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Calcium alters the sensitivity of intact horizontal cells to dopamine antagonists

(dopamine receptors/adenylate cyclase/retina)

ROBERT VAN BUSKIRK AND JOHN E. DOWLING

Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

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ABSTRACT **Horizontal cells of the carp retina possess dopamine receptors linked to adenylate cyclase. Isolated, intact horizontal cells respond to micromolar concentrations of dopamine, whereas nanomolar concentrations of haloperidol, (+)-butaclamol, and flupenthixol block the dopamine response. Preincubation in Ringer's solution containing increased levels of Ca^{2+} (5–110 mM) decreases the sensitivity of the cells to these antagonists by 1,000–10,000 times. Dopamine sensitivity of the cells is not affected by Ca^{2+} levels in the preincubation medium. Preincubation of the cells in Ringer's solution containing 500 μM L-glutamate, an agent that increases intracellular Ca^{2+} levels in intact horizontal cells, also decreases the sensitivity of the cells to haloperidol. These data suggest that antagonist sensitivity of intact horizontal cells may be regulated by intracellular Ca^{2+}.**

Horizontal cells are second-order retinal neurons that, in teleost fish, are known to receive two synaptic inputs. One input is from the photoreceptor cells, which may use glutamate or aspartate as their neurotransmitter (1–3). The second input is from the interplexiform cells, a centrifugal retinal neuron that uses dopamine as its neurotransmitter (4, 5). The teleost retina possesses a highly specific dopamine-sensitive adenylate cyclase that has pharmacological properties virtually identical to the dopamine-sensitive adenylate cyclases described elsewhere in the brain (6, 7).

To investigate more directly the nature of the dopamine receptors in the retina and the role of cAMP in retinal function, we have developed methods for isolating populations of horizontal cells from the carp (8). Retinas are mechanically dissociated after enzymatic treatment in Ringer's solution containing trypsin. The resulting cell suspension is applied to a sedimentation column containing 0.8–4% Ficoll. The horizontal cells sediment to the lower third of the gradient where they are collected and concentrated. When fractions highly enriched in horizontal cells are incubated with dopamine and isobutylmethylxanthine (iBuMeXan), a phosphodiesterase inhibitor, significant amounts of cAMP accumulate. cAMP is undetectable in unstimulated fractions of horizontal cells; therefore, we estimate that dopamine can increase cAMP levels by at least 400–500 times in horizontal cells.

Dopamine at 2–10 μM produces a half-maximal response in the isolated cells which is similar to that observed in homogenates or in the intact retina. The response of the isolated cells to dopamine also can be readily blocked by various dopamine antagonists, including haloperidol, (+)-butaclamol, and fluphenazine. Surprisingly, however, these antagonists are ≈1,000–10,000 times more effective in blocking the effects of dopamine on the intact, isolated cells (8) than they are in homogenates of whole retina (6). Antagonist concentrations of 1–40 nM inhibit by 50% the effects of 200 μM dopamine on the isolated cells.

We observed further that disruption of the isolated horizontal cells altered their sensitivity to antagonists, so that homogenates of isolated horizontal cells were inhibited only by micromolar concentrations of antagonists as are homogenates of the whole retina (6). These results indicated that the enhanced sensitivity of the isolated horizontal cells to antagonists may relate to the receptors being situated in intact membranes across which there are significant potentials or ion gradients, or both. In this paper, we describe experiments that suggest that the sensitivity of horizontal cells to antagonists is altered by changes in intracellular Ca^{2+}.

