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2-CHLOROADENOSINE REDUCES THE N CALCIUM CURRENT OF CULTURED MOUSE SENSORY NEURONES IN A PERTUSSIS TOXIN-SENSITIVE MANNER

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SUMMARY

1. The adenosine analogue 2-chloroadenosine (CADO) reduced the duration of calcium-dependent action potentials (CAPs) in mouse dorsal root ganglion (DRG) neurones in culture, by reducing voltage-activated calcium conductance (Macdonald, Skerritt & Werz, 1986). Using the single-electrode voltage clamp technique, we recorded three calcium current components in these neurones, the transient low-threshold (T), transient high-threshold (N) and slowly inactivating high-threshold (L) currents, as described previously (Nowycky, Fox & Tsien, 1985; Gross & Macdonald, 1987). CADO (100 μM) had no effect on the isolated T and L currents. In contrast, CADO reduced calcium currents evoked at clamp potentials positive to -20 mV from holding potentials (V_h) near the resting membrane potential; under these conditions, the calcium current consisted primarily of N and L calcium current components.

2. This effect of CADO was not voltage dependent. CADO reduced the magnitude of the calcium current without affecting the voltage dependence of the calcium current–voltage relation. In addition, similar reductions of calcium current were observed when currents were evoked from V_h of -60 or -80 mV.

3. In order to determine if a guanine nucleotide-binding (G) protein was involved in the CADO effect on calcium current, cultures were pre-treated with pertussis toxin (PT) for at least four hours. PT (100 ng/ml) reduced or abolished the CADO-induced reduction of CAP duration and calcium current.

4. Since CADO inhibits adenylate cyclase through the PT-sensitive G protein, G_i, we compared the effects of CADO and 8-Br-adenosine 3′,5′-cyclic-monophosphate (8-Br-cyclic AMP) on calcium current. The effect of 8-Br-cyclic AMP was voltage dependent, unlike that of CADO. 8-Br-cyclic AMP reduced calcium currents evoked from $V_h = -65$ mV, but had no effect on currents evoked from $V_h = -85$ mV.

5. We conclude that the adenosine agonist CADO reduced CAP duration in mouse DRG neurones by selectively reducing the N current component, and that the coupling between the adenosine receptor and the calcium channel required a PT-sensitive G protein. The CADO effect was unlikely, however, to be due to modulation of adenylate cyclase activity.

INTRODUCTION

Adenosine and the adenine nucleotides probably act as neurotransmitters in the peripheral and central nervous systems (e.g. Phillis & Wu, 1981; Williams, 1987). These substances are usually inhibitory and have been shown to reduce neurotransmitter release in a number of different preparations (e.g. Dunwiddie & Fredholm, 1984; Singh, Dryden & Chen, 1986). Adenosine and its analogues also have been shown to inhibit synaptosomal calcium influx (Wu, Phillis & Thierry, 1982) and to decrease the duration of calcium-dependent action potentials (CAPs) and the magnitude of voltage-activated calcium currents (Henon & McAfee, 1983; Macdonald *et al.* 1986; Dolphin, Forda & Scott, 1986). These observations are consistent with the idea that adenosine reduces neurotransmission through modulation of calcium current.

It has recently been shown, however, that multiple voltage-activated calcium current components exist in neurones (Nowycky *et al.* 1985; Fox, Nowycky & Tsien, 1987*a*, *b*; Gross & Macdonald, 1987), the transient low-threshold (T), the slowly inactivating high-threshold (L) and the transient high-threshold (N) calcium current components. It has not yet been determined which of these current components is affected by adenosine, or by what biophysical or biochemical mechanism(s).

Neurotransmitter receptors could be coupled directly to ion channels via G proteins, or indirectly, via second messenger systems. Recently G proteins have been demonstrated to regulate neurotransmitter modulation of potassium (Breitwieser & Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson & Hille, 1985) and calcium currents (Holz, Rane & Dunlap, 1986; Heschler, Rosenthal, Trautwein & Schultz, 1987). In addition, G protein involvement in adenosine agonist-induced inhibition of calcium current has been demonstrated utilizing non-hydrolysable GTP analogues; intracellular administration of GTP-γ-S enhanced, while GDP-β-S reduced, the adenosine effect (Scott & Dolphin, 1987). Adenosine has also been shown to inhibit or stimulate adenylate cyclase activity, presumably via G_i or G_s (Gilman, 1984), by binding to A_1 or A_2 receptors, respectively (Fredholm, Jonzon & Lindstrom, 1983). Previous work (Macdonald *et al.* 1986; Dolphin *et al.* 1986) suggested that the reduction of calcium current by adenosine was mediated through receptors that were most similar to the A_1 type. Thus, it is possible that adenosine could modify calcium channel activity directly via G proteins or indirectly by modulating cyclic AMP levels.

