Dopamine inhibits mammalian photoreceptor Na⁺,K⁺-ATPase activity via a selective effect on the $\alpha 3$ isozyme

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ABSTRACT The rat retina contains dopaminergic interplexiform cells that send processes to the outer plexiform layer where dopamine is released in a light-dependent manner. We report herein that physiologically relevant concentrations of dopamine inhibited ouabain-sensitive photoreceptor oxygen consumption in dark- and light-adapted rat retinas and inhibited Na⁺,K⁺-ATPase specific activity (EC 3.6.1.37) in a rat rod outer-inner segment preparation. Experiments with the selective D₁ agonist fenoldopam or D₂ agonist quinpirole and experiments with dopamine plus either the D₁ antagonist SCH23390 or D₂/D₄ antagonist clozapine showed that the inhibition of oxygen consumption and enzyme activity were mediated by D₂/D₄-like receptors. The amphetamine-induced release of dopamine, monitored by the inhibition of oxygen consumption, was blocked by L-2-amino-4-phosphonobutyric acid and kynurenic acid. Pharmacological and biochemical experiments determined that the IC₅₀ values of ouabain for the $\alpha 1$ -low and $\alpha 3$ -high ouabain affinity isozymes of photoreceptor Na⁺,K⁺-ATPase were $\approx 10^{-5}$ and $\approx 10^{-7}$ M, respectively, and that the D₂/D₄-like mediated inhibition of Na⁺,K⁺-ATPase was exclusively selective for the $\alpha 3$ isozyme. The dopamine-mediated inhibition of $\alpha 3$ first occurred at 5 nM, was maximal at 100 μ M (–47%), had an IC₅₀ value of 382 ± 23 nM, and exhibited negative cooperativity (Hill coefficient, 0.27). Prior homogenization of the rod outer-inner segment completely prevented the long-lasting inhibition, suggesting that the effect was coupled to a second messenger. Although the physiological significance of our findings to photoreceptor function is unknown, we hypothesize that these results may have relevance for the temporal tuning properties of rods.

The Na⁺,K⁺-ATPase (Na,K-ATPase; EC 3.6.1.37) is an integral plasma membrane protein that actively transports Na⁺ and K⁺ against their electrochemical gradients. This ubiquitous protein is present in high concentrations in neurons where it plays a fundamental role in maintaining cellular ionic gradients that are required for neural activity, transport of amino acids and glucose, and regulation of cell pH and volume (1). In the photoreceptor cell, the inner segment Na,K-ATPase maintains the dark current that is responsible for the light response (2). The enzyme is composed of two functional subunits: α and β . The catalytic α subunit has binding sites for Na⁺, K⁺, ATP, and ouabain. Three different isoforms of the α subunit ($\alpha 1$, $\alpha 2$, and $\alpha 3$), which differ most significantly in their sensitivity to cardiac glycoside inhibition, have been identified in different tissues (3).

In the rat retina, the $\alpha 1$ -low and $\alpha 3$ -high ouabain affinity isoforms of the enzyme account for $\geq 97\%$ of the Na,K-ATPase mRNA. The $\alpha 3$ isoform has been localized to rat photoreceptors, horizontal cells, and bipolar cells (4–6). Photoreceptors predominantly express the $\alpha 3$ mRNA ($\approx 85\%$), a

small amount of $\alpha 1$ mRNA ($\approx 15\%$), and almost no detectable $\alpha 2$ mRNA ($\leq 0.4\%$). Electron microscopic immunocytochemistry studies reveal that the $\alpha 3$ isoform is localized exclusively to the plasma membrane of the inner segments (5, 6). The photoreceptor Na⁺,K⁺-pump operates near $V_{\max}$ in the dark-adapted or depolarized state, due to the constant entry of Na⁺ into the cell and the leakage of K⁺ from the cell, and is slowed during maintained rod-saturating illumination in response to a decrease in the intracellular Na⁺ concentration (7, 8). Currently, the decrease in intracellular Na⁺ concentration is thought to be the only regulator of the photoreceptor Na⁺,K⁺-pump. Except for calcium (9–11), the short-term regulation of retinal or photoreceptor Na,K-ATPase activity by neuromodulators, hormones, or second messengers remains unexplored. Dopamine, however, inhibits the activity of total Na,K-ATPase in isolated neostriatal neurons (12) and in several segments of the rat renal nephron (13, 14) and inhibits ouabain-sensitive oxygen consumption in rat renal cortical tubule cells (15). It is noteworthy that the α isozyme specificity of the dopamine-mediated inhibition of brain Na,K-ATPase was not examined.

