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Depolarization- and Transmitter-Induced Changes in Intracellular Ca^{2+} of Rat Cerebellar Granule Cells in Explant Cultures

John A. Connor, Hsiu-Yu Tseng, and Philip E. Hockberger

Department of Molecular Biophysics, AT&T Bell Laboratories, Murray Hill, New Jersey 07974

Digital imaging of the Ca indicator fura-2 has been used to study the responses of developing granule cells in culture to depolarization and transmitter action. Unstimulated cells bathed in Krebs saline exhibited cytoplasmic Ca ion concentrations, [Ca^{2+}], that were generally in the 30–60 nM range. Exposure of cells to high-potassium (25 mM) saline depolarized the membrane potential and produced an immediate rise in [Ca^{2+}] that recovered within 2–3 min in normal saline. The response grew progressively larger over the first 20 d in culture. Transient increases in [Ca^{2+}] to levels > 1 μM were observed after 12–14 d *in vitro*, at which time the cells displayed intense electrical activity when exposed to high K. At this stage, the increases were attenuated by blocking action potential activity with TTX. In TTX-treated or immature cells, in which the transient phase of the Ca change was relatively small, a second exposure to high K typically produced a much larger Ca response than the initial exposure. The duration of this facilitation of the response persisted for periods longer than 5 min. Application of the neurotransmitter GABA induced a transient increase in membrane conductance, with a reversal potential near resting potential (approx. −60 mV), and caused an intracellular Ca^{2+} increase that outlasted the exposure to GABA by several minutes. Glutamate, or kainate, induced an increase in membrane conductance but with a reversal potential more positive than spike threshold. These agents also elevated intracellular Ca^{2+}, but unlike the case with GABA, this Ca response reversed rapidly upon removal of the transmitter. The facilitatory effect of repeated exposures to high-K saline, as well as the persistent Ca elevation following a brief GABA application, suggests that granule cells possess the capability of displaying activity-dependent changes in Ca levels in culture.

In the preceding paper (Hockberger et al., 1987) we described the development of electrophysiological and immunocytochemical characteristics of cerebellar granular cells maintained in explant culture. Although inward and outward currents of different types were described using whole-cell patch recording, it was difficult to make any clear demonstration of a Ca current. The important question of Ca involvement with physiological activity, and its possible development, was left unanswered. In this paper we approach the problem of measuring Ca influx by using a fluorescent indicator of Ca^{2+}, fura-2 (Grynkiewicz et al., 1985; Tsien et al., 1985), which is trapped within the cells and visualized using digital imaging of the fluorescence (Connor, 1985, 1986; Williams et al., 1985). Fura-2 is characterized by its bright fluorescence and high rejection to interference from Mg^{2+} and pH changes (Grynkiewicz et al., 1985). At the present time we are able to monitor spatially resolved changes in intracellular free Ca^{2+} (hereafter called the Ca response) with a time resolution of seconds.

The intracellular free Ca levels and membrane potential responses of cultured granule cells were also examined following application of various neurotransmitter-like molecules. We have concentrated our efforts on testing GABA and glutamate, since cerebellar granule cells contain receptors to these transmitters *in vivo* (Olsen and Mikoshiba, 1978; Palacios et al., 1980; Hill and Bowery, 1981; Greenamyre et al., 1984, 1985), as well as *in vitro* (Meier et al., 1984; Cull-Candy and Ogden, 1985; Wroblewski et al., 1985). We were particularly interested in determining whether there was any coupling (either positive or negative) between the inhibitory transmitter, GABA, and Ca influx, as has been suggested for other vertebrate neurons (Dunlap, 1981; Desarmenien et al., 1984). A preliminary report of these findings has been presented in abstract form (Connor et al., 1986a).

Materials and Methods

Explants from postnatal rats (P3–P5) were prepared and maintained as described in the preceding paper (Hockberger et al., 1987). Intracellular recordings and voltage clamping were performed using whole-cell patch recording techniques (Hamill et al., 1981). Iontophoretic electrodes were filled with 0.1 M solution of the neurotransmitter agent buffered to pH 7.0 in 10 mM HEPES (except kainate, pH 4.0). Recordings were done in a flowing bath of Krebs-HEPES saline.

Serum-supplemented cultures were washed in serum-free, defined media (Ahmed et al., 1983) for 15–30 min before loading with indicator. Fura-2/AM (Molecular Probes, Junction City, OR) was dissolved in dimethyl sulfoxide, 5 mg/ml, to make a stock solution. Loading solutions were made by adding the stock to serum-free defined medium to give nominal concentrations of 4–6 μM. Cells were bathed in the loading solution for 30–45 min at 36°C, then rinsed and given a 1.5–3 hr postincubation in defined medium to allow time for deesterification of the indicator. There was significant variation in the amount of indicator trapped into cells from different cultures under the same loading conditions. In general, older cultures (more than 30 d *in vitro*, DIV) trapped less indicator for a given exposure than younger ones. In all cases the large flat cells and glia present in the cultures exhibited almost no fura-2 fluorescence compared to the granule neurons (see Figs. 4, 11, 12). The reason for this difference between cells is unclear (i.e., whether due to differences in loading of the dye or thickness of cells), but it allowed experiments to be performed that might not have been possible otherwise.

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Correspondence should be addressed to John A. Connor, Department of Molecular Biophysics, 1C-423, AT&T Bell Laboratories, 600 Mountain Ave., Murray Hill, NJ 07974.

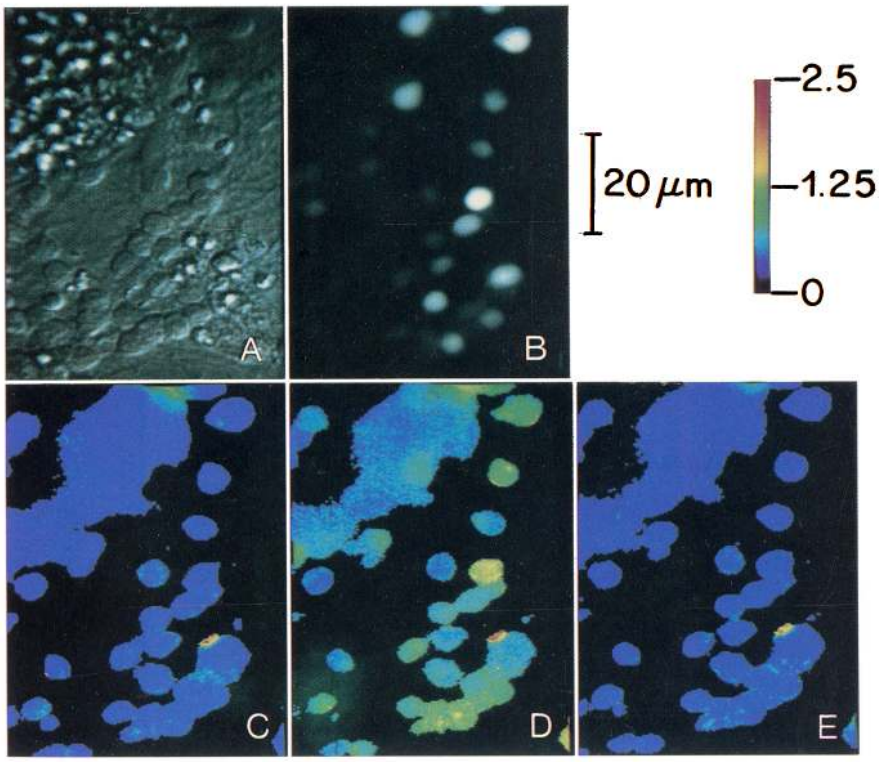


Figure 1. CCD images of granule cells in neuroblast stage of development. *A,* Nomarski image of neuroblasts spreading out from explant (*upper left*). Explant made on P4, 2 DIV. *B,* Fluorescence image of cells in same field at 360 nm excitation. The intensity is determined primarily by the cell size and amount of dye trapped. *C,* Fluorescence ratio image, using 340/360 nm excitation, of cells in normal saline (4.7 mM K). *D,* Cells exposed to 25 mM K for 2 min. The fluorescence ratio has increased significantly. *E,* Cells back in normal saline for 3 min. Ratio recovered in all cells. For this figure, color bar shows only the value of ratio.

Coverslips (#1) containing cultured cells were mounted in a temperature-controlled chamber (31–34°C), which allowed direct access to the coverslip underside, and placed on the stage of a Zeiss IM-35 microscope. Warmed Krebs-HEPES saline flowed through the chamber at approximately 1 ml/min, maintaining a depth of 0.1–0.3 mm. Deadtime for changing saline composition was approximately 45 sec. Flow regulation was downstream from the switching valve, insuring that this period was uniform for all solution changes. Krebs-HEPES (normal) saline contained: NaCl, 120 mM; KCl, 4.7 mM; $CaCl_2$, 1.8 mM; $MgCl_2$, 1.2 mM; HEPES buffer, 10 mM, at pH 7.4. High-K solutions were made by adding KCl from a 1 M stock to the normal saline.

A Nikon UV-F 40X glycerine immersion objective together with a 100-W Hg lamp (Osram) mounted in the Zeiss housing were used for epifluorescence measurements. Images were obtained using a CCD camera (model 81-A, Photometrics Ltd., Tucson, AZ), which employed an RCA SID 53612 chip, 320 × 512 pixels. Exposure times of 0.25–0.5 sec were used to obtain each image; actual times depended upon the size of the cell, indicator loading, and contrast needed. Apparent bleaching of fura-2 was less than 5% for control measurements, i.e., 20 sec exposures to 340 nm excitation at the same intensity used in experiments. Excitation wavelengths at 340 and 380 nm were obtained using interference filters (Melles-Griot, 10 nm ½ B.W.). Exposure times were controlled by a Uniblitz shutter mounted between the filters and the lamp condenser lens. A neutral density (1.5 O.D.) filter was used in conjunction with the 380 nm filter to obtain approximately equal fluorescence image intensity for the 2 excitation wavelengths. Most experiments were conducted with a 480-nm-long pass exit filter, but suitable controls were done using a broadband interference filter (50 nm B.W.) centered at the emission maximum of fura-2 (500 nm).

