0.1 μM	3	100 ± 1	97 ± 7	108 ± 7
Quinpirole, 1.0 μM	5	99 ± 5	101 ± 10	101 ± 5
Quinpirole, 10.0 μM	5	101 ± 5	110 ± 5	107 ± 3

After labeling for 2 hr with [^{3}H]inositol, rat striatal slices were incubated for 30 min in the presence of 10 mM LiCl and the presence or absence of the indicated concentrations of quinpirole. Values are means ± SE of N experiments, each performed in triplicate. No treatment had a statistically significant effect when analyzed by repeated measures. Control values (dpm IPs/10,000 dpm PI) were: IP_1, 2,360 ± 161; IP_2, 3,844 ± 201; and IP_3, 2,436 ± 207. Mean ± SE PI = 44,071 ± 1,380.

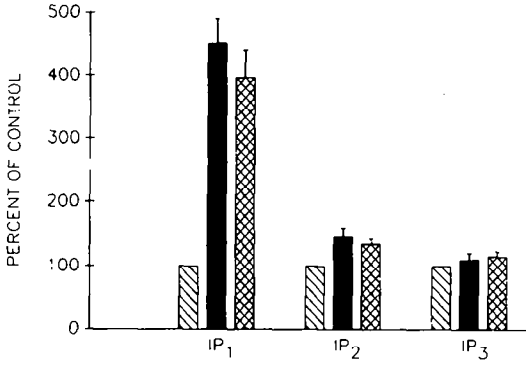

Fig. 1. Lack of effect of quinpirole on carbachol-stimulated accumulation of IPs. Labeled slices were incubated for 30 min with no drug (diagonal bars), 0.5 mM carbachol (solid bars), or 0.5 mM carbachol plus 1.0 μM quinpirole (cross-hatched bars). All incubations included 10 mM LiCl. Values are means ± SE of five separate experiments expressed as percent of control. Response in the presence of drugs was statistically different from control ($P < 0.0001$ for IP_1; $P < 0.005$ for IP_2 by repeated measures) but quinpirole + carbachol did not differ from quinpirole alone. Control values are shown in the legend to Table 1.

In a separate series of five experiments, 1 μM quinpirole, 10 μM (+)-SKF 38393 (a selective D_1 partial agonist), or both were added for 20 min to slices that had been preincubated with LiCl for 10 min. As shown in Table 2, none of these treatments significantly affected the accumulation of IPs.

In three experiments, slices were preincubated for 10 min with 5 μM scopolamine (a muscarinic receptor antagonist), 5 μM kynurenic acid (a glutamic acid receptor antagonist) and 0.5% glutathione (w/v), as well as with 10 mM LiCl. Quinpirole (1.0 μM) was then added and the incubation continued for 20 min. The accumulation of IP_1 in the presence of quinpirole and the above additions was 119 ± 7% (mean ± SE) of its level in the presence of the additions alone. For IP_2 and IP_3 these values were

Table 2. Lack of effect of D_1 and D_2 dopamine agonists on basal accumulation of inositol phosphates

Drug	IP_1	IP_2 (% of control)	IP_3
Quinpirole, 1 μM	97 ± 4	105 ± 6	111 ± 6
SKF 38393, 10 μM	115 ± 12	106 ± 6	117 ± 9
Quinpirole, 1 μM, + SKF 38393, 10 μM	104 ± 6	105 ± 4	108 ± 6

After labeling for 2 hr with [^{3}H]inositol, slices were preincubated for 10 min in the presence of 10 mM LiCl. Test drugs were then added as indicated, and the incubation was continued for an additional 20 min. Values are means ± SE of five experiments, each performed in triplicate. No treatment had a statistically significant effect. Control values (dpm IPs/10,000 dpm PI) were: IP_1, 2,058 ± 135; IP_2, 4,117 ± 258; and IP_3, 1,711 ± 266. Mean ± SE PI = 34,756 ± 1,080.

116 ± 8 and 103 ± 12% respectively. These differences were not statistically significant (df = 2, P = 0.14, 0.19 and 0.92, respectively, by paired *t*-test). However, we did note a 24–36% decrease in IP_3 accumulation in samples containing the above additions versus control containing 10 mM LiCl only (Table 3). In addition, we obtained inhibition of 13 and 24% in two experiments comparing scopolamine plus LiCl to LiCl alone.

