THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 42, Issue of October 16, pp. 27703–27707, 1998

Printed in U.S.A.

Regulation of Adenylyl Cyclase by Membrane Potential*

(Received for publication, June 1, 1998, and in revised form, July 6, 1998)

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Mammalian adenylyl cyclases possess 12 transmembrane-spanning domains and bear a superficial resemblance to certain classes of ion channels. Some evidence suggests that bacterial and sea urchin sperm adenylyl cyclases can be regulated by membrane depolarization. In the present study, we explored the effect of altering membrane potential on the adenylyl cyclase activity of cerebellar granule cells with acute potassium depolarization. A biphasic stimulatory and then inhibitory response is evoked by progressive increases in the extracellular [K]:[Na] ratio in the absence of extracellular Ca^{2+}. This effect does not mimic the linear increase in membrane potential elicited under the same conditions. Instead it appears as though membrane depolarization opens L-type (nimodipine-sensitive) Ca^{2+} channels, allowing the entry of Na^+, which directly stimulates adenylyl cyclase activity. Gramicidin, which generates pores that are permeable to monovalent cations, and concurrently eliminates the membrane potential, permits a similar stimulation by extracellularly applied Na^+. Although the results indicate no direct sensitivity of cerebellar granule cell adenylyl cyclase to membrane potential, they do demonstrate that, as a result of membrane depolarization, the influx of Na^+, as well as Ca^{2+}, will elevate cAMP levels.

Now that detailed structural information is emerging on parts of the catalytic regions of adenylyl cyclase that reside in the cytosol (1, 2) the complexity of the membrane-inserted portion of the enzyme stands out as an unresolved mystery. All cloned adenylyl cyclases possess 12 putative transmembrane-spanning domains (3). As originally noted by Krupinski *et al.* (4), these structures closely resemble the ATP-binding cassette superfamily of transporters, which include the cystic fibrosis transmembrane conductance regulator and P-glycoprotein (5). However, unlike ion channels, the transmembrane-spanning domains of the adenylyl cyclases possess few charged amino acids, nor do they share detailed homology among family members. The puzzle of the function of the membrane components is accentuated by the fact that parts of the cytosolic domains of adenylyl cyclases, when expressed separately from the membrane segments, can be regulated by G-protein subunits and forskolin (6, 7). Some years ago Schultz *et al.* (8) showed that adenylyl cyclase from *Paramecium* was stimulated by membrane depolarization. More recently, cAMP accumulation in sea urchin (*Lytechinus pictus*) sperm was also found to be stimulated in response to membrane depolarization (9). Both of these findings are quite tantalizing within the context of the transmembrane organization mentioned above. However, no structural information has yet emerged on the adenylyl cyclases from these sources, and indeed, it is clear that these cyclases differ in significant regulatory properties from mammalian enzymes, so whether they share the structural features of the mammalian adenylyl cyclases is unknown. A more recent report observed that as a result of long term (0.5 h) depolarization of cerebellar granular cells in the absence of extracellular Ca^{2+}, cAMP accumulation was elevated; this led the authors to suggest that the endogenous adenylyl cyclase of granule cells was voltage-sensitive (10). While voltage sensitivity was an attractive interpretation of those data, a number of other possibilities could also have been envisaged.

In the present study we have characterized the expression and regulation of adenylyl cyclase activity in primary cultured cerebellar granular cells. Adenylyl cyclase immunoreactivity was localized on or near the plasma membrane throughout the cell bodies, axons, and dendrites of these cells. In axons, cyclase immunoreactivity appeared clustered and concentrated on the growth cone. Such localization, coincident with voltage-sensitive Ca^{2+} channels (11), indicates that adenylyl cyclase is well positioned to respond to neuronal depolarization. We then explored cAMP accumulation in these cells in response to short term depolarization. We found that, indeed, when membrane depolarization acted as a driving force for Na^+ or Ca^{2+} entry, cAMP accumulation was stimulated. In the absence of Ca^{2+}, Na^+ entered the cells in response to membrane depolarization via nimodipine-sensitive Ca^{2+} channels. However, membrane depolarization by itself did not affect adenylyl cyclase activity. A similar stimulation was achieved by the pore-forming agent, gramicidin, in the presence of increasing concentrations of extracellular Na^+, where no change in membrane potential would be expected. Although these results demonstrate that the adenylyl cyclase activity of cultured granule neurons is not sensitive to membrane potential *per se,* they do indicate that as a result of membrane depolarization under normal circumstances the influx of Na^+, as well as Ca^{2+}, will elevate cAMP levels.

