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D_2, D_3, and D_4 Dopamine Receptors Couple to G Protein-Regulated Potassium Channels in *Xenopus* Oocytes

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SUMMARY

Human D_2, D_3, D_4 and dopamine receptors were individually coexpressed in *Xenopus* oocytes with a G protein-regulated inwardly rectifying potassium channel (GIRK1). At −100 mV in 96 mM potassium, dopamine (0.1–100 nM) evoked an inward current; the current showed inward rectification, reversed polarity at 0 mV, and was blocked by barium (50% inhibition by 10 μM). The concentrations of dopamine activating 50% of the maximal current (EC_{50}) were not different (2–4 nM) for D_2, D_3, and D_4 receptors, but the maximal current was 3-fold larger for D_2 and D_4 than for D_3 receptors. Dopamine evoked reproducible inward currents at D_2 and D_4 receptors when applied repeatedly, but second responses could not be observed in oocytes expressing D_3 receptors. 7-Hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin mimicked the effect of dopamine (EC_{50} of ~2, ~3, and ~19 nM at D_2, D_3, and D_4, respectively). (−)-Sulpiride reversibly blocked the dopamine-induced current with IC_{50} values of 5, 300, and 2000 nM for D_2, D_3, and D_4 receptors, respectively. Dopamine was ineffective in oocytes injected 2 hr previously with pertussis toxin. We concluded that all three D_2-like dopamine receptors share the potential to activate inwardly rectifying potassium channels.

The D_2, D_3, and D_4 receptors for dopamine are often grouped together as D_2-like receptors in contrast with the D_1 and D_5 (D_1-like) receptors; the distinction is made on the basis of primary structure but also applies to commonly observed second messenger pathways (1–3). The original distinction between D_1 and D_2 receptors (4) was made in large part on whether dopamine stimulated (D_1) or inhibited (D_2) adenylate cyclase. Correspondingly, activation of D_1-like receptors expressed from cDNAs typically leads to stimulation of adenylate cyclase (1–3), whereas activation of heterologously expressed D_2-like receptors can inhibit adenylate cyclase activity (5–7), although some groups have found functional coupling of D_3 receptors difficult to demonstrate (8–11).

D_2 receptors also couple to potassium channels. This was first observed in *Aplysia* (12) and characterized more fully in the dopamine cells of the rat midbrain (13, 14). Subsequent work has shown other examples of a dopamine-activated potassium conductance in pituitary melanotrophs and lactotrophs (15, 16), where the action underlies a hyperpolarizing postsynaptic potential (17). These studies (15, 16) further demonstrated that a pertussis toxin-sensitive G protein was involved in the transduction from dopamine receptor to potassium channel. In this respect, the D_2 receptor belongs to a large subclass of seven-transmembrane domain receptors at which agonists lead to potassium channel opening (18).

A cDNA clone has been isolated that encodes the potassium channel, or a component of it, that is activated by receptors of this subclass. This potassium channel subunit is known as GIRK1 (19) or as a potassium channel activated by G protein from atrium (KGA)(20). The native channel may be a heteromultimer comprising additional subunits (21), but in *Xenopus* oocytes expression of GIRK1 RNA is sufficient to endow responsiveness to agonists at M_2 muscarinic (19–21); μ- (22), δ- (20, 23, 24), and κ-opioid (23, 25); 5-hydroxytryptamine$_{1A}$ (20); and cannabinoid (26) receptors as long as the appropriate receptor RNA is coexpressed.

The purpose of the current study was to determine whether the dopamine D_2, D_3, and D_4, receptors would couple to GIRK1 in *Xenopus* oocytes; such information might be useful in the development of more selective agonists and antagonists at these receptors.