We therefore tested the effect of the adenosine analogue 2-chloroadenosine (CADO) on the T, L and N current components in mouse dorsal root ganglion (DRG) neurones in culture. The role of a G protein in regulating adenosine receptor-mediated effects on calcium currents was assessed using pertussis toxin (PT), an exotoxin from *Bordetella pertussis*, which inactivates G_i and G_o (Gilman, 1984; Hescheler *et al.* 1987). We also compared the effects of CADO and 8-Br-cyclic AMP to determine if the adenylate cyclase–cyclic AMP second messenger system was involved in the CADO-induced reduction of calcium current.

METHODS

Cell culture

Fetal DRG neurones were grown in culture with spinal cord neurones as previously described (Gross & Macdonald, 1987), except that the concentration of nerve growth factor in the plating medium was 100–500 ng/ml, and 10–200 ng/ml thereafter. Cultures were used for experiments after 4–12 weeks.

Intracellular recording of action potentials and single-electrode voltage clamp

Intracellular recordings were made at 35 °C in a recording solution of pH 7·3–7·4 and 320 mosM consisting of the following (in mM): NaCl, 142·4; KCl, 5·3; $CaCl_2$, 5·0; $MgCl_2$, 0·8; glucose, 5·6; Tris-base, 13·0; tetraethylammonium chloride (TEA-Cl), 5·0. Recording micropipettes (20–40 MΩ) were filled with 4 M-potassium acetate. Action potentials were recorded as previously described (Macdonald *et al.* 1986). Action potential duration was measured from the stimulus onset to the time when the original membrane potential was restored. After-hyperpolarizations were not included in the duration measurement.

For voltage clamp experiments recording micropipettes were filled with 3 M-CsCl. The recording solution consisted of the following (in mM): Tris-base, 13; choline chloride, 67; KCl, 5·0; $CaCl_2$, 2·0; $MgCl_2$, 0·8; glucose, 5·6; TEA-Cl, 100, at a pH of 7·3–7·4 and osmolarity of 320 mosM. Injection of the potassium channel blocker caesium into neurones from the recording micropipette and use of sodium-free, TEA-containing recording solution allowed recording of calcium currents virtually free of potassium or sodium currents. A single-electrode voltage clamp amplifier (Axoclamp-2, Axon Instruments, Burlingame, CA, USA) was used which switched between voltage sampling and current injection at 8–10 kHz with a 70% (recording)–30% (current injection) duty cycle. Headstage outputs were continuously monitored on a separate oscilloscope to ensure that voltage settling occurred prior to the voltage sample-and-hold measurement. Voltage step commands were generated by a microcomputer (IBM-XT) using the program pClamp (Axon Instruments, La Jolla, CA, USA). Current traces were sampled at 1·6–5·0 kHz, depending on the duration of the voltage step command (512 samples per sweep), and digitized, stored and analysed using this same program.

Leak currents were determined by applying hyperpolarizing voltage commands of magnitudes equal to the depolarizing commands used to evoke the inward currents being studied. These hyperpolarizing commands produced inward currents which were assumed to be equal and opposite in magnitude to leak currents. Alternatively, leak currents were recorded during depolarizing commands equivalent to those used to evoke inward currents after block of calcium currents by cadmium (100–200 μM). In either case, leak currents were subtracted digitally from the relevant inward currents to yield the calcium currents.

Voltage clamp recordings were made from neurones 40–50 μm in diameter. To obtain the best possible voltage clamp, neurones were selected that were nearly spherical and without large processes. If poor voltage clamp control occurred, currents were not included in the analyses.