MATERIALS AND METHODS

Neurons were dissociated and separated in the following manner. Carp (*Cyprinus carpio;* 10–20 cm in length) were maintained at room temperature in an aerated tank. Prior to an experiment, the carp were dark-adapted for 15–20 min. The eyes were enucleated, and the corneas and lenses were removed. Eight retinas were gently removed from the globe under dim white light and placed in 20 ml of a solution containing 0.08% trypsin (Sigma) in a phosphate-containing Ringer's solution (80 mM NaCl/22.7 mM $NaHCO_3$/0.8 mM Na_2HPO_4/3.5 mM KCl/0.1 mM KH_2PO_4/2.4 mM $MgSO_4$/10 mM dextrose/5 mM EGTA, pH 7.4). Retinas were bubbled in 95% O_2/5% CO_2 for 1.5 hr at room temperature and transferred to 10 ml of Hepes-containing Ringer's solution (80 mM NaCl/22.7 mM $NaHCO_3$/3.4 mM KCl/2.4 mM $MgSO_4$/2.3 mM $CaCl_2$/10 mM dextrose/10 mM Hepes, pH 7.4). The retinas were washed twice in this buffer and dissociated in 40 ml of the Hepes at 9°C by gently pipetting the retinal pieces up and down with a Pasteur pipette. Clumps of cells were allowed to settle for 20 min.

Aliquots (24 ml) were drawn from the top of the cell suspension and loaded onto a 0.8–4% Ficoll gradient column (diameter, 14 cm; depth, 9 cm; volume, 750 ml). The remaining 16 ml of Ringer's solution containing clumps of cells was discarded. Cells were allowed to sediment at 9°C. After 4 hr, retinal cells were distributed throughout the gradient. Fractions enriched with horizontal cells were collected as described (8) and divided into eight equal aliquots. Each aliquot contained ≈3,000 cells, and of these ≈95% were horizontal cells.

The cells in each aliquot were centrifuged into a pellet. The Ficoll from each centrifuge tube was decanted and replaced with 2.0 ml of Hepes/Ringer's solution containing various concentrations of $CaCl_2$ ranging from 0 to 200 mM or 500 μM L-glutamate. The cell pellets were routinely preincubated at 4°C for 30 min in Ringer's solution containing various Ca^{2+} concentrations or L-glutamate. At 4°C, the pellet and the cells within

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Abbreviation: iBuMeXan, isobutylmethylxanthine.

the pellet maintained their integrity for the duration of the preincubation period. After 30 min, the incubate was decanted and the pellets were either left intact or gently homogenized with a hand homogenizer. The intact pellets or homogenates were tested for their ability to accumulate cAMP in the presence of dopamine and dopamine antagonists by incubating for 3 min at 30°C in antagonist alone, followed by a 5-min incubation at 30°C in 200 μM dopamine and antagonist. Two to eight separate experiments were performed with each antagonist tested. All incubation Ringer's solutions contained 2.3 mM $CaCl_2$, except for those experiments in which the cell pellet was preincubated in solution lacking $CaCl_2$ or in solution lacking $CaCl_2$ but containing 5 mM EGTA. In these cases the dopamine/antagonist Ringer's solutions also lacked $CaCl_2$ or lacked $CaCl_2$ but contained 5 mM EGTA. iBuMeXan (2 mM) was also present during agonist or antagonist incubations, or both, to inhibit phosphodiesterase activity. Incubation volumes and conditions and the method of radioimmunoassay were similar to those described previously (8).

RESULTS

Fig. 1 shows that when pellets enriched in horizontal cells were incubated for 3 min in control Ringer's solution containing 200 μM dopamine, 2 mM iBuMeXan, and various concentrations of haloperidol, ≈2 nM of the antagonist was required to reduce the cAMP accumulation by half—i.e., the IC_{50} value for haloperidol was 2 nM (Fig. 1, open circles). When similar pellets containing horizontal cells were gently disrupted in a glass homogenizer, and the resulting homogenate was tested for its ability to accumulate cAMP in the presence of dopamine and haloperidol, the IC_{50} value for the antagonist was ≈20 μM (≈20,000 nM) (Fig. 1, closed circles).

If, prior to the incubation in Ringer's solution containing dopamine, dopamine antagonists, and iBuMeXan, pellets containing horizontal cells were preincubated for 30 min at 4°C in Ringer's solution containing various concentrations of $CaCl_2$, the sensitivity of the cells to the antagonists was found to alter substantially. For example, Fig. 1 shows that incubation of isolated horizontal cells in 11 mM $CaCl_2$ for 30 min altered the sensitivity of the cells to haloperidol by ≈10,000 times (open squares). That is, the IC_{50} value for haloperidol was ≈20 μM, whereas the same antagonist had an IC_{50} value of 2 nM in cells preincubated for 30 min in control Ringer's solution containing 2.3 mM Ca^{2+}. After preincubation in 11 mM $CaCl_2$, the intact isolated cells behaved, with regard to sensitivity to haloperidol, similarly to homogenates of horizontal cells (Fig. 1) or homogenates of the whole retina (6).