Materials and Methods

DNA templates for *in vitro* transcription. The GIRK1 coding region was cloned with the use of reverse transcription-polymerase chain reaction from rat heart poly(A)$^+$ RNA with oligonucleotide primers 5′-ATGTCTGCACTCCGAAGGAAA and 5′-CTATGTGAAGCGGTCAGAGTT derived from the published sequence (19, 20). The DNA product was subcloned into pBluescript SKII vector (Stratagene) and sequenced. *Not*I was used for linearization, and T7 RNA polymerase was used for in vitro transcription. A modified

ABBREVIATIONS: GIRK1, G protein-regulated, inwardly rectifying potassium channel; 7-OHDPAT, 7-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

human D_4 receptor cDNA construct with two 48-basepair repeats in the third intracellular loop (27) was subcloned into the *Nco*I and *Sal*I sites of the pSPUTK vector (Stratagene). The plasmid DNA was linearized with *Eco*RI and transcribed with SP6 RNA polymerase.

The coding regions of human D_{2L} (28) and D_3 (29) were transferred into an isogenic sequence background by polymerase chain reaction overlap extension (30): in both cases, the coding regions were flanked at the 5′-end by a T7 RNA polymerase promoter sequence followed by a short linker region and a Kozak consensus sequence and at the 3′-end by the 3′-untranslated region of the human D_2 receptor clone (~1 kb). The T7 RNA polymerase promoter was introduced by the primer T7GEM and the Kozak consensus sequence by the 5hD2 or 5hD3 primers. The first rounds of amplification were performed on 10 ng of human D_2 or D_3 receptor plasmid DNA with the primer pairs 5hD2/D2D2A and 5hD3/D3D2A, respectively, to amplify the coding regions and Univ3D2 in combination with D2D2S or D2D3S to amplify separately the 3′-untranslated tail. After 1:50 dilution, the DNA products were submitted pairwise to a second round of amplification with the primer pair T7GEM/Univ3D2. Vent polymerase (Biolabs) was used in 50 ml reaction mix, according to the supplier's protocol. Thirty temperature cycles were executed (1 min at 96°, 1 min at 50°, 2 min at 72°, or 4 min for the second round of amplification). After purification (filtration through spin-column CS-100 DEPC, Clontech), 20 ml of the polymerase chain reaction products or 2 mg of the linearized plasmids was transcribed *in vitro* in a Promega reaction mix containing cap analogue m7G(5)ppp(5)G.

Sequences of the oligonucleotide primers. The sequence for T7GEM was 5′GCACTGTAATACGACTCACTATAGGGCGAATTCGAGCTCGACCATG; 5hD2, 5 GGGCGAATTCGAGCTCGACCATGGATCCACTGAATCTGTCCTGGTA; 5hD3, 5′GGGCGAATTCGAGCTCGACCATGGCATCTCTGAGTCAGCTGAGTAG; D2D2S, 5′TCCTGAAGATCCTCCACTGCTGAGGTGGATCGGCCTCCTCTTCTTA; D3D2S, 5′TCCTCAAGATCCTGTCTTGCTGAGGTGGATCGGCCTCCTCTTCTTA; D2D2A, 5′TAAGAAGAGGAGGCCGATCCACCTCAGCAGTGGAGGATCTTCAGGA;D3D2A,5′TAAGAAGAGGAGGCCGATCCACCTCAGCAAGACAGGATCTTGAGGA; and Univ3D2, 5′CGACTAGTGAAGGTGACTCGTCAAAGTTTTATT.

Oocyte injection and recording. Female *Xenopus laevis* were anesthetized in 0.4% tricaine (MS222, Sigma) and decapitated. The ovaries were removed, and oocytes were defolliculated in a solution containing 87.5 mM NaCl, 2 mM KCl, 5 mM HEPES, pH 7.5, and 2 mg/ml collagenase IA (Sigma). Oocytes were injected with 50 nl of the RNA mixture (InjectMatic, Gabai, Switzerland) and incubated at 18° for 3–14 days in a solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 5 mM sodium pyruvate, pH 7.5. Recordings were made during continuous superfusion (10 ml/min) with this solution. Two glass microelectrodes containing 3 M KCl were used, with a GeneClamp amplifier and PClamp software (Axon Instrument, Foster City, CA). Unless otherwise specified, oocytes were clamped at a holding potential of −100 mV. Drugs were applied by changing the superfusing solution. The high potassium solution contained 96 mM KCl, 2 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5; intermediate potassium concentrations were obtained through mixing of the two solutions. Dose-response curves were fitted by logistic functions with the use of a Marquardt-Levenberg least-squares algorithm (Origin, Microcal software). Averaged values are expressed as mean ± standard error. Several agonist concentrations were usually applied to the same oocyte, so the mean value is that determined with the least-squares curve fit.

