Multiple Second-Messenger System Modulation of Voltage-Activated Calcium Currents in Teleost Retinal Horizontal Cells

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Pfeiffer-Linn, Cindy L. and Eric M. Lasater. Multiple second-messenger system modulation of voltage-activated calcium currents in teleost retinal horizontal cells. J. Neurophysiol. 80: 377–388, 1998. Two voltage-activated calcium currents, a transient T-type and a PL-sustained type, have been measured in isolated, cultured white bass horizontal cells. These two voltage-activated calcium currents were found to be modulated by two independent second-messenger systems. Furthermore, activation of either second-messenger system led to similar changes in calcium current activity. Activation of the cyclic AMP second-messenger pathway or the sn-1,2-diacylglycerol (DAG) second-messenger system resulted in a significant decrease in the amplitude of the transient current and a simultaneous large increase in the amplitude of the sustained current. Both second-messenger systems achieved their effects through protein phosphorylation. The cyclic AMP pathway resulted in the activation of protein kinase A (PKA) and the DAG pathway worked to activate protein kinase C (PKC). Two protein kinase inhibitors were analyzed in this study for their ability to inhibit second-messenger activated protein kinase activity and separate the two pathways. The peptide cyclic AMP-dependent protein kinase inhibitor and staurosporine were found to be nonspecific at high concentrations and inhibited both second-messenger pathways. At low concentrations however, staurosporine specifically inhibited only PKC, whereas adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase inhibitor was selective for PKA. Both second-messenger systems were activated by the neuromodulator, dopamine. Thus one agonist can initiate multiple second-messenger systems leading to similar changes in voltage-activated calcium current activity. The modulatory action on calcium currents produced by one second-messenger system added to the modulatory action resulting from activation of the other second-messenger system. The effect is to alter the magnitude of the horizontal cell calcium currents.

INTRODUCTION

Second-messenger systems can alter nerve cell activity by modifying characteristics of voltage-activated ion channels (Huganir 1987; Klein et al. 1982; Onozuka et al. 1988; Pfeiffer-Linn and Lasater 1993), ligand-gated ion channels (Browning et al. 1990; Greengard et al. 1991; Huganir 1986; Huganir and Greengard 1990; Kirkness et al. 1989; Miles and Huganir 1988; Porter et al. 1990; Wang et al. 1991), and neurotransmitter release (Castellucci et al. 1980; Greengard et al. 1972; Nestler and Greengard 1989). This often occurs as a result of signal transduction events where neurotransmitters or neuromodulators bind to synaptic receptors and initiate events that ultimately activate protein kinases to phosphorylate specific substrate proteins. One of the best studied examples of these events involves 3′,5′ cyclic AMP (cyclic AMP or cAMP). Cyclic AMP is produced by the activation of adenylate cyclase. Production of cAMP subsequently activates cyclic AMP-dependent protein kinase (PKA) by binding to the regulatory subunit of PKA, releasing the catalytic subunit that can then phosphorylate a wide variety of cytoplasmic and membrane proteins (Casperson and Bourne 1987; Erskine et al. 1989; Nestler and Greengard 1989; Schacher et al. 1988).

In teleost retinal horizontal cells, the cAMP second-messenger system has been linked to activation of a D1-type dopaminergic receptor (DeVries and Schwartz 1989; Lasater 1987; Lasater and Dowling 1985; O’Connor et al. 1989). Horizontal cells are second-order neurons in the retina that receive synaptic input from photoreceptors as well as from dopaminergic interplexiform cells (Trifonov 1968; Yang et al. 1988; Zucker and Dowling 1987). These retinal horizontal cells are tightly coupled electrically by gap junctions and are responsible for the lateral spread of information across the retina as well as surround inhibition seen in bipolar cells (Kaneko 1970, 1971; Sakuranaga and Naka 1985; Werblin and Dowling 1969).

Previous studies have shown that several aspects of horizontal cell activity are modified by dopamine including electrical coupling between horizontal cells (Lasater and Dowling 1985) and ionic conductances gated by L-glutamate (Knapp and Dowling 1987; Knapp et al. 1990). More recently, dopamine was found to modulate differentially two types of voltage-activated calcium currents found in isolated white bass horizontal cells (Pfeiffer-Linn and Lasater 1993, 1996a,b). These calcium currents have been well characterized on the basis of pharmacological and electrophysiological criteria.

One of these calcium currents has some pharmacological and biophysical properties in common with the transient or T-type calcium current described in a variety of other preparations (Fox et al. 1987; Nilius et al. 1985; Nowycky et al. 1985). The second voltage-activated calcium current is a unique type of sustained calcium current, which shares some biophysical and pharmacological properties in common with the P-type calcium current and some properties of the high-voltage-activated L-type calcium current. As a result, the current has been labeled as a PL-type calcium current (Pfeiffer-Linn and Lasater 1996a,b). Both the T-type transient current and the PL-type sustained calcium current have been studied for their role in neurotransmitter release, their possible role in contributing to formation of the light response, and their potential contribution to maintaining the cell’s membrane potential in the dark (Pfeiffer-Linn and Lasater 1993; Sullivan and Lasater 1992). Whole cell patch-clamp recordings revealed that dopamine modu-
lated these currents differentially via activation of the cAMP second-messenger system (Pfeiffer-Linn and Lasater 1993). The transient calcium current was reduced significantly in magnitude, whereas the sustained calcium current was potentiated simultaneously after dopamine application.

