BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **224,** 271–280 (1996)
ARTICLE NO. 1019

# Modulation by Polycationic $Ca^{2+}$-Sensing Receptor Agonists of Nonselective Cation Channels in Rat Hippocampal Neurons

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Received May 20, 1996

We recently cloned an extracellular calcium ($Ca_0^{2+}$)-sensing receptor (CaR) from bovine parathyroid. The CaR is also expressed in various regions of brain, suggesting that it could potentially mediate some of the well-known but poorly understood effects of $Ca_0^{2+}$ on neuronal function. We have now examined the effects of polycationic CaR agonists on the activity of nonselective cation channels (NCC) in cultured rat hippocampal neurons, using the cell-attached configuration of the patch clamp technique and applying CaR active agents to the external bath solution. The polycationic CaR agonist, neomycin (100 $\mu$M), as well as an elevated concentration of $Ca_0^{2+}$ (3 mM), which is known to activate the cloned CaR, significantly increased the probability of channel opening (Po). The polyamine, spermine (300 $\mu$M), which also mimics the actions of $Ca_0^{2+}$ on the cloned CaR, produced similar changes in Po in rat hippocampal neurons. Elevation of $Ca_0^{2+}$ also increased Po for a similar NCC in HEK293 cells transfected with the cloned human CaR but not in nontransfected HEK cells. Thus the CaR can regulate the activity of $Ca^{2+}$-permeable NCC in hippocampal neurons and could potentially modulate key functions of these cells, including neurotransmission and neuronal excitability. © 1996 Academic Press, Inc.

A variety of neuronal processes are regulated by calcium ions (1-8). These effects of calcium are generally thought to be mediated through $Ca^{2+}$ serving as a key intracellular second messenger (2,5-12). Small changes in the extracellular $Ca^{2+}$ concentration ($Ca_0^{2+}$), however, also modify essential neuronal processes (13-19), but the mechanisms underlying these actions of $Ca_0^{2+}$ are not well understood. A $Ca_0^{2+}$-sensing receptor (CaR) has recently been cloned from bovine parathyroid (20) and rat kidney (RaKCaR) (21), which is a central regulatory element in extracellular calcium homeostasis. This G protein-coupled receptor is linked to activation of phospholipase C and confers upon parathyroid and kidney cells the capacity to sense even minute ($\sim$2-3%) changes in $Ca_0^{2+}$.

By northern blot analysis, the CaR is also expressed in regions of brain not known to be involved in systemic mineral ion homeostasis, including hippocampus. Sequence analysis of the CaR reveals domains homologous to those within the metabotropic glutamate receptors (mGluRs) (22,23), another G protein-coupled receptor that plays important roles in hippocampal function. recently, a brain form of CaR has been cloned from a rat striatal cDNA library (24) that is identical to RaKCaR. Immunohistochemical studies showed that it is predominantly localized in nerve terminals, including those within the hippocampus.

The functional implications of the brain CaR are not yet known, but substantial neuronal activity-dependent changes in the concentrations of extracellular ions, including $Ca_0^{2+}$, have been documented in hippocampus and elsewhere in brain (14-18), which could regulate the activity of the CaR in nearby neurons. A potential role for the CaR in controlling neuronal excitability could be mediated through modulation of specific ion channels. For example, some NCC can be regulated not only by intracellular calcium but also by $Ca_0^{2+}$ (25,26). Indeed, recent studies have shown that mGluRs, which are not only structurally homologous to the CaR but also couple to similar signal transduction systems, modulate neuronal excitability by