EXPERIMENTAL PROCEDURES

Antibody and Western Blots—The antiserum raised against a C-terminal region common to adenylyl cyclases has been described (12, 13). For immunoblot analysis, 15 μg of particulate protein prepared from either whole cerebellum or phosphate-buffered saline-washed granule cell cultures was loaded per lane, separated on 8% gels, and then transferred to nitrocellulose. Antiserum was diluted 1:2000 and incubated with the blot overnight at 4 °C before visualization with chemiluminescence.

Cell Culture—We used the method of Slesinger and Lansman (14), with slight modifications. Growth media consisted of minimum essential media (Life Technologies, Inc.) containing 2 mM L-glutamine, Earle's salts, 10% fetal bovine serum, 25 mM KCl, and penicillin/streptomycin. Rat pups aged 6–9 days were euthanized by an overdose of

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§ Supported by National Research Service Award Fellowship F32 MH11609.

¶ Supported by a Medical Research Council Project Grant.

‖ Supported by the Royal Society.

CO_2, followed by decapitation. Whole cerebella were removed, washed with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, digested in 2.5% trypsin for 10 min, washed, and then gently triturated through three fire-polished pipettes of decreasing diameter in the presence of pancreatic deoxyribonuclease (Sigma, Poole, UK; 2000 Kunitz units/ml). The cell suspension was plated on poly-D-lysine-coated plastic six-well dishes at 5×10^6 cells/well for cAMP determinations or onto glass coverslips at 2×10^5 cells/coverslip for fluorescence immunocytochemistry. Cytosine arabinoside (Sigma, 10 μM) was added once to cells 3 days after plating to kill dividing cells. One-half of the media was replaced on days 3 and 6, and cells were used for experiments 6–8 days after plating. To characterize the cultures at this age, cells were fixed as described for immunofluorescence and stained with the pan-cyclase antibody (1:4000) using the immunoperoxidase method and then examined under phase-contrast microscopy to assess cell types. Cyclase-positive granule neurons constituted 97 ± 2% of the cells. About 1% of the cells were astrocytes, which also stained intensely for adenylyl cyclase in both their cell bodies and distal processes. The remaining cells were unidentified and stained only weakly.

Immunofluorescence Localization of Adenylyl Cyclase—All steps were carried out at room temperature. Cells grown on coverslips were washed twice in Dulbecco's phosphate-buffered saline containing Ca^{2+} and Mg^{2+} and then fixed in 4% freshly depolymerized paraformaldehyde, 0.1 M sodium phosphate, pH 7.4, for 1 h. Coverslips were then washed extensively, quenched in 0.25% NH_4Cl for 10 min, and permeabilized in 0.5% Triton X-100 for 5 min. All subsequent steps were carried out in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. Cells were blocked for 15 min in 4% normal goat serum and incubated for 1 h with the pan-cyclase antiserum (1:500). Visualization was with fluorescein isothiocyanate-coupled anti-rabbit Fab fragments (Jackson ImmunoResearch, West Grove, PA). Black and white micrographs of areas of interest were digitally scanned and presented using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Measurement of cAMP Accumulation—cAMP accumulation in intact cells was measured according to the method of Evans *et al*. (15) as described previously (16) with some modifications. [2-^{3}H]Adenine (Amersham International, Little Chalfont, UK; 1.5 μCi/well) was added to the culture medium of cerebellar neurons on 24-well plates to label the ATP pool (2 h, 37 °C). The cells were then washed once and incubated with a nominally Ca^{2+}-free Krebs buffer containing 135 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 11.5 mM glucose, 11.6 mM HEPES, and 0.1 mM EGTA adjusted to pH 7.4 with 2 M Tris base (900 μl/well). (A background Ca^{2+} concentration of 10 μM in such a buffer would yield a free Ca^{2+} concentration of 5 nM.) All experiments were carried out in triplicate at 37 °C in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (400 μM, Sigma), which was preincubated with the cells for 10 min prior to a 2-min assay. Cells were assayed in the presence of forskolin (Calbiochem, Nottingham, UK).