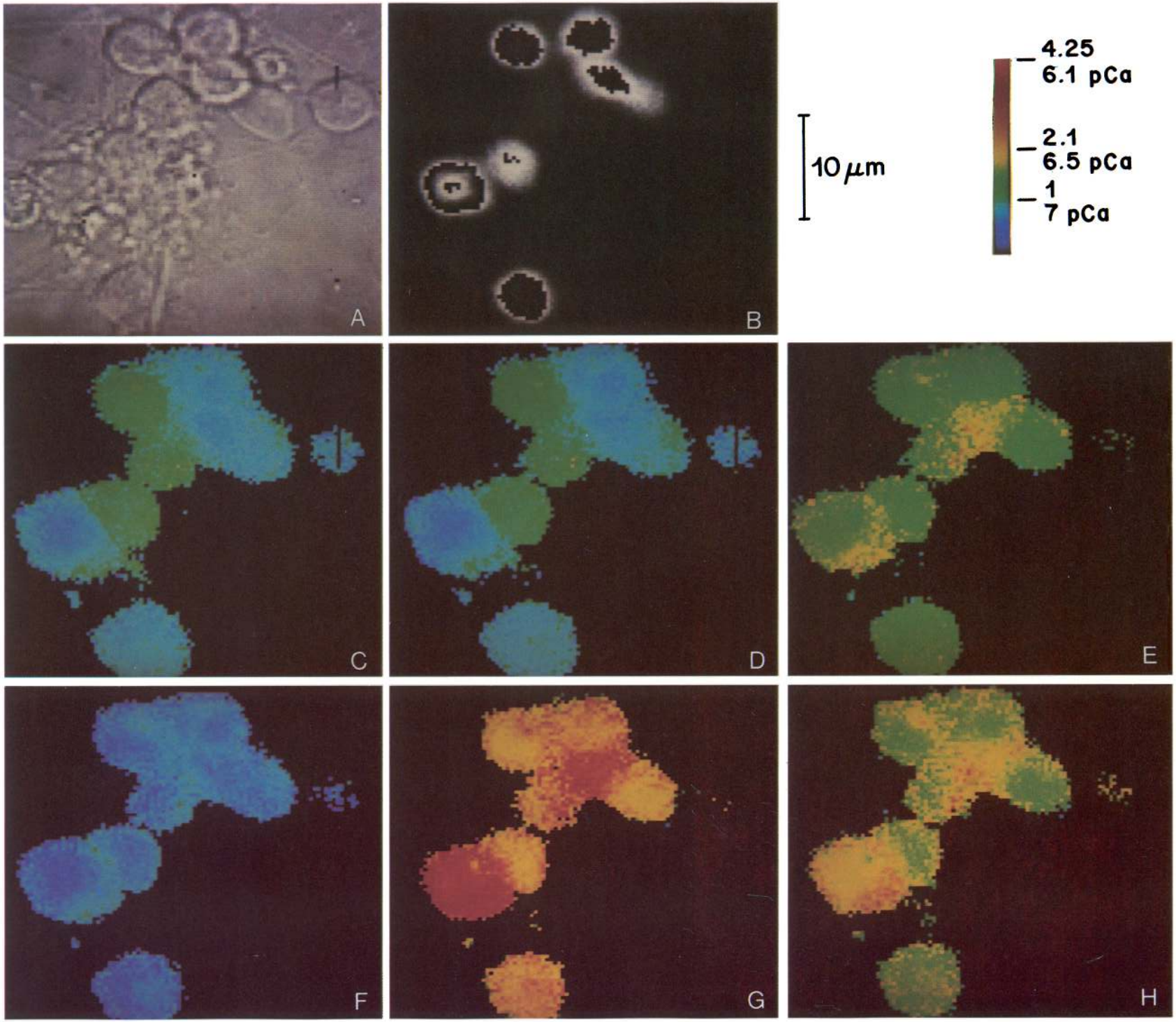
Paired exposures (using 340 and 380 nm excitation) were separated by approximately 1.5 sec, the time required to manually change excitation filters. Each image was corrected for camera dark current and background fluorescence by subtracting a frame of the same excitation wavelength made using a blank portion of the coverslip covered to an appropriate depth with saline. Blanks (see below) were recorded at the outset of each experimental day. Data were obtained by quantifying the intensity of fluorescence at 500 nm in response to excitation at 340 and 380 nm, and we have presented the difference in emission intensities as a ratio image (or graphically as a ratio of absolute intensities). Using ratios should, to a first approximation, normalize for differences in preparation geometry, as well as for the effects of light scattering, illumination nonuniformity, and indicator concentration differences, thereby resulting in a direct measurement of Ca concentration (see Grynkiewicz et al., 1985):

$$[Ca^{2+}] = K_D\left(\frac{R - R_{min}}{R_{max} - R}\right)\left(\frac{F_0}{F_s}\right). \quad (1)$$

Conversion of ratios to Ca concentrations, $[Ca^{2+}]$, were made using Eq. 1 with $R_{min} = 0.5$, $R_{max} = 11.7$, $F_0/F_s = 7.5$, and $K_D = 225 \times 10^{-9}$ M. R_{min} is the minimum value of the fluorescence ratio (340/380 nm) observed when no Ca^{2+} (and 5 mM EGTA) is added to calibrating saline; R_{max}, the maximum value of the ratio obtained at Ca^{2+} levels > 100 μM; F_0/F_s, the ratio of the 380 excitation fluorescence at low and saturating Ca^{2+} levels; and K_D, the dissociation constant of the indicator-Ca reaction.

Two experiments were run using the Ca iontophore ionomycin (Calbiochem) at a nominal concentration of 10 μM in buffered Ca salines. We were unable to reproduce the excellent titration curves of Williams et al. (1985) for smooth muscle. A given field of our cells often shows heterogeneous Ca^{2+} levels even at this high concentration of iontophore. The levels shift up and down along with the buffered external Ca^{2+}, but differences are often maintained between cells. These experiments have allowed us to confirm, though, that the extreme fluorescence ratios corresponding to very low and very high Ca^{2+} are approximately the same as the *in vitro* standards, giving considerable credibility to the method. That is, with cells bathed in saline containing 10 μM ionomycin and 3 mM EGTA at pH 7.4, the smallest ratio observed was 0.52. Replacing this saline with normal Krebs (1.8 mM Ca) without ionomycin caused the ratios in all cells to increase to 11 and above. Presumably, enough ionomycin remained in the cell membranes to be effective at the high external Ca levels. The upper limits on the fluorescence ratios were difficult to measure because the 380 nm excited signal is very faint. Nevertheless, the upper limit was approximately the same as that observed *in vitro.* Anomalously low resting ratios, as reported by Almers and Neher (1985), were not observed. Surprisingly, the cells tolerated several cycles of this treatment. Further details on the calibration of this imaging system have been published elsewhere (Connor, 1986).

Blank areas present a problem when using ratios, because the representations of light levels on the array are small numbers and are therefore subject to large *relative* variations due to noise. Ratioing a field of these numbers gave an extremely mottled picture. For display purposes, a threshold was therefore declared for the denominator field,


A
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7 pCa
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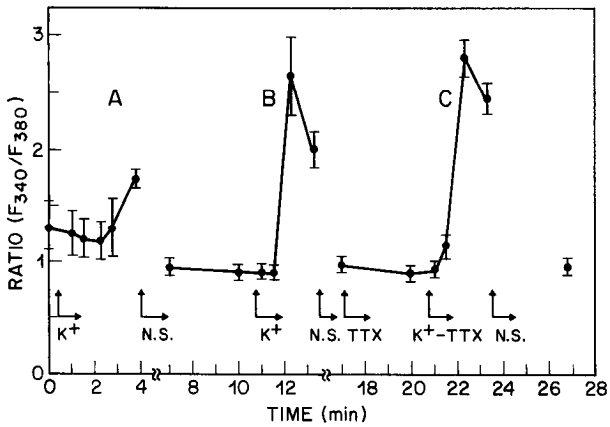

Figure 3. Graphic representation of the pooled data from 5 of the neurons illustrated in Figure 2 showing the mean Ca levels (with SD bar) of the neurons during 3 consecutive exposures to 25 mM K saline (normal saline, *N.S.*). During the third pass, TTX was present in the bathing solution (0.3 μM). Measurements were made only at the times indicated by the data points, which were connected by straight lines for illustration. Ca levels were not followed during recovery periods (*interrupted traces*) but were generally 80–90% complete within 1–2 min (see Fig. 7*A*).

and for pixel values below this, the ratio was replaced by a common, arbitrary value corresponding to a color or gray level. This is known as masking. Black was chosen for color displays and an intermediate shade of gray for noncolor displays. In Figures 1, 2, 4, 6, ratio images have been presented in color in order to maximize the resolution of intensity differences. A calibration bar is shown at the right-hand side of all figures.

Results

High K experiments

Changes in membrane potential were induced in granule cells by briefly exposing them to high extracellular K bathing solutions. Generally, the external K concentration was raised no higher than 25 mM, this being sufficient to depolarize resting membrane potential by 20–30 mV. There were no apparent adverse effects of high-K solutions on the cells as membrane potential responses were routinely reversible. In this regard, we note that neurons exhibited enhanced survival in media containing 25 mM K (see preceding paper).

The effects of high K on intracellular Ca^{2+} levels were strongly dependent upon the developmental stage of the granule cells, the magnitude increasing with age. Elevating K from its normal level, 4.7 mM, to 25 mM always increased internal Ca^{2+}; this was true even in the neuroblast stage. Figure 1 illustrates the effects of K depolarization on a field of approximately 40 cells in the neuroblast stage of development (i.e., 3 DIV). The high-density field of cells shown in the Nomarski image (Fig. 1*A*) typifies the immediate surroundings of the original explant material in cultures at this stage of development. Figure 1*B* shows the fluorescence of fura-2 trapped in these cells using 360 nm excitation. The emission using 360 excitation is relatively independent of Ca^{2+} levels (Grynkiewicz et al., 1985), making the cell-to-cell variation in fluorescence in Figure 1*B* due to differences in the amount of dye trapped and cell thickness. Taking the intensity of the cell in the center right of the field as 100%, the dimmest cells that showed up in the photograph were from 20 to 25%. The upper-left-hand region, where the highly refractive structures appear in the Nomarski picture, displayed a diffuse fluorescence (around 15%) not visible in this image. Regions of high cell density were avoided in most experiments since it was difficult to discriminate between background and cell signal. It is clear from the picture of Figure 1*B* that many of the cells trapped very little indicator since all cells appeared to be of roughly the same thickness. At slightly later stages of development, when cells had migrated further from the explant site, the trapping differences were less extreme. However, as pointed out in Materials and Methods, cells in cultures older than 30 DIV trapped less indicator.

Figure 1*C* shows the ratio image (340/360 nm excitation) of this field of cells in normal saline. The threshold level for display in this case was set at 8% of maximum for the 360 nm excitation signal; therefore, some somata, especially in the lower-left corner of Figure 1*C*, were imaged even though they did not show up in the low-resolution display of Figure 1*B*. The diffuse nature of the signal in the upper left is clearly apparent in the ratio image. In normal saline, the ratio signal was nearly uniform over the population of cells, even though the individual levels of fluorescence were very different. Exposing the cells to 25 mM K for 2 min gave rise to an increase in the ratio in nearly all the cells (Fig. 1*D*). The change was completely reversed by a 2–3 min wash in normal saline (Fig. 1*E*). The resting fluorescence ratio, averaged for 8 cells, corresponds to Ca^{2+} level of approximately 50 nM. During exposure to high K, even the most responsive cells remained below 200 nM. These responses were much smaller than the ones obtained when the cells were a few days older.