Several different studies have established that dopaminergic neurons are the only catecholaminergic containing neuron in the mammalian retina (rat, monkey, and human; refs. 16 and 17). In these species, dopamine is the neurotransmitter for interplexiform cells and a subpopulation of amacrine cells (18, 19) that is released in a light-dependent manner (16, 17). Dopamine reaches its targets in the outer retina via by diffusion (20), where it modulates neural circuitry, light and dark adaptation, retinomotor movements, and disc shedding (16, 17). In addition, immunocytochemical, cloning, and expression studies have shown that mouse, rat, and human retinal photoreceptors possess either D₄ or D₂ receptors (21–23) that are linked to the inhibition of adenylyl cyclase in intact retinal preparations (22, 24). In the studies reported herein, ouabain-sensitive oxygen consumption in pharmacologically isolated rat photoreceptors (photoreceptor oxygen consumption, QO_{PR}), a measure of Na,K-ATPase activity in intact retinas, and Na,K-ATPase specific activity in rat rod outer-inner segments (ROS-RISs) were measured. We show that dopamine, at nanomolar to micromolar concentrations, inhibits Na,K-ATPase activity and the specific activity of the $\alpha 3$ isozyme of Na,K-ATPase. Furthermore, these effects are mediated by D₂/D₄-like, and not D₁, dopamine receptors. An abstract describing these findings has been published (25).

MATERIALS AND METHODS

Materials. Ultrapure chloride salts of sodium, potassium, magnesium, and calcium were from Alfa Products (Ward Hill,

Abbreviations: Na,K-ATPase, Na⁺,K⁺-ATPase; QO_{PR}, photoreceptor oxygen consumption; APB, L-2-amino-4-phosphonobutyric acid; Kyn, kynurenic acid; ROS-RIS, rod outer-inner segment.

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MA). The D₁ agonist SKF82526 (fenoldopam mesylate) was a gift from Mustafa Lokhandwala (University of Houston), whereas the D₂ agonist LY171555 (quinpirole), D₁ antagonist R-(+)-SCH23390 hydrochloride and D₂/D₄ antagonist clozapine were from Research Biochemicals (Natick, MA). D-amphetamine sulfate was from Merck Sharp & Dohme. All chemicals were analytical quality or better (Sigma). All media were prepared fresh daily with 18.3 MΩ water, had a pH of 7.40 ± 0.01, and were 305 ± 5 mOsm. Dopamine, fenoldopam, quinpirole, and D-amphetamine were prepared in buffers containing 5 mM ascorbic acid to prevent oxidation.

Experimental Animals. Adult (90 day old) female Long-Evans hooded rats (Charles River Breeding Laboratories) maintained under a 12-hr light/12-hr dark cycle were used. QO_{PR} experiments used retinas from dark-adapted rats and were conducted under infrared illumination (26). The Na,K-ATPase enzyme studies were conducted under normal room light and began 1 hr after the onset of the light cycle. A total of 1029 rats was used.

Pharmacologically Isolated Photoreceptor Respiration. After decapitation, both dark-adapted retinas were removed rapidly and placed into an ice-cold Ringer's buffer (124 mM NaCl/26 mM NaHCO₃/5 mM KCl/1 mM MgCl₂/1.2 mM CaCl₂/6 mM glucose, aerated with 95% O₂/5% CO₂) where the vitreous and retinal pigment epithelium were removed. Each whole neural retina was placed into a 1.5-ml water-jacketed (37.0 ± 0.1°C) Gilson-type oxygen chamber containing Ringer's buffer with L-2-amino-4-phosphonobutyric acid (APB; 100 μM) plus kynurenic acid (Kyn; 5 mM) added. APB/Kyn were used to pharmacologically isolate photoreceptors from the remaining inner retina so that QO_{PR} could be determined (26, 27). QO_{PR} of each retina was determined polarographically and was recorded continuously in the dark and during the presentation of a half-saturating or rod-saturating light adapting stimulus (26). QO_{PR} rates were obtained as nmol of O₂ consumed per retina per min, converted to dry weight-adjusted values for photoreceptors, and expressed as μmol of O₂ per mg (dry weight) per hr.