$[Ca^{2+}]_i$ Measurements—$[Ca^{2+}]_i$[1] was measured in populations of cerebellar neurons plated on 9×22-mm glass coverslips, loaded with 2 μM fura-2-AM (Molecular Probes, Eugene, OR) at room temperature as described (17). The coverslips were mounted vertically in a polymethylmethacrylate cuvette with 2.5 ml of a Ca^{2+}-containing Krebs buffer (as described under "cAMP Accumulation") in a Perkin-Elmer LS50 spectrofluorimeter with the coverslip at a 30° angle to the incident light. Solution changes were effected by perfusing fresh solution into the bottom of the cuvette while continuously removing solution from the top of the cuvette. Estimation of the $[Ca^{2+}]_i$ was performed as described previously using the ratio of 510 nm emission values arising from alternating excitation at 340 and 380 nm (17).

Electrophysiology Methods—Culture medium was removed from the cells by washing three times in extracellular solution containing (mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES-NaOH, pH 7.4. Glass microelectrodes (Clarke Electromedical Instruments, Pangbourne, UK) of 5 MΩ resistance were fabricated on a Flaming and Brown P90 pipette puller (Sutter Instruments) and filled with intracellular solution containing (mM): 140 KCl, 5 NaCl, 1 $MgCl_2$, 0.1 EGTA, 10 HEPES-KOH, pH 7.2. All current recordings were made using an Axopatch 1D patch clamp amplifier (Axon Instruments) and acquired onto computer using a National Instruments PC1200 A/D interface (National Instruments, Newbury, UK) and V-Clamp software (Strathclyde, UK, John Dempster). Cell-attached patches of 2–10 GΩ resistance were routinely produced, and breakthrough to whole cell was monitored under voltage clamp with 20-mV imposed voltage steps. Immediately after formation of a whole cell, as judged by the increase in capacitance transients, the cells were held under current clamp and the membrane potential recorded. Extracellular K^+ was raised by 1:1 substitution with Na^+. Complete and rapid solution changes were effected by removal of the bathing medium and a 3-ml wash in new solution.

[1] The abbreviations used are: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; NMDG, *N*-methyl-D-glucamine.

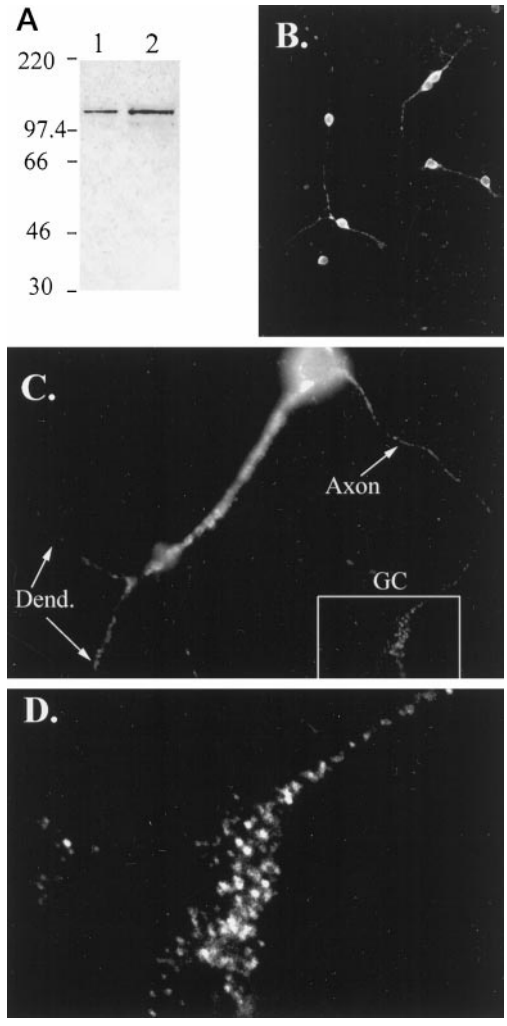

FIG. 1. **Localization of adenylyl cyclase in cerebellum using a pan-adenylyl cyclase antibody.** *A*, Western blot analysis of particulate preparations from whole cerebellum of a 300-g adult rat (*lane 1*) or cerebellar granule cells that were isolated from rats at postnatal day 8 and grown for 8 days before harvesting (*lane 2*). Each lane was loaded with 15 μg of protein. *B*, pattern of immunolabeling in granule cells. *C*, detail of a granule cell showing immunoreactivity in the cell body, dendrites (*Dend.*), axon (*Axon*), or growth cone (*GC, boxed*). *D*, localization of adenylyl cyclase to punctate structures on the growth cone.