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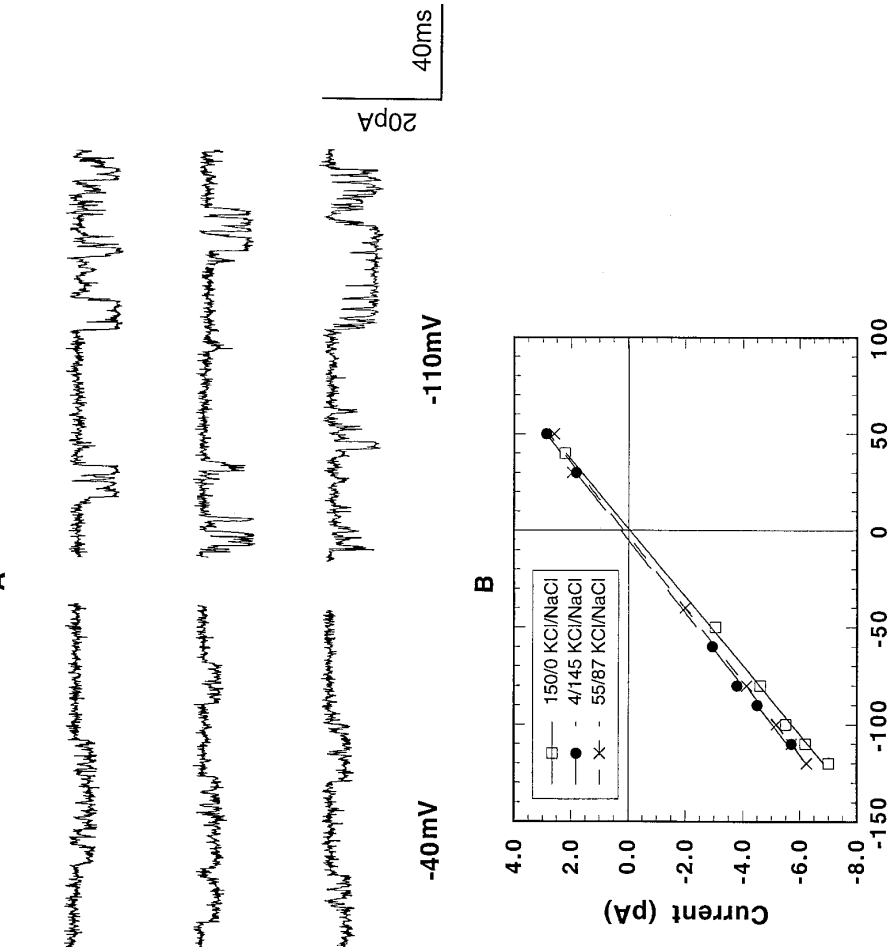


**FIG. 1.** Single channel currents (**A**) and current–voltage relations (**B**) taken at different monovalent cation ratios as indicated on the left sides of the current traces. In **A,** the records were taken at −40 mV (left column) or −110 mV (right column). Downward deflections represent negative inward currents due to the opening of the channels. In **B,** the current amplitudes are plotted as a function of voltage at the three different monovalent cation concentrations indicated in the inset.