Although modulation of the voltage-activated calcium currents in white bass horizontal cells could occur either by direct action on the ion channels or by an indirect effect, the direct phosphorylation of calcium channels by activation of the cAMP second-messenger system is best supported by biochemical evidence (Catterall 1991; Sculptoreanu et al. 1993a,b). Biochemical analyses of the phosphorylation events have demonstrated that multiple types of voltage-activated calcium channels are substrates for phosphorylation by cAMP-dependent PKA, as well as protein kinase C (PKC) (Catterall 1991). Activation of PKC usually is initiated by ligand binding to specific cell-surface receptors. This event activates phosphoinositide-specific phospholipase C (PLC) through an intervening guanine nucleotide-binding protein (G protein). Activated PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to yield sn-1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP$_3$). In some cases IP$_3$ releases calcium from intracellular stores that binds to cytosolic PKC, causing it to translocate to the membrane. DAG then activates PKC and results in phosphorylation of specific proteins.

In teleost horizontal cells, DAG and PKC have been shown to play a role in light adaptation and the formation of spinules at the synapse with photoreceptors (Rodrigues and Dowling 1990; Weiler et al. 1991). Otherwise, little else is known about the DAG second-messenger system and the functional role of PKC in these cells. Because it now is recognized that a variety of second-messenger systems can mediate the modulation of calcium channels, in this study, we have examined the effect of activating the DAG second-messenger system on calcium current activity in isolated, cultured white bass horizontal cells. When DAG analogues or PKC activators were applied to voltage-clamped cells under conditions favoring the expression of both calcium currents, we found that the transient current decreased, whereas the sustained current simultaneously increased in the same neuron. These same results are obtained when the cAMP second-messenger system is activated (Pfeiffer-Linn and Lasater 1993) and suggest that the calcium channels in white bass horizontal cells can be modulated in the same direction by two different second-messenger systems. Surprisingly, we also found that exogenous application of dopamine activated both second messengers. Thus activation of a single receptor stimulated two separate second-messenger systems to phosphorylate what are probably separate sites on the same calcium channel.

**METHODS**

**Cell dissociation**

White bass horizontal cells were isolated and cultured according to the procedures outlined by Dowling et al. (1985) and by Lasater (1986). Briefly, a white bass (*Roccus chrysops*) was dark adapted for 2 h. The animal was killed in accordance to guidelines set forth in the Guide for the Care and Use of Laboratory Animals published by the National Research Council. Both eyes were removed under dim red light, and the retinas were separated from the pigment epithelium after removal of the cornea and lens. Isolated retinas were incubated for 40 min in a modified L-15 (Leibowitz) culture medium (GIBCO BRL, Grand Island, NY) containing 40 units of papain/ml (Worthington Biochemicals, Freehold, NJ). The enzymatically treated retinas then were triturated with a large glass bore pipette using several washes of fresh L-15. This procedure acted to remove excess papain and to break the isolated retina into numerous tissue fragments. These retinal fragments were triturated again with fresh L-15 using a finer bore glass pipette and distributed into 35-mm culture dishes containing fresh L-15. After distribution, 500 units/ml of penicillin-streptomycin antibiotics were added to each dish, and the cells were kept in a low temperature incubator at 10°C until used. Experiments were performed on cells that had been maintained in culture usually between 1 and 3 days.

**Solutions**

At the beginning of each experiment, culture medium was exchanged for white bass Ringer containing the following (in mM): 130 NaCl, 10 CaCl$_2$ or BaCl$_2$ (to enhance the calcium currents), 2.8 KCl, 1 MgCl$_2$, 10 glucose, 8.4 N-2-hydroxyethylpipеразине-N’-2-етаноусульфониевый кислота (HEPES), 0.20 tetrodotoxin (to block the inward sodium current), and 10 4-aminopyridine (4-AP; to block outward potassium currents). The pH of the Ringer was adjusted to 7.4 using NaOH. The recording pipette solution contained (in mM): 120 potassium gluconate, 4 NaCl, 11 ethylene glycol-bis-(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 1 MgCl$_2$, 8.4 HEPES, 1 CaCl$_2$, and 20 tetraethylammonium (to block potassium currents). In previous studies in white bass horizontal cells, we found that calcium current results obtained using potassium gluconate in the recording pipette were identical to experiments using cesium chloride in the pipette (Pfeiffer-Linn and Lasater 1993). Test solutions, such as dopamine; the membrane permeable agents 8- (4-chlorophenylthio) adenosine 3’,5’-cyclic monophosphate (8-CPT); phorbol 12-myristate 13-acetate (PMA); 1-oleoyl-2-acetyl-rac-glycerol (OAG; all obtained from Sigma, St. Louis, MO); adenosine 3’,5’-cyclic monophosphothioate, Rp-Isomer, triethylammonium salt (rp-cAMPS; Calbiochemicals, La Jolla, CA); and staurosporine (Research Biochemicals, Natick, MA) were superfused over selected voltage-clamped cells at the rate of 1 ml/min through a gravity-fed system. Membrane impermeable substances such as the peptide cAMP-dependent protein kinase inhibitor (PKI, from Sigma), KT 5720, chelerythrine chloride, and bisindolylmaleimide (obtained from Calbiochemicals) were introduced to voltage-clamped cells through the recording pipette. For example, once a seal was made and the membrane ruptured, PKI was allowed to diffuse into the cell for 4 min before beginning an experiment.

Various concentrations of each second-messenger agent used in this study were tested for their effect on calcium current activity. Based on the dose-response obtained, care was taken to use comparable concentrations in experiments using second-messenger agents. This eliminated possible erroneous conclusions that could have been drawn if noncomparable concentrations were used.