Drug preparation and application

CADO (100 μM) and 8-Br-cyclic AMP (1 mM; both from Sigma Chemical Co., St Louis, MO, USA) were dissolved in recording medium and applied by pressure ejection (1·68–6·7 kPa) from micropipettes with tip diameters of 10–15 μm. Drug- or diluent-containing micropipettes were positioned approximately 20 μm from the cell body during the 1–2 s application, then removed to the oil phase. Micropipettes containing diluent had no effect on evoked action potentials, currents or holding currents. A concentration of 100 μM-CADO was used as this concentration was previously determined to elicit a maximum response (Macdonald *et al.* 1986).

Pertussis toxin pre-incubation

For some experiments neurones were pre-incubated with PT (Sigma Chemical Co.). Four microlitres of 50 μg/ml pertussis toxin in 0·1% bovine serum albumin (BSA) was added to neuronal cultures containing 2 ml of culture medium resulting in a concentration of 100 ng/ml PT. Control cultures received 4 μl of 0·1% BSA. Cultures were incubated for 4–16 h. Prior to intracellular recording, culture medium was exchanged for recording medium containing 100 ng/ml PT or diluent. Comparisons were made between treated and untreated cultures of the same plating.

Alternatively, the position of each neurone was marked following recording. The recording medium was then exchanged for growth medium plus PT or diluent as described above and the neurones were incubated 16–20 h. Each neurone was then relocated, and recordings made following reimpalement.

Statistics

Comparisons were made using Student's paired *t* test and Student's *t* test for correlated groups.

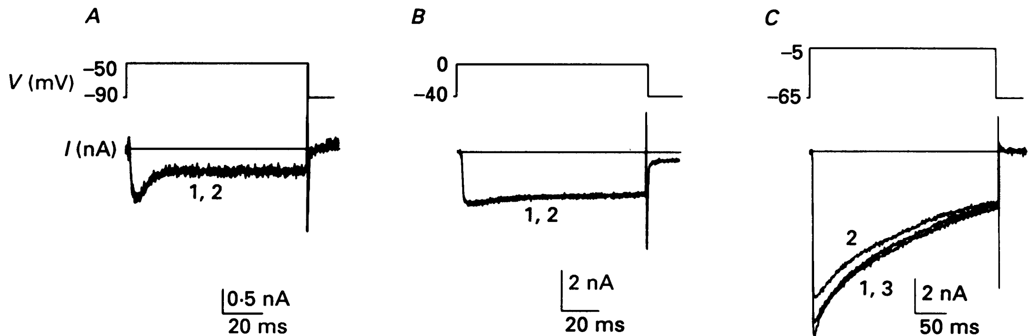

Fig. 1. CADO selectively reduced N current. The currents shown in *A* and *C* were recorded from the same neurone while those in *B* are from a different neurone. Illustrative voltage traces show the V_h and voltage step (mV). Digitized, leak-subtracted currents are shown; inward traces are downward. Current traces were obtained before (trace 1), immediately after (trace 2), and following recovery from (trace 3) application of 100 μM-CADO. The isolated T (*A*) and L (*B*) currents were unaffected by CADO. The peak calcium current was selectively reduced by CADO, suggesting an action on N current alone (*C*). Note in tracing *A* that the T current did not decay to the baseline. This was probably due to an overestimate of leak current or may reflect long-lasting T-type channel openings (Fox *et al.* 1987).

RESULTS

2-Chloroadenosine selectively reduced the N current in dorsal root ganglion neurones

The first series of experiments tested whether the CADO-induced reduction of voltage-activated calcium conductance in DRG neurones was due to a differential effect on the three calcium current components of these neurones. In the present studies, the isolated T current was 1·0–1·5 nA when evoked at -60 to -50 mV from $V_h = -90$ to -100 mV, and the isolated L current was 1·0 to 3·0 nA when evoked at -20 to 0 mV from $V_h = -50$ to -40 mV. The N and L current components were evoked together with commands positive to -20 mV from $V_h = -65$ mV. Using this protocol the N current component was estimated to be 5–15 nA (Fig. 1).

CADO (100 μM) had no effect on the isolated T (Fig. 1*A*) or L (Fig. 1*B*) currents. In contrast, CADO reduced the peak calcium current evoked positive to -20 mV from V_h near the resting membrane potential. There was a wide variability in the response rate and the extent of calcium current reduction among culture groups. In a typical culture group, CADO reduced the peak calcium current 10–38% (Fig. 1*C*) in twenty-two out of twenty-five neurones, with the mean reduction being 21 ± 2% ($\pm$S.E.M., $n = 22$). This effect of CADO was rapid in onset (within 5 s) and completely reversed in 1–3 min (Fig. 1*C*).