The sensitivity of the isolated horizontal cells to haloperidol altered dramatically over a relatively narrow range of Ca^{2+} levels in the preincubation Ringer's solution. Fig. 2 shows that increasing Ca^{2+} from the normal 2.3 mM to 5 mM altered the IC_{50} for haloperidol from ≈2 nM to ≈10 μM. Preincubation with 11 mM Ca^{2+} increased the IC_{50} somewhat further, to 20 μM, but further increases of Ca^{2+} in the preincubation medium did not alter the sensitivity of the cells to haloperidol. On the other hand, reducing the concentration of Ca^{2+} in the preincubation medium to <2.3 mM (i.e., 0 mM) did not further sensitize the cells to haloperidol. It appears, therefore, that there is a maximum and minimum sensitivity of the cells to haloperidol that may be altered by preincubation with Ca^{2+}.

The sensitivity of intact cells to (+)-butaclamol and flupenthixol was also altered by changing Ca^{2+} levels in the prein-

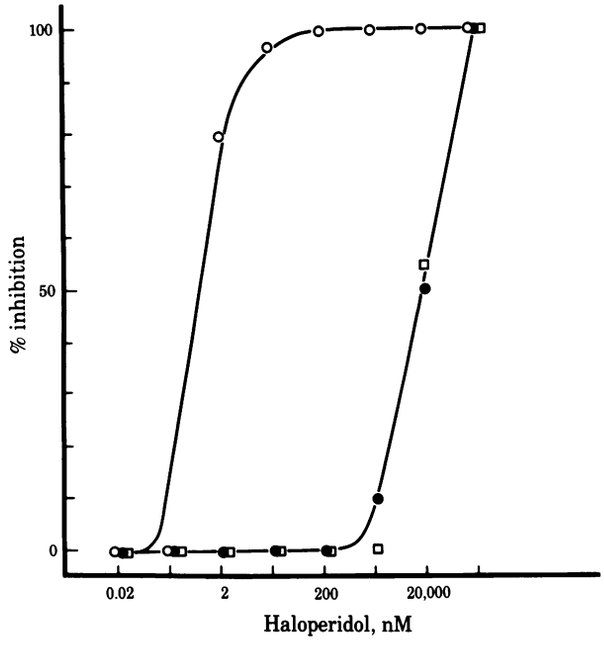

FIG. 1. Effects of homogenization and Ca^{2+} on the inhibition by haloperidol of dopamine-stimulated cAMP accumulation in pellets of horizontal cells. The cell pellets were preincubated for 30 min at 4°C in either 2.3 mM $CaCl_2$ (○, ●) or 11 mM $CaCl_2$ (□). Prior to incubation with dopamine and haloperidol, certain pellets were hand-homogenized (●), whereas others were left intact (○, □). Intact pellets or homogenized pellets were incubated for 3 min in Ringer's solution containing 2 mM iBuMeXan and the indicated concentrations of haloperidol, followed by incubation for 5 min in the same Ringer's solution containing 200 μM dopamine. Data are from single representative experiments involving duplicate assays. Preincubation in 11 mM Ca^{2+} or homogenization decreased the sensitivity of the cells 10,000-fold when compared to intact cells incubated in control Ringer's solution. Between experiments performed under similar conditions, the variability in IC_{50} values never varied more than 10-fold.