**Recording**

Patch pipettes were pulled from borosilicate glass using a Narishige vertical microelectrode puller (Narishige Instruments, Tokyo). Electrodes were used uncoated and unpolished. The resistance of the recording electrodes was between 4 and 8 MΩ when measured in Ringer bath solution. Series resistance and electrode and cell capacitance were compensated for electrophoretically. A small junction potential was measured (<3 mV) and was nulled.

Cells in culture were identified easily according to their unique characteristic morphologies (Dowling et al. 1985). White bass

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FIG. 1. Effect of second-messenger activators on calcium current amplitude. Protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA), was applied to a voltage-clamped H2 type cone horizontal cell (A). Transient calcium current was apparent when the cell was stepped to −20 mV (left, control) from the holding membrane potential, whereas the sustained current was elicited at step commands to +10 mV (A, right, control). Two traces have been superimposed for each figure section. One trace represents the control current response, whereas the 2nd trace was obtained 2 min after 50 nM PMA was superfused over the cell. PMA decreased the transient current and increased the sustained current. In B, current traces were obtained before and after the adenosine 3'-5'-cyclic monophosphate (cAMP) activator, 8-(4-chlorophenylthio) adenosine 3'-5'-cyclic monophosphate (8-CPT), was superfused over a 2nd cell. As we have shown previously (Pfeiffer-Linn and Lasater 1993), the left current traces superimposed on each other demonstrate that 50 μM 8-CPT decreased the transient current, whereas the right current traces show that 8-CPT increased the sustained current amplitude. Note that both PMA and 8-CPT had similar effects on the 2 types of voltage-activated channels. 

cone horizontal cells were voltage clamped using the whole cell patch-clamp technique described by Hamill et al. (1981). Current recordings were obtained using an Axopatch 200A amplifier (Axon Instruments). Cells were voltage clamped at a holding potential of −70 mV. To elicit calcium current activity, cells were 1) stepped to various depolarized membrane potentials between −70 and +50 mV, or 2) the membrane potential of the cell was changed in a rampwise manner from −90 to +50 mV during a 250-ms period of time. Following these stimulus parameters, peak transient calcium current occurred when the membrane potential of the voltage-clamped cell was about −20 mV. Peak sustained calcium current activity occurred when the membrane potential of the cell was at about +10 mV. Data was collected using a PC in conjunction with a LabMaster data acquisition board. Analysis of the data were carried out using Axon Instruments pClamp suite of programs.

RESULTS

Effect of PKC activators on calcium currents

To examine the effect of PKC on the two voltage-activated calcium currents found in white bass horizontal cells, specific activators of PKC were applied to voltage-clamped horizontal cells. Under pharmacological conditions favoring the expression of calcium currents, the membrane potential of a H2 cone horizontal cell was depolarized from a holding potential of −70 to −20 mV to elicit the transient calcium current (Fig. 1, left), or to +10 mV to elicit the PL-type sustained calcium current (Fig. 1, right). In Fig. 1A, a phorbol ester activator of PKC, PMA, was superfused over the voltage-clamped cell and current traces were obtained before and after 50 nM PMA application. Each figure in A represents the control current response superimposed on the current response obtained 2 min after PMA was applied. In the presence of nanomolar concentrations of PMA, the amplitude of the transient current significantly decreased by 55%, whereas the peak amplitude of the sustained calcium current was potentiated by 86%. In other words, activation of PKC modulated differentially the two types of voltage-activated calcium channels in white bass horizontal cells. This differential modulatory response due to activation of PKC was characteristic of the response recorded in 15 other voltage-clamped cells (Table 1). However, no modulatory effects on both types of calcium current were seen if 4-α-phorbol-12-myristate-13-acetate (4-α-PMA) was substituted for PMA (n = 6). 4-α-PMA is an inactive isomer of PMA that was used as a negative control for phorbol ester activation of PKC.

Another pharmacological activator of PKC is OAG. When the experiments described above were repeated with OAG instead of PMA (n = 6; Table 1), the transient calcium current significantly decreased and the sustained current significantly increased. OAG mimicked the effect of PMA on the two types of calcium current. PMA’s actions on the two types of voltage-activated calcium current were also similar to the effect of activating the
cAMP second-messenger system. In Fig. 1B, 50 μM 8-CPT was superfused over another voltage-clamped H2 cone horizontal cell. 8-CPT is a membrane permeant cAMP derivative and activates cAMP-dependent PKA. As shown in Fig. 1B, 8-CPT significantly decreased the transient calcium current and simultaneously increased the sustained calcium current in the same cell. As mentioned previously, this modulatory effect of activating the cAMP second-messenger system has been previously well characterized and well documented (Pfeiffer-Linn and Lasater 1993, 1996a). However, we were surprised to find that two second-messenger systems modified the voltage-activated calcium channels by increasing the sustained current and decreasing the transient current.

To determine the concentration range over which PMA was active, a dose-response curve was generated and is illustrated in Fig. 2. To generate data points, the peak sustained calcium current amplitudes were measured under control conditions when the cell’s membrane potential was shifted to +10 mV. Control current traces then were compared with current traces after various concentrations of PMA were superfused over the clamped cells. The percent change in peak current amplitude subsequently was plotted against the various PMA concentrations. As can be seen from this figure, concentrations of PMA >5 nM significantly potentiated the amplitude of the sustained calcium current. Data points were curve fit using the Hill equation. The EC_{50} was calculated to be 7.5 nM, and the maximum percent change in sustained current was an increase of 120%. A PMA dose-response curve was not generated for the transient current due to the fact that a number of cells did not show a transient current (see METHODS). However, 10 nM PMA decreased transient current activity by a mean of 49 ± 12% (mean ± SD, Table 1). This decrease in transient current activity represents the maximal change due to PMA, as similar results were obtained using 50 nM PMA.