This laboratory has previously demonstrated that CADO reduced calcium currents evoked at potentials positive to −50 mV and had no effect on the voltage dependence of the calcium current–voltage relation (Macdonald *et al.* 1986). This observation was confirmed in the present study in three neurones in which commands were applied from V_h = −80 mV in the presence and absence of 100 μM-CADO (data not shown). In addition, CADO reduced peak calcium current to a similar extent whether currents were evoked at −20 mV from V_h = −80 or −60 mV.

Pertussis toxin pre-incubation prevented the 2-chloroadenosine-induced reduction of calcium-dependent action potential duration

Previous work in this laboratory has established that the CADO-induced reduction of CAP duration was due to a decrease in calcium current (Macdonald *et al.* 1986). Because the responsiveness of the neurones to CADO varied among culture lots, we used CAP duration as an assay of the responsiveness of culture groups to CADO. This technique allowed large numbers of neurones to be sampled, and was therefore employed in the initial experiments testing the effect of PT pre-incubation on CADO responses.

Two experiments were done to test the effect of CADO (100 μM) on CAP duration following pre-incubation of DRG neurones with PT. In the first experiment, paired cultures from the same plating were treated with 100 ng/ml PT or control medium for 4 or 16 h, and the effect of CADO on CAP duration was determined. In control cultures from the 4 h treatment protocol, thirteen of twenty-three (57%) neurones responded to CADO with a mean decrease in CAP duration of 30±5% (±S.E.M.). In the cultures treated for 4 h with PT only seven of twenty-four (29%) neurones responded to CADO with a mean reduction in CAP duration of 20±6% (±S.E.M.). In control cultures from the 16 h treatment protocol, forty-five of sixty-nine (65%) neurones responded to CADO with a mean reduction in the CAP duration of 36±3% (±S.E.M.). In cultures treated with PT for 16 h, none of the thirty-three neurones tested responded to CADO with a reduction in CAP duration.

In a second experiment, thirty DRG neurones in which CADO (100 μM) elicited a reduction in CAP duration were identified, and their positions in the culture dish were marked as described in Methods. These DRG neurones were then treated with 100 ng/ml PT or control medium for 4 or 16 h. Of these neurones, three control and five PT-treated neurones were viable following treatment. The neurones were then reimpaled and CAPs evoked. The CADO-induced reduction in CAP duration which ranged from 20 to 40% in control neurones was unchanged following reimpalement (Fig. 2*A*). The CADO-elicited decrease in CAP duration was reduced from 57 to 20%, a 65% reduction, in a neurone treated for 4 h with PT. In four neurones in which the CADO-elicited decrease in CAP duration ranged from 10 to 28%, CADO no longer elicited a decrease in CAP duration following 16 h of PT treatment (Fig. 2*B*).

Pertussis toxin prevented the 2-chloroadenosine-elicited reduction of calcium current

After establishing that PT pre-treatment blocked the CADO-elicited reduction of CAP duration, we used the voltage clamp technique to test if PT pre-treatment blocked the CADO-elicited reduction of calcium current. Paired cultures from the same plating were treated with 100 ng/ml PT or control medium for 7 or 16 h.