For all pharmacological experiments, stock solutions of all chemical agents were diluted in normal Ringer's solution, maintained at 37.0°C, and delivered in 1–10 μl through an inlet port on the Gilson chamber. Either 100 nM or 10 μM dopamine was used. Ouabain, at 100 nM or 1 mM, was used as a positive control to inhibit selectively the activity of the α3 isozyme or the activity of all the isozymes of the Na,K-pump, respectively (11, 28). The Na⁺ ionophore monensin (10 μM) was used to produce a rapid, stable, and long-lasting increase in the intracellular ROS-RIS Na⁺ concentration (29). D-amphetamine (50 μM) was used to induce a calcium-independent release of dopamine by reversing its transport across the plasma membrane (30, 31). To examine dopamine receptor subtype specificity, retinas were first exposed to SCH23390 (1 μM) or clozapine (1 μM) for 5 min in the chamber prior to the addition of 10 μM dopamine. The maximum effect of each agent was reached within 5 min and was maintained throughout the recording period. Each retina was used for only one experiment.

For light-adaptation experiments, a dark-adapted retina was stimulated for 120 sec with either half rod-saturating or rod-saturating white light (32): the latter maximally reduced the dark-adapted QO_{PR} (26). The stimulus intensities measured at the retina were equivalent to 170 Rh* per ROS per sec (2.8 × 10² photons per μm² per sec) or 1800 Rh* per ROS per sec (3.0 × 10³ photons per μm² per sec), respectively (where Rh* is photoexcited rhodopsin).

ROS-RIS Isolation and Characterization. A simple and rapid mechanical isolation procedure, based on ref. 33, was developed to localize and assess the effects of dopamine on ROS-RIS Na,K-ATPase activity since ROS-RIS isolated using enzymatic dissociation had decreased plasma membrane in-

tegrity and Na,K-ATPase activity. Unless otherwise noted, all procedures were conducted at 4°C.

After decapitation, both retinas from three light-adapted rats were removed rapidly and placed into a Petri dish containing Tris/EGTA buffer (TBE = 105 mM NaCl/5 mM KCl/2 mM MgCl₂/25 mM Tris/25 mM NaHCO₃/10 mM glucose/1 mM EGTA) where the vitreous and retinal pigment epithelium were removed. Retinas were placed in microcentrifuge tubes containing 500 μl of TBE and triturated 40 times with a fire-polished 1000-μl pipette tip. After 60 sec, the remaining retinas were removed. The sample was triturated 5 more times and allowed to settle for 60 sec. A 20-μl aliquot was mixed with 20 μl of buffered glutaraldehyde/paraformaldehyde (34) for cellular identification and ROS-RIS cell counts with a hemocytometer and differential interference optics (*n* = 11 preparations). A 450-μl aliquot was centrifuged at 350 × *g* for 10 min. The pellet (intact ROS-RIS) was resuspended in various media for different experiments.

ROS-RIS Rhodopsin Assays, Didansyl-Cystine Dye Exclusion, and Janus Green B Dye Staining. To provide a direct correlative measure of the number of photoreceptors, rhodopsin was extracted from the ROS-RIS of dark-adapted rats and measured using an Hewlett-Packard 8452A diode array spectrophotometer (35). The absorbance of rhodopsin at its λ_{max} was calculated from difference spectra. Values are expressed as nmol of rhodopsin per preparation (*n* = 5).

N,N'-Didansyl-cystine dye exclusion studies were conducted (36) to evaluate quantitatively the integrity of the ROS. Isolated ROS-RIS were resuspended in 1.0 ml of TBE, incubated for 0, 15, 30, 45, or 60 min at 37.0°C, and stained for 1 min in an equal volume of freshly prepared dye (10 μM). A sample was placed on the hemocytometer slide and examined with an Olympus BH2 microscope equipped for epifluorescence. Intactness was determined by the ability of ROS-RIS to exclude the fluorescent dye. A minimum of 500 ROS-RIS were counted at each time point (*n* = 3–5 preparations per time point). Values are expressed as the percentage of intact cells.

The number of ROS-RIS, free RIS, and other cells with intact mitochondria were counted on the hemocytometer slide after staining with the mitochondrial dye Janus green B (*n* = 7 preparations) (37).