Intracellular Ca^{2+} changes in more developed granule cells (>5 DIV) were examined using 340/380 nm ratio images. Cells were more dispersed on the plates of older cultures, and we have analyzed more than 300 individual granule cells from these cultures. Resting Ca^{2+} levels for cells 10 DIV or less were always in the range of 50–80 nM. Levels in individual cells were constant when exposed to flowing Krebs saline for up to 40 min. Resting levels in more mature cells also fell within this range for the

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Figure 2. Ca^{2+} changes in granule cells at early, immature stage of development. *A*, Nomarski image of 9 granule neurons (6 DIV). *B*, Fluorescence image of same field, 380 nm excitation. Somata appear black or ringed in center because the display scale has been intentionally set to show faint fluorescence levels. This causes a "wraparound" on the video monitor, in which the very bright areas, such as the central somata, appear black as the numbers work their way up the gray scale for the second or higher time. The fluorescence of the neuron farthest to the left was so intense that the scale was traversed 2×. Rings may be viewed as contour maps of intensity. *C*, Fluorescence ratio image (340/380 nm) of same field, of cells bathed in normal saline. Fluorescence of the cell farthest to the right was too faint so show up in *B* but was sufficient to exceed the mask threshold (see Materials and Methods) and give a ratio image. *D*, Cells in 25 mM K for 75 sec displayed ratios not significantly changed. *E*, Cells in high-K saline 2 min 15 sec. *F*, Four minute recovery in normal saline. *G*, Second pass in high-K saline, 75 sec as in *B*. Ca levels increased to nearly 1 μM in 2 of the neurons. The right-hand cell almost dropped out of the image because pixel values in the 380 nm image were below the masking threshold. *H*, Cells in high-K saline for 2 min 15 sec (as in *E*) on second pass. Color bar gives both ratios (*upper values*) and calcium concentrations (*pCa*) estimated from Eq. 1 (Materials and Methods).

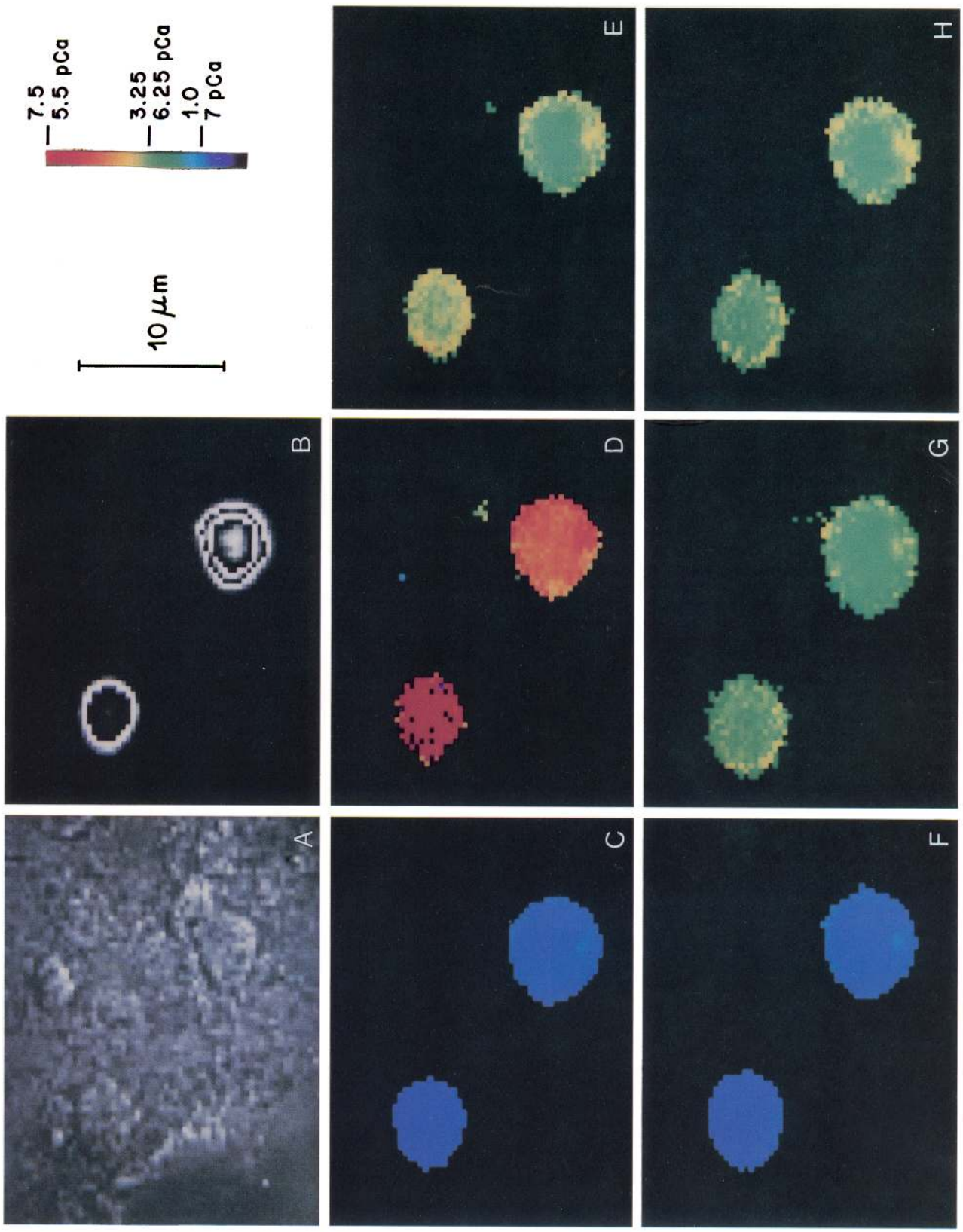

Figure 4. Ca^{2+} changes in granule cells in the intermediate stage of development. *A*, Nomarski image of 2 cells (14 DIV) in which the resolution was hampered by the presence of dense connective tissue around cells. *B*, Fluorescence image at 380 nm excitation showing that surrounding tissue trapped almost no indicator. *C*, Fluorescence ratio image in normal saline. *D*, Thirty second exposure to 25 mM K. *E*, Ninety second exposure to high K. *F–H*, Same sequence as *C–E* with 0.3 μM TTX added to saline. These data are plotted in Figure 5.

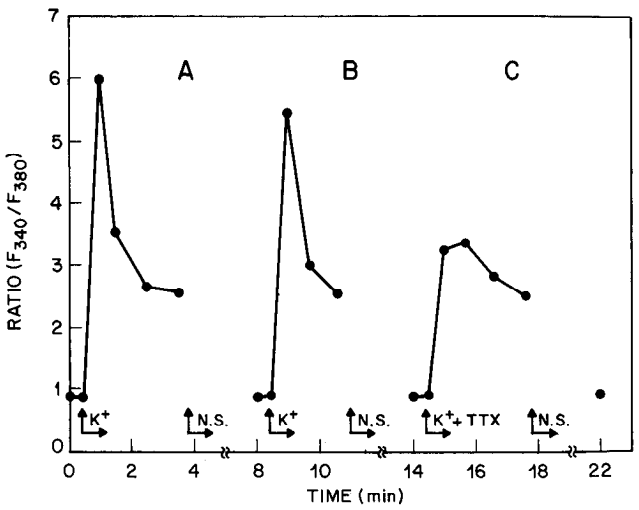

Figure 5. Averaged numerical data for the 2 cells of Figure 4. Two high-K runs were made (*A, B*) before TTX was added to the bath. Runs *A* and *C* are illustrated in Figure 4.

great majority (>75%), while the remainder were slightly higher. Three coverslips that contained cells showing higher Ca^{2+} levels were examined specifically for the effects of TTX. In these cells Ca^{2+} levels were reduced from the range 100–120 μM to below 100 nM (see below). Since TTX blocks the spontaneous electrical activity that we have often observed in these preparations, it is reasonable to assume that the elevated levels were due to Ca influx due to spontaneous electrical discharge.

Figure 2 contains images of a cluster of 9 immature neurons (6 DIV) that were given a total of 3 exposures to 25 mm K in succession with 4 min recovery periods in normal saline between. Figure 2, *A* and *B*, shows the Nomarski and 380 nm fluorescence images. Figure 2, *C–H*, illustrates changes in internal Ca^{2+} for the first 2 exposures. Figure 2*C* shows Ca^{2+} levels in the cells in normal saline, and Figure 2*D* was made 75 sec after high-K saline reached the cells. During that period the Ca levels had not changed significantly. Data of Figure 2*E* were taken 1 min later and show significant Ca increases in the cells. Following a 4 min recovery, Ca^{2+} levels were at or below initial values (Fig. 2*F*).

Reexposure to high K led to quite different Ca^{2+} changes. At the 75 sec point the Ca^{2+} levels had increased dramatically in most of the cells (Fig. 2*G*), and 1 min later values had fallen to intermediate levels that were maintained for the duration of the high-K exposure (Fig. 2*H*). Data pooled from 5 of the neurons are plotted in Figure 3 for all 3 high-K runs and show that the second and third passes produced nearly the same Ca^{2+} change, each much larger than the first. When individual exposure times to high K were longer than the 2–3 min shown here, Ca^{2+} levels settled to a plateau that was maintained for at least 10 minutes (cf. Fig. 9*A*). Generally, though, exposures were limited to less than 4 minutes. The records of Figures 3 and 4 are typical of the more than 80 neurons examined at this stage of development, i.e., immature granule cells.

The facilitation of the Ca response apparent in Figures 2 and 3 may indicate a very important nonlinearity in cellular response to high K. The effect was present at both early and late stages of development. In immature neurons, like those displayed in Figure 3, the first 3–4 min in high K always gave a smaller increase in Ca^{2+} levels than subsequent, identical exposures even though the levels returned to baseline between exposures. In the more differentiated cells, this same effect could be demonstrated only when the initial Ca response had been damped by TTX (see below). The presence or absence of TTX in the bathing saline had no effect on the responses to high K in younger cells (Fig. 3*C*).

Granule cells in culture longer than 12 d (i.e., intermediate cells) responded to an initial exposure to 25 mM K with a rapid, massive increase in internal Ca^{2+}. Figure 4*A* shows the Nomarski image of 2 granule neurons after 14 DIV. Figure 4*B* shows the 380 nm excitation fluorescence and demonstrates that the 2 neurons were the only cells to trap a significant amount of indicator. Figure 4*C* shows Ca^{2+} levels in the unstimulated cells, while Figure 4, *D* and *E*, shows the responses at approximately 30 and 90 sec in 25 mM K, respectively. Given the slow nature of changing external solutions and the limited speed of reading and storing the CCD data, it is quite probable that the peak of the Ca^{2+} transient was missed in these cells. Even so, peak fluorescence ratios of 6.5–7 were observed, corresponding to Ca^{2+} levels of approximately 2 μM. This peak rapidly decayed to a plateau, graphically displayed in Figure 5*A*, as the average response of both cells from Figure 4. After a large initial response such as this, subsequent Ca^{2+} elevations were either the same (as illustrated in Figure 5*B*) or smaller. At this stage of development, TTX caused a pronounced reduction in the size of either the initial or subsequent Ca response. The ratio images of Figure 4, *F–H*, show the effect of 0.6 μM TTX plus high K after 2 previous exposures to high-K saline. Figure 5*C* graphically shows that the amplitude of this response was reduced by approximately one-half, and the rise time was slowed considerably. It can also be seen that the plateau levels were nevertheless approximately the same in all 3 runs. This was generally the case in the older cells examined.