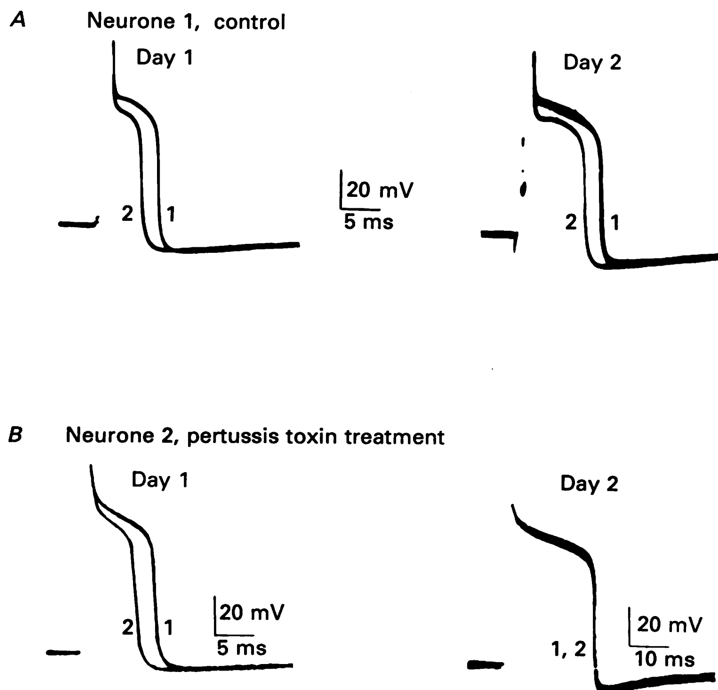

Fig. 2. PT pre-treatment blocked the CADO-induced reduction in CAP duration. CAPs were evoked every 15 s prior to (trace 1) and subsequent to (trace 2) application of 100 μM-CADO. *A*, CAPs recorded from the same neurone before (day 1) and after (day 2) a 16 h treatment with control medium. *B*, CAPs were recorded from a different neurone before (day 1) and after (day 2) a 16 h treatment with PT-containing (100 ng/ml) medium. PT treatment blocked the ability of CADO to reduce the CAP duration. The increase in the duration of the CAP following PT treatment (as shown) was not usually observed.

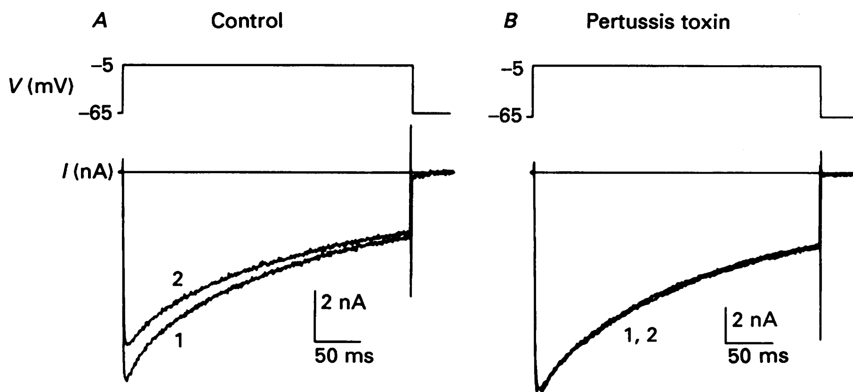

Fig. 3. PT blocked CADO-elicited reduction of calcium current. Digitized, leak-subtracted currents from two neurones are shown. Currents are shown before (trace 1) and after (trace 2) a 2 s pressure ejection of CADO (100 μM). CADO reduced calcium current in a neurone treated for 16 h with control medium (*A*) but had no effect on calcium current in a neurone treated for 16 h with 100 ng/ml PT (*B*).