To verify that activation of PKC is involved in the modulation of the two voltage-activated calcium currents, a potent inhibitor of PKC, staurosporine, was superfused over selected voltage-clamped cells before PMA application. Not only did staurosporine eliminate PMA’s effect, but staurosporine also acted to decrease the amplitude of the voltage-activated sustained current under control conditions. This is illustrated in Fig. 3A. Figure 3A, left, shows four superimposed current traces. There is no difference among the four traces. Figure 3A, center, represents four current responses

### Table 1: Effect of various second-messenger agents on calcium current amplitude

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Antagonist</th>
<th>Transient</th>
<th>Sustained</th>
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<tbody>
<tr>
<td>PMA</td>
<td>—</td>
<td>↓ 49 ± 12 (8)</td>
<td>↑ 85 ± 12 (15)</td>
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<td>4-α-PMA</td>
<td>—</td>
<td>↓ 9 ± 8 (4)</td>
<td>↑ 8 ± 9 (6)</td>
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<td>OAG</td>
<td>—</td>
<td>↓ 25 ± 10 (4)</td>
<td>↑ 50 ± 16 (6)</td>
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<tr>
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<td>PKI</td>
<td>↓ 39 ± 11 (4)</td>
<td>↑ 72 ± 18 (9)</td>
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<tr>
<td>PMA</td>
<td>Staurosporine</td>
<td>↓ 4 ± 10 (5)</td>
<td>↑ 5 ± 12 (8)</td>
</tr>
<tr>
<td>PMA</td>
<td>BIS</td>
<td>↓ 1 ± 2 (2)</td>
<td>↑ 4 ± 5 (5)</td>
</tr>
<tr>
<td>PMA</td>
<td>Chelerythrine</td>
<td>↓ 1 ± 2 (5)</td>
<td>↑ 11 ± 18 (5)</td>
</tr>
<tr>
<td>PMA/CPT</td>
<td>—</td>
<td>↓ 52 ± 12 (3)</td>
<td>↑ 180 ± 25 (6)</td>
</tr>
<tr>
<td>8-CPT</td>
<td>—</td>
<td>↓ 38 ± 12 (6)</td>
<td>↑ 135 ± 53 (11)</td>
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<tr>
<td>8-CPT</td>
<td>PKI</td>
<td>↓ 5 ± 6 (6)</td>
<td>↑ 2 ± 16 (12)</td>
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<tr>
<td>8-CPT</td>
<td>Staurosporine</td>
<td>↓ 30 ± 18 (3)</td>
<td>↑ 85 ± 21 (8)</td>
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<tr>
<td>8-CPT</td>
<td>rp-cAMP</td>
<td>↓ 5 ± 10 (3)</td>
<td>↑ 18 ± 48 (4)</td>
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<tr>
<td>8-CPT</td>
<td>KT-5720</td>
<td>↓ 8 ± 15 (4)</td>
<td>↑ 12 ± 20 (5)</td>
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<tr>
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<tr>
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<tr>
<td>DA</td>
<td>BIS</td>
<td>↓ 8 ± 10 (3)</td>
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<td>DA</td>
<td>Chelerythrine</td>
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<td>rp-cAMP</td>
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<td>BIS &amp; Rp-cAMP</td>
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<td>↑ 5 ± 3 (3)</td>
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The results presented here were obtained when cone horizontal cells were held at a membrane potential of −70 mV and the membrane potential was stepped to −20 mV to activate the transient current or to +10 mV to activate the sustained current. Similar results were obtained when the cell’s membrane potential was changed in a rampwise fashion from −70 to +50 mV over 0.5 s. The values are mean percent change ± SD from control transient and sustained calcium currents. The direction of the arrow signifies either a decrease in current amplitude (↓) or an increase (↑). Number of cells tested for each of the various agents are in parentheses. Note that all voltage-clamped horizontal cells showed a sustained calcium current when depolarized to +10 mV. However, only approximately one-half of selected cells displayed a measurable transient current when the membrane potential was depolarized to −20 mV. This is not unusual and the lack of measurable transient currents in some cells may be due to the isolation procedure (Pfeiffer-Linn and Lasater 1993; Sullivan and Lasater 1992). PMA, phorbol 12-myristate 13-acetate (10 nM); 4-α-PMA, 4-α-phorbol 12-myristate 13-acetate (10 nM); OAG, 1-oleoyl-2-acetyl-rac-glycerol (20 μM); 8-CPT, 8-(4-chlorophenylthio) adenosine 3’,5’-cyclic monophosphate (50 μM); PKI, adenosine 3’,5’-cyclic monophosphate (cAMP)-dependent protein kinase inhibitor (10 μM); DA, dopamine (50 μM); BIS, bisindolylmaleimide (20 μM); and RP-cAMP, adenosine 3’,5’-cyclic monophosphothioate, Rp-Isomer, triethylammonium salt (75 nM). Other chemical concentrations were staurosporine (1 nM); chelerythrine chloride (250 nM); and KT 5720 (100 nM).
FIG. 3. Effect of staurosporine on calcium current activity. A: H2 cone horizontal cell was voltage-clamped, and the membrane potential was stepped from −70 to +10 mV to elicit sustained calcium current activity. Left: 4 superimposed current traces obtained under control conditions. Center: effect of superfusing 1 nM staurosporine over the same cell. Bottom: current trace obtained immediately after the start of superfusion. After perfusion of staurosporine for 1 min, the current decreased (middle trace). Top 2 current traces were obtained 2 and 3 min after staurosporine application. Staurosporine further decreased the amplitude of the sustained calcium current. Note that there is no difference between current traces obtained after 2 min. After the stabilization of this current, 10 nM PMA was applied to the same cell in conjunction with staurosporine. In the presence of staurosporine, PMA did not affect calcium current activity. B, left: 4 control current traces are shown that were obtained from a voltage-clamped horizontal cell whose membrane potential was stepped to ±20 mV from a holding potential of ±70 mV. Center: 4 current traces obtained after superfusion of 1 nM staurosporine. Top trace was obtained shortly after staurosporine application. Middle trace was obtained 1 min after superfusion of staurosporine; bottom 2 superimposed traces were obtained 2 and 3 min after superfusion. After the stabilization of this current, 10 nM PMA was applied to the same cell in conjunction with staurosporine (right). Staurosporine blocked PMA’s modulatory effect on the transient calcium current. Representation of the voltage step used to activate the calcium currents (not to scale) is below each set of traces.