Figure 6 illustrates that facilitation of the response to high K could be demonstrated in intermediate neurons in the presence of TTX. Figure 6*A* shows a Nomarski picture of a field of 6 granule cells (11 DIV), and Figure 4*B* shows the fluorescence image at 380 nm. Figure 6, *C–E*, shows the Ca^{2+} levels in normal saline (6*C*) and at 40 sec (6*D*) and 1.5 min (6*E*) after switching to 25 mM K saline plus TTX. Figure 6, *F–H*, illustrates ratio images at equivalent times for the second pass in high K plus TTX, the initial frame having been made after a 4 min recovery period in TTX saline. Again the transient swing in Ca^{2+} was much greater for the second pass. The facilitation decreased measurably over 10 min, but the recovery time course has not been adequately analyzed at this time.

Exposure of intermediate granule cells to 15 mM K induced a large rise in internal Ca^{2+} (Fig. 7*A*). For this smaller K elevation, the difference between intermediate and immature cells (Fig. 7*B*) was more noticeable than with 25 mM K. Unlike the immature cells, the Ca response of intermediate cells was still impressive with 15 mM K, most probably due to Ca entry during action potential activity. The latter was due not only to the K depolarization, but also to the excitatory coupling between these cells (Fig. 4*A*, preceding paper).

We have not performed simultaneous electrophysiology and Ca imaging, but we have monitored companion populations of granule cells using whole-cell patch recording. After 10–14 DIV, most of the cells generated large action potentials, and there was generally evidence of excitatory synaptic interaction. Exposure of these cells to 25 mM K triggered a high rate of action potential discharge that persisted for 20–30 sec, after which the cells re-

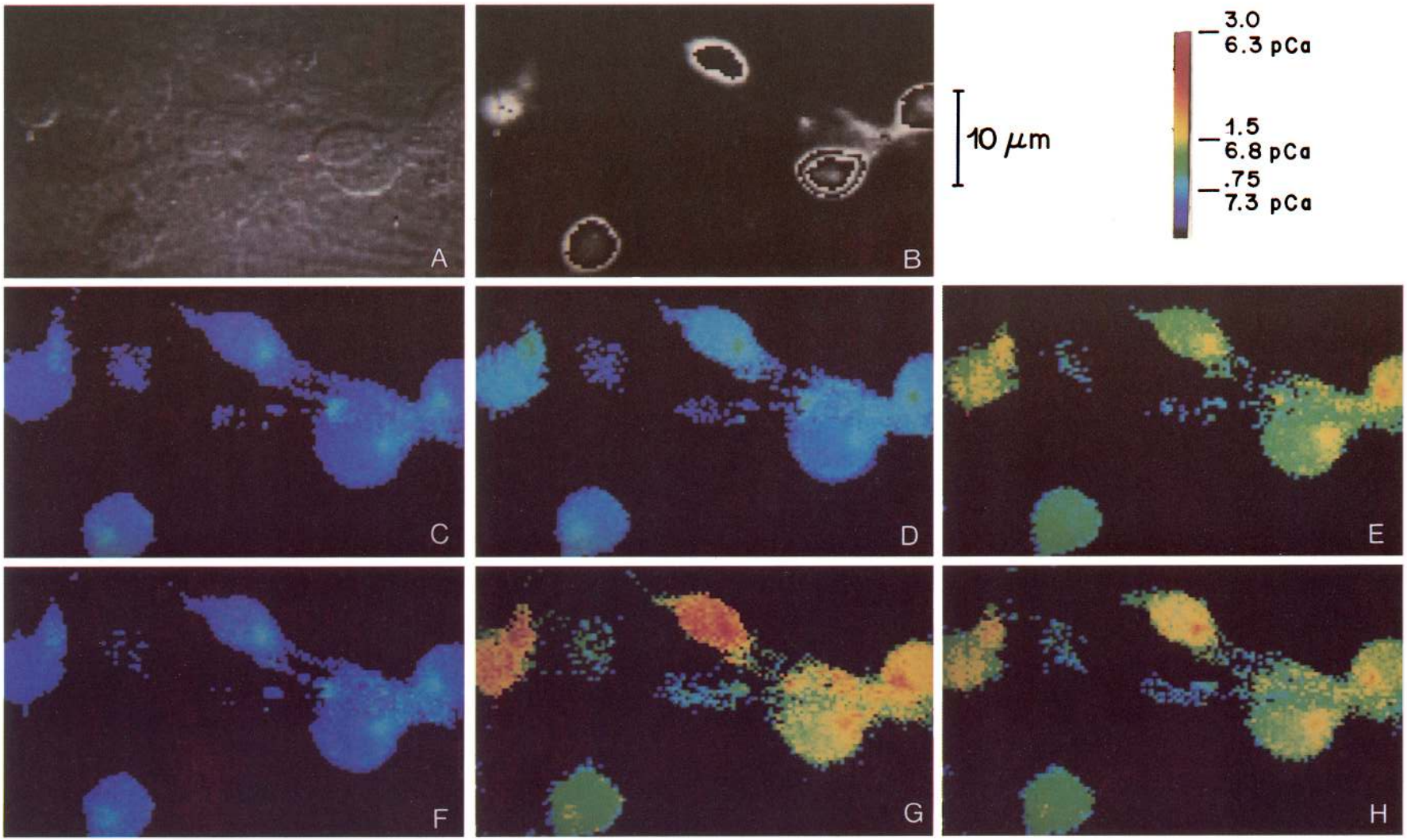

Figure 6. Facilitating response to 25 mM K in intermediate cells (11 DIV) treated with TTX. *A,* Nomarski image of 6 neuron field. *B,* Fluorescence at 380 nm excitation. *C,* Fluorescence ratio image (340/380 nm) in normal saline. *D, E,* Exposures of 40 and 90 sec in 25 mM K. *F–H,* Second pass through at similar times following a 4 min recovery period. The Ca transients were visibly larger.

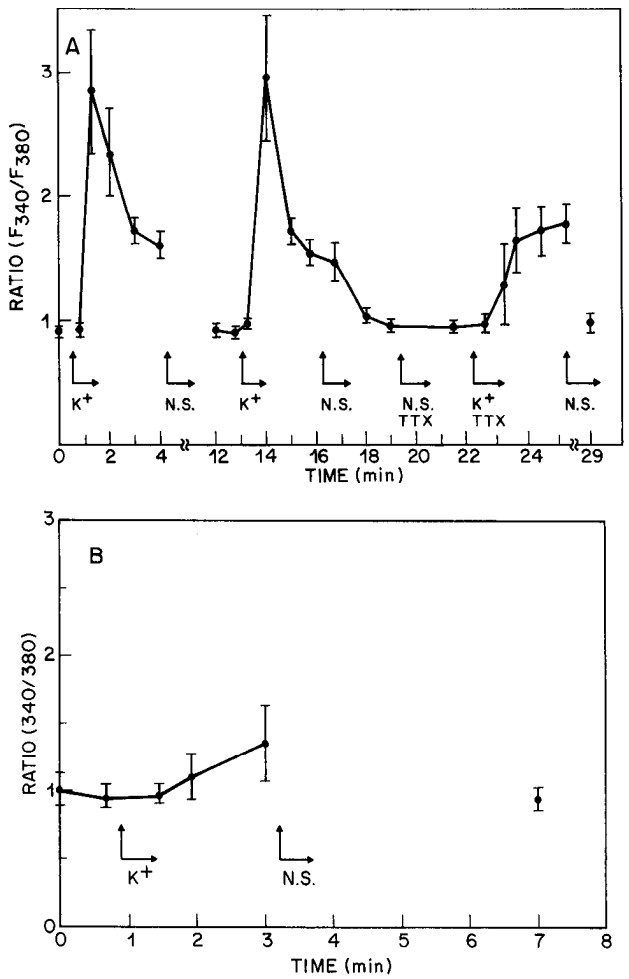

Figure 7. Graphic representation of Ca responses to 15 mM K saline in intermediate and immature granule cells. *A*, Pooled responses induced by 15 mM K saline in intermediate stage neurons (12 DIV) from the same field. The results are plotted for 2 exposures to high K and a subsequent exposure to high K plus TTX saline. The cells had been given a brief exposure to high K before the first data were taken, so the initial response shown was maximal. The continuous record between exposures illustrates the usual time course of recovery Ca levels. *B*, Slower and smaller Ca response pooled from 8 immature cells (2 DIV) to 15 mM K.

mained depolarized at approximately −30 mV. This intense period of activity was abolished by TTX, but the cells still depolarized to the vicinity of −30 mV. The neuroblasts and immature granule cells, by our criteria, have no action potentials. Raising external K to 15 mM produced action potentials in a fraction of the intermediate cells we examined, but generally it caused depolarizations of about 10 mV. We believe that the action potentials were inducing a large influx of Ca^{2+} into cells either through Ca channels, as described in mammalian CNS (for review, see Miller, 1985), or through an Na–Ca exchange mechanism, as carefully documented in the squid giant axon