Results

Dopamine evokes potassium current. Oocytes that had been coinjected with GIRK1 and any of the three dopamine receptor subtypes responded to dopamine (0.1–100 nM) when tested after 5–10 days. The response at −100 mV in high potassium solution was an inward current that developed within a few seconds of dopamine reaching the oocyte (Fig. 1). The current persisted throughout the dopamine application (≤2 min) and, for D_2 and D_4 receptors, declined when the dopamine application was discontinued. Dopamine did not evoke any such current in oocytes that had been injected only with the RNA for GIRK1 or for a dopamine receptor (10 oocytes). Responses to dopamine in oocytes expressing D_3 receptors differed in three ways from those seen in oocytes expressing D_2 or D_4 receptors. First, the maximal currents were smaller in oocytes expressing D_3 receptors (54 ± 4 nA, 47 oocytes) than in oocytes expressing D_2 (160 ± 17 nA, 40 oocytes) or D_4 (180 ± 21 nA, 18 oocytes). Second, in oocytes expressing D_3 receptors, the current evoked by dopamine did not reverse fully when the dopamine was washed out. Third, oocytes expressing D_2 and D_4 receptors responded repeatedly to dopamine when it was reapplied at intervals of a few minutes, whereas oocytes expressing D_3 receptors produced a second response to dopamine only when the interval between applications was ≥2 hr.

Barium strongly inhibited the inward currents evoked by dopamine in cells expressing the D_2 receptor (Fig. 2). The block was voltage dependent and increased with hyperpolarization, and at −100 mV in high potassium solution, the concentration causing 50% block (IC_{50}) was 9.2 ± 0.2 μM (four oocytes) (Fig. 2B). In comparison, 10 μM barium also inhibited approximately half of the current in cells expressing D_3 or D_4 receptors (three or four oocytes; Fig. 2B). A change to the high potassium solution always caused a significant inward potassium current (at −100 mV) in oocytes expressing GIRK1; this current was also inhibited by 47 ± 1% (four oocytes) with barium (10 μM), even in the absence of dopamine.

The current induced by dopamine was inwardly rectifying (Fig. 3) and reversed polarity close to zero mV (D_2, −5 ± 3 mV, four oocytes; D_3, 0 ± 3, three oocytes; D_4, −3 ± 2 mV, three oocytes) (Fig. 3). This reversal potential became more negative in higher potassium concentrations (Fig. 3C), equal to a shift of 51 mV for 10-fold change in potassium concentration.

Pertussis toxin sensitivity. Responses to dopamine (100 nM) were abolished by injection of pertussis toxin (2.5 ng in 10 nl) into the oocytes 2 hr before the recording (4–10 oocytes, two batches of oocytes) (Fig. 4). This was true for all three receptors. The current induced by changing to the high po-

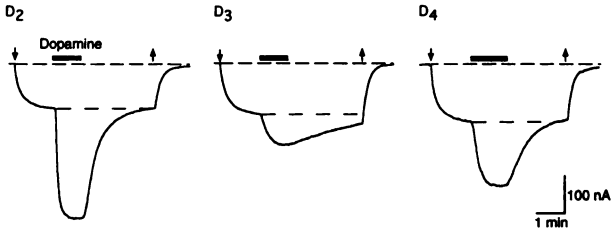

Fig. 1. Dopamine induces inward currents in oocytes previously injected with cRNAs coding for D_2, D_3, or D_4 dopamine receptor, along with GIRK1. The first inward current occurs (↓) when the solution is changed to 96 mM potassium; this is later washed out (↑). *Filled bars*, dopamine (30 nM) applied for periods indicated. All oocytes were from the same ovary. Responses in oocytes expressing D_3 receptors were smaller than those seen in D_2 and D_4 oocytes.