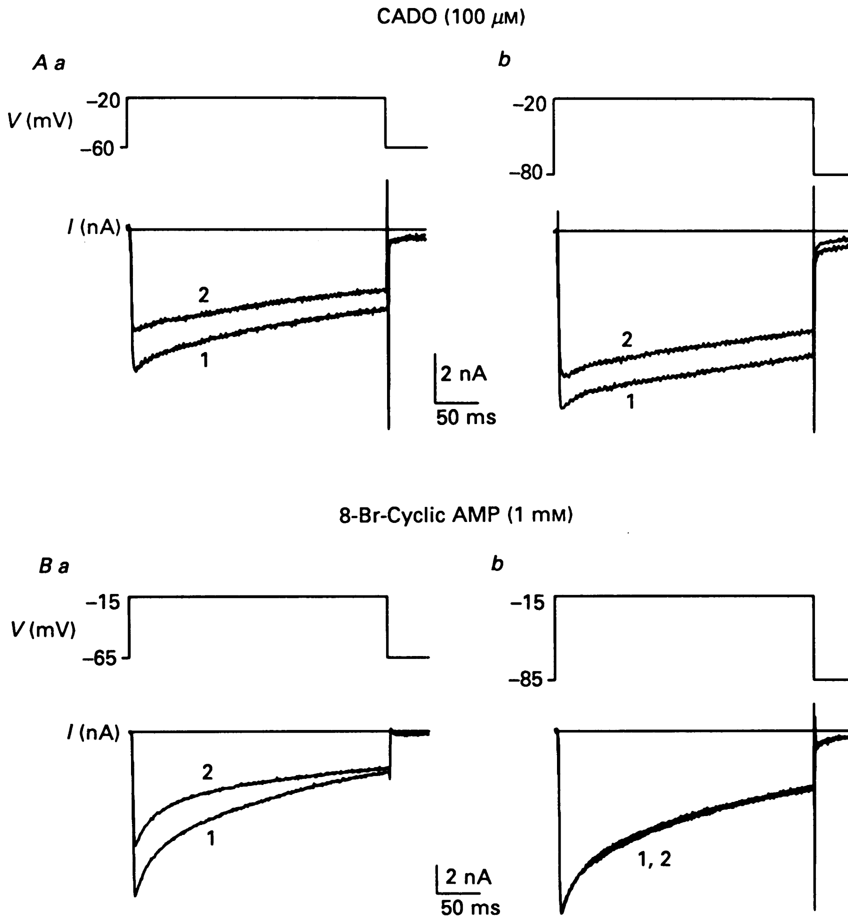

Fig. 4. CADO reduced peak calcium current in a voltage-independent manner while 8-Br-cyclic AMP reduced peak calcium current in a voltage-dependent manner. The currents shown in *Aa* and *b* are from the same neurone while those in *Ba* and *b* are from two different neurones. Digitized, leak-subtracted currents are shown. Currents are shown before (trace 1) and after (trace 2) a 2 s pressure ejection of CADO (100 μM; *A*) or 8-Br-cyclic AMP (1 mM; *B*). CADO reduced peak calcium current equally when evoked from $V_h = -60$ mV (*Aa*) or -80 mV (*Ab*). 8-Br-cyclic AMP reduced peak calcium current when evoked from $V_h = -65$ mV (*Ba*) but not from $V_h = -85$ mV (*Bb*).

Calcium currents were evoked with 300 ms depolarizing commands to -5 mV from $V_h = -65$ mV. In PT-treated neurones, the reduction of peak calcium current by 100 μM-CADO was less than that in control neurones (Fig. 3). CADO reduced peak calcium current in control neurones 22 ± 2 % ($\pm$S.E.M., $n = 14$) and PT-treated neurones 7 ± 2 % ($\pm$S.E.M., $n = 15$; significantly different from control, $P < 0.05$). The reduction in peak calcium current induced by CADO in PT-treated cells was not different from that seen after application of diluent, 6 ± 1 % ($\pm$S.E.M., $n = 4$).

8-Br-Cyclic AMP reduced peak calcium current in a voltage-dependent manner

The above experiments showed that a PT-sensitive G protein was required to mediate the reduction of N current by CADO. One possible pathway would be through G_i-mediated inhibition of adenylate cyclase. If this were the case, it would be expected that an increase in cyclic AMP would have the opposite effect of CADO. To test this, we compared the effect of CADO to that of 8-Br-cyclic AMP on calcium currents in DRG neurones.

CADO (100 μM) reduced peak calcium current to a similar extent when evoked at -20 mV from $V_h = -60$ or -80 mV, 21 ± 2% ($n = 22$) and 19 ± 3% ($n = 5$), respectively (Fig. 4*A*). In contrast, 8-Br-cyclic AMP reduced peak calcium current 24 ± 6% ($n = 4$) when evoked at -15 mV from $V_h = -65$ (Fig. 4*Ba*) but had no effect on currents evoked from $V_h = -80$ ($n = 10$, Fig. 4*Bb*). The reduction of calcium current evoked from $V_h = -65$ mV was maximal 3–5 min after the application of 8-Br-cyclic AMP.

DISCUSSION

The present study confirms and extends the previous report that CADO shortened CAP duration and reduced a voltage-dependent calcium conductance in mouse DRG neurones (Macdonald *et al.* 1986). CADO had no effect on isolated T or L currents, but reduced calcium currents containing the N current component, suggesting that its effect was due to a selective reduction of the N current. A similar effect of CADO in hippocampal CA3 neurones has been reported in preliminary form by Madison and his colleagues (1987). CADO did not alter the voltage dependence of the calcium current–voltage relation, the apparent time dependence of current activation, or the rate of N current inactivation. In addition, the peak calcium current was reduced to a similar degree by CADO when neurones were held at $V_h = -60$ to -80 mV, suggesting that CADO did not alter the voltage dependence of steady-state inactivation of N current. It is nevertheless possible that CADO reduced calcium currents by increasing steady-state inactivation, i.e. by causing a negative shift in the steady-state inactivation curve. This shift would have been greater than -15 mV to account for the similarity in current reduction at V_h of -65 and -80 mV, however. Clearly, further experiments will be needed, perhaps including single-channel current analysis, to unequivocally determine the biophysical mechanism of calcium current reduction by CADO.