ROS-RIS Pharmacology. The ROS-RIS were incubated in medium containing dopamine and/or specific D₁ or D₂/D₄ agonists or antagonists. For the initial experiments, the ROS-RIS were resuspended in 1.0 ml of (i) TBE, (ii) TBE plus ascorbic acid, or (iii) 10 μM dopamine and then incubated for 0, 5, 10, 15, and 20 min at 37°C. After the incubation, the samples were centrifuged at 350 × *g* for 10 min at 4°C. The ROS-RIS were resuspended in 1 ml of ice-cold TBE and homogenized, and 50-μl aliquots were assayed for α1 and α3 specific activity as described in detail below. There were no differences between the TBE and TBE plus ascorbic acid results on total, α1, or α3 Na,K-ATPase activity (data not shown), so the combined data represent the control ROS-RIS data.

In subsequent experiments, ROS-RIS were resuspended in 1.0 ml of (i) TBE plus ascorbic acid, (ii) dopamine (10⁻⁹ to 10⁻³ M), (iii) fenoldopam (100 nM) and/or quinpirole (100 nM), or (iv) dopamine (10 μM) plus SCH23390 (1 μM) and/or clozapine (1 μM) and then incubated for 10 min at 37°C. After the incubation, the samples were assayed for α1 and α3 specific activity as described below. The drug concentrations were determined from preliminary experiments and produced maximal effects. Positive control experiments were conducted to examine whether the effects of dopamine were direct or mediated by second messengers. In these experiments, ROS-RIS were homogenized prior to 10-min incubation with 10 μM dopamine.

ROS-RIS Na,K-ATPase Assays. Total Na,K-ATPase activity and isozyme specific activities were determined after the incubations. Na,K-ATPase activity was measured spectrophoto-

tometrically by determining P_i liberated from ATP (11, 38). Isolated ROS-RIS were resuspended in 500 μ l of ice-cold TBE and homogenized in a 2-ml Dounce-type glass homogenizer using 30 strokes. The assay mixture contained 125 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM Tris-ATP, 30 mM Tris, 1 mM EGTA, 50 μ l of ROS-RIS homogenate, and either the absence or presence of ouabain (initially 10^{-10} to 10^{-3} M) in a final volume of 250 μ l. The enzyme was activated by ATP and proceeded for 45 min at $37.0 \pm 0.1^\circ C$. Hydrolysis was linear for up to 75 min with 20–30% of the total substrate utilized. Assays were performed in triplicate. Total Na,K-ATPase specific activity was the difference between the activities recorded in the absence (total ATPase) and presence (ouabain insensitive-ATPase) of 10^{-3} M ouabain. The method for determination of the specific activity of the $\alpha 1$ and $\alpha 3$ isozymes is described below. Values are expressed as μ mol of P_i hydrolyzed per mg of protein per hr ($\pm$ SEM). Protein content was determined by the method of Bradford (38) using BSA as a standard.

Photoreceptors essentially contain only the $\alpha 1$ -low and $\alpha 3$ -high ouabain isozymes of Na,K-ATPase (4–6, 28). Therefore, a computer program based on a mathematical model designed to distinguish and determine the IC_{50} values of two differential noncompeting ligand binding sites was used to fit the data and to obtain the IC_{50} values for the ROS-RIS $\alpha 1$ and $\alpha 3$ isozymes from a full ouabain inhibition curve (UltraFit, Biosoft, Cambridge, U.K.). The determination of distinct IC_{50} values of ouabain for ROS-RIS $\alpha 1$ and $\alpha 3$ allowed us (i) to accurately and independently assess the specific activity of $\alpha 1$ and $\alpha 3$, (ii) to eliminate the need for generating full ouabain inhibition curves for each experiment such that, subsequent experiments used tubes containing no ouabain (total ATPase), 10^{-7} M ouabain ($\alpha 3$), 10^{-5} M ouabain ($\alpha 1$), and 10^{-3} M ouabain (ouabain insensitive-ATPase and thus total Na,K-ATPase), and (iii) to assess the effects of dopaminergic agonists and antagonists on the $\alpha 1$ and $\alpha 3$ ROS-RIS isozymes.

Statistical Analysis. All values are presented as the mean $\pm$ SEM. All data analysis were performed on untransformed

data and analyzed using the appropriate analysis of variance (ANOVA) and post hoc multiple comparisons were made on the original data using Tukey's honestly significant difference test according to procedures provided by the SAS statistical package (SAS Institute, Cary, NC). Differences from controls were regarded as significant if $P < 0.05$.

