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ACETYLCHOLINE RELEASE BY BRADYKININ, INOSITOL 1,4,5-TRISPHOSPHATE AND PHORBOL DIBUTYRATE IN RODENT NEUROBLASTOMA CELLS

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SUMMARY

1. The action of bradykinin (BK), inositol 1,4,5-trisphosphate ($InsP_3$), and phorbol dibutyrate (PDBu) on the release of acetylcholine (ACh) was studied electrophysiologically on short-distance ($< 20\ \mu m$) synapses formed between cultured NG108-15 mouse neuroblastoma × rat glioma hybrid cells and rat muscle cells. Action potentials in NG108-15 cells did not usually evoke an excitatory junction potential (EJP) in the muscle cell in this system.

2. Ionophoretic application of BK onto the somatic surface of an NG108-15 cell produced an increase in frequency of miniature end-plate potentials (MEPPs) for 40–50 s in the paired myotube. Some MEPPs were evoked during BK-induced hyperpolarization (10–20 s) of the hybrid cell soma. A few MEPPs were also elicited during BK-induced depolarization.

3. Ionophoretic injection of Ca^{2+} into an NG108-15 cell soma generated MEPPs for a very brief period (less than 3 s), coincident with somatic hyperpolarization. No increase was observed during a subsequent somatic depolarization induced by a larger current of Ca^{2+}.

4. Ionophoretic injection of $InsP_3$ into the cytoplasm of an NG108-15 cell soma transiently evoked MEPPs during the $InsP_3$-induced hyperpolarizing phase. A large $InsP_3$ injection caused sustained generation of MEPPs for 2–4 min, associated with $InsP_3$-evoked depolarization.

5. Within 3–5 min after exposure of NG108-15–myotube pairs to 1 μM-PDBu, the MEPP frequency increased by 2–5 times and reached a plateau after 8 min. The increase continued after wash-out of the drug. The PDBu-induced increase of MEPPs was still observed when the membrane potential of the NG108-15 cell was clamped at -30 mV.

6. The data suggest that the BK-induced facilitation results from the action of two intracellular second messengers: an $InsP_3$-dependent release of Ca^{2+} from the intracellular storage sites and protein phosphorylation by diacyclglycerol (DAG)-activated protein kinase C.

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INTRODUCTION

The role of Ca^{2+} ions in the transmitter release from presynaptic terminals evoked by a *depolarization* is well established (Katz & Miledi, 1968). However, the mechanism for *receptor*-mediated presynaptic facilitation has not been extensively studied, because of lack of suitable systems.

Neuroblastoma × glioma NG108-15 hybrid cells contain high concentrations of acetylcholine (ACh) and choline acetyltransferase (McGee, Simpson, Christian, Mata, Nelson & Nirenberg, 1978; Wilson, Higashida, Minna & Nirenberg, 1978), and readily form synapses with cultured muscle cells (Nelson, Christian & Nirenberg, 1976; Puro & Nirenberg, 1976; Higashida, Wilson, Adler & Nirenberg, 1978; Nirenberg, Wilson, Higashida, Rotter, Krueger, Busis, Ray, Kenimer, Adler & Fukui, 1983). In such synapse pairs effective contact is signalled by the appearance of miniature end-plate potentials (MEPPs). The NG108-15 hybrid–myotube synapse provides a simple system to elucidate the mechanism of receptor-evoked neurotransmitter secretion, because there is less interaction by other inputs or by feedback regulations (Christian, Nelson, Bullock, Mullinax & Nirenberg, 1978; Nakagawa, Higashida & Miki, 1984). It has been previously reported that the application of bradykinin (BK) to such synaptically connected hybrid cells increases the frequency of MEPPs recorded from the muscle cells (Yano, Higashida, Inoue & Nozawa, 1984).

BK produces two sequential potential changes in the NG108-15 cells: an initial hyperpolarization, followed by a depolarization, with a substantial decrease and increase in spontaneous action potential frequency, respectively (Higashida *et al.* 1978; Reiser & Hamprecht, 1982; Yano *et al.* 1984; Higashida, Streaty, Klee & Nirenberg, 1986). The two membrane potential changes are due to the sequential activation and inhibition of two membrane K^+ currents, one Ca^{2+} dependent and the other voltage dependent (Higashida & Brown, 1986; Brown & Higashida, 1988*a*, *b*). Previous experiments indicate that these two effects of BK might arise from a single primary biochemical action: the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate to form inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG; Yano *et al.* 1984; Yano. Higashida, Hattori & Nozawa, 1985*a*; Yano, Higashida & Nozawa, 1985*b*; Brown & Higashida, 1988*b*). The question addressed in these experiments is whether these two intermediate substances might trigger the increased MEPP frequency, playing a role as second messengers for BK receptor-mediated facilitation. Part of this work has been presented in a preliminary form (Higashida, 1986).

METHODS

Co-culture. Hindlimb muscle cells of eight to ten newborn rats were minced and treated for 30 min with 0·2% trypsin (Worthington, Freehold, NJ, U.S.A.) dissolved in Dulbecco's modified Eagle's medium (DMEM). The cells were collected by centrifugation (500 *g* for 5 min), and suspended in 14 ml DMEM supplemented with 10% fetal calf serum and 10% horse serum, then dissociated into single cells by repetitive (20 times) pipetting. The cells in three culture dishes (60 mm in diameter) were pre-incubated for 40 min at 37 °C to remove fibroblasts (Puro & Nirenberg, 1978). Muscle cells (1×10^6 cells/35 mm dish) were plated on collagen-coated dishes in the above medium. After 3 days the cells were exposed to 10 μM-5-fluorodeoxyuridine and 100 μM-uridine in DMEM plus 10% horse serum for 2 days to suppress the growth of non-fused muscle cells

and mitotic fibroblasts (MacDermot, Higashida, Wilson, Matsuzawa, Minna & Nirenberg, 1979). After washing out the drugs, the cells were incubated for another day in DMEM plus 10% horse serum for recovery. $2–5 \times 10^4$ NG108-15 cells, which had been treated with 10 μM-PGE_1 (prostaglandin E_1) and 1 mM-theophylline (Higashida, Kato, Kano-Tanaka, Okuya, Miyake & Tanaka, 1981; Nirenberg *et al.* 1983) for 5 days, were added on the already fused myotube culture and maintained in DMEM supplemented with 3% horse serum, 100 μM-hypoxanthine, 16 μM-thymidine and 1 mM-dibutyryl cyclic AMP (Boehringer, Indianapolis, U.S.A.) for 1–3 weeks. A cell pair where a round hybrid cell (50–150 μm in diameter) settled on top of, or beside, a thick myotube, and thus made a synaptic connection with a very short process or directly with the hybrid's cell body, was chosen for the experiments (see Plate 1), in order to minimize the distance between $InsP_3$ formation and ACh release sites. Morphological evidence shows that the cell soma or near-neurites of NG108-15 hybrid cells contain clear or dense core vesicles (Daniels & Hamprecht, 1974; Nelson, Christian, Daniels, Henkart, Bullock, Mullinax & Nirenberg, 1978; Furuya & Furuya, 1983; Furuya, Sawada, Nagatsu, Suzuki & Higashida, 1985).