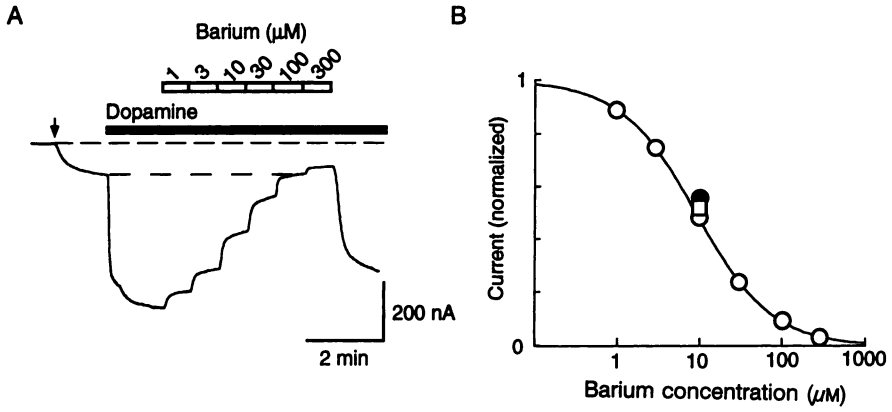

Fig. 2. Barium inhibits the dopamine-induced current. A, Onset of superfusion with high potassium solution (↓) (see legend to Fig. 1). *Filled bar*, dopamine (100 nM) applied for the period indicated. *Open bars*, barium applied in the concentrations indicated (μM) during the periods indicated. B, Averaged data obtained from three oocytes expressing D_2 receptors (○, standard error is less than the size of the symbols). ● and □, inhibition by barium (10 mM) in oocytes expressing D_3 and D_4 receptors (four oocytes).

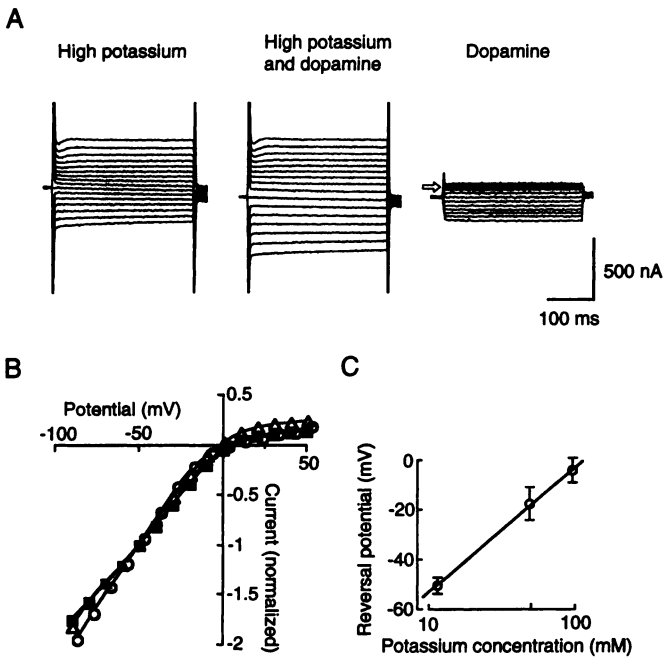

Fig. 3. Dopamine activates an inwardly rectifying K^+ conductance. A, Responses to voltage steps from −100 to −50 mV in high potassium concentration (96 mM); *left*, control; *middle*, dopamine (100 nM); *right*, difference; *arrow*, zero current level. B, Current induced by dopamine plotted versus voltage for oocytes expressing D_2 (○), D_3 (■), or D_4 (△) receptors along with GIRK1. Currents are normalized to the value at −50 mV. C, Relationship between external potassium concentration and reversal potential for oocytes expressing D_2 receptors and GIRK1 (five oocytes).