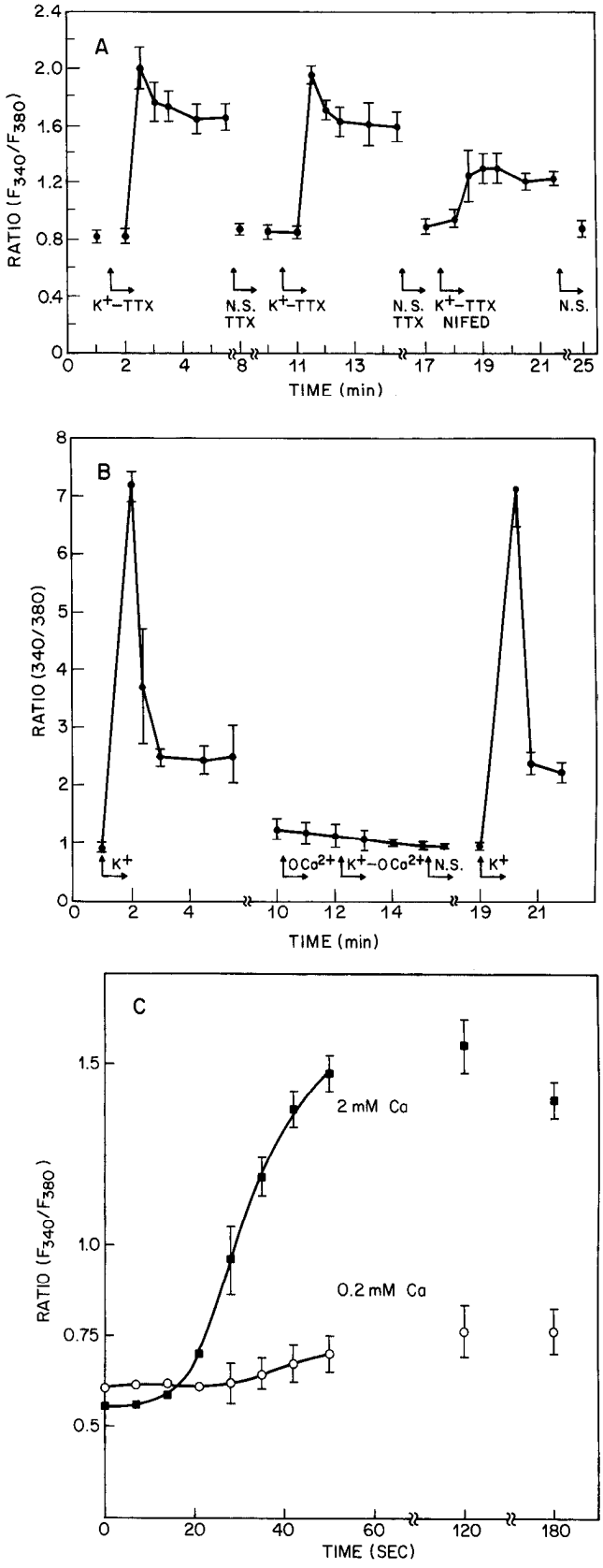

Figure 8. Graphic representation of Ca responses in the presence of nifedipine (*NIFED*) and Ca-free saline. *A*, Nifedipine decrease in high K-induced changes in Ca were pooled from 4 intermediate neurons (9 DIV) in the same field. Nifedipine experiments were done in the presence of TTX and after stable responses to the high-K exposures had been established. *B*, Absence of Ca response to 25 mM K in Ca-free saline (3 mM EGTA). Note very large Ca changes with high K before and after the Ca-free run (*middle*). Data pooled from 3 intermediate neurons (13 DIV) in the same field. *C*, Data pooled from 3 neurons showing the effects of lowering external [Ca] from 2 to 0.2 mM on the K-induced Ca^{2+} response.

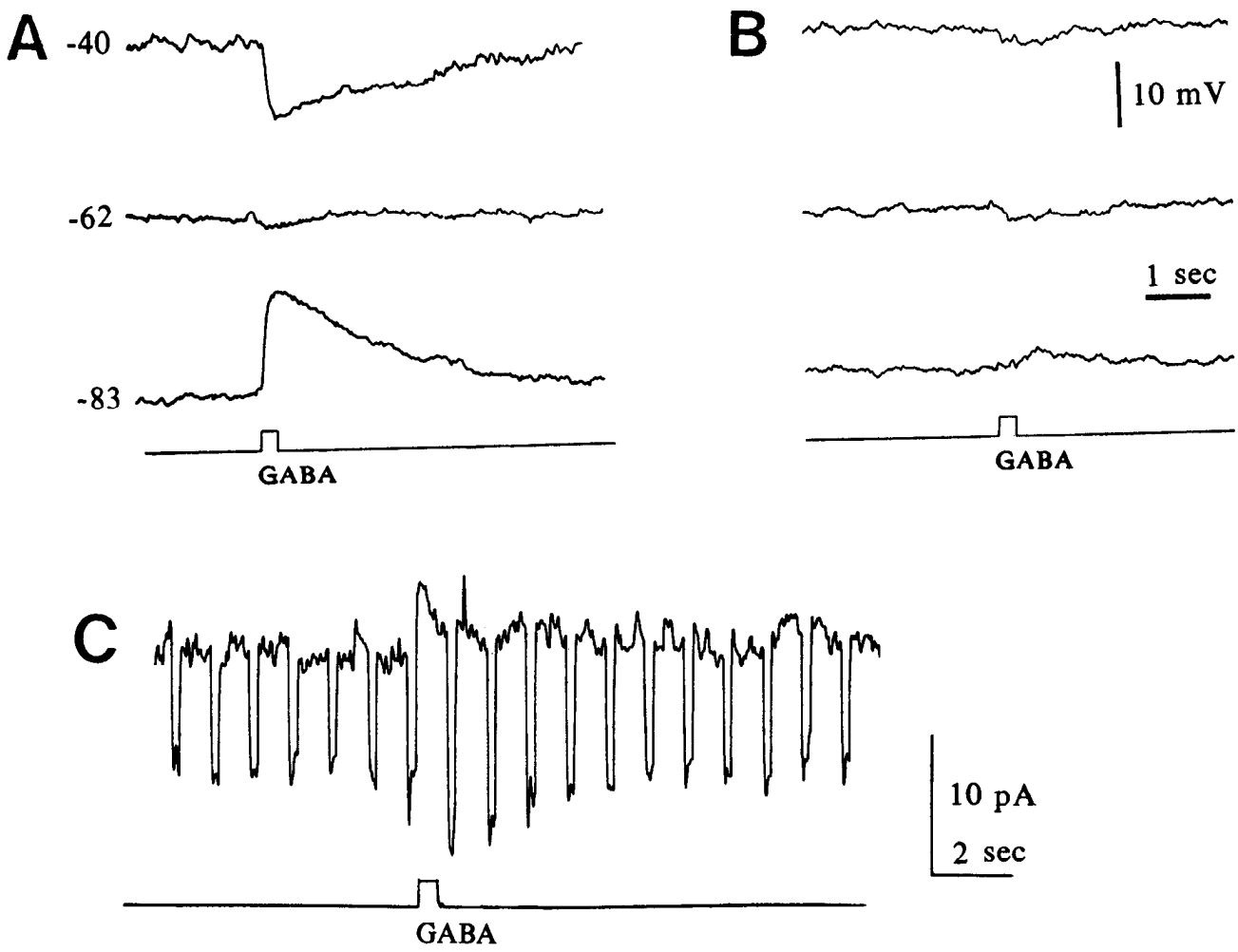

Figure 9. Electrical responses of intermediate granule cells to GABA measured using whole-cell patch recording. *A,* Iontophoretic application of GABA at 3 different membrane potentials showing that the reversal potential for the response was approximately −60 mV. *B,* Blockage of the response at the same potentials by 10^{-6} M-bicuculline. *C,* Current pulses necessary to produce a constant voltage change of −15 mV before and after GABA application (holding potential was −45 mV). The results demonstrate an approximately 2-fold, transient conductance increase brought about by GABA.

(Mullins et al., 1985), or possibly through the Na channels themselves.

We have investigated the effects of standard Ca-channel blockers (Cd, Mn, and nifedipine) on the K-stimulated Ca^{2+} increase. Cadmium and Mn have been used widely in mammalian CNS neurons, as well as in invertebrates, to block Ca currents. Both of these divalent ions have proven unsatisfactory as blockers for the type of experiments done here. At concentrations as low as 100 μM, Cd invariably produced a slow, steady increase in the fluorescence ratio of fura-2. At present we do not know whether the ratio increase was due to Ca or Cd entering the cell and binding to the indicator, since Cd binding to fura-2 also increases the 340:380 fluorescence ratio (J. A. Connor, unpublished observations). In either case, the introduction of high K produced a sharp increase in the fluorescence ratio superimposed upon the baseline drift in the presence of Cd. It would appear from data such as these that the K depolarization was opening divalent channels in the presence of Cd.

Manganese gave somewhat different results. Within a minute or two of exposure to the Mn saline (1 mM), fluorescence at both wavelengths decreased rapidly to about 10% of initial values. The ratio of the 2 wavelengths showed a small decrease. Since the paramagnetic ion Mn^{2+} should quench the fluorescence of fura-2, the decrease was not surprising; however, it showed that, even in the resting cells, the entry rate of Mn was high enough to quench a large fraction of the fura-2 fluorescence. Studies on other mammalian CNS neurons in culture have shown that Mn can enter cells through Ca channels (Peacock and Walker, 1983).

Nifedipine (6 μM) blocked approximately 50% of the K-induced Ca^{2+} increase in granule cells. Figure 8*A* shows the results from a field of 4 cells (9 DIV) given 2 exposures to 25 mM K in the absence of nifedipine, followed by 1 exposure in the presence of nifedipine. It can be seen that the time course of the response was slowed and the amplitude reduced. In each of the cells examined the nifedipine blocked at least 40% of the Ca response. Extended washing, 10–15 min, failed to reverse the block completely.

The response to high K was completely absent in Ca-free saline. Figure 8*B* shows the averaged response of 3 granule cells (13 DIV) to a 25 mM K challenge in normal Ca saline, followed by 3 min washout in Ca-free saline (1 mM EGTA) with normal K, and during a 4 min exposure to high-K, Ca-free saline. The