Synapses were studied in DMEM supplemented with 2 mM-$CaCl_2$, 100 μM-choline chloride (Nelson *et al.* 1976). Tetrodotoxin (1 μM) was sometimes added. Membrane potentials or currents from NG108-15 cells were recorded with a KCl (3 M)-filled micropipette (resistance 10–40 MΩ) via an Axoclamp-2 amplifier (Axon Instruments, Burlingame, Ca, U.S.A.), as described by Brown & Higashida (1988*a*). Muscle cells were penetrated with 1 M-potassium-citrate-filled microelectrodes (10–20 MΩ resistance) and potentials were amplified via a WPI 707 amplifier (WPI Inc., CT, U.S.A.).

Methods of ionophoresis of BK (Sigma, 0·1 mM in 0·1 mM-HCl), $InsP_3$ (Sigma or Calbiochem, 0·5 mM in distilled water) or Ca^{2+} (0·5 M) were the same as described previously (Higashida *et al.* 1986; Higashida & Brown, 1986; Brown & Higashida, 1988*a*, *b*). Phorbol dibutyrate (PDBu, Calbiochem) was added to the perfusion fluid as 1:10000 dilution of a 10 mM solution in ethanol as described previously (Higashida & Brown, 1986).

Data are presented as mean ± S.E.M. unless stated otherwise.

RESULTS

In agreement with previous reports (Puro & Nirenberg, 1976; Higashida *et al.* 1978; Higashida *et al.* 1981; De Blas, Adler, Shih, Chiang, Cantoni & Nirenberg, 1984; Busis, Daniels, Bauer, Pudimat, Sonderegger, Schaffner & Nirenberg, 1984; Yano *et al.* 1984), spontaneous miniature end-plate potentials (MEPPs) were regularly recorded in rat myotubes co-cultured with NG108-15 cells. The MEPP frequency varied from one synapse to the next and the mean frequency scattered over a wide range from 0·01 to 9 events/s (with a mean value of 0·88 ± 0·15 events/s, $n = 153$). The intervals between successive discharges also varied from a few milliseconds to 10–30 s. Amplitudes were generally small, the majority being < 1 mV, but occasional large potentials up to 10–20 mV were concomitantly recorded. Amplitude histograms showed a monotonic distribution, skewed towards the baseline noise with a mean of 1·3 ± 0·12 mV ($n = 168$). The rise times were 2–8 ms, and did not vary with size (see Fig. 6*B*). Addition of *d*-tubocurarine (< 1 μM) reversibly suppressed all MEPPs.

Action potentials in NG108-15 cells induced by direct stimulation occasionally evoked excitatory postsynaptic potentials (EPPs) of varying amplitudes in the muscle cells (see Fig. 1 of Nelson *et al.* 1976 and Figs 1 and 2 of Puro & Nirenberg, 1976), but the failure rate was high (82–98%) and quantal content accordingly very low (0·02–0·2), as previously reported (Nelson *et al.* 1976).

These electrophysiological properties of NG108-15–myotube synapses accord with the morphologically immature state (Furuya & Furuya, 1983) and resemble those

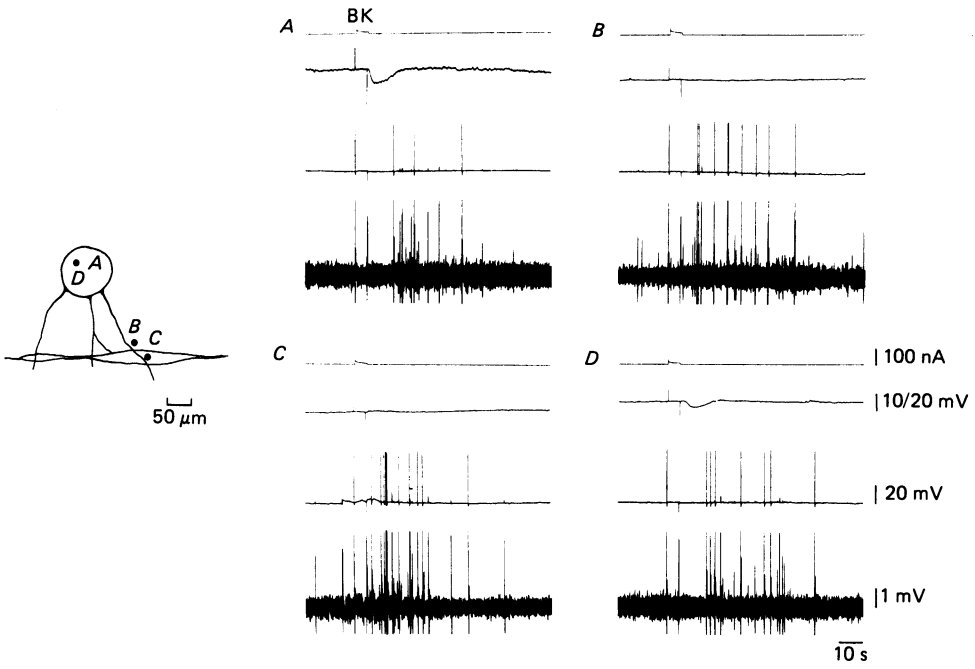

Fig. 1. ACh release evoked by BK stimulation at different locations on an NG108-15 cell. Sequential ionophoretic application of BK onto the cell soma (*A*), a neurite close to a muscle cell (*B*) and on the muscle cell (*C*), and again on the cell soma (*C*). The points of application are indicated by the dots in the schematic drawing on the left. In each panel (*A–C*) records show from above downwards: ionophoretic current (30 nA × 5 s); pen chart of membrane potential of the NG108-15 hybrid cell (hyperpolarization downwards); and (lower two traces) DC- and AC-coupled potentials recorded from the muscle cell. Each upward deflection corresponds to a variable size of muscle depolarizing responses (MEPPs) and action potentials (regular size). Resting membrane potentials of the hybrid and muscle cells were −56 and −66 mV, respectively. Calibration of the hybrid membrane potential, 10 mV for *A* and 20 mV for *B–D*.

seen at botulinum-poisoned or regenerating adult motor nerve junctions (Harris & Miledi, 1971; Dennis & Miledi, 1974*a*, *b*; Kim, Lomo, Lupa & Thesleff, 1984).