Previous work has demonstrated the involvement of a G protein in the CADO-induced inhibition of cortical neurones (O'Regan & Phillis, 1987) and the reduction of calcium current (Scott & Dolphin, 1987). The present studies confirm and extend these observations by showing that the selective effect of CADO on calcium current components was mediated through a PT-sensitive G protein. PT ADP-ribosylates both G_i, which links neurotransmitter receptors to adenylate cyclase in an inhibitory manner, and G_o, a neuronal G protein recently shown to link neurotransmitter receptors to calcium channels (Hescheler *et al.* 1987). PT renders G_i non-functional and has a similar effect on G_o (Gilman 1984; Sternweis & Robishaw, 1984; Ewald, Sternweis & Miller, 1988). Either of these G proteins could, therefore, potentially mediate the effect of CADO on N current.

If G_i mediated the CADO-induced reduction of calcium current, than it could do so by inhibiting adenylate cyclase and reducing cyclic AMP levels. We consider this to be unlikely, however, since the action of 8-Br-cyclic AMP on calcium current was similar, rather than opposite, to that of CADO. Furthermore, while 8-Br-cyclic AMP also reduced N current selectively (Gross & Macdonald, 1988, 1989), it apparently did so by a mechanism different to that of CADO – the reduction of N current by 8-Br-cyclic AMP was voltage dependent while the reduction produced by CADO was not. These results are consistent with the idea that the reduction of N current by CADO may be mediated by G_i, and presumably by associated A_1 receptors, but that this effect was probably not mediated by modulation of cyclic AMP levels (cf. Dunwiddie & Fredholm, 1984).

Alternatively, a PT-sensitive G protein could link the adenosine receptor directly to the calcium channel. This type of mechanism has been described for the coupling of adenosine and cholinergic receptors to potassium channels (Kurachi, Nakajima & Sugimoto, 1986; Yantani, Codina, Brown & Birnbaumer, 1987). G proteins also couple a variety of transmitter receptors to calcium channels (e.g. Dolphin & Prestwich, 1985; Holz, Rane & Dunlap, 1986; Hescheler *et al.* 1987; Wanke, Ferroni, Malgaroli, Ambrosini, Pozzan & Meldolesi, 1987; Ewald *et al.* 1988). Further study will be required to determine the identity of and the mechanism by which the G protein mediates the CADO-induced reduction of N current.

The reduction of N current by adenosine could have important short- and long-term effects on neuronal function. Voltage-activated calcium channels not only contribute to neuronal depolarization, but allow the influx of calcium which can, in turn, activate potassium conductance (Meech, 1978), activate second messenger system components (Berridge & Irvine, 1985; Johnson & Mills, 1986) and initiate neurotransmitter release.

Thus, the reduction of N current by adenosine or its analogues could reduce neuronal excitability and modify other calcium-dependent neuronal processes. Of interest in this regard are other reports demonstrating effects of other compounds on N-type currents, including the neurotransmitters acetylcholine (Wanke *et al.* 1987), dynorphin A (Gross & Macdonald, 1987), neuropeptide Y (Ewald *et al.* 1988; Wiley, Gross, Fox & Macdonald, 1988), noradrenaline acting at α-receptors (Lipscombe & Tsien, 1987), and activators of second messenger systems (Gross & Macdonald, 1988, 1989). Furthermore, N current has recently been demonstrated to have a dominant role in evoked release of noradrenaline from sympathetic neurones (Hirning, Fox, McCleskey, Olivera, Thayer, Miller & Tsien, 1988). Adenosine inhibits the release of a variety of neurotransmitters from neuronal preparations (Fredholm & Hedqvist, 1980; Hollins & Stone, 1980; Phillis & Wu, 1981), an effect that has been shown to be blocked by PT in cerebellar and cortical neurones (Dolphin & Prestwich, 1985; Fredholm, Fastbom & Lindgren, 1986). As N current is the predominant calcium current in DRG neurones, our data would be consistent with the proposal that adenosine reduces neurotransmitter release through a reduction of N current that is mediated by a PT-sensitive G protein.

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