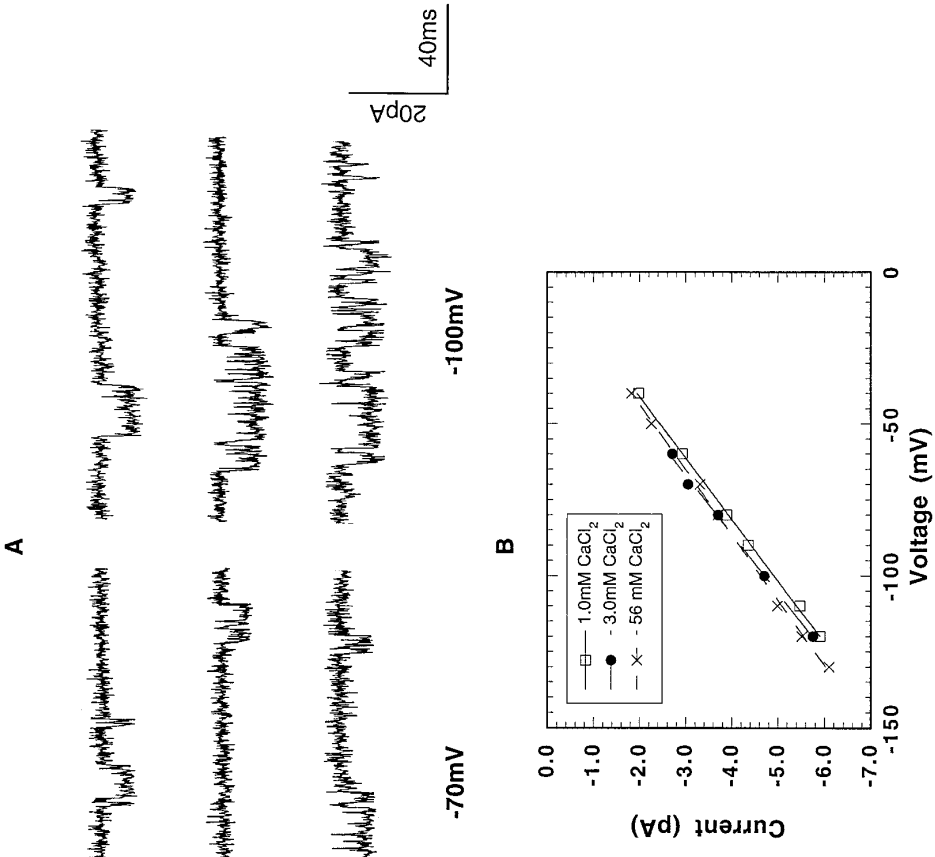


**FIG. 2.** Channel activities in cell-attached patches at three different $Ca^{2+}$ concentrations in the pipette solution as indicated on the left side of the tracings. In (**A**), the current sweeps are taken at −70 mV (left column) or −100 mV (right column). In (**B**), current–voltage relations were determined in the presence of three different $Ca^{2+}$ concentrations in the pipette as indicated in the inset.