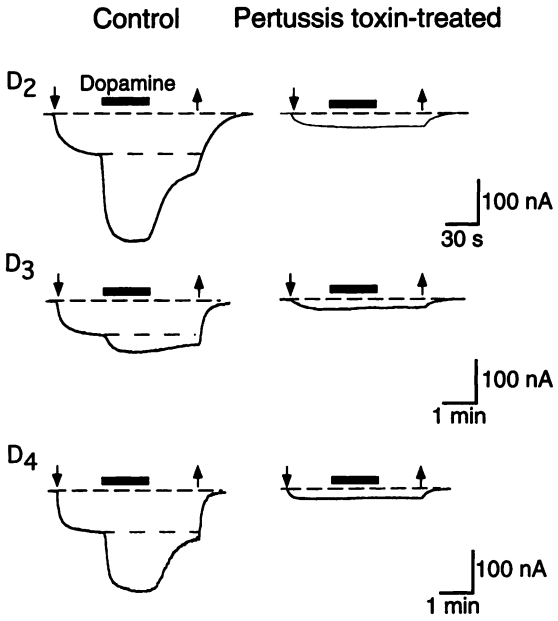

Fig. 4. Pertussis toxin blocks dopamine-induced currents. The first inward current occurs (↓) when the solution is changed to 96 mM potassium; this is later washed out (↑). The three rows show paired recordings from oocytes that had been previously injected with cRNAs for GIRK1 and D_2, D_3, or D_4 receptors. Oocytes were injected again 2 hr before the recordings shown, with water (10 nl) (*left*) or pertussis toxin (2.5 ng in 10 nl) (*right*). Dopamine concentration was 100 nM.

tassium solution was also less in pertussis-injected oocytes. Water injections (10 nl) had no effect on the dopamine-induced currents.

Agonist and antagonist sensitivity. We constructed cumulative dose-response curves (Fig. 5). All three receptors showed similar sensitivity to dopamine; the EC_{50} values were 2.3 ± 0.3 (eight oocytes), 1.8 ± 0.1 (five oocytes), and 3.5 ± 0.2 nM (five oocytes) for D_2, D_3, and D_4 receptor, respectively. 7-OHDPAT, which has been suggested to have some selectivity for D_3 receptors (30), was rather less effective at D_4 than at D_2 and D_3 receptors [EC_{50} values were 1.5 ± 0.1 nM (five oocytes), 2.9 ± 0.4 nM (five oocytes), and 18.8 ± 1 nM (four oocytes) at D_2, D_3, and D_4 receptors, respectively].

Sulpiride blocked the action of dopamine at all three receptors. For D_2 and D_4 receptors, this could be readily observed as a reversal of the inward current when the solution was changed from one containing dopamine to one containing both dopamine and (−)-sulpiride (Fig. 6A). For the D_3 receptor, it was necessary to compare dose-response curves to dopamine in the absence and the presence of (−)-sulpiride. The concentration of (−)-sulpiride causing 50% inhibition of the current evoked by dopamine (10 nM) was 5.1 ± 0.9 nM (six oocytes), 300 ± 60 nM (10 oocytes), and 2.2 ± 0.5 mM (six oocytes), for D_2, D_3, and D_4 receptors, respectively (Fig. 6B).

Discussion

The main finding of the current study was that D_2, D_3, and D_4 receptors can each couple to GIRK1 potassium channels when a receptor RNA and GIRK1 RNA are coexpressed in *Xenopus* oocytes. The principal features of this coupling are similar to those described previously for muscarinic M_2 (19–21); 5-hydroxytryptamine$_{1A}$ (20); cannabinoid (26); μ-, δ-, and