DISCUSSION

In this paper we present our attempts to (1) replicate the findings of Pizzi *et al.* [18, 19] showing that D_2 DA agonists inhibit PI hydrolysis and (2) determine if certain methodological differences may account for the negative findings reported by Kelly *et al.* [20]. One difference between the methods of these two groups was that Pizzi *et al.* [18, 19] included 10 mM LiCl in all their assays, whereas Kelly *et al.* [20] used either 5 mM or none. Lithium is used frequently in assays of IPs to inhibit the metabolism of IP_1 to free *myo*-inositol [23, 24], thus causing IP_1 to accumulate. Further evidence appears to indicate, however, that lithium may have additional effects on other aspects of the PI cycle [25]. In fact, two groups [26, 27] have reported recently that in cerebral cortex, lithium not only increases the accumulation of IP_1 in response to carbachol but also decreases the IP_4 level. Kelly *et al.* [20] have shown that this also occurs in rat striatum. Lithium may also have direct effects on the guanine nucleotide binding proteins thought to couple PI-linked receptors to PLC [28]. Therefore, while our experiments were not designed to study the effects of lithium on the coupling of G-proteins to either DA receptors or PLC, small differences in the concentration and duration of exposure to lithium were considered as potential sources of experimental variance. All of our experiments included 10 mM LiCl. In the studies presented in Table 2, the slices were exposed to lithium for 10 min prior to the addition of DA agonists for an additional 20 min. This mimicked the conditions reported by Pizzi *et al.* [18, 19], but we were nevertheless unable to find DA-mediated reduction of basal levels of IPs.

In addition to the presence of lithium in the assay buffer, Pizzi *et al.* [18, 19] also included scopolamine (SCO), kynurenic acid (KYN) and glutathione (GSH). Since both muscarinic agents and excitatory amino acids are known to stimulate PI hydrolysis (in the CNS in general [29] and in the corpus striatum in particular [15, 30, 31]), they reasoned that by inclusion of these antagonists inhibition by DA could be shown to be direct, rather than as a result of decreased acetylcholine or glutamic acid release [18] and also that an inhibition by DA agonists could be seen more easily if stimulation by endogenous ligands was decreased [19]. While we do not disagree with their reasoning, we were unable to replicate their results using these additions in the concentrations reported in their 1988 paper. We did see some inhibition of IP_3 levels with the addition itself of SCO + KYN + GSH. While this could most easily be explained by an inhibition of "tonic" muscarinic and glutaminergic stimulation, we also found a corresponding increase in IP_1 accumulation such that

Table 3. Effects of quinpirole on basal accumulation of inositol phosphates in the presence of scopolamine, kynurenic acid and glutathione

Drug	IP_1	IP_2 (% of control)	IP_3
Scopolamine, kynurenic acid, glutathione	144 ± 9*	98 ± 5	69 ± 7†
Scopolamine, kynurenic acid, glutathione + quinpirole	172 ± 18*	114 ± 11	70 ± 4†

Labeled striatal slices were incubated in the presence of 10 mM LiCl and the presence or absence of 5 μM scopolamine, 5 μM kynurenic acid and 0.5% glutathione (w/v). After 10 min, 1.0 μM quinpirole was added in the indicated samples and the incubation continued for an additional 20 min. Values are means ± SE of three experiments, each performed in triplicate. Control values (dpm IPs/10,000 dpm PI) were: IP_1, 1,990 ± 139; IP_2, 4,553 ± 97; and IP_3, 2,704 ± 9. Mean ± SE PI = 38,061 ± 1,089.

*,† P values: (*) P = 0.026, and (†) P = 0.016 by repeated measures analysis compared to control. Scopolamine, kynurenic acid, glutathione plus quinpirole was not significantly different from scopolamine, kynurenic acid, glutathione.

the total of all IPs remained essentially unchanged. Thus, it is possible that these drugs are having a post-receptor effect, such as increasing the rate of metabolism from IP_3 to IP_1. Additionally, these effects were most pronounced when GSH was included, rather than when only either SCO or SCO + KYN was present. Used at a concentration of 0.5% (16 mM), GSH, in addition to functioning as an antioxidant, may also reduce disulfide bonds and thus have direct effects on membrane receptors [32, 33] and other cellular proteins such as PLC [34]. While we do not fully understand the complex and probably multiple effects of the addition of SCO + KYN + GSH to the assay mixture, their inclusion does not explain the differences in the results obtained with respect to the effects of D_2 agonists since the addition of quinpirole had no further effect.

In addition to experiments on basal effects, we also stimulated the production of IPs with carbachol and attempted to inhibit this increased production with quinpirole. This approach was used since in those studies in pituitary tissue which found DA inhibition of PI hydrolysis, this inhibition was on production of IPs in response to stimulation by other neuromodulators rather than on basal production [6–8]. The situation in our preparation is more complex, however, since striatal slices contain a number of cell types whose synaptic pattern [35] make it unlikely that cholinergic and dopaminergic receptors modulating the PI response would be located on the same neurons. In contrast, the pituitary studies used a culture preparation enriched in lactotrophs having receptors for all the agonists involved. With that caveat in mind, we show that in a striatum quinpirole did not inhibit carbachol-induced accumulation of IPs in the presnce of lithium.