Bradykinin-induced transmitter release

Effect of bradykinin on transmission in synapses with long neurites. To determine whether BK-induced ACh release occurs from presynaptic regions, BK was applied to different parts of an NG108-15 cell connected with long neurites to a muscle cell, as shown in Fig. 1. Ionophoretic application of BK to the extracellular surface of the cell soma of the NG108-15 cell produced a hyperpolarization followed by a depolarization (Fig. 1*A* and *D*), as reported previously (Yano *et al.* 1984; Higashida *et al.* 1986). Many MEPPs and action potentials of the muscle cell were evoked 16 s after BK application between the late phase of the hyperpolarization and the subsequent depolarization of the hybrid cell. When BK was applied at two spots on one of the neurites > 200 μm away from the cell soma (distant from, or in contact with, the muscle cell), several muscle responses were evoked within 1–6 s, although

no apparent membrane potential response was recorded from the soma (Fig. 1*B* and *C*). It was confirmed in two other cells that receptors mediating soma membrane potential changes were restricted to the cell soma and to the first 50–100 μm of the cell processes (see Brown & Higashida, 1988*b*). ACh release was not observed after three BK applications onto the muscle cell outside synaptic regions and the BK-induced ACh response was never detected from non-innervated myotubes (tested in more than fifty cells). Also BK application had little or no effect on input membrane resistance in six muscle cells (control, $6{\cdot}8 \pm 0{\cdot}5$ MΩ; after BK, $7{\cdot}5 \pm 0{\cdot}6$ MΩ). Hence this BK effect is due to the activation of BK receptors in the neurite close to synapse regions.

Effect of bradykinin on transmission in synapses with short neurites. To avoid complications arising from the time necessary for signal conduction through long neurites and to minimize the distance from receptor activation to ACh release sites, effects of BK on ACh release were examined in NG108-15 cells that formed synapses either with very short neurites or directly with their cell body (pair 1 in Plate 1). Ionophoretic application of BK onto the cell soma of one such hybrid cell generated many MEPPs in the muscle cell during the early phase of the hyperpolarization of the NG108-15 cell and fewer MEPPs during the later phase (Fig. 2*A*). In these 'short synapses', the latency was greatly reduced to 1–2 s after BK application. The time course of the increase of MEPPs in response to ionophoretically applied BK in twenty-eight such hybrid–myotube pairs is shown in Fig. 2*B*. The frequency increase started 1–5 s after ionophoretic application of BK, and peaked at 7–14 s when most of the hybrid cells underwent hyperpolarization, then gradually returned to the initial level over 20–50 s. This time course corresponds well to that of a previous report (Yano *et al.* 1984), where BK was topically applied so that BK could activate almost all BK receptors presented on one NG108-15 cell. The MEPP frequency increased by a factor of 1·3–27 times (mean $= 6{\cdot}9 \pm 1{\cdot}2$ events/s, $n = 29$) over the control ($0{\cdot}32 \pm 0{\cdot}7$ events/s). The MEPP amplitude distribution after stimulation was also skewed towards the baseline. The mean amplitude of MEPPs before and after five BK applications in one given synapse was $1{\cdot}37 \pm 0{\cdot}12$ mV ($n = 188$) and $1{\cdot}49 \pm 0{\cdot}12$ mV ($n = 192$; the resting membrane potential of the myotube, −48 mV), respectively.

Though the frequency usually was higher than the control level (Fig. 2*B*), fewer MEPPs were evoked in the later phase of the BK response (20–50 s after BK application), even though the cell was more excitable and showed repetitive firings, as shown in Fig. 2*A*. The residual rise in MEPP frequency during this depolarization was not due to the action potential discharge, since the number of MEPPs coincident with action potentials did not exceed those induced by 100 action potentials elicited by current injection before the BK application.

The results show that facilitation by activating BK receptors in the cell soma of NG108-15 cells (which formed synapses within a very short distance) has properties similar to BK-induced presynaptic facilitation from a tip of long neurites. Subsequent experiments were carried out in NG108-15 cells that formed synapses directly or with short neurites in order to gain an insight into how bradykinin-induced facilitation is chemically transduced.