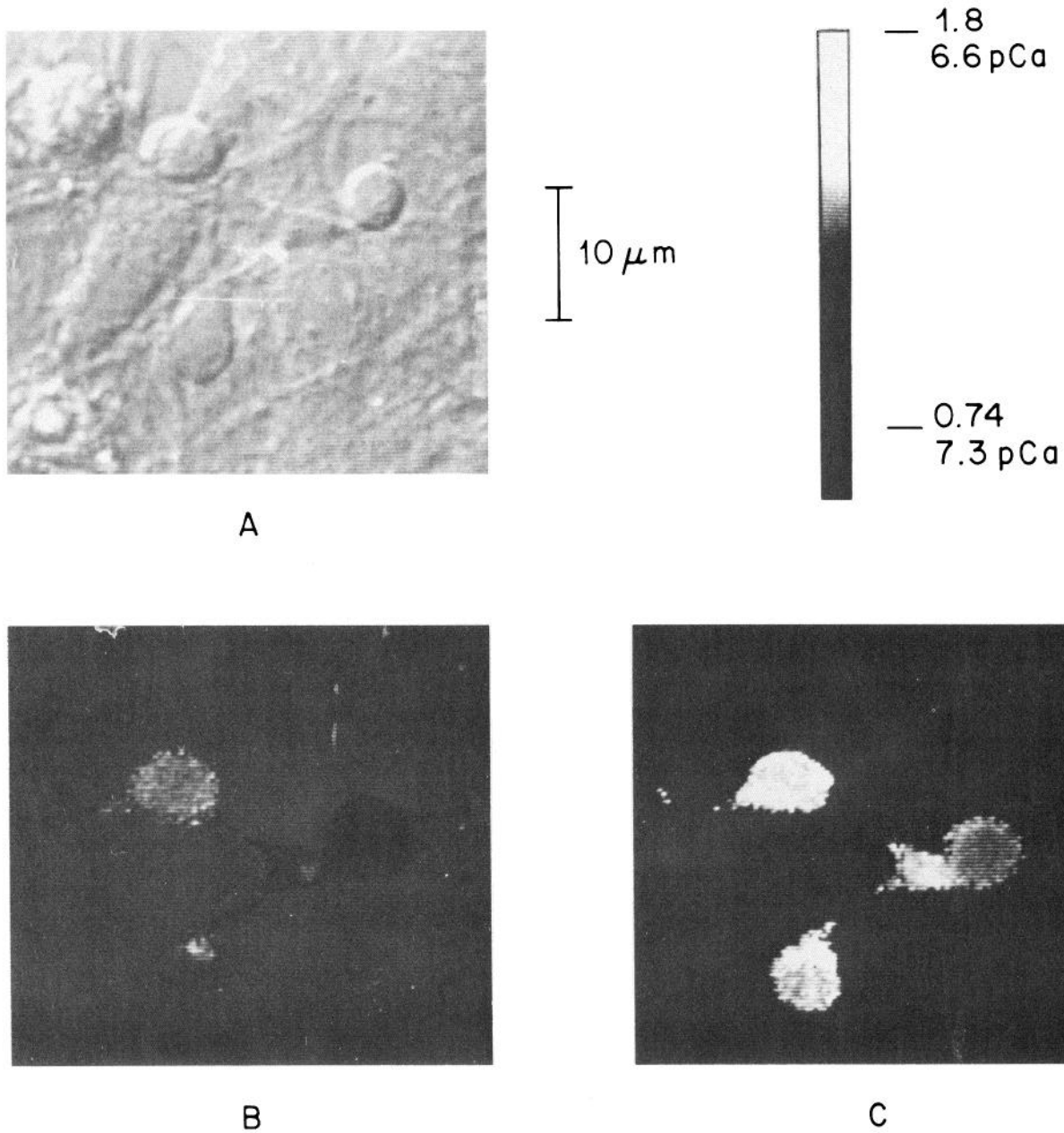

Figure 10. Indicator imaging of the effects of bath-applied GABA on Ca levels in 3 neurons (9 DIV). *A,* Nomarski image of the field. *B,* 340/380 nm ratio image in normal saline containing TTX. *C,* Elevated ratio image after 3 min in GABA–TTX saline. Gray scale images have been used for this and subsequent figures. Ca level changes are described in greater detail in text.

depressed intracellular levels of Ca^{2+} recovered within 3–4 min in normal, Ca-containing saline (not shown). Five coverslips containing a total of 30 neurons were examined in this fashion, and none showed any Ca^{2+} elevation in high-K saline in which external Ca was not present. We also noted no indicator loss or other deterioration of the cells that might have been assignable to EGTA effects.

Less extreme reductions in external [Ca] were also tested on a total of approximately 50 neurons. Figure 8*C* shows data pooled from 3 neurons (17 DIV) first exposed to 25 mM K and 2 mM external Ca (large response) and then to 25 mM K, 0.2 mM Ca (small response). The cells were given a 1 min exposure to 0.2 mM Ca, normal-K saline between the runs in order to equilibrate the external Ca before the K challenge. Longer exposures to the low-Ca salines caused a slow decrease in intracellular [Ca^{2+}]. We found that where the external [Ca] was below about 50 μM there was no appreciable increase in internal Ca^{2+} during exposure to 25 mM K in the great majority of cells. There is still a considerable gradient driving Ca into the cells even at these lower external Ca levels, so that there should be an increased influx during the K depolarizations. We would conclude that the lack of a response at low external Ca levels reflects the ability of the granule cells to buffer intermediate loads of Ca.

Neurotransmitter experiments

Figure 9 shows membrane responses of intermediate granule cells (18 DIV) to iontophoretic application of GABA. The characteristics are identical to GABA responses recorded in several

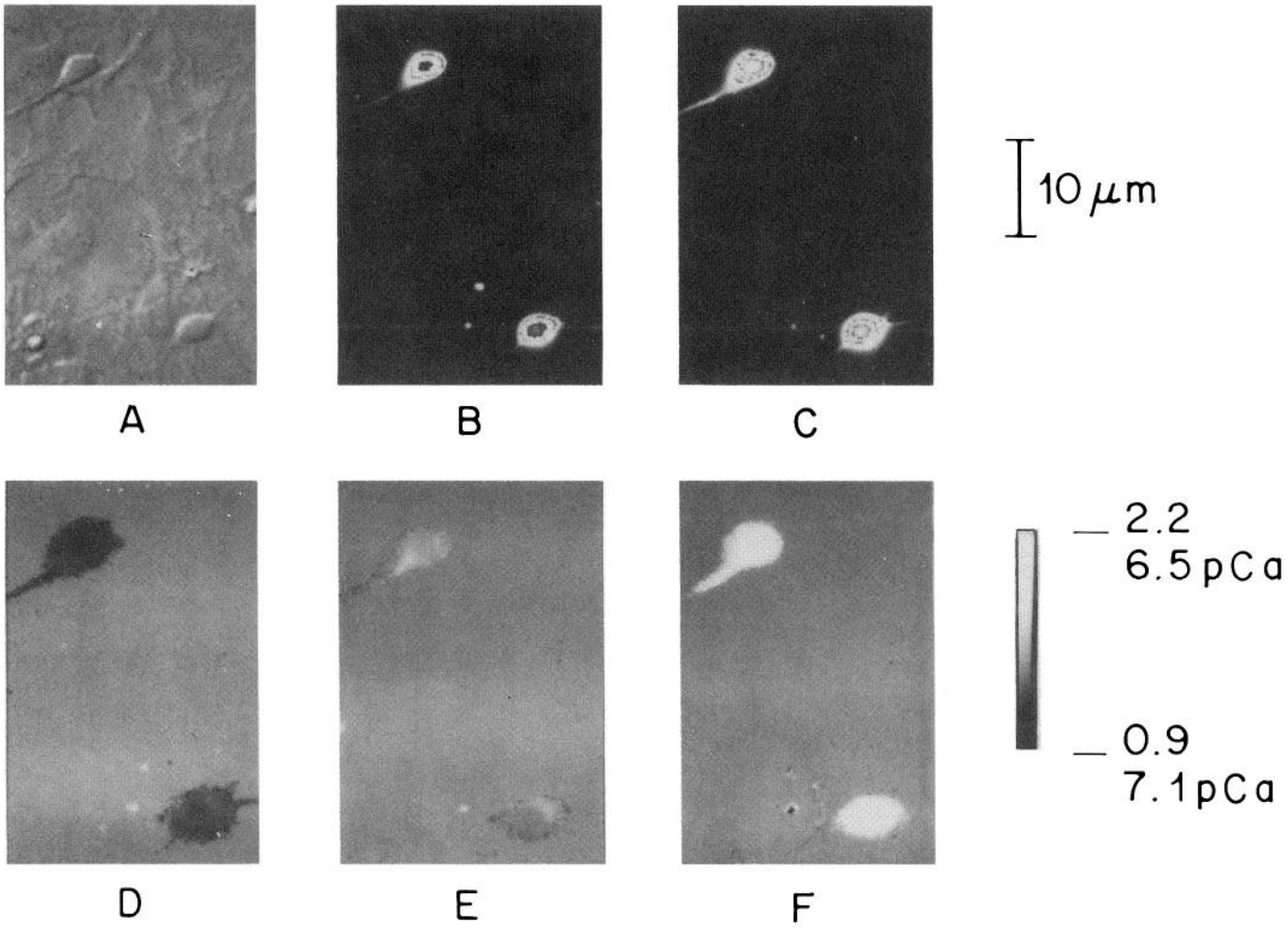

Figure 11. Augmentation of the GABA-induced Ca response in high-K saline. *A,* Nomarski image of field containing 2 granule cells (8 DIV) in addition to several large cells and their processes. *B* and *C,* 340/380 nm fluorescence images of field at a very sensitive display scale showing that only the granule cells trapped a significant quantity of indicator. Dark-corrected pixel values in the central somata were around 100 intensity units, while no area in the background was greater than 2 units. *D,* 340/380 nm ratio image in TTX saline with normal K. *E,* Ratio image after 3 min in 25 mM K with TTX. The plateau level for Ca increase had been reached. *F,* Thirty seconds after exposure to GABA in the high-K–TTX saline.

other types of mammalian CNS neurons (for review, see Nistri and Constanti, 1979): (1) the reversal potential for the response was within a few millivolts of the resting potential (approximately −60 mV in the differentiated cells of Fig. 9*A*, less in developing cells); (2) the response was blocked by either picrotoxin or bicuculline in micromolar amounts (Fig. 9*B*); and (3) GABA caused a rapid conductance increase under voltage clamp (Fig. 9*C*). The iontophoretic responses to GABA were observed even in neuroblasts after 2 DIV, and larger responses were evoked in mature granule cells after 30 DIV. We did not find the systematic disappearance of the membrane potential response to GABA as with glutamate (see below).

Bath application of GABA at 10 μM concentration caused only small changes in resting membrane potential, usually less than 5 mV, depending on the resting potential of the cell (also see Cull-Candy and Ogden, 1985). Because this voltage change is small and because GABA responses typically involve an increase in Cl conductance, we did not expect to observe a significant effect of this transmitter on internal Ca^{2+} levels. Despite this rationale, we found consistent, long-lasting changes in intracellular Ca^{2+} levels with GABA. This finding is illustrated in Figure 10, which shows the fluorescence ratio image for a field of 3 granule cells (9 DIV) before and after GABA exposure. In normal saline the ratio change indicated an increase in Ca^{2+} from resting levels of 60 and 100 nM to 150 nM in the 2 cells on the left. The cell on the right underwent a change from 50 to 125 nM. In over 40 neurons similarly observed, drawn from 4 different explant cultures, GABA increased Ca levels to the range of 120–160 nM. This Ca^{2+} increase generally required 1–2 min to develop and did not show significant reversal even after 10–15 min wash in normal saline.