obtained after 1 nM staurosporine was superfused over the voltage-clamped cell. The largest current (bottom) was obtained shortly after the start of staurosporine superfusion. The amplitude of the sustained current trace decreased 1 min after staurosporine superfusion (center trace) while the smallest current traces were obtained 2 and 3 min after superfusion (top 2 traces). After 2 or 3 min in staurosporine, sustained calcium current activity stabilized in all voltage-clamped cells examined (n = 9). At this point, application of PMA produced no enhancement of calcium current. This is demonstrated in Fig. 3A, right. Four current traces were obtained between 2 and 4 min after PMA application in the presence of staurosporine. Staurosporine blocked PMA’s modulatory action on the sustained calcium current in white bass horizontal cells.

In addition to affecting the sustained current, staurosporine increased the transient current in all voltage-clamped horizontal cells by an average of 30 ± 15% (n = 6), suggesting that PKC is active under normal conditions. In Fig. 3B, four control current traces were obtained from a voltage-clamped horizontal cell whose membrane potential was stepped to −20 mV to elicit the transient calcium current (left). Figure 3B, center, represents the effect of staurosporine on transient current activity. The smallest current was obtained shortly after staurosporine application (Fig. 3B, top). In the presence of staurosporine, the amplitude of the transient current increased. A current trace was obtained 1 min following staurosporine application (Fig. 3B, middle trace) and then again at 2 and 3 min (bottom 2 traces). There was no difference between current traces obtained after 2 min. Figure 3B, right, demonstrates that staurosporine blocked PMA’s modulatory action on the transient calcium current in white bass horizontal cells. Current traces were obtained 2, 3, and 4 min after PMA application in the presence of staurosporine.

Unlike PKC, PKA does not appear to be active under control or resting conditions. In previous experiments, when cAMP-dependent PKI was introduced into voltage-clamped horizontal cells through the recording pipette, there was no significant change in the amplitude of the transient or sustained calcium current (Pfeiffer-Linn and Lasater 1993).

**Activation of the DAG second-messenger system**

Activation of the DAG second-messenger system occurs as a result of ligand binding to specific cell-surface receptors.
In the white bass horizontal cells, we have shown that modulation of the voltage-activated calcium currents occurs with activation of the DAG second-messenger system. However, the neurotransmitter or neuromodulator that initiates activation of the DAG system here is unknown. Because it is well documented that dopamine receptors exist on teleost horizontal cells (DeVries and Schwartz 1989; Lasater 1987; Lasater and Dowling 1985; O’Connor et al. 1989), we performed experiments in an attempt to determine if dopamine activated the DAG second-messenger system in white bass horizontal cells as it seems to in bullhead catfish (Rodrigues and Dowling 1990).

Although previous studies done in this laboratory have demonstrated that D1-type dopaminergic receptors are linked to activation of the cAMP second-messenger system, other studies have shown that activation of a single receptor type can activate multiple signal transduction systems (Evans and Robb 1993). This can be achieved by a single receptor through activation of different G proteins in coupled receptors (Bylund 1992; Hosey 1992; Robb et al. 1994), or by activating single receptor subtypes that are linked directly to multiple second-messenger pathways (Thompson 1992). To determine if dopamine activated the DAG second-messenger system, dopamine was applied to voltage-clamped horizontal cells in the presence of a PKA inhibitor (the peptide inhibitor PKI) that should block any modulatory effect arising from activation of the cAMP second-messenger system. With 20 μM cAMP-dependent PKI in the cell cytoplasm, dopamine failed to affect calcium current activity. This suggested that dopamine did not initiate the DAG second-messenger system in teleost horizontal cells. However, high concentrations of protein kinase inhibitors have been found to lack specificity by inhibiting several types of protein kinase (Fujita-Yamaguchi and Kathuria 1988; Nakano et al. 1987; Niggli and Keller 1991). To determine whether 20 μM PKI blocked PKC as well as PKA, experiments were performed in the presence of various concentrations of cAMP-dependent PKI. In the studies summarized in Fig. 4 (middle), different concentrations of cAMP-dependent PKI were loaded into selected voltage-clamped cells from the recording electrode for 4 min. After this period of time, calcium current responses were obtained from cells before and after application of 10 nM PMA (Fig. 4A) or 50 μM 8-CPT (Fig. 4B). PKI concentrations of 20, 1, and 0.1 μM were found to eliminate 8-CPT’s effect on calcium current activity as well as PMA’s effect. However, when a lower concentration of PKI was present (0.01 μM), cAMP-dependent PKI selectively blocked the cAMP second-messenger system (Fig. 4B). This suggested that, at high concentrations, cAMP-dependent PKI is not specific for PKA.