RESULTS

Adenylyl Cyclase Expression in Primary-cultured Cerebellar Granule Cells—Using a pan-specific antibody (18), adenylyl cyclase immunoreactivity was seen to be enriched in homogenates of cerebellar granule cell cultures relative to whole cerebellum (Fig. 1*A*). Based on earlier functional analyses, this species would be expected to be predominantly Ca^{2+}-stimulable (19), and from *in situ* hybridization analysis, it would be expected to be AC1 rather than AC8 (20, 21). At the individual cell level, a very discrete labeling pattern was apparent. Adenylyl cyclase immunoreactivity was most prominent on the soma, but was also abundant on both axonal and dendritic processes (Fig. 1*B* and *C*). While the immunoreactivity on the soma and dendrites was distributed diffusely, on axons and on growth cones we observed a much more punctate pattern, with a remarkable concentration in discrete boutons on developing presynaptic terminals (Fig. 1*D*). Growth cones contain clusters

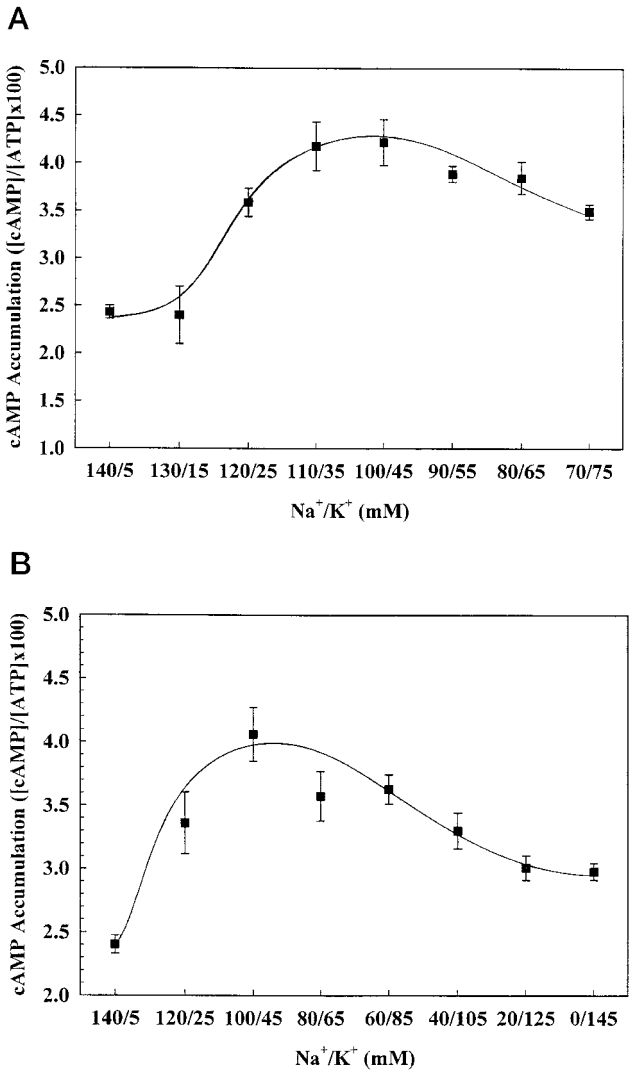

FIG. 2. **Effect of increasing the ratio of extracellular KCl to NaCl on cAMP accumulation by cerebellar neurons.** Cerebellar granular cells were exposed to the combinations of NaCl and KCl indicated (in mM) in Ca^{2+}-free Krebs buffer for 2 min in the presence of 10 μM forskolin. *A*, the [NaCl][:KCl] ratio was altered in 10 mM increments to 70:75. *B*, the [NaCl]:[KCl] ratio was modified in 20 mM increments to 0:145. cAMP accumulation was determined as described under "Experimental Procedures." (The osmolality of these various solutions was ~300 mosmol.)