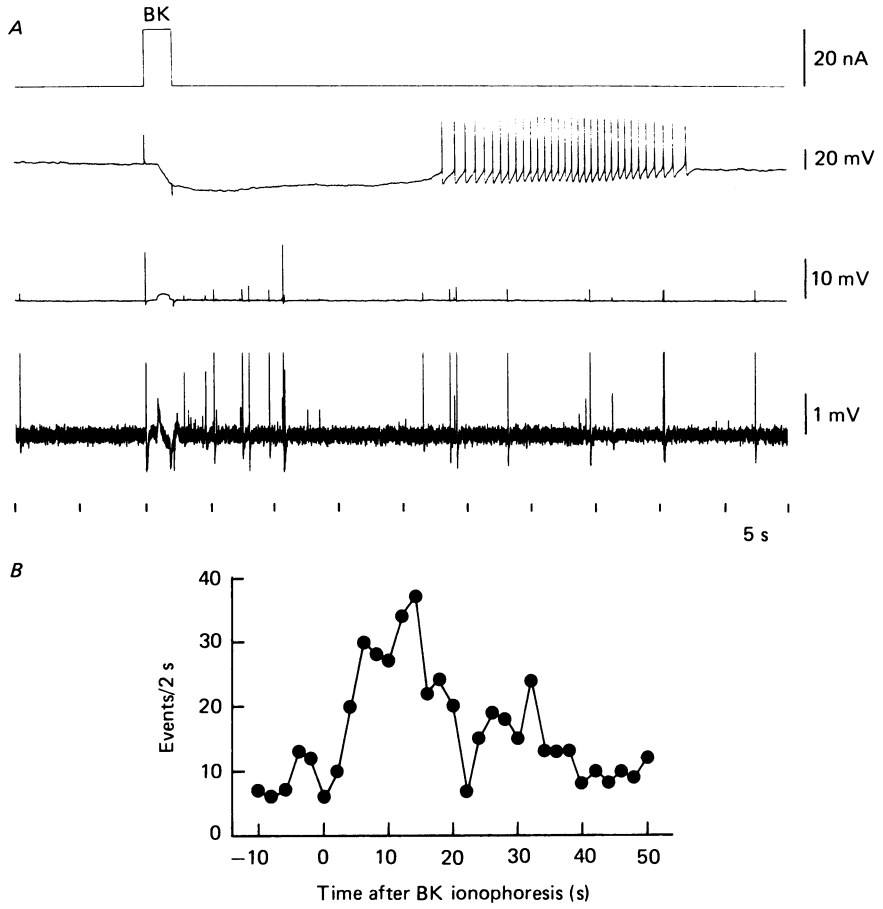

Fig. 2. ACh release accompanied the hybrid cell response to BK. *A*, simultaneous recording from one NG108-15 hybrid and a paired muscle cell connected with short neurite(s). Uppermost record, ionophoretic current of BK application on the extracellular surface of the cell soma of the hybrid cell (20 nA for 2 s) as indicated. Upper record, membrane potential of the hybrid cell. (Resting membrane potential, −52 mV. Note that spontaneous and repetitive action potentials were elicited during the recovery phase from the BK-induced hyperpolarization.) Lower two traces, DC- and AC-coupled recording of the muscle potentials, respectively. Resting membrane potential, −60 mV. Many MEPPs were evoked during hyperpolarization of the hybrid cell. Time marker interval, 5 s. *B*, frequency plot of synaptic events in response to ionophoretic application of BK accumulated from twenty-eight hybrid–myotube pairs.

Ca^{2+}-injection-evoked MEPPs

The period of the BK-induced increase in MEPP frequency corresponds well with the initial transient elevation of cytosolic Ca^{2+} concentrations, reported previously using fluorescence quin-2 measurements (Reiser & Hamprecht, 1985; Osugi, Uchida, Imaizumi & Yoshida, 1986*b*), and with the measurement of $^{45}Ca^{2+}$ influx and efflux reported by Yano *et al.* (1984) and Higashida *et al.* (1986). To test the role of Ca^{2+}, Ca^{2+} was ionophoretically injected into the cytoplasm of an NG108-15 cell soma

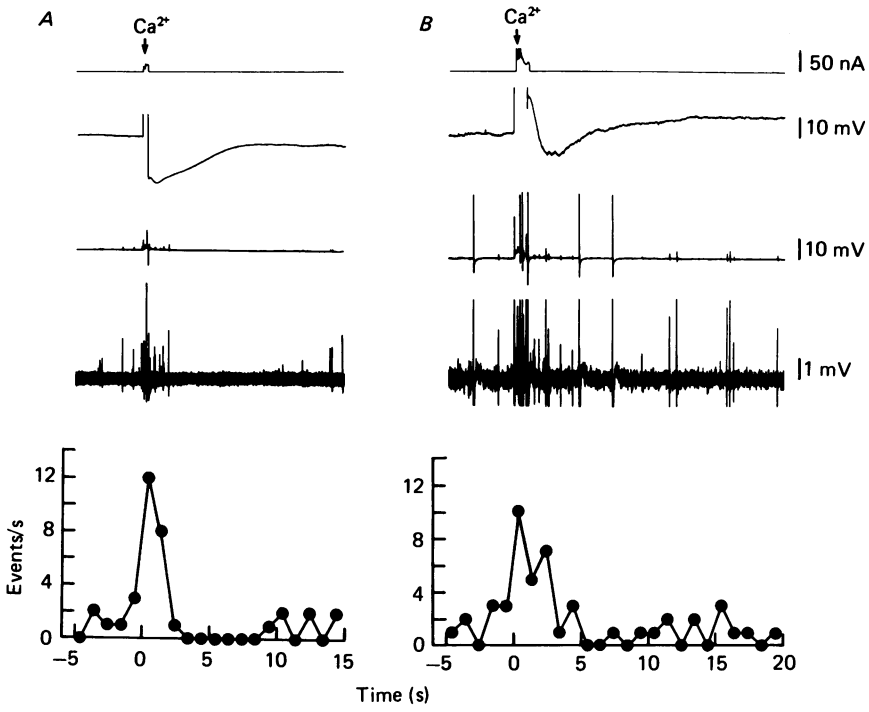

Fig. 3. MEPPs evoked by intracellular injection of Ca^{2+} into NG108-15 cells. Records in *A* and *B* were obtained from two different pairs. Uppermost record, ionophoretic current for Ca^{2+} injection: *A*, +20 nA for 0·5 s; *B*, +50 nA for 1 s. Upper record, hybrid cell response after Ca^{2+} injection; hyperpolarization (*A*) or hyperpolarization followed by depolarization (*B*). Resting membrane potentials of *A* and *B* were −44 and −43 mV, respectively. Lower two traces, muscle responses (MEPPs and action potentials) in DC and AC recordings. Resting membrane potential, −56 (*A*) and −60 (*B*) mV, respectively. Graphs, frequency plots of synapse responses in the above recordings.

through a second electrode filled with 0·5 M-$CaCl_2$. A burst of MEPPs was evoked during the depolarizing current, but this persisted only for a few seconds after the application when the cell was hyperpolarized (Fig. 3*A*). There was no further increase of MEPP frequency during the subsequent depolarizing phase when larger injection doses were given (Fig. 3*B*). The initial hyperpolarization results from activation of a Ca^{2+}-dependent K^+ current (Higashida & Brown, 1986; Brown & Higashida, 1988*a*) and hence provides evidence for effective release of Ca^{2+} from the pipette into the cytoplasm. The mean MEPP frequency increased by 3·9-fold (±0·61, $n = 6$) following Ca^{2+} injections over the control frequency (0·19±0·05 events/s). Similar ionophoretic currents of K^+ (from 3 M-KCl-filled pipettes) did not produce a comparable effect.

Inositol 1,4,5-trisphosphate-induced MEPPs

Since BK stimulates the formation of $InsP_3$ in NG108-15 cells (Yano *et al.* 1985*a*) and injected $InsP_3$ raises intracellular Ca^{2+} concentrations (Osugi *et al.* 1986*b*), the effect of $InsP_3$ on ACh secretion from NG108-15 cells was examined by intracellular