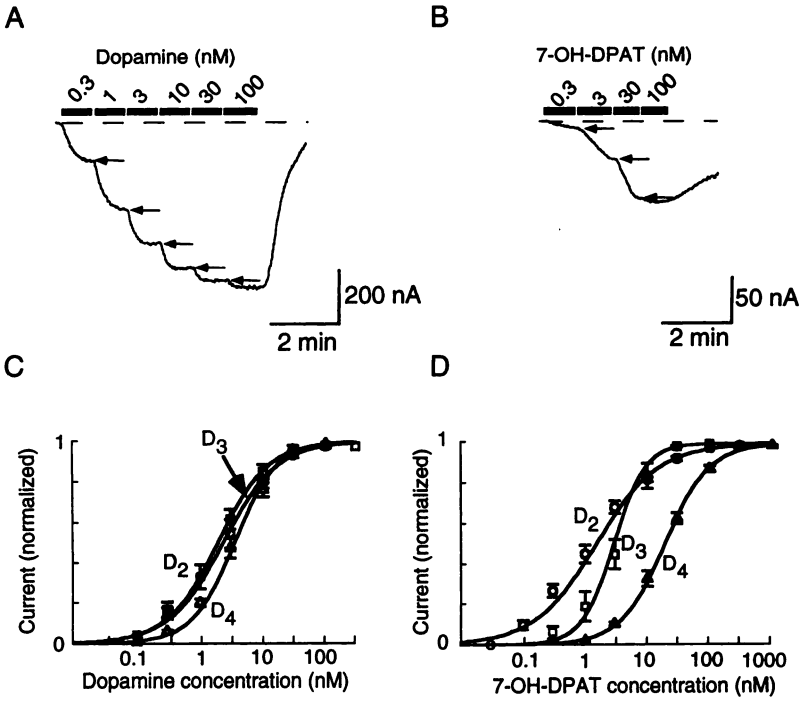

Fig. 5. Dose-response relations for dopamine and 7-OHDPAT. A, Cumulative application of dopamine to oocyte expressing D_2 receptor. B, Cumulative application of 7-OHDPAT to oocyte expressing D_3 receptor. Records shown are in high potassium solution. Arrows, current amplitude before the application of the next higher concentration. C and D, averaged data from three to five applications of each concentration in single oocytes (D_2 and D_4) or from single applications in five oocytes (D_3). Currents are normalized to the maximum response in each oocyte.

κ-opioid (20, 22–25), and adenosine A_1[1] receptors. These features include readily reversible, reproducible, concentration-dependent currents and, although this is variable in oocytes (20), block by pertussis toxin. The absolute amplitudes of the currents (measured in hundreds of nA) and their inward rectification and barium sensitivity are also similar to those previously reported (19–26).

All three receptors were similarly sensitive to dopamine, with EC_{50} values in the low nanomolar range. Similarly low EC_{50} values have been reported for D_2-like receptors in experiments in which inhibition of cAMP accumulation or mitogenesis was measured in transfected Chinese hamster ovary cells (7), mouse fibroblasts (9), and a mesoencephalic neuronal cell line (MN9D) (9). A direct comparison of the EC_{50} values with those found in native cells is difficult without independent estimates of the density of receptor and G protein in the membrane. The other agonist tested was 7-OHDPAT because of reports that it showed selectivity for D_3 receptors in binding (31) or behavioral (32) studies. We found no marked difference in the potency of 7-OHDPAT in activating D_2 and D_3 receptors, although 10-fold higher concentrations were required at D_4 receptors; a similar lack of selectivity in functional assays has been reported (6, 33, 34). Sulpiride completely blocked the responses to dopamine at all three receptors; the order of affinity ($D_2 > D_3 > D_4$) was similar to that reported by others for transfected receptors (3, 6, 7). The block by pertussis toxin implies the involvement of G_i or G_o in the transduction from all three receptors; this has been reported previously for dopamine activation of potassium currents in anterior pituitary cells (15, 16) as well as inhibition of calcium currents in anterior pituitary cells (16) and D_3 receptor-transfected NG108–15 cells (35). It seems unlikely that the pertussis toxin is directly blocking the potassium channel, because it is known that coupling from receptors to potassium channels can be restored by application of G proteins to pertussis toxin-treated cells (36).

D_3 receptors have 57% amino acids that are identical to D_2, and D_4 receptors have only 43% residues that are identical to D_2. However, it was clear from the results of the current study that responses at D_2 and D_4 receptors were most similar, whereas responses at D_3 receptors differed in three important ways. The first difference was that dopamine did not induce such large currents in oocytes expressing D_3 receptors as in oocytes expressing D_2 or D_4 receptors. A similar finding has been made for D_2 and D_3 receptor coupling to extracellular acidification (7). Care was taken to minimize differences in RNA stability and translation efficiency by creating D_2 and D_3 receptor cRNAs with identical 5′- and 3′-untranslated regions, although these precautions cannot guarantee similar densities of proteins in the plasma membrane. In three oocytes, both D_3 and A_1 receptors were expressed; the responses to adenosine were similar in magnitude to the responses to dopamine in oocytes expressing D_2 or D_4 receptors,[2] which implies that the weak responses at D_3 receptors did not result from poor GIRK1 expression. The second difference between D_3 responses compared with D_2 and D_4 was the slow reversal of the current when the dopamine application was discontinued. The third difference was the lack of response of D_3 receptor-expressing oocytes to a second application of dopamine within 5–60 min of the first application. When A_1 receptors were coexpressed, the responses to adenosine were similar before and after the dopamine application[3]; this suggests that the loss of response was not due to down-regulation of GIRK1 (see Ref. 37). Although not investigated systematically, these two differences might suggest that the D_3 receptor is undergoing covalent modification in the membrane as a result of dopamine action, such