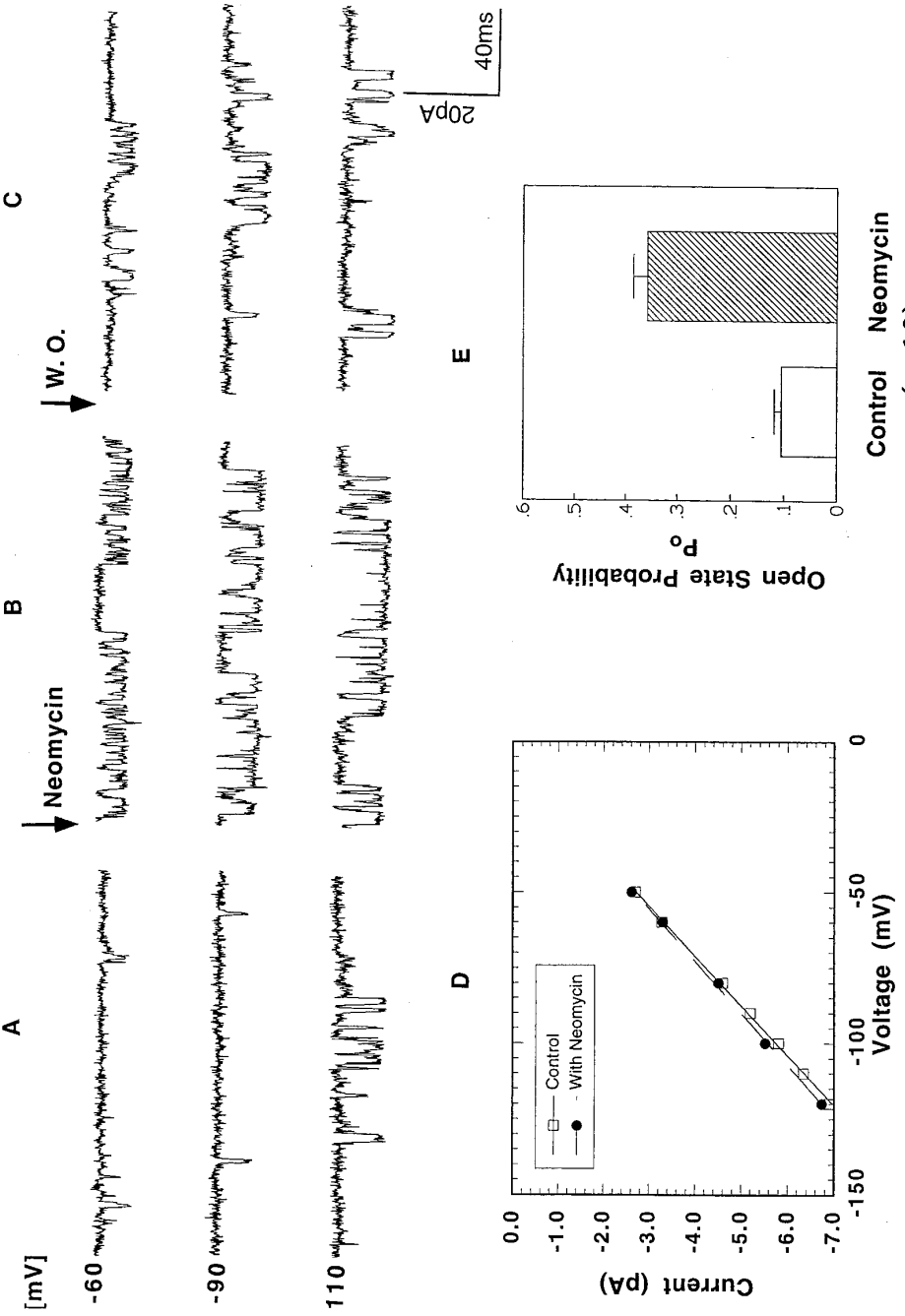


**FIG. 3.** Activation of NCC by neomycin. Single channel traces were taken at three different voltages as indicated on the left side of each row of records before (**A**) and after (**B**) addition of 100 $\mu$M neomycin as well as after washing out neomycin from the bath solution (**C**). Current–voltage relations and Po (mean +/− SEM, $n$=10) before and after addition of neomycin are shown in (**D**) and (**E**), respectively.

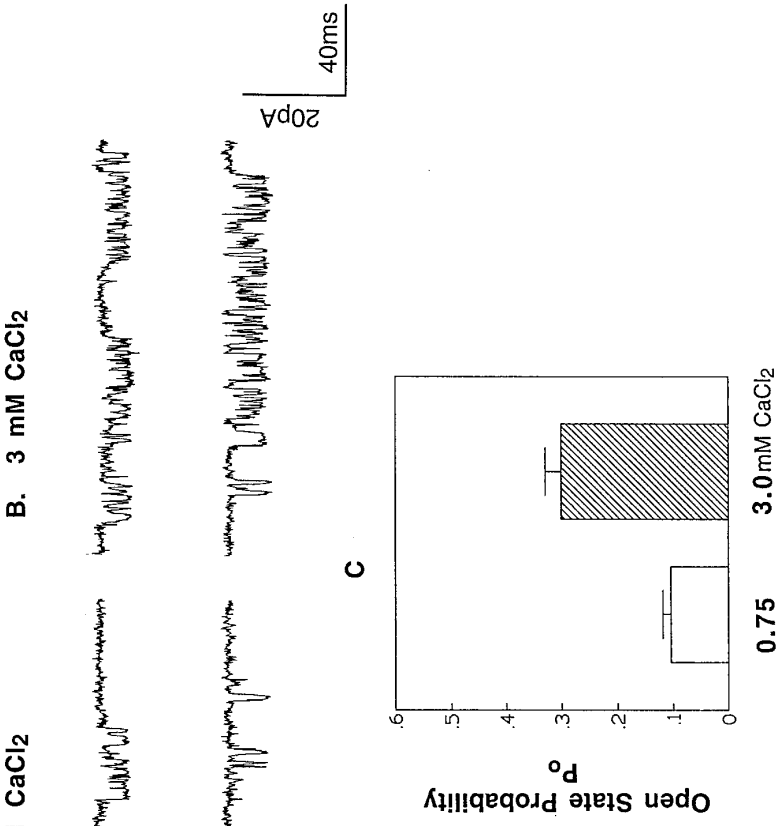


**FIG. 4.** Stimulation of channel activity in cell-attached patches by elevating external $Ca^{2+}$ in the bath solution from 0.75 mM (**A**) to 3.0 mM (**B**). The traces were taken at two different voltages as indicated on the left side of the records. The change in Po (mean +/− SEM, $n$=6) mediated by high $Ca_0^{2+}$ is shown in (C).