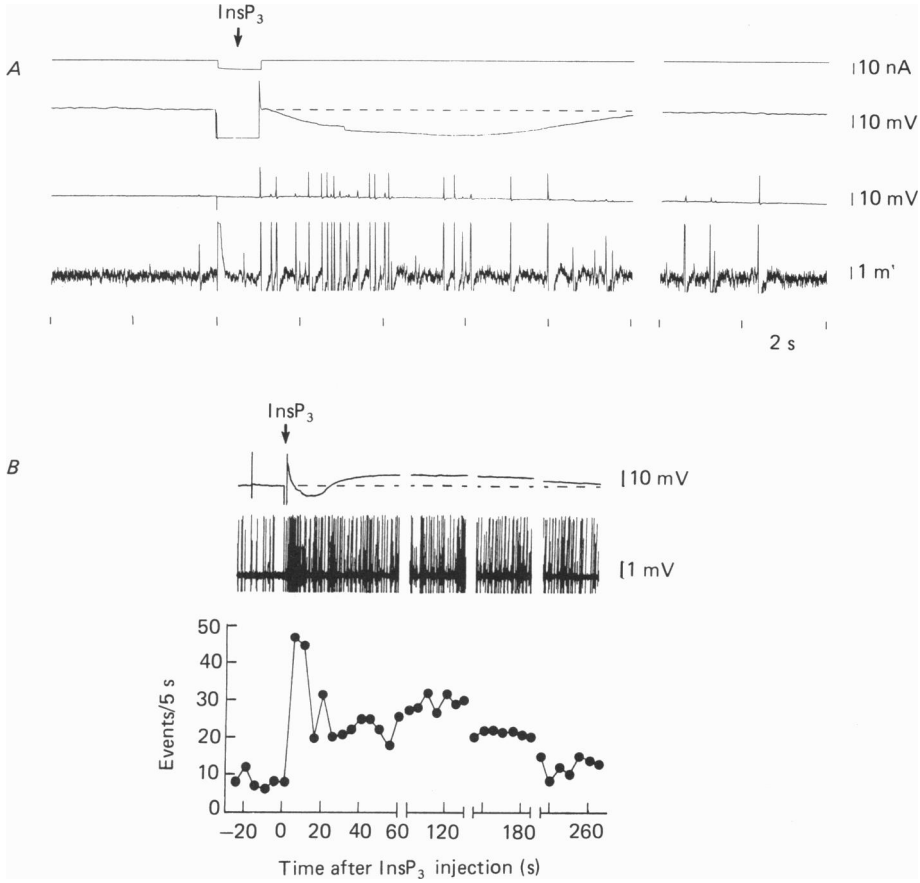

Fig. 4. MEPPs triggered by $InsP_3$ injection into an NG108-15 hybrid cell. Simultaneous recordings of one hybrid–myotube pair with different injection currents. Resting membrane potentials of the hybrid and muscle cells were −61 and −70 mV, respectively. *A*, records show from above downwards: injection current of $InsP_3$ (−10 nA, 1 s); membrane potential of the NG108-15 cell; DC- and AC-coupled recordings of the muscle cell. Note that many MEPPs were generated in response to injection of $InsP_3$ during the hyperpolarization of the hybrid cell. Time marker interval, 2 s. Record was interrupted by 10 s. *B*, $InsP_3$ was injected by a negative current of −50 nA × 1 s at time zero. Upper record, hyperpolarization followed by prolonged depolarization in the hybrid cell. Lower record, AC-coupled recording of MEPPs. Bottom, frequency histogram of MEPPs of the above recording. Events were calculated from expanded recordings re-run from the FM tape-recorder. Note the two phases of the frequency increase.

injection. Successful injections of $InsP_3$ into the cytoplasm of an NG108-15 cell soma elicited cell hyperpolarization (Fig. 4*A*), as described previously (Higashida *et al.* 1986). During the early phase of the hyperpolarization of the cell soma, MEPPs were initiated in the muscle cell, similar to those induced by BK application (Fig. 2*A*). A large injection of $InsP_3$ was usually accompanied by an additional long-lasting depolarization (Fig. 4*B*). In contrast to the Ca^{2+} injection, the MEPPs persisted during the subsequent depolarization (for 3 min). The mean frequency increase was

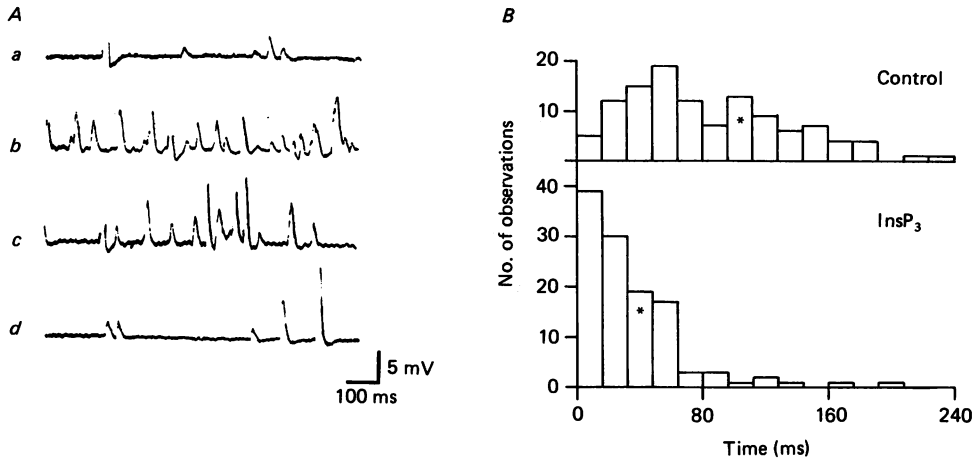

Fig. 5. MEPP frequency and interval change after $InsP_3$ injection. *A*, oscilloscope traces of MEPPs before and after injection of $InsP_3$ into the same NG108-15 cell as in Fig. 4. $InsP_3$ was applied by a current pulse (-20 nA × 1 s) into the hybrid cell. *a*, before injection; *b*, *c* and *d* are 10, 30 and 60 s after injection, respectively. The peak potential in three responses in each of *a*, *b* and *c* exceeded 20 mV and so it is not shown. *B*, histogram of time intervals of MEPPs before (upper) and after (lower) injection of $InsP_3$ following the previous one (*A*). 110 MEPPs were recorded for 4·8 s during $InsP_3$-induced MEPPs and 12·3 s during the control period. The observations were grouped in classes of 16 ms. The mean interval of the control and $InsP_3$ injection was 105 and 40 ms, respectively, as indicated (*).

3·71 ± 0·60-fold ($n = 26$) over the control value (1·54 ± 0·53 events/s). The minimum latency for the appearance of MEPPs after $InsP_3$ injection was 200–500 ms.

In the experiment illustrated in Fig. 4 there was a slight increase in the proportion of larger potentials after the $InsP_3$ injection: the mean amplitude before and after the $InsP_3$ injection was 2·61 ± 0·19 and 3·76 ± 0·23 mV ($n = 205$, $P < 0·01$; resting membrane potential of the myotube, -71 mV). Similar effect was seen in two other cells. Except for several superimposed muscle depolarizations, most of the potentials showed a monotonic rise (Fig. 5*A*). This suggests that each MEPP after the injection of $InsP_3$ is a single event. The intervals between MEPPs greatly shortened after the injection as shown in Fig. 5*B*. The mean interval time before and after the injection was 105 ± 7·4 and 40·1 ± 3·2 ms ($n = 111$, $P < 0·005$), respectively.