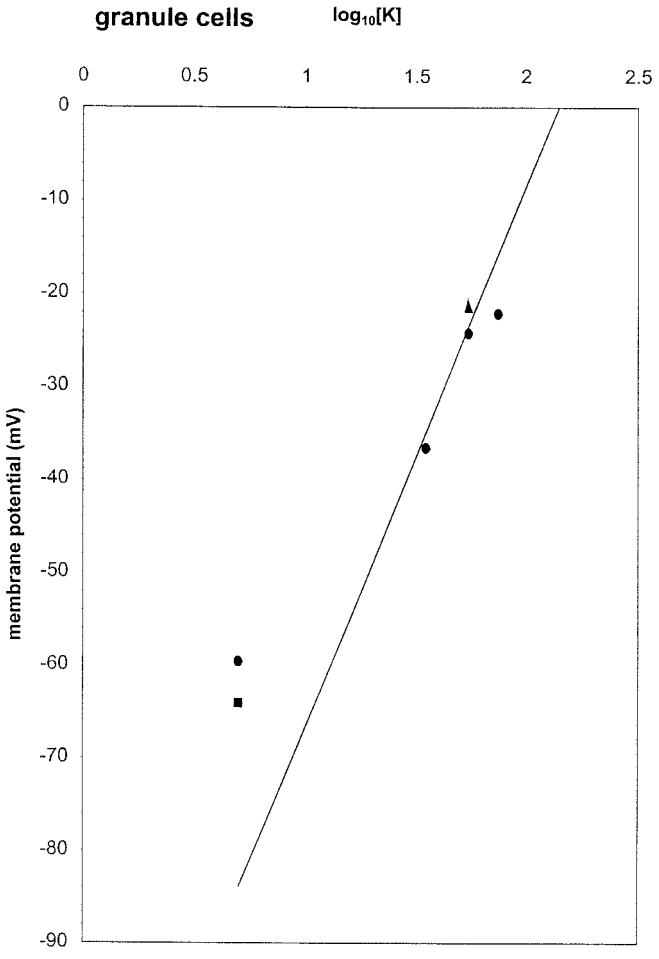

FIG. 3. **Measurement of resting membrane potential of single granule cells over a range of extracellular K^+ concentrations.** Whole cell current clamp recording of the membrane potential shows a roughly linear relationship between extracellular K^+ and membrane potential (*circles*). This relationship is approximated by the Nernst equation (*drawn line*). We have not made any attempt to fit the data to take account of Cl^- and Na^+ permeabilities. Neither Ca^{2+}-free (*squares*) nor the presence of 10 μM nimodipine (*triangles*) in the extracellular solution affect the membrane potential.

of voltage-activated Ca^{2+} channels (11), and Ca^{2+}-activated adenylyl cyclases located there would be expected to respond readily to depolarization. Indeed, Ca^{2+}-activated adenylyl cyclase activity has been implicated in the control of growth cone guidance (22).

Cerebellar Granular Neuron cAMP Accumulation Is Sensitive to Increasing Extracellular K^+—The simplest means of globally elevating membrane potential in a population of neurons is to increase extracellular [KCl] relative to [NaCl]. Cerebellar granular neurons were exposed to a range of progressively increasing extracellular K^+ concentrations in the absence of Ca^{2+}, by substituting KCl for NaCl in the bathing medium. As extracellular K^+ was increased in 10 mM increments, cAMP accumulation steadily increased. However, beyond 45 mM, the stimulation began to diminish (Fig. 2*A*). When K^+ concentrations were increased in 20 mM increments up to 145 mM, a clearly biphasic response was evident, resulting in almost no stimulation relative to 5 mM K^+ at the highest concentration (Fig. 2*B*). The initial stimulation at lower K^+ levels could be interpreted to reflect a voltage-dependent effect, since membrane potential is expected to depolarize as a function of a K^+ gradient. Since no Ca^{2+} is present in the bathing medium, the observed stimulation cannot reflect the Ca^{2+}-dependent stimulation of the endogenous Ca^{2+}-stimulable adenylyl cyclase. Indeed, this effect mimics rather closely what had been reported previously over a more protracted time course (30 min; Ref. 10) and which was interpreted to reflect a voltage sensitivity of the adenylyl cyclase. However, the diminution in the effect with higher [K^+] is not consistent with what would be expected to be a steady depolarization of the membrane potential with increasing [K^+]. (Indeed when the inert *N*-methyl-D-glucamine is substituted for NaCl, no significant stimulation of cAMP accumulation was observed as a function of increasing extracellular [K^+]; results not shown.) To confirm our assumptions with regard to the effect on membrane potential of increasing extracellular K^+, we measured the resting membrane potential in single cells over a range of extracellular [K^+] (Fig. 3).