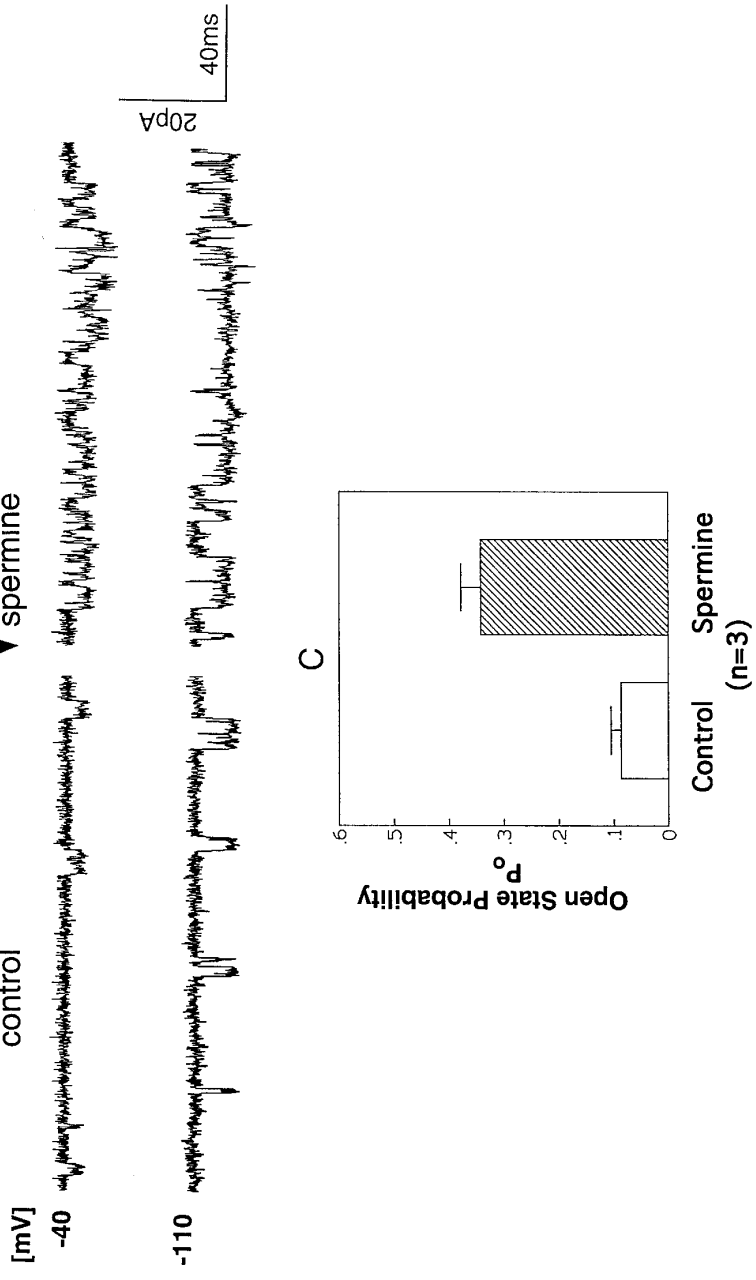


**FIG. 5.** Effect of spermine on channel activity in cell-attached patches of rat hippocampal neurons. Current sweeps were taken at two different voltages before (**A**) and after (**B**) addition of 300 $\mu$M spermine to the bath solution. The change in Po (mean +/− SEM, $n$=3) elicited by spermine is shown in (**C**).

changing the activities of certain ion channels (27-29), particularly nonselective cation channels (NCC) (29,30). We and others have previously employed a variety of polycationic CaR agonists (e.g., neomycin, high $Ca_0^{2+}$, $Gd_0^{3+}$, spermine and other polyamines) to characterize the pharmacological properties of cloned CaRs (20,21,31). In the present study, we show that some of these same CaR agonists also modulate the activity of NCC both in rat hippocampal neurons as well as in human embryonic kidney (HEK293) cells stably transfected with the cloned CaR, strongly suggesting they are regulated by the same receptor.

## MATERIALS AND METHODS

*Cell preparation.* Hippocampi were dissected from 1-12 day old rat brains, cut into 2-3 mm pieces, and four to six pieces were placed in 3.5 cm, uncoated Falcon petri dishes, filled with 2 ml of medium containing the following: Dulbecco's modified Eagle's Medium (DMEM), 5% fetal calf serum, 10 $\mu$g/ml penicillin and 20 $\mu$g/ml streptomycin. We avoided the use of polycationic agents (e.g., polylysine) as substrates for neuronal growth to avoid possible interactions of these with the neuronal CaR. Furthermore, no proteolytic enzymes were used in order to minimize damage to ion channels and receptors, including the CaR. Hippocampal cells were maintained in culture for up to several weeks under 5% $CO_2$ at 37°C. Tissue fragments adhere to the dishes within 2-4 days, and later neurons extend outward from the explants. These cultured cells remain viable for 1-4 weeks but are generally studied within 5-10 days after isolation. Cells having the typical shape of pyramidal neurons were mainly used in the present studies. The following solution superfuses the cells (in mM): NaCl, 140; KCl, 4; $CaCl_2$, 1 or 0.75 were indicated; $MgCl_2$, 0.5; glucose, 5; and HEPES, 5, pH 7.4. Test solutions containing CaR agonists or other agents were applied by superfusion.