We also studied the effects of the D_1 agonist SKF 38393 alone and in combination with quinpirole, since in a number of paradigms it has been shown that what are otherwise considered to be D_2 effects require the presence of some D_1 activity for full efficacy [36, 37]. Addition of the D_1 agonist, however, did not affect significantly levels of IPs either alone or in combination with quinpirole. Alternatively, it might be suggested that the inability to find a D_2 effect could be due to basal inhibition by endogenous DA in the slices blocking any further effect of exogenous agonists. This is highly unlikely, however, given the extensive preincubations and washings. Additionally, direct evidence against this possibility is the absence of any effect by the selective D_2 antagonist eticlopride (data not shown). Furthermore, similarly prepared tissue was able to respond as expected to selective D_1 and D_2 agonists with increased and decreased cyclic AMP accumulation [38].

Since our extraction method was not readily compatible with measurement of protein levels, we used the amount of labeled PI as a measure of tissue concentration to adjust for differences in the precise amount of tissue in aliquots of slices. This also corrected for variation in the degree of labeling in experiments performed on different days. It might be argued that this could introduce error if any of the test drugs affected additional PI labeling during the assay. To confirm that this was not the case, we re-computed our data using the gross dpm unadjusted by labeled PI. While this increased the amount of variation between experiments, there was no difference in results that would affect the conclusion that DA agonists do not modulate PI hydrolysis.

Basal levels of IP_2 and IP_3 in our samples were unusually high relative to the basal level of IP_1. We believe this is a result of contaminants co-eluting with these fractions. The alternative, that IP_1 "overflowed" into the IP_2 and IP_3 fractions, is unlikely given the chromatographic procedure employed. Two 10-mL portions of 200 mM AF/100 mM FA were used to elute IP_1. Even when IP_1 was stimulated to very high levels with carbachol, over 90% of the [^{3}H]IP_1 was recovered in the first 10-mL portion of the fraction. Additionally, when samples collected from the IP_1 fraction were desalted, dried, resuspended and re-applied to the column, only 5.8% of the total recovered was found in the IP_2 fractions and less than 1% was in the IP_3 fraction. Furthermore, the apparent excess of IP_2 and IP_3 is essentially accounted for by the higher proportions of these species in the blanks—samples which were "stopped" at the time the incubation with test drugs would have otherwise begun. Blank values represented approximately 36% (IP_1), 92% (IP_2), and 55% (IP_3) of the total radioactivity in controls. To relieve the system of this background noise, one could subtract the value of the blanks from the experimental samples. While this might make the basal values "look better," we avoided this since in addition to any impurities, the radioactivity in the blanks also represents [^{3}H]IPs present at the start of the experimental incubation. Since IP_2 and IP_3 are being metabolized as well as produced, the values of IP_2 and IP_3 present at the start of incubation do not represent an absolute baseline as subtraction of blanks would usually imply. We therefore chose not to manipulate the data by subtracting the blanks but rather simply present the total control values.

Although we have not thoroughly exhausted all the methodological differences between the reports of Kelly *et al.* [20] and Pizzi *et al.* [18, 19], we consider it unlikely that remaining differences, such as in tissue concentration or extraction procedure, would produce qualitative differences in our hands. We have also studied the PI response in rats chronically treated with the D_2 antagonist haloperidol. Such treatment has been shown to increase the density of D_2 receptors in the striatum [39]. This increase in receptor number potentially could increase the chances of finding a recalcitrant D_2 response; however, in this group as well, we were unable to find any effect of quinpirole [40]. Thus, we are forced to concur with Kelly *et al.* [20] that DA receptors are not linked to PI hydrolysis in the rat striatum.

While our data directly address DA receptor coupling to PI hydrolysis only in rat striatum, it is important to note that inhibition of basal PI hydrolysis by acute treatment with D_2 agonists has never been replicated in any tissue. Even in pituitary where the evidence for DA inhibition is strongest, significant inhibitory effects have been demonstrated only on PI hydrolysis stimulated by other agonists [6–8] rather than on basal activity. In reviewing the actions

of D_2 receptors in pituitary, Vallar and Meldolesi [41] have postulated recently that D_2 receptors do not inhibit immediate agonist-induced PI hydrolysis but rather indirectly inhibit only sustained stimulation [42] via modulation of intracellular calcium concentrations through direct effects on ion channels and cyclic AMP [43]. The bulk of the evidence thus suggests that D_2 DA receptors are not directly coupled to PLC.

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