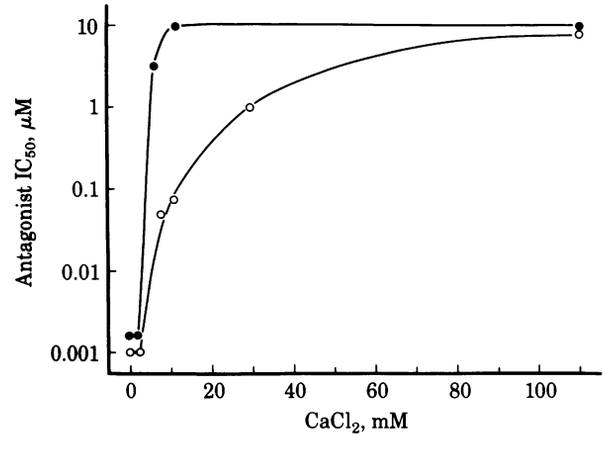

FIG. 2. Effects of various concentrations of Ca^{2+} on the inhibition by haloperidol (●) and (+)-butaclamol (○) of dopamine-stimulated cAMP accumulation in pellets of intact horizontal cells. Cell pellets were preincubated for 30 min at 4°C in the indicated concentrations of Ca^{2+}. Pellets were incubated for 3 min in Ringer's solution containing 200, 20, 2, 0.2, 0.02, 0.002, 0.0002, or 0.00002 μM haloperidol or (+)-butaclamol, followed by incubation for 5 min in Ringer's solution containing 2 mM iBuMeXan, 200 μM dopamine, and antagonist. Each point represents IC_{50} values from single experiments, each using the eight concentrations of haloperidol or (+)-butaclamol. The sensitivity of the intact horizontal cells both to haloperidol and (+)-butaclamol decreased with increasing Ca^{2+} concentrations in the preincubation Ringer's solution. However, the sensitivity of the cells to haloperidol altered over a narrower range of Ca^{2+} concentrations than was the case for (+)-butaclamol.

cubation Ringer's solution. However, the sensitivity of the cells to these antagonists did not alter over as narrow a range of Ca^{2+} concentrations as was the case with haloperidol. For example, Fig. 2 shows also the effects of various Ca^{2+} levels in the preincubation medium on the sensitivity of horizontal cells to (+)-butaclamol. Increasing Ca^{2+} levels to 11 mM decreased sensitivity of the cells to (+)-butaclamol by 100 times; increasing Ca^{2+} levels to 30 mM decreased the sensitivity by 1,000-fold. However, preincubation in 110 mM Ca^{2+} was required to reduce the sensitivity to the lowest level exhibited by the cells to (+)-butaclamol (i.e., to an IC_{50} of 10 μM).

In one set of experiments, the preincubation time in Ca^{2+} was varied between 30 sec and 60 min. The concentration of Ca^{2+} in the preincubation Ringer's solution was kept constant at 30 mM, and the effect of 0.1 μM flupenthixol on the accumulation of cAMP induced by 200 μM dopamine was determined. Initially and after 30 sec of preincubation, no measurable amount of cAMP accumulated in the cells (i.e., 0.1 μM flupenthixol completely inhibited the response). After 1, 2, 5, and 10 min of preincubation in 30 mM Ca^{2+}, the amount of cAMP that accumulated increased exponentially in a time-dependent fashion, indicating that the cells were becoming progressively less sensitive to 0.01 μM flupenthixol. With longer preincubation periods, there was only a relatively small increase in cAMP levels. In other control experiments, cells were examined by light microscopy after preincubation for 30 min at 4°C in 30 mM and 110 mM $CaCl_2$. After this period of preincubation in these concentrations of Ca^{2+}, the horizontal cells appeared intact and virtually indistinguishable from cells preincubated for 30 min at 4°C in 2.3 mM $CaCl_2$.

Homogenates of horizontal cells also showed altered sensitivity to antagonists that depended on Ca^{2+} levels in the preincubation and incubation medium. However, horizontal cell homogenates did not show the same extent of sensitivity changes as did intact cells. For example, homogenates of horizontal cells incubated in control Ringer's solution required 10 μM haloperidol and 1 μM (+)-butaclamol to inhibit by 50% the cAMP accumulation induced by 200 μM dopamine. When cells before homogenization were preincubated in solution lacking $CaCl_2$ or in solution lacking $CaCl_2$ and containing 5 mM EGTA and, after homogenization, were incubated with dopamine and antagonists in Ringer's solution lacking $CaCl_2$ or lacking $CaCl_2$ and containing 5 mM EGTA, some sensitization of the homogenates to antagonists was seen. For example, haloperidol sensitivity was altered slightly from an IC_{50} of 20 μM to $\approx$8 μM, whereas (+)-butaclamol sensitivity was changed from 1 μM to $\approx$0.1 μM. However, these sensitivities were far from those exhibited by intact cells to these antagonists after preincubation and incubation in Ringer's solution containing 0–2.3 mM Ca^{2+} [i.e., IC_{50} values of 2 nM for haloperidol and 1 nM for (+)-butaclamol]. Increasing Ca^{2+} levels in the preincubation and incubation Ringer's solution above those in control Ringer's solution decreased the sensitivity of the homogenates to (+)-butaclamol but not to haloperidol. In both cases, there appeared to be a minimum sensitivity of the homogenates to antagonist that was close to that of homogenates of whole retinas and similar to the lowest sensitivity observed in intact cells preincubated in high-Ca^{2+} Ringer's solution.