Membrane Potential of Granule Cells Increases as a Function of Increasing [KCl]/[NaCl]—Whole cell membrane potentials of cerebellar granular cells were measured under current clamp with various extracellular [K^+] as described under "Experimental Procedures." An approximately linear relationship was observed between membrane potential and extracellular K^+ (Fig. 3), which approximated the prediction of the Nernst

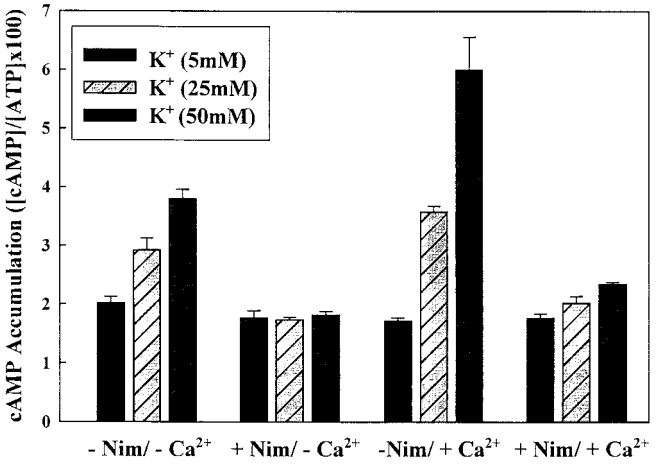

FIG. 4. **Nimodipine blocks both Ca^{2+}-dependent and Ca^{2+}-independent stimulation of cAMP accumulation in response to membrane depolarization.** Cerebellar granular cells were exposed to the concentrations of KCl indicated, along with NaCl to yield a total monovalent cation concentration of 145 mM. $CaCl_2$ (1 mM) and (10 μM) nimodipine were also included where indicated. Assays were conducted in the presence of forskolin (5 μM) for 2 min.

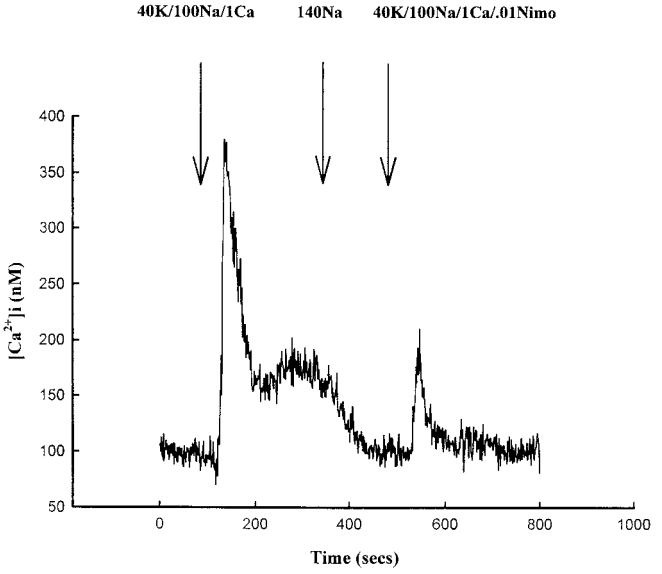

FIG. 5. **Depolarization of cerebellar granule cells induces a $[Ca^{2+}]_i$ rise that is attenuated by nimodipine.** Cerebellar granular cells on coverslips were loaded with fura-2-AM as described under "Experimental Procedures." The cells were then equilibrated in a spectrofluorimeter cuvette in Ca^{2+}-free Krebs for 2 min, after which they were perfused with Krebs containing 40 mM KCl/100 mM NaCl and 1 mM $CaCl_2$, where shown (*first arrow*). Perfusion with 140 mM NaCl-containing Krebs (*second arrow*) brought the $[Ca^{2+}]_i$ to basal. Perfusion of a Krebs solution containing 40 mM KCl/100 mM NaCl/1 mM $CaCl_2$ and 10 μM nimodipine (*third arrow*) yielded a very muted response, although without nimodipine the second response would have been virtually identical to the first (not shown).

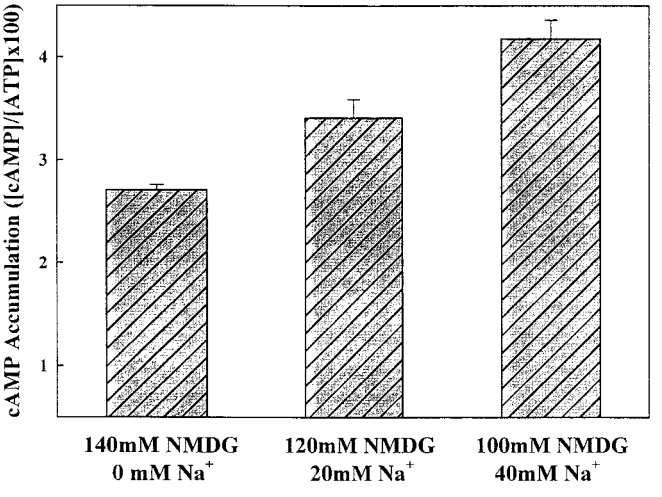

FIG. 6. **Gramicidin treatment permits direct stimulation of adenylyl cyclase by Na^+.** Cerebellar granular cells were pretreated with gramicidin (4 μM) in a Krebs buffer made up without NaCl, but with 140 mM NMDG, for 10 min. The cells were then exposed to the indicated combinations of NMDG and NaCl for 2 min in the presence of 5 μM forskolin with 400 μM 3-isobutyl-1-methylxanthine, and cAMP accumulation was assessed.