[1] E. Kawashima and R.A. North, unpublished observations.

[2] N. Hussy and K.A. Jones, unpublished observations.

[3] N. Hussy and K.A. Jones, unpublished observations.

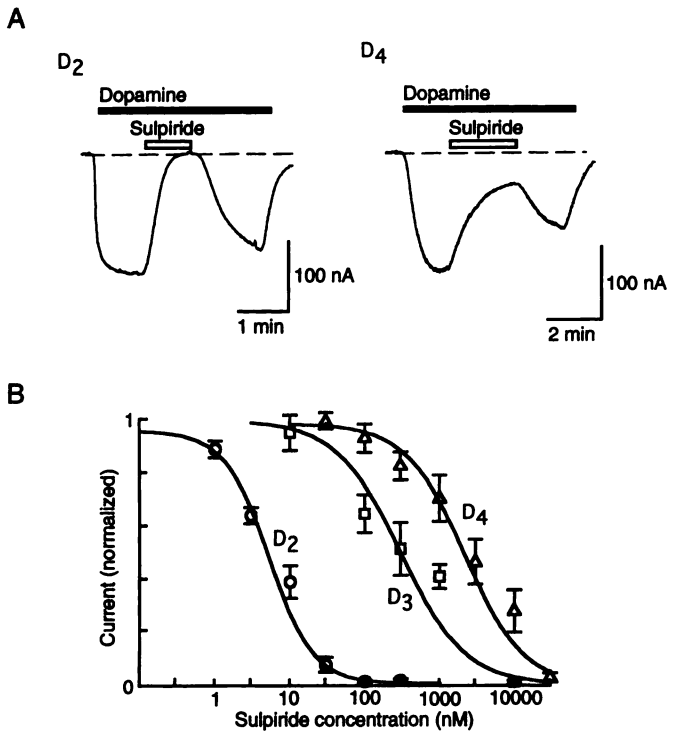

Fig. 6. Sulpiride inhibits responses to dopamine. A, (−)-Sulpiride reverses the current induced by dopamine. *Open bar*, 300 nM (−)-sulpiride; *filled bar*, 10 nM dopamine. Dopamine-induced current develops again as sulpiride is washed out. B, Averaged data for sulpiride inhibition in many oocytes. Dopamine concentration was 10 nM. For oocytes expressing D_2 and D_4 receptors, the number of applications of each concentration ranged from three to six (six oocytes with each receptor). For D_3 receptors, the number of applications at each point ranged from three to five, with 10 oocytes in dopamine (10 nM) alone and 10 different oocytes in dopamine and sulpiride.

as has been reported for other G protein-coupled receptors, including D_1 receptors (38). However, the difference could also arise as an artifact of the expression system, given that there is no control over the relative concentrations of receptor and G protein in the oocyte.

Dopamine activation of inwardly rectifying potassium channels has been described in *Aplysia*, endocrine cells, and mammalian brain (see introduction paragraphs). Experiments were carried out before cDNA clones for the dopamine receptor were isolated, although some clearly identified the receptor as being D_2-like rather than D_1-like (13, 15). The subsequent detection of D_3 mRNA in substantia nigra and ventral tegmental area, together with the current finding that D_3 receptors can couple to potassium channels, raises the possibility that D_3 receptors could contribute to dopamine-mediated inhibition of dopamine-containing neurons. The best way to resolve issues of this kind will be to develop agonists and antagonists that are highly selective for the receptors as expressed in their native environment. Coexpression of D_2, D_3, and D_4 receptors with GIRK1 in *Xenopus* oocytes may be helpful in this respect.

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