Figure 11 illustrates an augmentation of the GABA effect by high-K saline. Here, TTX was added to all solutions to avoid the possible effects of action potential firing. The 2 cells (8 DIV) were first bathed in normal saline (Fig. 11*D*) and then exposed to 25 mM K saline for 4 min and the plateau response determined (Fig. 11*E*). The Ca^{2+} level increased from approximately 60 nM to approximately 120 nM in both cells. The bathing solution was then changed to one containing 10 μM GABA in addition to the high K and TTX, and the response shown in Figure 11*F* was measured 30 sec later. The ratio image shows that there was a considerable GABA-induced increase in intracellular Ca,

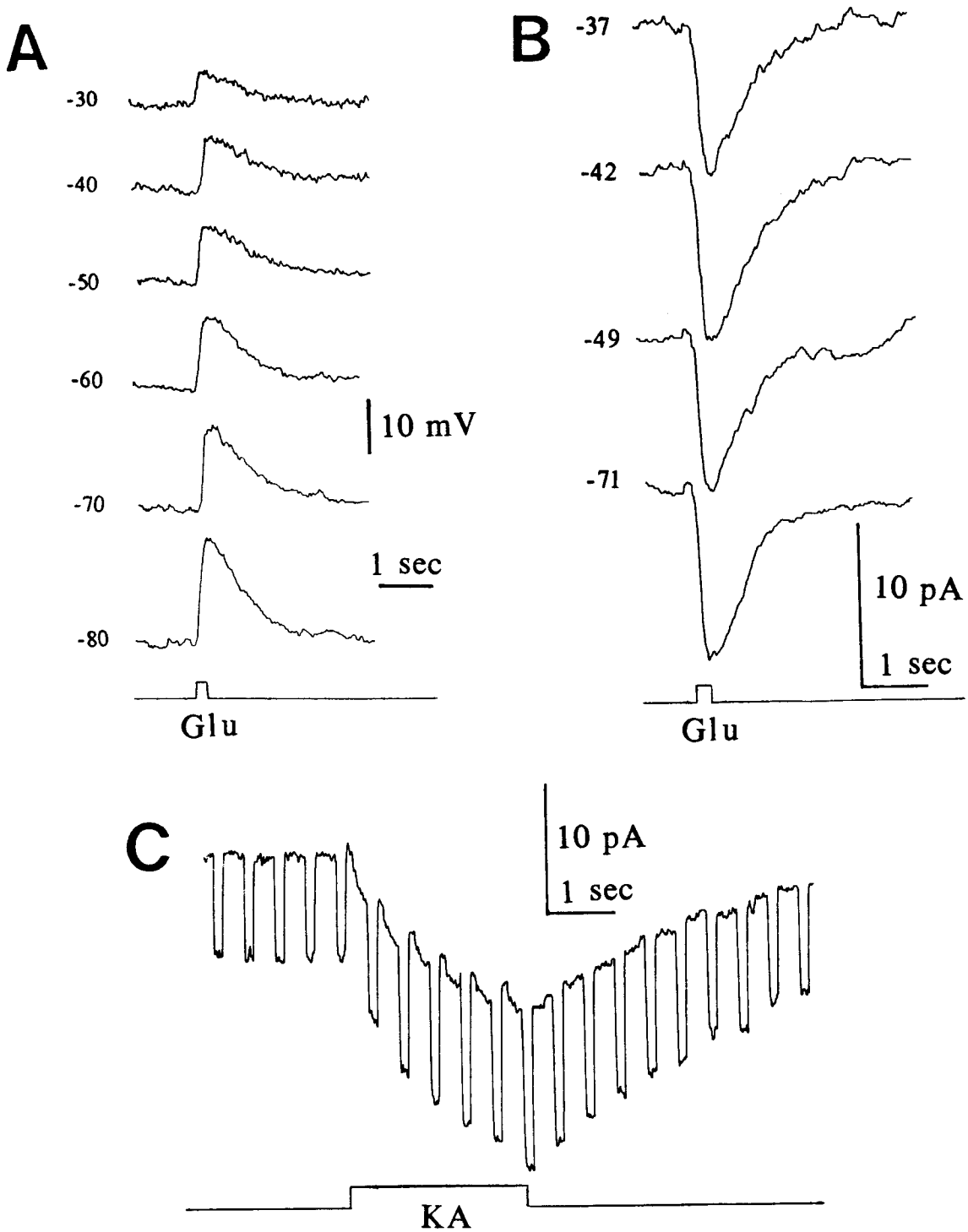

Figure 12. Effects of glutamate and kainate on membrane potential and conductance of intermediate cells. *A,* Membrane potential changes produced by iontophoretic application of glutamate at different holding potentials. *B,* Cell was voltage-clamped at different levels (−37 to −71 mV) and current responses to glutamate were measured. *C,* Conductance measurements under voltage clamp (holding potential, −50 mV) during response to kainate (*KA*) iontophoresis. Constant-voltage pulses (−15 mV) were applied. Kainate produced an approximately 10 nA inward current and a 50% conductance increase in this experiment.

the levels reaching approximately 220 nM. This increase was not due to high-K facilitation since high K was present all along and had reached a plateau in Figure 11*E*.

In addition to the GABA–high-K effect, Figure 11 also illustrates the extreme heterogeneity of indicator trapping by different cell types. The Nomarski picture of Figure 11*A* clearly shows 3 flat cells, in addition to the granule neurons, and many large processes. These structures are invisible in the fluorescence pictures of Figure 11, *B* and *C,* even though the display intensity was deliberately increased to a point where the proximal neurites of the granule neurons are visible and the somata levels are badly distorted (i.e., wrapped-around). At present we do not understand the reasons for the differential trapping, but its existence has proved invaluable in studying the granule neurons.

The intracellular Ca response to GABA described above was not expressed as early in development as the GABA-induced conductance change manifested in the electrical records. Postnatal explants required 4–5 d in culture before the granule cells began to show GABA-induced Ca responses. Therefore, we would assume that the Ca response represents a second, and probably distinct, phase of the GABA response (see Discussion).

Glutamate, applied iontophoretically, evoked transient depolarizations in roughly one-half of the granule cells tested during the first week in culture. This percentage dropped to below 10% in cultures older than 3 weeks. The response to kainate was found more often than to glutamate at all stages tested. In fact, in experiments employing double-barrel iontophoresis, many granule cells responded to kainate while being unaffected by glutamate.

Figure 12*A* shows a set of records where the response to glutamate was elicited under current clamp from holding potentials ranging from −80 to −30 mV. The reversal potential for this response extrapolated to −5 mV. However, under voltage clamp the response was less voltage-dependent over the same range of potentials (Fig. 12*B*), demonstrating the involvement of delayed rectification in modifying the voltage dependence of the sequence in Figure 12*A*. The same results were found using kainate, and similar current–voltage relations have been reported for mouse CNS neurons (Wojtowicz et al., 1981; Mayer and Westbrook, 1984; Nowak et al., 1984). Both glutamate and kainate responses involved membrane conductance increases under voltage clamp (Fig. 12*C*).

Bath exposure to glutamate (10 μM) caused only small changes in resting potential even in cells with large iontophoretic responses. Nevertheless, an increase in internal Ca^{2+} was detected in some cells following bath application that persisted for the duration of the exposure. The rise showed considerable cell-to-cell variation, and as with the electrical response, it was rare in older cultures. Exposure to 1 μM glutamate gave responses that were barely above detection threshold (5–10% ratio change was the approximate fluctuation range of cells under constant bath conditions).

Kainate (10 μM), on the other hand, consistently evoked Ca^{2+} level changes in more cells than glutamate, results that are in agreement with electrical measurements. Figure 13 shows images of a cluster of granule cells (12 DIV) in TTXsaline (Fig. 13*C*), and the small elevation in some cells after 2.5 min in kainate (Fig. 13*D*). After 5 min, all cells displayed increased Ca^{2+} levels (Fig. 13*E*), which recovered to initial levels after 10 min in normal saline (Fig. 13*F*). Glutamate and kainate responses, unlike the GABA response, reversed over the usual time course of experiments. However, the recovery from kainate or glutamate was still much slower than the recovery from the even larger Ca^{2+} changes induced by high K. Adenosine, *N*-methyl-D-aspartate, and quisqualate were ineffective in changing membrane potential or intracellular Ca^{2+} in approximately 30 neurons examined in normal saline.

Figure 14 illustrates differential effects of GABA and glutamate on 2 granule cells (18 DIV), bathed in TTX saline. The initial exposure was to glutamate (10 μM) and caused a small but detectable Ca^{2+} rise in the lower cell over time (Fig. 14,*C–E*). The upper cell was relatively unaffected. After recovery (Fig. 14*F*), 10 μM GABA was applied, causing a Ca response in both the upper and lower cells (Fig. 14*G*). Although we have not performed simultaneous membrane potential and Ca-imaging experiments, we have often recorded from cells that have shown different electrical responsiveness to GABA and glutamate applied using double-barrel iontophoresis.

Discussion

We have used CCD imaging of the Ca indicator fura-2 to study $[Ca^{2+}]$ changes in developing granule cells in culture. While the indicator method used here does not give a quantitative measure of the Ca flux or permit an unequivocal comparison of fluxes at different stages of development (because of possible differences in internal Ca buffering), we consider the technique extremely powerful for the following reasons: (1) Ca currents or channel activity has proved extremely sensitive to the internal dialysis that necessarily accompanies suction-electrode or whole-cell patch recording (Fedulova et al., 1981; Byerly and Hagiwara, 1982; Fenwick et al., 1982); and (2) other methods of looking at Ca currents, such as microelectrode voltage clamping (single or double electrode), are impractical for the small granule neurons. The indicator method reports directly the quantity of most interest anyway, since one is ultimately interested in the $[Ca^{2+}]$ perturbation produced by a given influx. Taken at face value the indicator signals are consistent with the notion that Ca currents increased in size during development of the granule cells. This observation is consistent with our results showing that other types of ionic currents also increased with cell maturation (see preceding paper).

A large fraction of the Ca entry is by way of channels blocked by the dihydropyridine, nifedipine (see Fig. 8*A*). For a given, long exposure to high K, the reduction in the Ca^{2+} response over time was in the vicinity of only 50%. However, if one considers the rates of change involved, which are more closely related to instantaneous flux, the effect was much greater. For example, in the unblocked preparations, the rate of change of $[Ca^{2+}]$ in the first 30 sec after high-K exposure was >10× that after exposure to nifedipine. That is, the large phasic entry was almost completely blocked. In other nerve culture preparations, dihydropyridine block has been associated with the "high threshold" channels (Takahashi and Ogura, 1983), as well as the action of dyhydropyridine Ca agonists (Nowycky et al., 1985), but it is difficult to make any clear association here with one type of channel or another because of the slow time scale of the measurements and the fact that Cd and Mn could not be used as contrasting blockers.

The difficulties encountered with Cd and Mn should serve as a caution on their use as Ca channel blockers, since even brief exposures allowed appreciable intracellular accumulation. The case for Mn is clearer, since roughly 90% of the fura-2 fluorescence was quenched within 3–5 min of exposure to a commonly used level of Mn (1 mM). This level of quenching implies that the Mn was present in the cytoplasm at approximately the same concentration as the fura-2, 0.8–200 μM, a high level considering that Mn^{2+} can compete with Ca^{2+} at a number of important binding sites *in situ*. The extent of Cd^{2+} invasion was more difficult to assess because it produced fluorescence changes similar to Ca. We would note, however, that the ratio increases observed in Cd^{2+}-containing saline were largely nonreversible.