The above data suggest that the antagonist receptor sites on horizontal cells may exist in two states: a high-affinity state at relatively low Ca^{2+} levels and a low-affinity state at relatively high Ca^{2+} levels. Evidence for antagonist receptor sites alternating between two affinity states was obtained in experiments with the antagonist flupenthixol. In control Ringer's solution, the IC_{50} for flupenthixol was $\approx$ 1 nM, whereas in cells incubated for 30 min in 110 mM $CaCl_2$, the IC_{50} was almost 10 μM. Fig. 3 shows that the inhibition curves for these two conditions were monophasic and essentially parallel. However, after preincubation of horizontal cells for 30 min with 5.8 mM Ca^{2+} or 30 mM Ca^{2+}, the inhibition curves were distinctly biphasic. The lower limbs of these curves had a half-maximum in the nanomolar range of antagonist, whereas the upper limbs of the curves were half-maximal in the micromolar range. The difference between the two curves lay in the relative contribution of the two components to the inhibition curves; much more low-affinity component was present in cells incubated with 30 mM Ca^{2+} than in cells incubated with 5.8 mM Ca^{2+}.

Incubating pellets of intact horizontal cells for 30 min at 4°C in various concentrations of Ca^{2+} did not affect the sensitivity of the cells to dopamine. Fig. 4 *Inset* shows that the dose–response curves for cells incubated in 0 and 110 mM Ca^{2+} were virtually identical. On the other hand, the amount of cAMP that accumulated in response to a fixed concentration of dopamine altered significantly in response to 30-min preincubations in various concentrations of Ca^{2+}. Fig. 4 shows that cells preincubated for 30 min in 10 mM Ca^{2+} accumulated approximately twice as much cAMP as cells preincubated without Ca^{2+} for 30 min. Increasing Ca^{2+} concentrations in the preincubation medium above 30 mM did not lead to further increases in cAMP accumulation. Basal levels of cAMP, on the other hand, did not appear to be affected by preincubation of cells in various concentrations of Ca^{2+}. Indeed, basal levels were virtually undetectable in all experiments involving isolated and intact horizontal cells.