*General electrophysiological measurements.* Patch-clamp methodology has been employed for single channel measurements using cell-attached and excised membrane patches. Patch pipettes were prepared from 7052 Corning (Garner Glass Co.) glass capillaries. They were pulled and fire-polished to a tip diameter $< 1$ $\mu$m and were usually coated with Sylgard. The pipette solution contained in mM (unless otherwise specified): NaCl, 87; KCl, 55; $CaCl_2$, 0.5; $MgCl_2$, 0.5, glucose, 10; Hepes 5.0 (pH 7.4). When filled with this external solution, the pipette tip resistances were 5-10 M$\Omega$. Seals with resistances of $>10$G$\Omega$ were used in single channel experiments, and currents were measured with an integrating patch-clamp amplifier. Single channel currents were filtered at 3 kHz through a 8-pole Bessel filter. In cell-attached patches, the resting potential corresponds to holding the patches at 0 mV.

*Data acquisition and analysis.* Voltage stimuli were applied and single channel currents were digitized (50-200 $\mu$s per point) and analyzed suing an IBM computer, a Labmaster board, and programs based on pClamp (Axon Instr,, Sunnyvale, CA),

## RESULTS

We identified NCC in hippocampal neurons by their typical electrophysiological properties (i.e., permeability to $Na^+$, $K^+$, and $Ca^{2+}$). When studied using the cell-attached mode, the amplitude of their single channel currents at defined voltages does not change substantially with alterations in the ratio between different cations in the pipette solution (Figure 1A). The nonselective properties of these channels can also be shown by the absence of change in their current-voltage relationships and the minimal shift in reversal potential with alterations in the cation content of the pipette solution (Figure 1B). When $Ca^{2+}$ in the pipette solution was varied, only small changes in current amplitudes (Figure 2A) and current-voltage relations (Figure 2B) were observed, showing that these channels have a permeability for $Ca^{2+}$ similar to that for $Na^+$ and $K^+$. The conductance was 45-60 pS.

The CaR agonist, neomycin (100 $\mu$M), dramatically activates the channel (Figure 3A and B), with only a small change in its current-voltage relationship (Figure 3C). Figure 3D illustrates that neomycin elicits a statistically significant increase in the probability of channel opening (Po) (Figure 3E). Elevating $Ca_0^{2+}$ from 0.75 to 3 mM in the bath solution, while recording channel activities in the cell-attached mode, produces a similar change in channel activity (Figure 4A and B) with a significant increase in Po (Figure 4C).

We also tested the effects of another polycationic agent on channel activity, the polyamine spermine, which was recently characterized as a CaR agonist (S. Quinn and P.M. Vassilev, manuscript in preparation). It also potentiates the activation of the CaR by low levels of $Ca_0^{2+}$ in bovine parathyroid cells and stably transfected HEK293 cells. Spermine (300 $\mu$M)

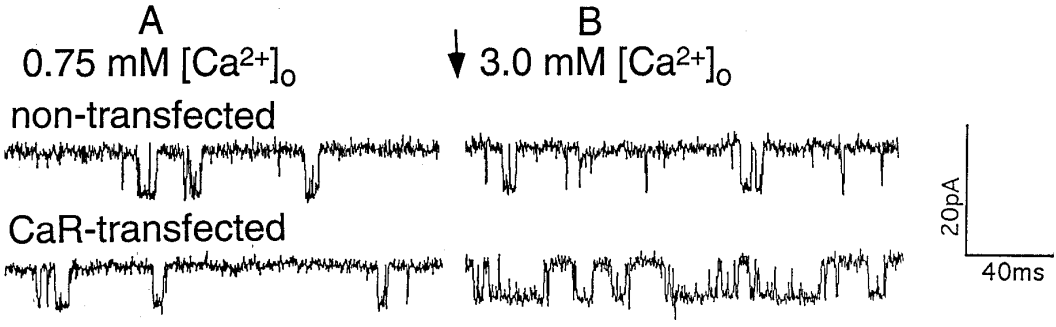


**FIG. 6.** Single nonselective cation channel activities in nontransfected HEK 293 cells and HEK cells stably transfected with the CaR before (**A**) and after (**B**) elevating external $Ca^{2+}$ in the bath solution from 0.75 mM to 3.0 mM.

substantially activates these channels in rat hippocampal neurons (Figure 5A and B). This experiment has been performed in the presence of 1 mM $Ca_0^{2+}$, which in the absence of spermine is not able to maintain high channel activity.