The $[Ca^{2+}]$ changes produced by GABA were a consistent finding in experiments using postnatal explant cultures. The GABA-induced Ca response was present in cells only after 3–4 d in culture, during which time responses to high K could be demonstrated. The response does not seem to be a simple effect of either depolarization or Ca influx through Cl channels for several reasons. First, the reversal potential for GABA was very

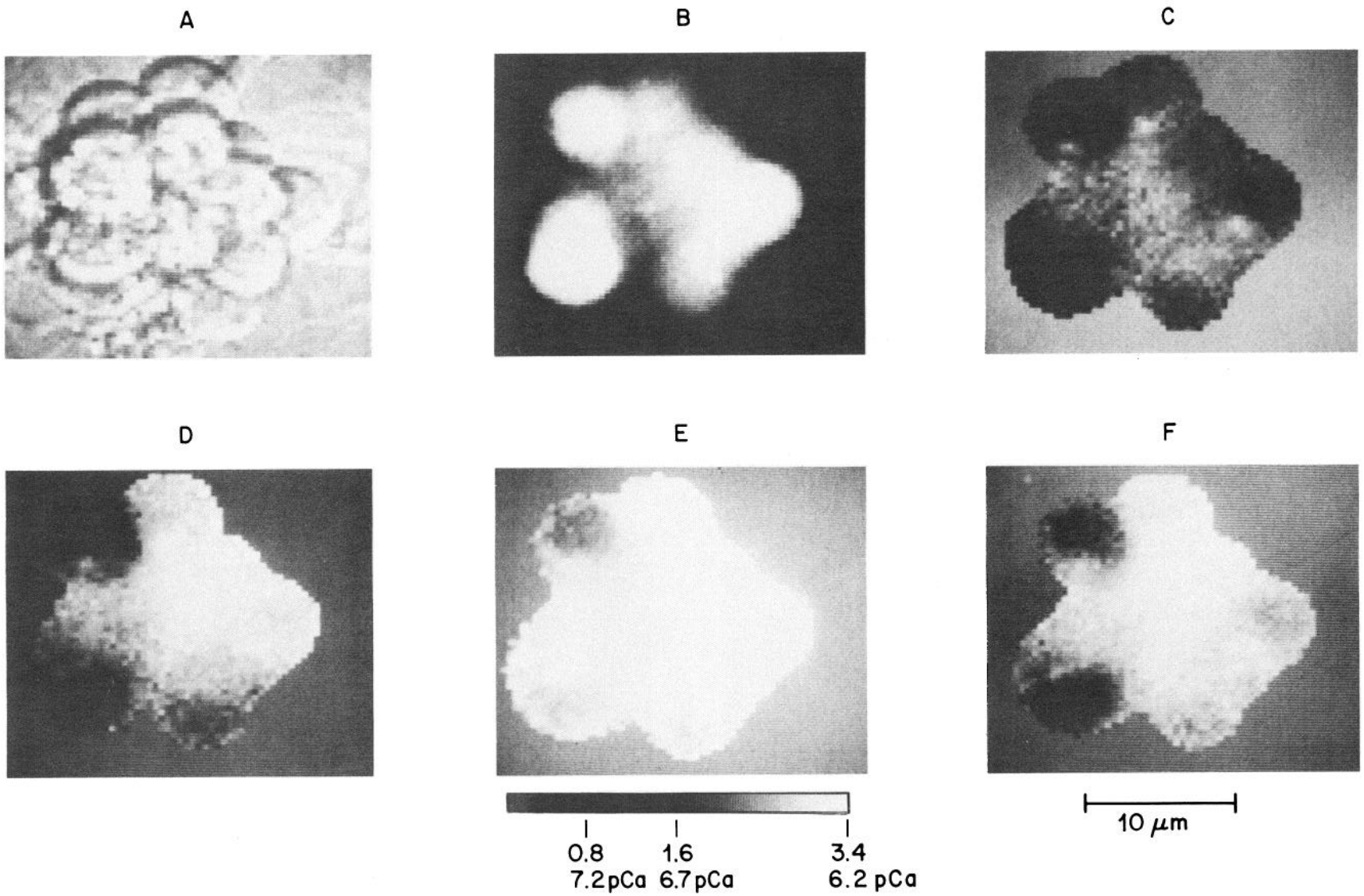

Figure 13. The effect of bath-applied kainate (10 μM) on Ca concentration in a cluster of intermediate neurons (12 DIV). *A,* Nomarski image of field. *B,* 380 nm excitation fluorescence image. *C,* Ratio image in normal saline. *D,* Kainate, 2.5 min. *E,* Kainate, 5 min. *F.*, Ten minute recovery. All solutions contained TTX (0.3 μM).

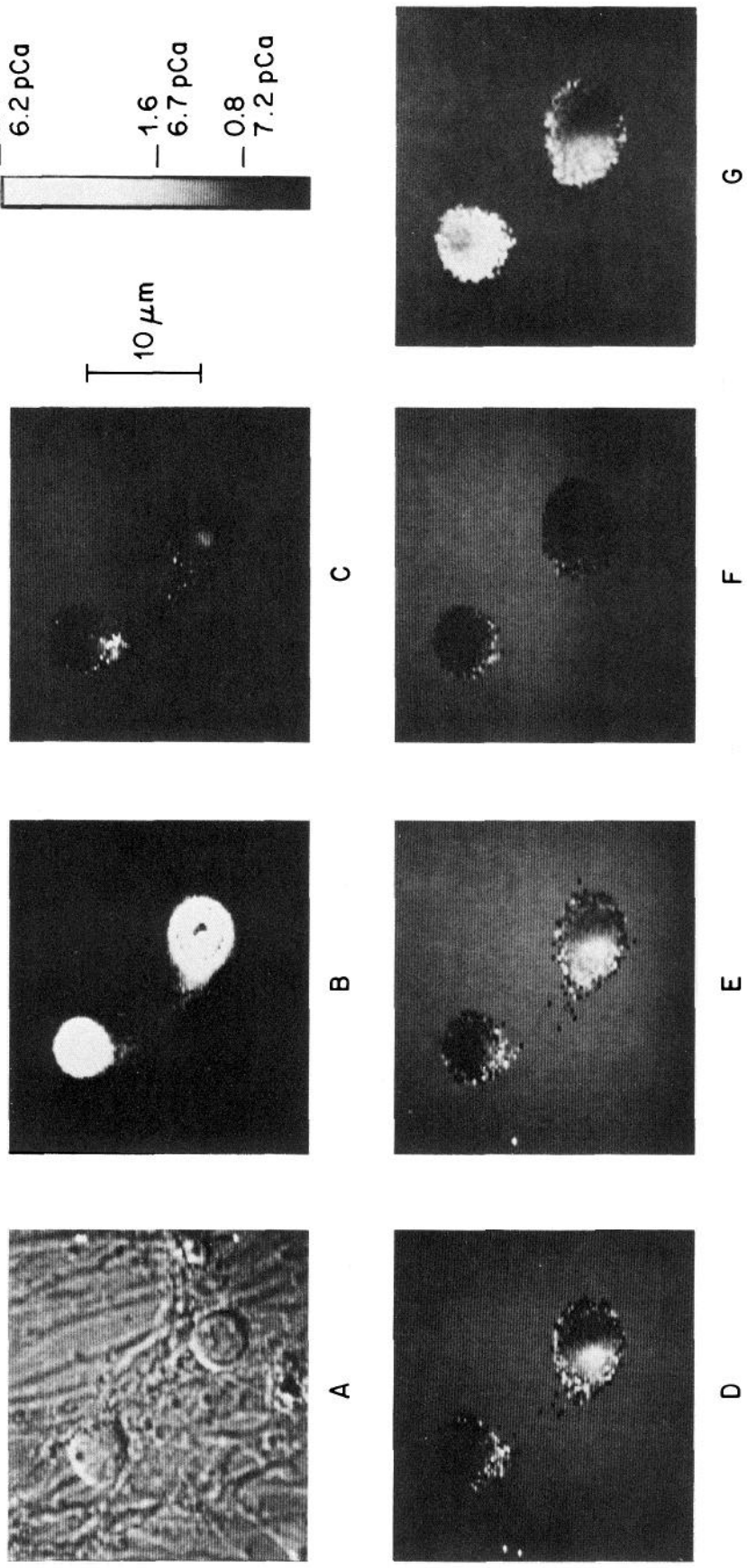

Figure 14. Differential action of glutamate and GABA on 2 neighboring intermediate neurons (18 DIV). *A* and *B*, Nomarski and 380 nm fluorescence images. *C*, Ratio image; cells in TTX saline. *D*, Ratio image after 1 min in glutamate (10 μM). *E*, Ratio image after 2 min in glutamate showed that the lower cell was significantly brighter. *F*, Ratio after 5 min recovery. *G*, Ratio after 1.5 min in GABA (10 μM) showed that both cells were significantly brighter.

near the cell resting potential, and bath application produced very small changes in membrane potential. Second, the Ca^{2+} changes induced by GABA far outlasted the exposure period, while any conductance change quickly reversed. In contrast, the more impressive $[Ca^{2+}]$ changes induced by high K were largely restored after 2–3 min in normal saline. Third, the voltage response to GABA was present in development before the Ca response. Thus, the Ca response induced by bath application of GABA may result from a direct effect on the voltage-dependent Ca current, although this effect would be the opposite of what is found in cultured embryonic sensory neurons (Dunlap, 1981).

The Ca responses to glutamate and kainate, on the other hand, could be due simply to Ca influx resulting from membrane depolarization, since onset and recovery were rapid and the transmitter produced sizable depolarizations (see also Wroblewski et al., 1985). Whether Ca conductance contributes directly to the conductance increase measured under voltage clamp (see Fig. 12*C*), however, remains to be seen. The gradual decline of glutamate responsiveness in older cultures may reflect culture conditions, but it is tempting to speculate that in some cases the receptors may have moved to the terminals where they reside in the adult cerebellum *in situ* (cf. Greenmayre et al., 1985).

The facilitation of the Ca^{2+} response to high K is extremely interesting in that it provides a mechanism for the cell to "remember" over periods of several minutes that it has undergone previous activity. Also, if one considers Ca-modified processes, such as transmitter release or enzymatic activity, it is quite plausible (given appropriate equilibrium constants) that the first of a train of stimuli might give a negligible response, while subsequent stimuli produce disproportionately larger responses (although see Levi et al., 1984). Something analogous to this Ca^{2+} facilitation effect was thought to have been demonstrated in molluscan neurons several years ago using the Ca^{2+} indicator aequorin (Eckert et al., 1977; Lux and Heyer 1977). In this case, however, it turned out to be an artifact arising from the nonlinear response of the Ca indicator (Ahmed and Connor, 1979; Smith and Zucker, 1980). Fura-2 should have no such problem over the range of Ca^{2+} levels encountered.

The critical difference between the Ca^{2+} facilitation observed here and conditions analogous to the "residual Ca^{2+} hypothesis" for synaptic transmitter release (see Katz and Miledi, 1968; Rahamimoff, 1968) is that the cytoplasmic $[Ca^{2+}]$ returns completely to baseline between stimuli, rather than remaining at an elevated level. Such a condition of residual Ca has been shown, for example, during posttetanic potentiation in *Aplysia* neuron L-10 (Connor et al., 1986b). At present we do not know whether the basis of the facilitation resides in modification of the Ca channel population with repeated use, as shown for adrenal chromaffin cells (Hoshi et al., 1984), or whether the granule cells have a moderately low buffer capacity for Ca that takes relatively long periods (over 10 min) to empty. We are currently attempting to determine whether Ca facilitation can be demonstrated using a controlled electrical stimulus.

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