As noted above, it has been postulated that glutamate or as-

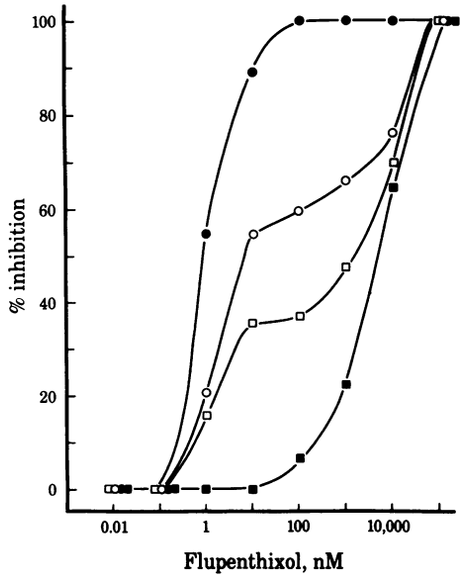

FIG. 3. Effects of Ca^{2+} on the inhibition by *cis*-flupenthixol of dopamine-stimulated cAMP accumulation in intact cells. Cells were preincubated in 2.3 (●), 5.8 (○), 30 (□), or 110 (■) mM $CaCl_2$ for 30 min at 4°C. The cells were incubated for 3 min in Ringer's solution containing 2 mM iBuMeXan and the indicated concentrations of *cis*-flupenthixol and finally for 5 min in Ringer's solution containing 2 mM iBuMeXan, 200 μM dopamine, and antagonist. Data are from single representative experiments involving duplicate assays. The sensitivity of intact horizontal cells to flupenthixol decreased with increasing Ca^{2+} concentrations. The inhibition curves for cells preincubated in 15 and 30 mM Ca^{2+} were biphasic, with the IC_{50} of the lower portion of each curve $\approx$1 nM and the IC_{50} of the upper portion of each curve $\approx$10 μM. In all experiments utilizing *cis*-flupenthixol, biphasic inhibition curves were observed when Ca^{2+} concentrations in the preincubation Ringer's solution were between 5 and 60 mM. In these experiments, <10-fold variability in the IC_{50} values for the two limbs was found.

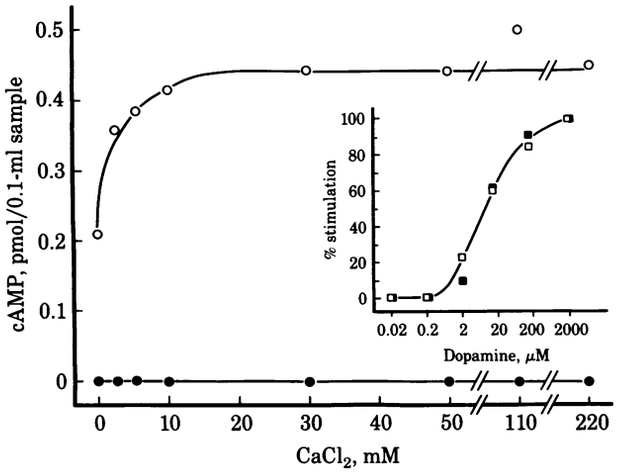

FIG. 4. Effects of Ca^{2+} on cAMP accumulation induced by 200 μM dopamine in isolated carp horizontal cells. Cells were separated, collected, and pelleted. Horizontal cell pellets were preincubated for 30 min at 4°C in various concentrations of Ca^{2+}. All pellets were incubated for 3 min in Ringer's solution with 2 mM iBuMeXan and then for 5 min in Ringer's solution containing 2 mM iBuMeXan and 200 μM dopamine (○) or in Ringer's solution containing 2 mM iBuMeXan (●). Each point represents a single pellet assayed in duplicate. (*Inset*) Effects of low and high concentrations of Ca^{2+} on the sensitivity of intact horizontal cells to dopamine. Cells were prepared and preincubated in either 0 (□) or 110 mM $CaCl_2$ (■) for 30 min at 4°C. Pellets were incubated for 3 min in Ringer's solution containing 2 mM iBuMeXan and finally for 5 min in Ringer's solution containing 2 mM iBuMeXan at the specified concentrations of dopamine. Data are from single representative experiments assayed in duplicate. Increasing Ca^{2+} concentration in the preincubation Ringer's solution increased the total accumulation of cAMP but did not affect the basal level or the sensitivity of these cells to dopamine.

partate may serve as the photoreceptor transmitter in the carp (1–3). On isolated carp horizontal cells maintained in culture, L-glutamate powerfully depolarizes the cells and induces a prolonged Ca^{2+}-dependent action potential in them (3). An important question is whether the application of L-glutamate to isolated horizontal cells, with the concomitant increase of intracellular Ca^{2+}, alters the sensitivity of the cells to antagonists. Fig. 5 shows that this is the case. Preincubation of cells with 500 μM glutamate in normal Ringer's solution alters their sensitivity to haloperidol in a fashion similar to preincubation in Ringer's solution containing 10–15 mM Ca^{2+} (Fig. 1). In preliminary experiments, we also have tested the effect of the calcium ionophore A23187 on the sensitivity to haloperidol of intact horizontal cells preincubated in Ringer's solution containing 2.3 mM Ca^{2+}. We found that the IC_{50} of the cells treated in this way was ≈10 μM. In other words, the calcium ionophore A23187, like L-glutamate, decreased the sensitivity of cells to haloperidol by ≈10,000-fold.