To document further the role of the CaR in regulating NCC, we compared the effects of CaR agonists on the activity of these channels in HEK293 cells stably transfected with the human CaR as well as in control, nontransfected HEK293 cells. We have recently identified such channels in this cell line and found that their conductance and other properties are very similar to those in hippocampal pyramidal neurons (C. Ye and P. Vassilev, unpublished observations). Figure 6 shows that elevating $Ca_0^{2+}$ from 0.75 to 3 mM substantially increased channel activity in the CaR-transfected but not in the nontransfected HEK293 cells (Figure 6). Similar effects were observed in three other experiments and other CaR agonists elicited similar activation of NCC only in CaR-transfected but not in the nontransfected HEK293 cells (C. Ye and P. Vassilev, unpublished observations).

## DISCUSSION

This study shows that the CaR can activate a nonselective cation channel in cultured hippocampal pyramidal neurons as well as in HEK cells stably transfected with the CaR in the absence of neurotransmitter receptor agonists in the pipette solution. Channels with similar conductance and other properties are also known to be regulated by some neurotransmitters. For example, both NMDA and non-NMDA ionotropic glutamate receptor channels are actually nonselective channels with varying permeabilities for calcium ions. Under physiological conditions, $Na^+$ and $Ca^{2+}$, driven by their concentration gradients, enter hippocampal neurons through these nonselective channels, while $K^+$ passes through the same channels in the opposite direction due to its concentration gradient. The dominant currents are generally inward, due to the influx of $Na^+$ and $Ca^{2+}$, and tend to depolarize the membrane. The apical dendrites of pyramidal neurons are densely packed with mGluRs and glutamate receptor channels and could also contain a substantial density of the CaR (24). Activation of nonselective channels by spermine and other CaR agonists in the apical dendrite would cause neuronal depolarization, leading to stimulation of glutamate receptor channels, particularly those of the NMDA-type. At the resting membrane potential, these channels are closed due to a voltage-dependent block by external $Mg^{2+}$, which is relieved at depolarizing voltages. Therefore, activation of NCC by spermine and other polycationic CaR agonists would be expected to stimulate neurotransmission and could potentially impact positively on key functions carried out by hippocampal pyramidal neurons, such as the induction of long term potentiation (LTP) which can be modulated by polyamines.

CaR agonists added to the bath solution activate NCC in pyramidal neurons and HEK cells transfected with the CaR (but not in nontransfected HEK cells) when studied using the patch clamp technique in the cell-attached mode. Therefore, activation of the channels occurs through an indirect cytosolic transduction pathway, because the tight seal of the patch clamp pipette does not permit access of the agonists to $Ca_0^{2+}$-sensing receptors within the membrane patch. Indeed, the activities of nonselective glutamate receptor channels are strongly dependent on several intracellular second messenger systems. For instance, forskolin, which stimulates adenylate cyclase and protein kinase A (PKA), substantially increases the amplitude and decay time of a non-NMDA-type channel in hippocampal neurons (33). Moreover, activation of adenylate cyclase increases spontaneous and evoked neurotransmitter release in excitatory synapses of field CA1 neurons in hippocampal slices (34). Protein kinase C (PKC) also modulates the activity of AMPA/kainate channels in hippocampal slices (34). The effects of PKC, PKA, and calmodulin-dependent protein kinase on these channels appear to play an important role in the induction of long term potentiation (LTP) and long term depression (LTD) in the hippocampus (34-36). The involvement of various intracellular messenger systems as transduction pathways for the action of spermine, $Ca_0^{2+}$ and other CaR agonists in regulating ion channel activities, excitability and other key functions of hippocampal and other neurons must be elucidated in further studies.

## ACKNOWLEDGMENTS

The work was supported by generous grants from the USPHS (DK41415 to E.M.B. and P.M.V. as well as DK44588 and DK48330 to E.M.B.), NPS Pharmaceuticals, Inc., and the St. Giles Foundation.