RESULTS

Several techniques have been employed to assess Na,K-ATPase activity in intact cells: ouabain-sensitive oxygen consumption (15, 39), ouabain-sensitive $^{86}Rb^+$ uptake (15, 39), and [3H]ouabain binding (40, 41). To assess the physiological and pharmacological effects of dopamine on ouabain-sensitive QO_{PR} , we measured QO_{PR} during dark, half-saturating, and rod-saturating light adaptation. Prior results with this preparation demonstrated its sensitivity and reliability in determining the effects of several diverse function-specific chemical agents on QO_{PR} (26). Fig. 1A reveals that dopamine produced concentration-dependent decreases in dark-adapted QO_{PR} that were similar to those produced by half-saturating light adaptation (–13%), rod-saturating light adaptation (–37%), 100 nM ouabain (–11%), or 1 mM ouabain (–31%). That is, 100 nM and 10 μ M dopamine decreased QO_{PR} 14% and 32%, respectively. At 100 nM dopamine plus 100 nM ouabain, their approximate IC_{50} values for inhibition of the $\alpha 3$ isozyme of Na,K-ATPase (see data below), the inhibitory effects of these drugs were additive (–26%). This suggests that these agents inhibit Na,K-ATPase by different mechanisms. Finally, the inhibition of dark-adapted QO_{PR} by 10 μ M dopamine was completely blocked by preincubation with 1 μ M clozapine but not by 1 μ M SCH23390, suggesting that D_2/D_4 -like receptors mediate the decrease in QO_{PR} .

Clozapine, SCH23390, or D-amphetamine (50 μ M) alone had no effect on the dark- or light-adapted QO_{PR} (96–100% of control; $n = 3$ –4 preparations per drug/light condition). These latter findings suggest that little, if any, endogenous

FIG. 1. Dopamine-, light-, and ouabain-induced decreases in rat photoreceptor oxygen consumption (QO_{PR}) during (A) dark adaptation and (B) two different levels of rod light adaptation; half-saturating and rod-saturating. The photoreceptors were pharmacologically isolated from the inner retina using a combination of APB/Kyn. Dopamine produced concentration-dependent decreases in QO_{PR} that were equivalent to those produced by different levels of light and concentrations of ouabain. Each retina was used for only one experiment. Values represent the mean $\pm$ SEM for 5–12 retinas per measure. In A, groups with a single asterisk differed from the dark-adapted controls at $P < 0.05$. In B, groups with a single asterisk differed from the half-saturated controls at $P < 0.05$, while groups with a double asterisk differed from the rod-saturated controls at $P < 0.05$.

dopamine was present or released in the pharmacologically isolated photoreceptor preparation and that APB/Kyn produced a relatively complete pharmacological isolation of the photoreceptors. This is consistent with data showing that both competitive and noncompetitive *N*-methyl-D-aspartate antagonists inhibit the amphetamine-induced release of dopamine (42–44). In contrast, 50 μ M D-amphetamine added to the dark-adapted whole retinal preparation (absence of APB/Kyn; ref. 26) decreased whole retinal oxygen consumption by $\approx 40\%$ ($n = 5$), which suggests that the released dopamine is capable of decreasing the QO_{PR} .

Fig. 1*B* shows that physiologically relevant concentrations of dopamine (100 nM) or ouabain (100 nM) further decreased QO_{PR} in photoreceptors exposed to half-saturating illumination: -18% and -13% , respectively. Higher concentrations of dopamine (10 μ M) or ouabain (1 mM) were capable of further decreasing the fully light-adapted QO_{PR} by 10% or 13%, respectively. To explore the possibility that dopamine produced its inhibitory effect by reducing the conductance of the cGMP-gated channel and thereby decreasing the intracellular Na^+ concentration, the effect of dopamine in the presence of 10 μ M monensin, a Na^+ ionophore (29), was examined. Monensin (10 μ M) was selected because it enabled the dark-adapted rate of QO_{PR} to be maintained during rod-saturating light-adaptation (Fig. 1*B*). The decrease in QO_{PR} after the addition of 10 μ M dopamine to the monensin-treated fully light-adapted retina was similar to that produced by 10 μ M dopamine alone (Fig. 1*B*) or by 10 μ M dopamine during dark adaptation (Fig. 1*A*). This result shows that the effect of dopamine was not secondary to altered Na^+ entry and indicates that the effect was independent of the intracellular Na^+ concentration. The percentage decrease in QO_{PR} produced by dopamine in the presence of monensin (-43%) was similar to that produced by 10 μ M dopamine during dark adaptation.