equation (*solid line*) assuming K^+ to be the only charge carrier. Neither the absence of Ca^{2+} nor the presence of the L-type Ca^{2+} channel blocker, nimodipine, in the bathing medium exerted any significant effect on the measured membrane potential (Fig. 3). Clearly, given the time required in making the various membrane potential measurements, the potentials established by the various extracellular $[K^+]$ are sustained for extended periods of time, including the duration of the cAMP determinations. However, a paradox is apparent in assuming a simple relationship between membrane potential and adenylyl cyclase activity, given that the effect of increasing extracellular $[K^+]$ on membrane potential is linear, but the consequence for adenylyl cyclase is biphasic. Thus, other mechanisms for the effect of increasing extracellular $[K^+]$ on adenylyl cyclase were considered.

L-type Ca^{2+} Channel Blockers Attenuate the Effect of Increasing KCl on cAMP Accumulation—We considered the possibility that the effect of increasing membrane potential on adenylyl cyclase actually reflected the entry of Na^+ into the neurons. We reasoned that in the presence of extracellular Ca^{2+}, as membrane potential is increased, voltage-activated Ca^{2+} channels gate mainly Ca^{2+}, allowing it to flow into neurons. However, in the absence of extracellular Ca^{2+}, Na^+ would be the predominant charge carrier through these channels, since at low divalent cation concentrations, monovalent cations permeate efficiently through Ca^{2+} channels (23). As extracellular $[K^+]$ is increased relative to $[Na^+]$ (*e.g.* beyond 80 mM), the driving force for Na^+ decreases, and less of the cation would enter the cell. Thus, the consequences of incremental increases in the extracellular ratio of [K]:[Na] from 0.03 to 6.25 (*viz.* 5:145 to 125:20) would be a biphasic response, an increase and then a decrease in intracellular Na^+. To explore this possibility, we examined the effect of the L-type Ca^{2+} channel blocker, nimodipine, on the cAMP accumulation in response to an elevated extracellular ratio of [K]:[Na]. To confirm that the nimodipine was effective under these conditions, we first examined its actions in the presence of extracellular Ca^{2+}. Under such circumstances, increasing extracellular K^+ from 5 to 45 mM caused a profound stimulation of adenylyl cyclase (Fig. 4). In the presence of nimodipine, this effect was largely eliminated. When a similar experiment was performed in the absence of extracellular Ca^{2+}, the approximately 2-fold stimulation in response to 45 mM K^+ persisted. However, this effect was eliminated by the inclusion of nimodipine in the assay (Fig. 4).

These results establish that Ca^{2+} entering through L-type channels prominently stimulates cerebellar neuron adenylyl cyclase. They also strongly support our hypothesis that in the absence of extracellular Ca^{2+}, it is the entry of Na^+ through the same L-type channels that stimulates cAMP accumulation.

Increasing Extracellular KCl Elevates $[Ca^{2+}]i$ via L-type Channels in Cerebellar Granule Cells—To confirm the assumption that nimodipine was exerting its effect as a consequence of blocking L-type channels, the response of fura-2-loaded granule cells to increasing extracellular K^+ was examined. Exposure of

granule cells to a medium including 40 mM K^+ and 1 mM Ca^{2+} caused an immediate sharp rise in $[Ca^{2+}]_i$, which settled down to an elevated plateau, as long as Ca^{2+} was retained in the bathing medium (Fig. 5). Inclusion of nimodipine (10 μM) in the medium largely precluded this effect (Fig. 5). Thus, it seems fair to conclude that L-type channels carry cations in response to depolarization by elevated K^+ in the granule cells.