This is illustrated in further detail in Fig. 5 where voltage-clamped horizontal cells, loaded with 10 nM PKI, were exposed to 50 μM 8-CPT or 10 nM PMA. In Fig. 5A, the normalized sustained calcium current increase to 8-CPT is plotted over time. After application of 8-CPT, the amplitude of the sustained calcium current increased by a mean 92% of control within 2–3 min. When 10 nM PKI was introduced into the voltage-clamped cells, 8-CPT’s effect on the sustained calcium current was eliminated (Fig. 5B). However, 10 nM PKI did not affect PMA’s modulation of the sustained current. In Fig. 5C, with 10 nM PKI in the cell, PMA increased the sustained calcium current by an average of 70%. This supports the previous finding that PKI is specific for the cAMP second-messenger system at concentrations <0.1 μM.

To determine the specificity of staurosporine on protein kinase activity, the above experiments were repeated using various concentrations of staurosporine instead of cAMP-dependent PKI (Fig. 4, right). Although staurosporine often is referred to as a specific PKC inhibitor (Tamaoki et al. 1986), it is a nonspecific kinase inhibitor that has been shown to inhibit several other kinases including the insulin receptor tyrosine kinase (Fujita-Yamaguchi and Kathuria 1988), cAMP-dependent protein kinase, cGMP-dependent protein kinase (Niggli and Keller 1991), calcium-calmodulin-dependent kinase (Yanagihara et al. 1991), and a tyrosine kinase (Nakamo et al. 1987). We found that concentrations of 50, 10, and 5 nM staurosporine eliminated both PMA’s and 8-CPT’s modulation of the sustained calcium current in white bass horizontal cells. Therefore, at high concentrations, staurosporine did not act as a specific blocker of PKC but blocked cAMP-dependent protein kinase as well in horizontal cells supporting the findings of Niggli and Keller (1991). However, at lower concentrations, staurosporine was much more specific for the DAG second-messenger system (Fig. 6). In Fig. 6A, 10 nM PMA was applied to voltage-clamped horizontal cells, and the increase in sustained calcium current was measured and plotted against time. Under control conditions, PMA increased calcium current activity during the course of 2 min. At the peak response, the mean sustained calcium current increased by 80%. When 1 nM staurosporine was superfused over voltage-clamped cells, staurosporine selectively inhibited PMA’s effect on sustained calcium current activity (Fig. 6B) without significantly affecting the cAMP second-messenger cascade’s ability to increase I_{Ca} (Fig. 6C).

The results of these experiments provide evidence that the protein kinase inhibitors, cAMP-dependent PKI, and staurosporine, are specific for PKA and PKC, respectively, when specific concentrations of each inhibitor are used. With this knowledge, we repeated the experiments designed to determine if dopamine could initiate the DAG second-messenger cascade. Figure 7 illustrates the effect of applying dopamine to a voltage-clamped horizontal cell after blocking PKA activity with 10 nM PKI. Under these conditions, dopamine decreased the transient current and simultaneously increased the sustained PL-type calcium current. In other words, dopamine mimicked the effect of PMA on calcium current activity and therefore is likely the neuromodulator activating the DAG second-messenger system in white bass horizontal cells (see discussion).

To determine if dopaminergic modulation of the voltage-activated calcium currents involved any other second-messenger systems, dopamine was applied to voltage-clamped cells loaded with 10 nM cAMP-dependent PKI in conjunction with 1 nM staurosporine. In the presence of the two protein kinase inhibitors, dopamine had no effect on calcium current activity when compared with control conditions. Similar results were obtained from eight other cells (Table 1). This illustrates that dopamine indeed does work through PKC and probably does not activate more than two second-messenger systems to modulate calcium channel activity (see discussion).
FIG. 4. Specificity of 2 protein kinase inhibitors. Bar graphs illustrate the effect that various concentrations of 2 protein kinase inhibitors had on blocking sustained calcium current activity. Each bar represents the mean peak calcium current amplitude obtained after the membrane potentials of voltage-clamped horizontal cells were changed in a rampwise fashion from −70 to +50 mV. A, left: bars represent the mean peak sustained calcium current amplitude obtained under control conditions and after application of PMA. Center: bars represent the effect of PMA (10 nM) on calcium current activity in voltage-clamped horizontal cells loaded with various concentrations of the PKA inhibitor, PKI. Right: bars demonstrate the mean sustained calcium current amplitude obtained after PMA’s application from another group of voltage-clamped horizontal cells exposed to various concentrations of staurosporine. B, left: mean peak sustained calcium current amplitude obtained under control conditions and after application of 8-CPT. Middle: bars demonstrate the mean result of applying 8-CPT to voltage-clamped cells loaded with various concentrations of PKI. Right: bars illustrate 8-CPT’s effect in the presence of various staurosporine concentrations for another group of voltage-clamped cells. Note that PKI differentially blocked or was specific for the cAMP second-messenger system when 0.01 μM was used. Likewise, staurosprine was specific for the sn-1,2-diacylglycerol (DAG) second-messenger system when 1 nM was used. At higher PKI and staurosporine concentrations, both second-messenger systems were blocked. Standard deviations are represented by the vertical error bars. Each bar was generated from between 5 and 8 different cells.