The identification, yield, purity, and integrity of the isolated ROS-RIS were characterized by quantitative morphometry, Janus green B dye staining, rhodopsin concentration, and didansyl-cystine dye studies. ROS-RIS were identified easily by their long thin outer segments and mitochondria-rich ellipsoid inner segments (Fig. 2). The number of ROS-RIS obtained from six retinas (one preparation) was $20.9 \pm 0.3 \times 10^6$. The percentage of ROS-RIS per preparation was $92 \pm 2\%$. Remaining cells were either photoreceptor outer segments or inner segments only (6%) or nonphotoreceptor cells. The rhodopsin concentration was 1.8 ± 0.2 nM, which supports the ROS-RIS yield data. Didansyl-cystine dye exclusion studies revealed that the ROS remained almost completely intact

FIG. 2. A representative differential interference contrast micrograph of mechanically dissociated adult rat ROS-RIS. A 20- μ l aliquot of freshly isolated ROS-RIS was diluted 1:1 with mixed aldehydes (photographed using a $\times 40$ objective). Note the homogeneity of the ROS-RIS cell population. ($\times 1250$)

throughout the entire incubation procedure. At 0–45 min, the number of intact cells per preparation was $96 \pm 4\%$, whereas at 60 min it was $90 \pm 6\%$. The mitochondria in the RIS were fully intact and appeared similar to those from perfused light-adapted rat retinas observed at high magnification under the electron microscope (34).

The ROS-RIS total Na,K-ATPase specific activity was 34.1 ± 1.2 μ mol of P_i per mg of protein per hr (Fig. 3). These values are ≈ 3 -fold higher than whole retinal values obtained from similarly age-matched female Long-Evans rats (11). This appears to reflect the 2- to 3-fold greater ouabain-sensitive oxygen consumption in the dark-adapted outer retina relative to the whole or inner retina, respectively (26). Using a computer program based on a mathematical model for differential noncompeting ligand binding sites, the ouabain concentration-response curve for the inhibition of ROS-RIS Na,K-ATPase, like that in whole retina (28) and brain (41), could be resolved into two apparently homogenous components with distinctly different IC_{50} values of ouabain (Fig. 3). The IC_{50} values for the $\alpha 3$ -high and $\alpha 1$ -low ouabain affinity isozymes were 9.5×10^{-8} and 8.7×10^{-6} M, respectively. The photoreceptor ouabain-insensitive ATPase specific activity was 25.5 ± 1.2 μ mol of P_i per mg of protein per hr and was unaffected by any experimental treatment.

To investigate whether dopamine inhibited the $\alpha 1$ and/or $\alpha 3$ isozyme of Na,K-ATPase and to determine the time course of this inhibition, the activity of the $\alpha 1$ and $\alpha 3$ isozymes was examined as a function of incubation time in the absence or presence of 10 μ M dopamine. Fig. 4*A* presents the results as a percentage of the no dopamine control values for the $\alpha 1$ and $\alpha 3$ isozymes at time zero: 10.61 ± 0.61 and 26.08 ± 0.85 μ mol of P_i per mg of protein per hr, respectively ($n = 29$). There were no differences in $\alpha 1$ or $\alpha 3$ activity at any time in preparations incubated without dopamine: $97 \pm 4\%$ and $98 \pm 3\%$ of zero time control, respectively ($n = 5$ –18 preparations per time point). The time course data reveal that (i) 10 μ M dopamine selectively inhibited the $\alpha 3$ isozyme, (ii) the onset of the inhibition was slow, and (iii) the maximal inhibition occurred at 10 min ($-41 \pm 5\%$) and persisted for at least 20 min. For all subsequent experiments, the effects of dopamine were examined using a 10-min incubation period. Fig. 4*B* illustrates the concentration-dependent inhibition of the $\alpha 3$ isozyme by dopamine. The first significant inhibition of $\alpha 3$ occurred at 5 nM dopamine while maximal inhibition of $47 \pm 6\%$ was observed at 100 μ M dopamine. A similar 5 log unit range of

FIG. 3. Concentration-dependent inhibitory effects of ouabain on isolated ROS-RIS Na,K-ATPase specific activity. The data were fit by a computer program based on a mathematical model for differential noncompeting ligand binding sites. The ouabain concentration-response curve was resolved into two apparently homogenous components with distinctly different IC_{50} values of ouabain. The IC_{50} values for the $\alpha 3$ -high and $\alpha 1$ -low ouabain affinity Na,K-ATPase isozymes were determined to be 9.5×10^{-8} and 8.7×10^{-6} M, respectively. Values represent the mean $\pm$ SEM for triplicate samples of 7–15 preparations per ouabain concentration.