Phorbol dibutyrate-induced MEPPs

Within 3–5 min after perfusion with 1 μM-PDBu, the frequency of MEPPs started to increase, and reached a plateau after 8–10 min (Fig. 6*A* and *C*). PDBu at 1 μM caused an average increase in MEPP frequency of 3·0 times (± 0·17, $n = 5$) from the control value (0·24 ± 0·05 events/s). Some MEPPs after PDBu exposure are shown in Fig. 6*B*. Larger-amplitude responses were frequently recorded after the drug exposure. The input resistance of muscle cells was unchanged before and after PDBu (three cells).

PDBu depolarizes NG108-15 cells (Brown & Higashida, 1988*b*). However, the frequency increase was also observed when the cell was voltage clamped at -30 mV

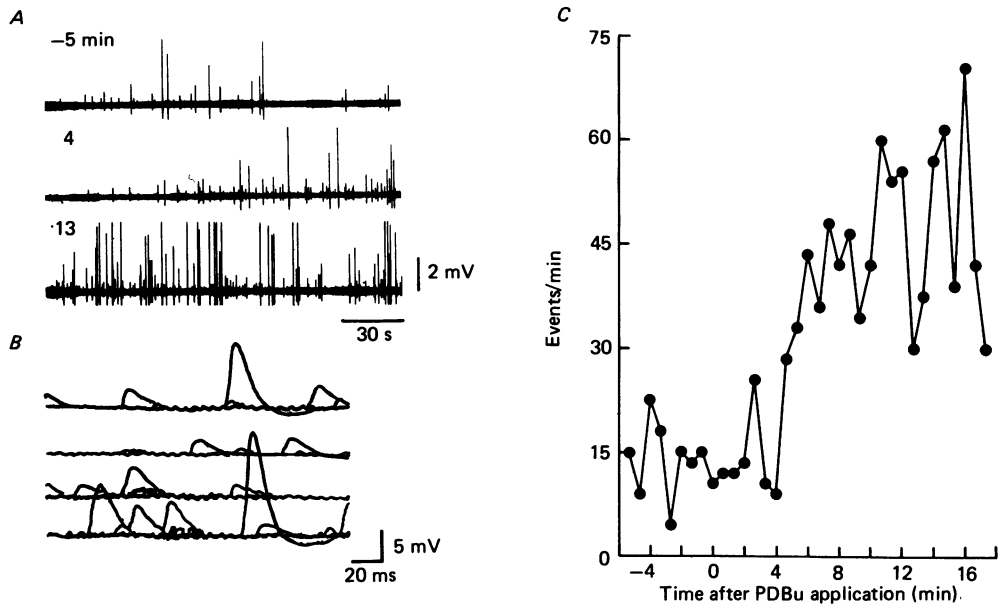

Fig. 6. Effect of phorbol dibutyrate (PDBu) on MEPPs. An NG108-15 hybrid–myotube pair was exposed to 1 μM-PDBu at time zero. Resting membrane potential of the muscle cell, −59 mV. *A*, examples of MEPPs (AC-coupled record) measured at 5 min before (upper), 4 min (middle) and 13 min (lower) after PDBu treatment. *B*, oscilloscope traces (DC-coupled record) of MEPPs recorded 9 min after PDBu. *C*, frequency plot of MEPPs after PDBu treatment.

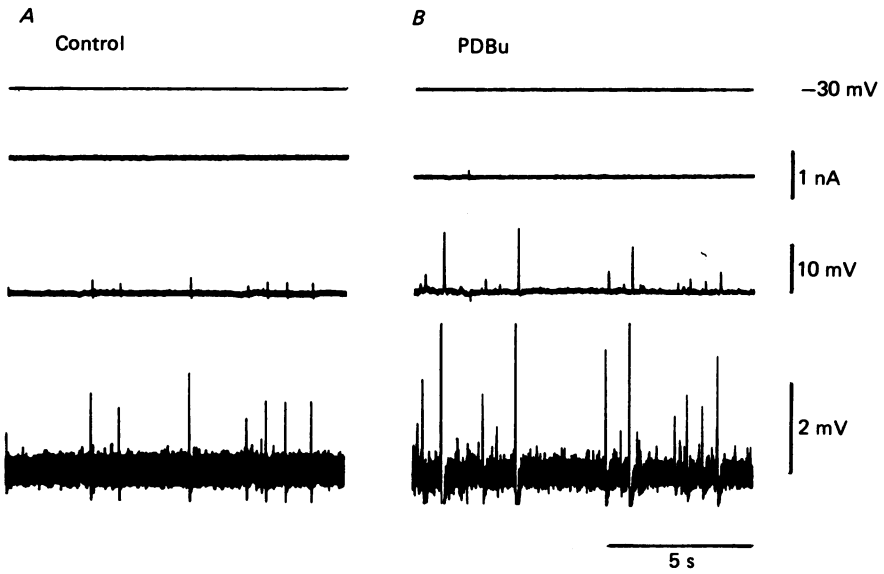

Fig. 7. Effect of PDBu on MEPPs produced from a voltage-clamped NG108-15 cell. Records were obtained 3 min before (*A*) and 20 min after (*B*) PDBu treatment. Uppermost trace, the hybrid cell potential clamped at −30 mV. Second trace down, PDBU-induced inward current of the hybrid cell. Two lower traces, membrane potential and MEPPs of the muscle cell. Resting membrane potential, −58 mV.

(Fig. 7), indicating that the increase of MEPP frequency after PDBu treatment is not simply due to the cell depolarization.

DISCUSSION

The results show that BK receptor activation in NG108-15 cells generates an increase in ACh release. Although there existed the difference that no membrane potential change was detected by application of BK on neurites in synapse regions while marked membrane potential changes were recorded by BK application on the cell soma, BK applied on the cell soma can release ACh as effectively as when applied onto neurites. Therefore, synapses formed with a short distance which were used in these experiments provided a system to analyse the mechanism for BK-induced presynaptic facilitation at the intracellular second-messenger level.

The ACh release evoked by BK can be dissected into two phases. Muscle MEPP frequency increased during both hyperpolarizing and depolarizing phases of BK action in synaptically connected NG108-15 hybrid cells; indeed, the peak increase in MEPP frequency coincided with the initial hyperpolarization. This suggests that the changes in MEPP frequency were not secondary to the membrane potential change or to the consequential changes in spontaneous action potential discharge rate. The latter can further be discounted on other grounds: changes in MEPP frequency were seen in the absence of spike discharges, and the quantal content of evoked EPPs ($< 0{\cdot}2$, i.e. one pulse in five causes release of a quantum) was too low to yield a significant increase in frequency.