Inhibition of second-messenger systems

Although we have demonstrated that PKI and staurosprorine can be specific at certain concentrations in white bass horizontal cells, we also have demonstrated that there is a lack of specificity when high concentrations are used. Because of this, we repeated a number of experiments using more specific inhibitors of PKA and PKC. Figure 8A illustrates the effects of dopamine and 8-CPT on sustained calcium current activity in the presence of the more specific PKA inhibitor, rp-cAMP. Mean current/voltage plots were generated from horizontal cells, the membrane potentials of which were changed in a rampwise fashion to elicit sustained calcium current activity. As shown in the figure, after application of 75 nM rp-cAMP, 8-CPT failed to significantly increase the sustained calcium current amplitude (n = 4, Table 1). However, when another group of voltage-clamped cells were exposed to rp-cAMP, dopamine significantly increased the peak calcium current activity by a mean of 83% (n = 4, Table 1). Although this represents a substantial change, it is significantly less than the change observed when dopamine is applied in the absence of a PKA inhibitor.

Similar results are also obtained when another more specific PKA inhibitor was introduced to voltage-clamped horizontal cells. In the presence of 100 nM KT-5720, 8-CPT had no significant effect on calcium current activity (n = 5, Table 1). However, when dopamine was applied to another group of voltage-clamped cells exposed to KT-5720, the mean peak sustained calcium current amplitude increased by 64% (n = 4, Table 1). Taken together, these results support the hypothesis that rp-cAMP and KT-5720 inhibit the cAMP second-messenger system. Because dopamine’s effect on calcium current amplitude is not eliminated in the presence of these PKA inhibitors, these results also support the idea that modulation of the voltage-activated calcium current is not solely controlled by PKA.

In Fig. 8B, similar experiments were performed using a more specific inhibitor of PKC, chelerythrine chloride, instead of PKA. As shown by the mean I-V plots, PMA had no significant effect on calcium current amplitude in the
FIG. 5. Specificity of 10 nM cAMP-dependent PKI. This figure demonstrates that 10 nM PKI selectively inhibited the cAMP second-messenger cascade. A: normalized control, sustained calcium current responses to 50 μM 8-CPT were obtained and plotted at various times. Sustained calcium currents were elicited by depolarizing each voltage-clamped cell from −70 to +10 mV. After application of the cAMP activator, 8-CPT, the amplitude of the sustained current increased for 2 min. B: sustained calcium currents were evoked after 10 nM PKI was allowed to diffuse into the voltage-clamped cells. With 10 nM PKI in the cells, 8-CPT failed to modulate the sustained calcium current amplitude. C: 10 nM PKI did not block modulation of the calcium current due to PMA. Shortly after PMA application, the amplitude of the sustained current increased steadily for 2 min in the presence of 10 nM PKI. Each triangle represents the mean change in normalized sustained calcium currents from control conditions obtained from 9 to 20 cells. Vertical bars represent SD.

FIG. 6. Specificity of 1 nM staurosporine. This figure shows that 1 nM staurosporine selectively inhibited the DAG second-messenger system. A: increase in sustained calcium current activity after application of 10 nM PMA. Sustained calcium currents were elicited by stepping the membrane potential of each cell from the holding potential of −70 to +10 mV. Data points (∆) are represented as the mean change in normalized sustained calcium currents from control conditions. Shortly after PMA was applied to a voltage-clamped cell the amplitude of the evoked calcium current steadily rose for ~2 min until it reached a plateau. B: same voltage-clamped cells were exposed to 1 nM staurosporine before application of PMA. In the presence of 1 nM staurosporine, all modulation caused by activation of PKC was eliminated and in fact the current was reduced from control levels in many cells similar to what was seen in Fig. 3. C: 1 nM staurosporine did not block the action of 8-CPT to modulate calcium current activity. Each data point represents the mean value obtained from 8 cells. Standard deviations are represented by the vertical error bars.

DISCUSSION

In this study, we have shown that signal transduction events mediated by two second-messenger systems can mod-
PL calcium current and simultaneously decreased the amplitude of the transient T calcium current. Modulation of these currents did not involve a shift in activation range.

Dopamine is the agent initiating modulation of calcium current activity. This is based on the finding that dopamine can activate either second-messenger system individually and from previous knowledge that dopamine’s modulatory action is eliminated in the presence of the D1 specific antagonist, SCH23390 (Pfeiffer-Linn and Lasater 1993, 1996a).

However, activation of multiple second-messenger systems through a single receptor type can be achieved if the receptor is coupled to different G proteins (Bylund 1992; Hosey 1992; Robb et al. 1994) or by activation of single receptor subtypes that are linked directly to multiple second-messenger systems (Thompson 1992). Therefore, we have proposed two receptor strategies for dopamine binding. In Fig. 9A, dopamine binds to two separate dopamine receptor subtypes to initiate activation of the two separate second-messenger systems. Previous studies have shown that in teleost horizontal cells, activation of adenylate cyclase increases cAMP levels and leads to activation of cAMP-dependent PKA. Activated PKA then phosphorylates a site(s) on each of the voltage-activated calcium channels. In this study, we have shown that the DAG second-messenger system also is activated by dopamine. This leads to activation of PLC, which cleaves PIP2 into DAG and IP3. DAG subsequently activates PKC to most likely phosphorylate sites on the two voltage-activated calcium channels (Ma et al. 1992).

The other possible pathway involves dopamine binding to a single dopamine receptor subtype, which activates two separate G proteins (Fig. 9B). One of the G proteins is linked to activation of adenylate cyclase and PKA, whereas the other G protein leads to activation of PLC. The net effect of either model increases the sustained voltage-activated calcium current and simultaneously decreases the transient calcium current.