FIG. 4. Time-dependent (A) and concentration-dependent (B) effects of dopamine on the relative activity of the $\alpha 1$ -low and $\alpha 3$ -high ouabain affinity ROS-RIS Na,K-ATPase isozymes. (A) The ROS-RIS were incubated in dopamine for various times and then assayed for Na,K-ATPase activity. Only the $\alpha 3$ isozyme was inhibited by dopamine. The data were fitted by interpolation. (B) The ROS-RIS were incubated in various concentrations of dopamine for 10 min and then assayed for Na,K-ATPase activity. The data were fitted to a power function using the method of least squares. The IC_{50} value of dopamine was 382 ± 23 nM. Values presented represent the mean $\pm$ SEM of triplicate samples for 5–18 preparations per time point or dopamine concentration and are expressed as the percent of control (no dopamine) for the ROS-RIS $\alpha 1$ or $\alpha 3$ specific activity. There were no differences in $\alpha 1$ or $\alpha 3$ activity at any time in preparations incubated without dopamine. Mean values marked by an asterisk differed from the no dopamine controls at $P < 0.05$.

dopamine-induced inhibition of total Na,K-ATPase has been reported (12, 13). Although the exact mechanism responsible for this large range is unknown, it corresponds to a Hill coefficient of 0.27 ± 0.04 , indicating negative cooperativity. This negative cooperativity is physiologically relevant since it will result in an increased inhibition of the $\alpha 3$ isozyme at very low concentrations of dopamine over that expected if the Hill coefficient was 1.0 (45). The IC_{50} value of dopamine was 382 ± 23 nM, a value very similar to that measured next to the photoreceptors of light-adapted *Xenopus laevis* retinas (20). Furthermore, and importantly, when the ROS-RIS were homogenized prior to incubation with $10 \mu M$ dopamine for 10 min, there was no inhibition of the $\alpha 3$ isozyme: $25.37 \pm 1.15 \mu mol$ of P_i per mg of protein per hr ($n = 5$).

To determine whether the selective inhibition of the $\alpha 3$ isozyme of Na,K-ATPase was mediated by D_1 and/or D_2/D_4 -like dopamine receptor subtypes, a series of pharmacological experiments was performed using selective dopamine agonists and antagonists (Fig. 5). The data are plotted relative to the no dopamine control values for $\alpha 1$ and $\alpha 3$ and for reference the selective inhibitory effect of $10 \mu M$ dopamine on the $\alpha 3$ isozyme of Na,K-ATPase is illustrated (-39%). Fenoldopam (D_1 agonist) had no significant effect on the $\alpha 1$ or $\alpha 3$ isozyme

FIG. 5. Effects of dopamine, D_1 or D_2 agonists, and dopamine plus D_1 or D_2/D_4 antagonists on the ROS-RIS Na,K-ATPase $\alpha 1$ and $\alpha 3$ isozyme specific activities. The D_2/D_4 -like-mediated inhibition of Na,K-ATPase was exclusively selective for the $\alpha 3$ isozyme. The ROS-RISs were incubated for 10 min in $10 \mu M$ dopamine, a D_1 agonist (100 nM fenoldopam), a D_2 agonist (100 nM quinpirole), or $10 \mu M$ dopamine plus either a D_1 ($1 \mu M$ SCH23390) or D_2/D_4 -like ($1 \mu M$ clozapine) antagonist. The samples were then assayed for Na,K-ATPase activity. Values represent the mean $\pm$ SEM for triplicate samples from 5–18 preparations per pharmacological treatment and are expressed as the percent of control (no dopamine) for the ROS-RIS $\alpha 1$ or $\alpha 3$ specific activity. Mean values marked by an asterisk differed from the no dopamine $\alpha 3$ controls at $P < 0.05$.