Entry of Na^+ through Gramicidin-generated Pores Mimics the Effect of Membrane Depolarization—To separate unequivocally the effect of membrane depolarization from the proposed action of Na^+ entry, we used the bacterial pore-forming agent, gramicidin. Gramicidin incorporates into eukaryotic membranes and generates large pores that allow the equilibration of ions on both sides of the membrane and, consequently, is expected to collapse the membrane potential (24). Granule cells were treated with gramicidin in 140 mM *N*-methyl-D-glucamine (NMDG) for 10 min prior to assay in various combinations of Na^+ and NMDG. As Na^+ was increased to 40 mM a clear stimulation in activity was apparent (Fig. 6). Given that the presence of gramicidin brings the membrane potential to zero, this effect of Na^+ must reflect a direct effect of the cation on the adenylyl cyclase.

DISCUSSION

The present study has explored the possibility that adenylyl cyclase in cultured cerebellar granular cells is regulated by membrane potential. The localization of adenylyl cyclase immunoreactivity noted along the axons and dendrites of these cells, along with the striking concentrations in growth cones, would appear to predispose the enzyme to such regulation. When the cells were depolarized by increasing the external [K]:[Na] ratio, a clear stimulation of cAMP accumulation was observed both in the presence and absence of external Ca^{2+}. However, this stimulation was biphasic, peaking at approximately 45 mM K^+ and declining thereafter. Such a biphasic stimulation of cAMP accumulation was incompatible with a simple membrane potential-dependent stimulation, since we were able to show (and as would be predicted by the Nernst equation) that membrane potential increased linearly as a function of increasing the external [K]:[Na] ratio. Nevertheless, it might be argued that the initial stimulatory response to membrane depolarization did reflect a sensitivity to membrane potential and that the loss of the response at higher K^+ reflected some additional deleterious effect of high K^+. These possibilities are resolved by the observation that, in fact, it is due to the activity of L-type channels that adenylyl cyclase activity is stimulated either in the absence or presence of external Ca^{2+}. Earlier studies had established that primary-cultured cerebellar granule cells (as used in the present study) express predominantly L-type Ca^{2+} channels at early cell ages (25), although neurons from more developed animals show a significant representation of other channel types (26). The conclusion that it is the entry of cations though L-type channels that stimulates adenylyl cyclase is confirmed by the fact that nimodipine, which exerts no effect on membrane potential, reverses the depolarization-dependent stimulation of cAMP accumulation, either in the absence or presence of extracellular Ca^{2+}. This conclusion is strengthened by the fact that gramicidin-generated pores in the membranes permit the membrane potential-independent entry of sodium ions into the cell, which mimics the effect of depolarization. Thus, it must be concluded that it is not membrane potential *per se*, but membrane potential-driven entry of sodium into cerebellar granular cells that permits the stimulation of cAMP accumulation. It should also be noted that in the absence of extracellular Ca^{2+}, no increase in $[Ca^{2+}]_i$ was elicited either by depolarization or the treatment of cerebellar granule cells with gramicidin and Na^+ (not shown). Thus, the effect of sodium could not reflect any release of Ca^{2+} from intracellular stores.

The one earlier suggestion that cerebellar granular cell adenylyl cyclase was sensitive to membrane potential utilized different assay conditions (*e.g.* extended assay times, 30 min, and hyperosmotic treatments with extracellular KCl) from those used presently (10). Nevertheless, those data were qualitatively similar to those reported here, and they can readily be incorporated with the present results and interpretation. Thus, although the possibility that mammalian adenylyl cyclase might be sensitive to membrane potential remains attractive, particularly in the light of the complex membrane-insertion profile of adenylyl cyclases, the present data do not support such a prospect.

It is possible that the reports of membrane potential sensitivity of adenylyl cyclase from *Paramecium* and *L. pictus* sperm do indeed reflect sensitivity to membrane potential, since the assays were performed in the absence of either K^+ or Na^+ (8, 9). Nevertheless, other functions must be sought for the complex organization of mammalian adenylyl cyclases in membranes. One possibility is a requisite association of adenylyl cyclases with Ca^{2+} entry channels (27, 28). In nonexcitable cells, Ca^{2+}-sensitive adenylyl cyclases are regulated exclusively by capacitative Ca^{2+} entry and not at all by nonspecific elevations of Ca^{2+} in the cytosol. It is possible that adenylyl cyclases are physically associated with Ca^{2+} channels via the transmembrane regions (28).

Acknowledgments—We thank Dr. Ravi Iyengar for the adenylyl cyclase antibody used in this study, Dr. Colin Taylor for use of the LS50 spectrofluorimeter and assistance with $[Ca^{2+}]_i$ measurements, Dr. Steve Hladky for gramicidin A, and Andy Letcher for assistance.

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J. Biol. Chem. 1998, 273:27703-27707.
doi: 10.1074/jbc.273.42.27703