The data presented in this paper support the idea that only
available for all dopamine receptors, and therefore it is not possible for us to evaluate whether dopamine is acting at multiple receptors linked to different second-messenger systems.

An alternative explanation as to how the multiple second-messenger systems are activated may involve cross-reactivity between the two pathways. Several different studies have demonstrated that activation of protein kinases associated with one second-messenger cascade can initiate activation of another second-messenger pathway (Bell et al. 1985; Fredholm et al. 1987; Laufer and Changeux 1989; Puurunen et al. 1987; Sugden et al. 1987). For instance, it may be that in white bass horizontal cells dopamine binds to a D1-type dopaminergic receptor that ultimately activates the cAMP-dependent PKA. Activation of PKA then could subsequently lead to activation of PLC, which would then cleave PIP2 into DAG and IP3, thus initiating the DAG arm of the second-messenger system (Bell et al. 1985; Laufer and Changeux 1989; Puurunen et al. 1987). However, we do not believe this to be the case in teleost horizontal cells as specific inhibition of one second-messenger pathway did not affect the modulation of calcium currents through activation of the other second-messenger system.

Modulation of calcium channel activity

We found that staurosporine, applied by itself to voltage-clamped cells, reduced the amplitude of the sustained calcium current in white bass horizontal cells. As low concentrations of staurosporine were found to be specific for PKC, the decrease in the sustained calcium current amplitude suggests that PKC is active under resting conditions and that activation of PKC occurs through an internal mechanism. Other electrophysiological studies of voltage-activated calcium currents also have demonstrated that the activity of calcium channels can be modulated in vivo by specific protein kinases (Armstrong and Eckert 1987; Kostyuk 1984; Reuter 1983; Tsien et al. 1972). But, if PKC is active under control conditions in white bass horizontal cells, why does initiation of the DAG second-messenger system with dopamine change the amplitude of the calcium currents further? One possible explanation of these results is that an internal regulatory mechanism exists that acts to limit PKC activation. Subsequent application of dopamine then fully activates the DAG second-messenger pathway and removes the limitations placed on PKC activation. Alternatively, although 1 mM staurosporine did not block the cAMP-dependent PKA, staurosporine still could inhibit another undiscovered protein kinase involved in modulation of calcium current activity. At this point, we have not ruled out either possibility.

Unlike PKC, PKA does not appear to be active under control conditions. However, because cAMP-dependent PKI was introduced through the patch pipette, control values for each cell could not be obtained. Therefore we cannot entirely rule out the possibility that PKA is active under control conditions and cAMP-dependent PKI’s action occurred before we could obtain a true calcium current measurement.

Dopaminergic receptor subtypes

At present, dopamine receptors are divided into two main groups, dopamine D1-like receptors and dopamine D2-like receptors. In the study of these receptors, molecular biological techniques have identified several structurally distinct receptor subtypes. Dopamine D1-like receptors are composed of D1 and D5 subtypes, whereas D2-like receptors are composed of D2A, D2B, D3, and D4 receptor subtypes (Jackson and Westlind-Danielsson 1994; Sokoloff and Schwarz 1995). However, antagonists are not generally

FIG. 9. Schematic diagram representing possible receptor binding strategies. Our data indicates that dopamine simultaneously activates 2 second-messenger systems to modulate differentially 2 voltage-activated calcium channels present in white bass horizontal cells. This could occur through one of 2 pathways. A: dopamine binds to 2 different dopamine D1 receptor subtypes. Each subtype is linked to activation of a separate second-messenger system. In the scheme illustrated in B, dopamine binds to a D1-type dopamine receptor that activates 2 separate G proteins. One G protein is linked to the cAMP second-messenger system, whereas the other G protein activates the DAG system. In the presence of dopamine, both of these proposed receptor binding strategies lead to an increase in the amplitude of the sustained current and a simultaneous decrease in the amplitude of the transient current. D1, D1-type dopaminergic receptor.
channels. Surprisingly, activation of either second-messenger system led to similar changes in calcium current activity. Why would such redundancy be built into a retinal neuron? We have ruled out the possibility that both systems must be activated for modulation to occur because modulation occurred even when one second-messenger pathway was blocked pharmacologically. Thus it appears that each individual second-messenger system contributes to a fraction of the total possible modulatory effect. As seen in Table 1, dopamine can increase the amplitude of the PL calcium current by a mean of 195% from control conditions. However, activation of neither the cAMP pathway nor the DAG pathway alone changed the amplitude of the calcium currents to the degree that dopamine did. To determine if simultaneous activation of both pathways produced the same magnitude of modulatory action on calcium currents as dopamine, we performed experiments using white bass Ringer containing 10 nM PMA as well as 50 μM 8-CPT (Table 1). Sustained calcium currents were generated before and after addition of the second-messenger mixture from six different voltage-clamped cells. Two minutes after the activators were applied, the mean increase in sustained calcium current activity rose 180% from control conditions which is statistically similar to the increase measured in the presence of dopamine.

Thus each second-messenger system in white bass horizontal cells contributes to the modulatory effect, but both second-messenger pathways must be activated to achieve the maximum magnitude of effect. Therefore the white bass horizontal cells have two distinct mechanisms available to them that helps regulate voltage-activated calcium currents. As these currents are thought to play important roles in horizontal cell physiology, it is likely that regulation of these channels underlie significant aspects of horizontal cell plasticity, such as that which might occur with light and dark adaptation (Rodrigues and Dowling 1990; Weiler et al. 1991; Yang et al. 1988).

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REFERENCES


KNAPP, A. G. AND DOWLING, J. E. Dopamine enhances excitatory amino