The initial membrane hyperpolarization results from the activation of a Ca^{2+}-dependent K^+ current, and is therefore itself only a secondary consequence of a rise in intracellular Ca^{2+} (Brown & Higashida, 1988*a*). A transient rise in Ca^{2+} following application of BK has been detected using quin-2 fluorescence (Reiser & Hamprecht, 1985; Osugi *et al.* 1986*b*; Jackson, Hallam, Downes & Hanley, 1986). This appears to result from the formation of $InsP_3$ from phosphatidylinositol bisphosphate breakdown and consequent liberation of Ca^{2+} from intracellular stores, since (a) rapid BK-induced formation of $InsP_3$ has been detected (Yano *et al.* 1985*a*) and (b) intracellular application of $InsP_3$ reproduced the equivalent rise in Ca^{2+} (Osugi *et al.* 1986*b*) and the activation of the Ca^{2+}-dependent K^+ current (Brown & Higashida, 1988*b*). An $InsP_3$-induced rise in Ca^{2+} therefore provides a plausible explanation for the increased MEPP frequency. In support of this, intracellular injection of $InsP_3$ readily increased MEPP frequency after an appropriately short latency (Fig. 4). It seems that the Ca^{2+}-releasing effect of $InsP_3$ overrides the normal inhibitory effect expected for the membrane hyperpolarization.

However, both the formation of $InsP_3$ and the increase in intracellular Ca^{2+} produced by BK are transient effects, and seem unlikely to account for the more sustained increase in MEPP frequency. A more likely possibility is that this results from the associated formation of DAG and consequent activation of protein kinase C (see Nishizuka, 1986), since PDBu produced a more sustained release. Phorbol esters also increase $[Ca^{2+}]_i$ in NG108-15 cells (Osugi, Imaizumi, Mizushima, Uchida & Yoshida, 1986*a*), but this is secondary to depolarization and consequent Ca^{2+} entry. Since PDBu still induced a release under voltage-clamp conditions, this is

more likely to reflect a direct activation of transmitter release by C-kinase, as indicated in other tissues (Di Virgilio, Lew & Pozzan, 1984; Putney, McKinney, Aub & Leslie, 1984; Rink & Sanchez, 1984). Phorbol esters have also been reported to potentiate transmission at the frog neuromuscular junction (Publicover, 1985; Eusebi, Molinaro & Caratsch, 1986; Shapira, Silberberg, Ginsburg & Rahamimoff, 1987) and in rat hippocampal slice (Malenka, Madison & Nicoll, 1986). The increased MEPP amplitude is probably not postsynaptic in origin since the postsynaptic action of phorbol esters is depressant (Caratsch, Grassi, Molinaro & Eusebi, 1986).

In summary, the initial increase of MEPPs induced by BK may be attributed to the formation of $InsP_3$ and the consequent increase of cellular Ca^{2+} concentration by release from the intracellular store sites. This Ca^{2+} rise activates Ca^{2+}-dependent K^+ channels which leads to cell membrane hyperpolarization. In addition, simultaneously formed DAG activates protein kinase C which might contribute to the late phase of ACh secretion. This effect might be reinforced by an additional intracellular rise in Ca^{2+} due to the membrane depolarization and influx of Ca^{2+}.

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REFERENCES

BROWN, D. A. & HIGASHIDA, H. (1988*a*). Membrane current responses of NG108-15 mouse neuroblastoma × rat glioma hybrid cells to bradykinin. *Journal of Physiology* **397**, 167–184.

BROWN, D. A. & HIGASHIDA, H. (1988*b*). Inositol 1,4,5-trisphosphate and diacylglycerol mimic bradykinin effects on mouse neuroblastoma × rat glioma hybrid cells. *Journal of Physiology* **397**, 185–207.

BUSIS, N. A., DANIELS, M. P., BAUER, H. C., PUDIMAT, P. A., SONDEREGGER, P., SCHAFFNER, A. E. & NIRENBERG, M. (1984). Three cholinergic neuroblastoma hybrid cell lines that form few synapses on myotubes are deficient in acetylcholine receptor aggregation molecules and large dense core vesicles. *Brain Research* **324**, 201–210.

CARATSCH, C. G., GRASSI, F., MOLINARO, M. & EUSEBI, F. (1986). Postsynaptic effects of the phorbol ester TPA on frog end-plates, *Pflügers Archiv* **407**, 409–413.

CHRISTIAN, C. N., NELSON, P. G., BULLOCK, P., MULLINAX, D. & NIRENBERG, M. (1978), Pharmacological responses of cells of a neuroblastoma × glioma hybrid clone and modulation of synapses between hybrid cells and mouse myotubes. *Brain Research* **147**, 261–276.

DANIELS, M. P. & HAMPRECHT, B. (1974). The ultrastructure of neuroblastoma glioma somatic cell hybrids. Expression of neuronal characteristics stimulated by dibutyryl 3′,5′ cyclic monophosphate. *Journal of Cell Biology* **63**, 691–699.

DE BLAS, A., ADLER, M., SHIH, M., CHIANG, P. K., CANTONI, G. L. & NIRENBERG, M. (1984). Inhibitors of CDP-choline synthesis, action potential calcium channels, and stimulus–secretion coupling. *Proceedings of the National Academy of Sciences of the U.S.A.* **81**, 4353–4357.

DENNIS, M. J. & MILEDI, R. (1974*a*). Non-transmitting neuromuscular junctions during an early stage of end-plate reinnervation. *Journal of Physiology* **239**, 553–570.

DENNIS, M. J. & MILEDI, R. (1974*b*). Characteristics of transmitter release at regenerating frog neuromuscular junctions. *Journal of Physiology* **239**, 571–594.

DI VIRGILIO, F., LEW, D. P. & POZZAN, T. (1984). Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels. *Nature* **310**, 691–693.

EUSEBI, F., MOLINARO, M. & CARATSCH, C. G. (1986). Effects of phorbol ester on spontaneous transmitter release at frog neuromuscular junction. *Pflügers Archiv* **406**, 181–183.

FURUYA, S. & FURUYA, K. (1983). Ultrastructural changes in differentiating neuroblastoma × glioma hybrid cells. *Tissue and Cell* **15**, 903–919.

FURUYA, S., SAWADA, M., NAGATSU, T., SUZUKI, O. & HIGASHIDA, H. (1985). Localization of [^{3}H]serotonin in neuroblastoma × glioma hybrid cells. *Brain Research* **361**, 77–90.

HARRIS, A. J. & MILEDI, R. (1971). The effect of type D botulinum toxin on frog neuromuscular junctions. *Journal of Physiology* **217**, 497–515.