DISCUSSION

We showed that preincubating isolated and intact carp horizontal cells in various concentrations of Ca^{2+} alters the sensitivity of the cells to dopamine antagonists by 3–4 orders of magnitude. That intracellular levels of Ca^{2+} are likely to be critical for these alterations is shown by the experiments in which L-glutamate and the calcium ionophore A23187 were applied to the cells. L-Glutamate induces a prolonged Ca^{2+} spike in carp horizontal cells (3), thereby increasing Ca^{2+} levels within the cells. After the application of L-glutamate to isolated cells, the sensitivity to haloperidol was altered as if Ca^{2+} levels in the preincubation medium were raised from 2.3 to 11 mM (Fig. 5).

The same result was observed after preincubation of the cells in the calcium ionophore A23187. The conclusion that intracellular Ca^{2+} levels regulate antagonist sensitivity of the intact horizontal cells is supported also by the observation that homogenizing isolated horizontal cells in Ringer's solution containing normal levels of Ca^{2+} (2.3 mM) also dramatically decreased the sensitivity of the system to antagonists.

Of the antagonists tested, the effects of haloperidol were most sensitive to changes of Ca^{2+} in the preincubating medium. Varying the Ca^{2+} concentrations in the preincubation Ringer's solution by <10 mM maximally altered the sensitivity of the cells to haloperidol (i.e., by 10,000 times). The sensitivity of the cells to (+)-butaclamol and flupenthixol was also dramatically altered by increases in Ca^{2+} levels in the preincubation Ringer's solution, but much larger changes of Ca^{2+} (up to 100 mM) were required to desensitize the cells maximally. With all antagonists tested, there appeared to be a maximum and minimum sensitivity of the cells to the agents—for example, lowering Ca^{2+} in the preincubation medium below control levels (i.e., 2.3 mM) did not enhance sensitivity of the cells to any of the antagonists. There also appeared to be a minimum sensitivity of the cells to the antagonists regardless of Ca^{2+} levels in the preincubation medium. These results suggest that the receptor sites interacting with the antagonists may exist in two states, a high-affinity state and a low-affinity one. The experiments using flupenthixol provide support for this hypothesis. That is, when cells were preincubated in Ringer's solution containing concentrations of Ca^{2+} intermediate between those inducing maximal and min-

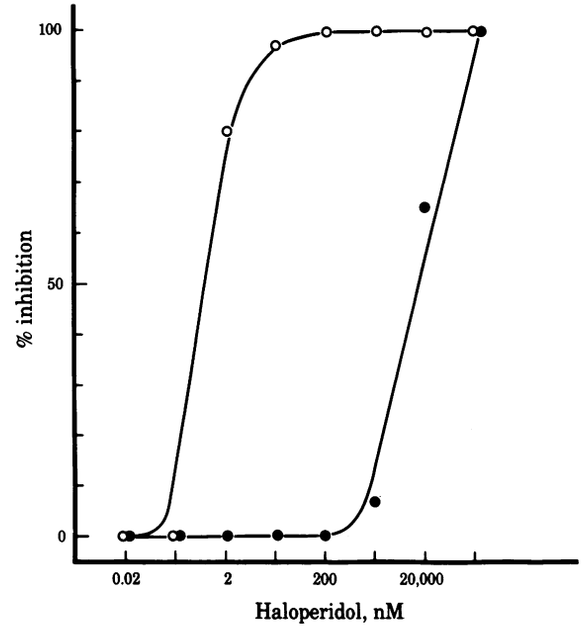

FIG. 5. Effects of L-glutamate on the inhibition by haloperidol of dopamine-stimulated cAMP accumulation in intact horizontal cells. Cells were prepared and preincubated for 30 min at 4°C in Ringer's solution containing normal levels of Ca^{2+} (2.3 mM) and 500 μM L-glutamate (●). The cells were incubated for 3 min with Ringer's solution containing 2 mM iBuMeXan and the specified concentrations of haloperidol, followed by a 5-min incubation with Ringer's solution containing 2 mM iBuMeXan, 200 μM dopamine, and antagonists. Data are from a single representative experiment involving duplicate assays and are compared with a control experiment (from Fig. 1) in which intact cells were preincubated in Ringer's solution containing normal levels of Ca^{2+} (2.3 mM) and no glutamate (○). The addition of L-glutamate in the preincubation Ringer's solution shifted the sensitivity of the intact horizontal cells to haloperidol by 10,000-fold. Among three experiments utilizing 500 μM L-glutamate, the IC_{50} values for haloperidol varied less than 10-fold—i.e., they ranged from 1 to 10 μM.