## REFERENCES

1. Zucker, R. S. (1993) *J. Physiol.* **87,** 25–36.
2. Malenka, R. C., Kauer, J. A., Zucker, R. S., and Nicoll, R. A. (1988) *Science* **242,** 81–84.
3. Parfitt, K., and Madison, D. V. (1993) *J. Physiol.* **471,** 245–268.
4. Alford, S., Frenguelli, B. G., Schofield, J. G., and Collingridge, G. L. (1993) *J. Physiol.* **469,** 693–716.
5. Clapham, D. E. (1995) *Cell* **80,** 259–269.
6. Neher, E. (1987) *Nature* **326,** 242.
7. Muller, W., and Connor, J. A. (1991) *Nature* **354,** 73–76.
8. Guthrie, P. B., Segal, M., and Kater, S. B. (1991) *Nature* **354,** 76–80.
9. Fasolato, C., Innocenti, B., and Pozzan, T. (1994) *TIPS* **15,** 77–83.
10. Ghosh, A., and Greenberg, M. E. (1995) *Science* **268,** 239–247.
11. Nelson, P. G., and Fields, R. D. (1994) *J. Neurobiol.* **25,** 219.
12. Regehr, W., and Tank, D. W. (1990) *Nature* **345,** 807–810.
13. Dunwiddie, T. V., and Lynch, G. (1979) *Brain Res.* **169,** 103–110.
14. Nicholson, C., Ten Bruggencate, G., Steinberg, R., and Strokle, H. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1287–1290.
15. Ficker, E., and Heinemann, U. (1992) *J. Physiol.* **445,** 4310–455.
16. Hamon, B., and Heinemann, U. (1986) *Exp. Brain Res.* **64,** 27–36.
17. Lucke, A., Kohling, R., Staub, H., Moskopp, D., Wassman, H., and Speckmann, E.-J. (1995) *Brain Res.* **671,** 222–226.
18. Ares, J., Stabel, J., and Heinemann, U. (1992) *Can. J. Physiol. Pharmacol.* **70,** S194–S205.
19. Armstrong, C. M., and Lopez-Barneo, J. (1987) *Science* **236,** 712–714.
20. Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J., and Hebert, S. C. (1993) *Nature* **366,** 712–714.
21. Riccardi, D., Park, J., Lee, W.-S., Gamba, G., Brown, E. M., and Hebert, S. C. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 131–135.
22. Nakanishi, S. (1994) *Science* **258,** 6597–603.
23. Nakanishi, S. (1994) *Neuron* **13,** 1031–1037.
24. Ruat, M., Molliver, M. E., Snowman, A. M., and Snyder, S. H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3161–3165.
25. Nakazawa, K., Fujimori, K., Takanada, A., and Inoue, K. (1990) *J. Physiol.* **428,** 257–272.
26. Li, J. Q., Prod'hom, B., and Kucera, P. (1994) *Pfleug. Arch. Eur. J. Physiol.* **429,** 183–192.

27. Charpak, S., Gahwiler, B. H., Do, K. Q., and Knopfel, T. (1990) *Nature* **347,** 765–767.
28. Shirasaki, T., Harata, N., and Akaike, N. (1994) *J. Physiol.* **475,** 439–453.
29. Guerineau, N. C., Bossu, J.-L., Gahwiler, B. H., and Gerber, U. (1995) *J. Neurosci.* **15,** 4395–4407.
30. Vignes, M., Blanc, E., and Recasens, M. (1995) *Eur. J. Neurosci.* **7,** 1791–1802.
31. Hammerland, M. G., Brown, E. M., Hebert, S. C., and Nemeth, E. F. (1993) *J. Bone Mineral Res.* **8**(Suppl. 1), S133.
32. Nemeth, E. F. (1995) $Ca^{2+}$-receptor-dependent regulation of cellular functions. *New Physiol. Sci.* **10,** 1–5.
33. Reale, V., Hales, C. N., and Ashford, M. L. J. (1995) *J. Memb. Biol.* **145,** 267–278.
34. Bliss, T. V. P., and Collingridge, G. L. (1993) *Nature* **361,** 31–39.
35. Ben-Ari, Y. (1995) *J. Neurobiol.* **26,** 295–298.
36. Ghosh, A., Ginty, D. D., Bading, H., and Greenberg, M. E. (1994) *J. Neurobiol.* **25,** 294–303.