activity. In contrast, quinpirole (D_2 agonist) significantly decreased the $\alpha 3$ (-30%), but not the $\alpha 1$, isozyme activity. Consistent with these results, clozapine, used as a D_2/D_4 antagonist, completely blocked the inhibitory effect of $10 \mu M$ dopamine on the $\alpha 3$ isozyme without affecting the $\alpha 1$ isozyme. Moreover, SCH23390 (D_1 antagonist) was unable to reverse the selective inhibitory effect of $10 \mu M$ dopamine. The combination of fenoldopam plus quinpirole ($71 \pm 5\%$ of control; $n = 3$) or dopamine plus SCH23390 and clozapine ($99 \pm 3\%$ of control; $n = 3$) had the same effects on $\alpha 3$ isozyme activity as quinpirole alone or dopamine plus clozapine. SCH23390 or clozapine in the absence of dopamine had no effect on the $\alpha 3$ isozyme activity ($98 \pm 4\%$ of control values; $n = 3$).

DISCUSSION

The present results show that the inhibitory effects of physiologically relevant concentrations of dopamine on ouabain-sensitive QO_{PR} and on the $\alpha 3$ isozyme of Na,K-ATPase in isolated ROS-RIS are mediated exclusively by D_2/D_4 -like receptors. In addition, the concentration-dependent effects of dopamine on QO_{PR} are similar to those produced by physiologically relevant levels of light and concentrations of ouabain. These results are consistent with the demonstrated presence of the $\alpha 3$ isozyme of Na,K-ATPase (4–6) and D_4 receptors on rod photoreceptor inner segments (22, 23). The biochemical mechanism underlying the inhibition of the $\alpha 3$ isozyme of Na,K-ATPase by dopamine is unknown. The data obtained from the dopamine plus rod-saturating light and dopamine plus monensin experiments suggest that dopamine does not inhibit the enzyme by decreasing the intracellular Na^+ concentration. In addition, we interpret the findings obtained from the dopamine plus ouabain experiments to suggest that dopamine and ouabain inhibit the enzyme by different cellular mechanisms. Furthermore, the results suggest that a second messenger system was involved since dopamine had no effect in homogenized ROS-RIS preparations, the onset of the inhibition was slow, and the inhibition persisted for at least 20 min. One likely candidate is cAMP. This suggestion is sup-

ported by pharmacological and *in situ* hybridization studies demonstrating that mouse photoreceptors possess D₄ receptors linked to the inhibition of adenylyl cyclase (22) as well as the well-known coupling of some D₂ receptors to the inhibition of adenylyl cyclase (46). Our preliminary pharmacological data, however, suggest a distinctly different signaling pathway (47).

The selective inhibition of photoreceptor Na,K-ATPase by activation of the D₂/D₄-like receptors is distinctly different from the dopaminergic-mediated inhibition of Na,K-ATPase in isolated neostriatal neurons and renal proximal tubules. In these preparations, the inhibition of total Na,K-ATPase activity was produced by the simultaneous activation of both D₁ and D₂ receptors, which were assumed to be on the same cell (12, 48), or by activation of the D₁ receptor (14). The reason for these differences is currently unknown.

The physiological relevance of our findings to photoreceptor function is presently unknown. One possibility is that the light-mediated release of dopamine (16, 17, 40) partially contributes to the inhibition of the photoreceptor Na, K-pump (this paper) and, thus, like ouabain (8), effectively increases the relative extracellular K⁺ around the rod outer and inner segments by preventing its reaccumulation during maintained illumination. Furthermore, our results show that dopamine, like light and ouabain, can produce graded levels of inhibition of ouabain-sensitive QO_{PR} and, thus, by extension, possibly graded levels of extracellular K⁺. Increases in the extracellular K⁺ have been demonstrated to increase the amplitude of the hyperpolarization-activated inward current (*I_h*) of the rod (49). The dopamine-mediated K⁺-induced increase in current would presumably add to the light-induced inward current, further depolarizing the membrane and, thus, increase the transient response of rods to bright flashes of light. In support of this hypothesis are the results of two studies. (i) In isolated mammalian photoreceptors, dopamine reversibly decreased the half-activation voltage of *I_h* (50). (ii) It was shown in isolated *Xenopus laevis* retinal eyecups that activation of D₂, but not D₁, receptors increased the rod flicker fusion frequency, whereas D₂, but not D₁, antagonists in the presence of dopamine markedly reduced the rod flicker fusion frequency (51). Alternatively, it has been suggested that an increase in extracellular K⁺ may cause an increase in the number of available *I_h* channels and thereby increase the inward current (49).

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