imal flupenthixol sensitivities, biphasic inhibition curves were observed. The lower limb was in the nanomolar range of antagonist sensitivity, whereas the upper limb was in the micromolar range of antagonist sensitivity. Varying preincubation Ca^{2+} levels in the intermediate concentration ranges simply altered the relative proportion of these two limbs.

Homogenates of isolated horizontal cells also showed some changes in sensitivity to antagonists, depending on Ca^{2+} levels in the preincubation and incubation media. The minimum sensitivity of the homogenates to the various antagonists was similar to the minimum sensitivity of intact cells incubated in high-Ca^{2+} Ringer's solution. However, lowering Ca^{2+} levels in the preincubation and incubation medium never sensitized homogenates to antagonists to the same extent as was observed with intact cells. In some experiments using Ringer's solution lacking Ca^{2+}, 5 mM EGTA was added to the incubating medium to chelate any Ca^{2+} released from organelles upon cell homogenization. When such homogenates were tested for antagonist sensitivity, they behaved similarly to those homogenates incubated in solution lacking both Ca^{2+} and added EGTA. Why homogenates never showed antagonist sensitivity similar to that observed in intact cells is not clear. It may suggest that homogenization of cells induces irreversible changes in the receptor–membrane complex.

It is significant that dopamine sensitivity of the intact cells was similar to that observed in homogenates of the whole retina (6) and that alteration of Ca^{2+} levels in the preincubation and incubation media did not appear to affect agonist sensitivity of isolated cells. The amount of cAMP produced with maximal agonist concentrations did vary to some extent, depending on Ca^{2+} concentration in the incubating medium. This effect has been reported before and is thought to reflect a role by Ca^{2+} and/or calmodulin in the coupling of the dopamine receptor with adenylate cyclase (9–11).

Our observations that antagonist sensitivity of isolated cells is dramatically altered by Ca^{2+}, but agonist sensitivity is not, and that nanomolar concentrations of antagonist are sufficient to inhibit by 50% the effects of dopamine at 200 μM, suggests that there are separate dopamine agonist and antagonist binding sites on horizontal cells. It has been proposed earlier that there may be separate dopamine agonist and antagonist binding sites on cells (12–14), and the present experiments appear to provide significant evidence in favor of this notion.

The present experiments also may provide some insights on the nature of dopamine receptor sites and their classification. For example, it has been proposed that there may be as many as four separate classes of dopamine receptors, and one of the criteria used to distinguish these receptor types is their sensitivity to dopamine antagonists (15). For example, the D1 and D3 receptors are characterized by micromolar sensitivity to dopamine antagonists, whereas the D2 and D4 receptors are sensitive to nanomolar concentrations of antagonists. Our experiments have shown that antagonist sensitivity of intact horizontal cells can be altered from the nanomolar to the micromolar range by altering intracellular Ca^{2+} levels. Thus, the receptor sites on intact cells incubated in normal Ringer's solution behave, with regard to antagonist sensitivity, like D2 or D4 receptors, whereas the receptors on cells preincubated in high-Ca^{2+} Ringer's solution behave like D1 or D3 receptors. These data suggest, therefore, that binding sites with different antagonist sensitivities may not necessarily be separate entities but different states of the same receptor complex.

Finally, we may ask if the alteration of antagonist sensitivity by intracellular Ca^{2+} levels in horizontal cells has any physiological significance. None is known at the present time. However, the fact that antagonist sensitivity can potentially be regulated by horizontal cells *in vivo*, and that separate agonist and antagonist binding sites on cells may exist, does suggest some physiological function. For example, it is possible that there are endogenous dopamine antagonists (16, 17); if so, the existence of separate binding sites for these substances and the ability of cells to regulate their sensitivity to antagonists may be explained.

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