HIGASHIDA, H. (1986). Bradykinin or inositol 1,4,5-trisphosphate increase Ca^{2+} dependent acetylcholine secretion from NG108-15 neuroblastoma–glioma hybrid cells. *Federation Proceedings* **45**, 1690.

HIGASHIDA, H. & BROWN, D. A. (1986). Two polyphosphatidylinositide metabolites control two K^+ currents in a neuronal cell. *Nature* **323**, 333–335.

HIGASHIDA, H., KATO, T., KANO-TANAKA, K., OKUYA, M., MIYAKE, A. & TANAKA, T. (1981). Proliferation and synapse formation of neuroblastoma × glioma hybrid cells: Effect of glia maturation factor. *Brain Research* **214**, 287–299.

HIGASHIDA, H., STREATY, R. A., KLEE, W. & NIRENBERG, M. (1986). Bradykinin-activated transmembrane signals are coupled via No or Ni to production of inositol 1,4,5-trisphosphate, a second messenger in NG108-15 neuroblastoma–glioma hybrid cells. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 942–946.

HIGASHIDA, H., WILSON, S. P., ADLER, M. & NIRENBERG, M. (1978). Synapse formation by neuroblastoma and hybrid cell lines. *Society for Neuroscience Abstracts* **4**, 591.

JACKSON, T. R., HALLAM, T. J., DOWNES, C. P. & HANLEY, M. R. (1987). Receptor coupled events in bradykinin action: rapid production of inositol phosphates and regulation of cytosolic free Ca^{2+} in a neural cell line. *EMBO Journal* **6**, 49–54.

KATZ, B. & MILEDI, R. (1968). The role of calcium in neuromuscular facilitation. *Journal of Physiology* **195**, 481–492.

KIM, Y. I., LOMO, T., LUPA, M. T. & THESLEFF, S. (1984). Miniature end-plate potentials in rat skeletal muscle poisoned with botulinum toxin. *Journal of Physiology* **356**, 587–599.

MACDERMOT, J., HIGASHIDA, H., WILSON, S. P., MATSUZAWA, H., MINNA, J. & NIRENBERG, M. (1979). Adenylate cyclase and acetylcholine release regulated by separate serotonin receptors of somatic cell hybrids. *Proceedings of the National Academy of Sciences of the U.S.A.* **76**, 1135–1139.

MCGEE, R., SIMPSON, P., CHRISTIAN, C., MATA, M., NELSON, P. & NIRENBERG, M. (1978). Regulation of acetylcholine release from neuroblastoma × glioma hybrid cells. *Proceedings of the National Academy of Sciences of the U.S.A.* **75**, 1314–1318.

MALENKA, R. C., MADISON, D. V. & NICOLL, R. A. (1986). Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature* **321**, 175–177.

NAKAGAWA, Y., HIGASHIDA, H. & MIKI, N. (1984). A single class of neurotensin receptors with high affinity in neuroblastoma × glioma NG108-15 hybrid cells that mediate facilitation of synaptic transmission. *Journal of Neuroscience* **4**, 1653–1661.

NELSON, P. G., CHRISTIAN, C. N., DANIELS, M. P., HENKART, M., BULLOCK, P., MULLINAX, D. & NIRENBERG, M. (1978). Formation of synapses between cells of a neuroblastoma × glioma hybrid clone and mouse myotubes. *Brain Research* **147**, 245–259.

NELSON, P., CHRISTIAN, C. & NIRENBERG, M. (1976). Synapse formation between clonal neuroblastoma × glioma hybrid cells and striated muscle cells. *Proceedings of the National Academy of Sciences of the U.S.A.* **73**, 123–127.

NIRENBERG, M., WILSON, S. P., HIGASHIDA, H., ROTTER, A., KRUEGER, K., BUSIS, N., RAY, R., KENIMER, J., ADLER, M. & FUKUI, H. (1983). Synapse formation by neuroblastoma hybrid cells. *Cold Spring Harbor Symposium on Quantitative Biology* **48**, 707–715.

NISHIZUKA, Y. (1986). Studies and perspective of protein kinase C. *Science* **233**, 305–312.

OSUGI, T., IMAIZUMI, T., MIZUSHIMA, A., UCHIDA, S. & YOSHIDA, H. (1986*a*). 1-Oleoyl-2-acetylglycerol and phorbol ester stimulate Ca^{2+} flux through Ca^{2+} channels in neuroblastoma × glioma hybrid NG108-15 cells. *European Journal of Pharmacology* **126**, 47–51.

OSUGI, T., UCHIDA, S., IMAIZUMI, T. & YOSHIDA, H. (1986*b*). Bradykinin-induced intracellular Ca^{2+} elevation in neuroblastoma × glioma hybrid NG108-15 cells; relationship to the action of inositol phospholipids metabolites. *Brain Research* **379**, 84–89.

PUBLICOVER, S. J. (1985). Stimulation of spontaneous transmitter release by the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate, an activator of protein kinase C. *Brain Research* **333**, 185–187.

PURO, D. G. & NIRENBERG, M. (1976). On the specificity of synapse formation. *Proceedings of the National Academy of Sciences of the U.S.A.* **73**, 3544–3548.

PUTNEY JR, J. W., MCKINNEY, J. S., AUB, D. L. & LESLIE, B. A. (1984). Phorbol ester-induced protein secretion in rat parotid gland. Relationship to the role of inositol lipid breakdown and protein kinase C activation in stimulus–secretion coupling. *Molecular Pharmacology* **26**, 261–266.

REISER, G. & HAMPRECHT, B. (1982). Bradykinin induced hyperpolarizations in rat glioma cells and in neuroblastoma × glioma hybrid cells. *Brain Research* **239**, 191–199.

REISER, G. & HAMPRECHT, B. (1985). Bradykinin causes a transient rise of intracellular Ca^{2+}-activity in cultured neural cells. *Pflügers Archiv* **405**, 260–264.

RINK, T. J. & SANCHEZ, A. (1984). Effects of prostaglandin I_2 and forskolin on the secretion from platelets evoked at basal concentrations of cytoplasmic free calcium by thrombin, collagen, phorbol ester and exogenous diacylglycerol. *Biochemical Journal* **222**, 833–836.

SHAPIRA, R., SILBERBERG, S. D., GINSBURG, S. & RAHAMIMOFF, R. (1987). Activation of protein kinase C augments evoked transmitter release. *Nature* **325**, 58–60.

WILSON, S., HIGASHIDA, H., MINNA, J. & NIRENBERG, M. (1978). Defects in synapse formation and acetylcholine release by neuroblastoma and hybrid cell lines. *Federation Proceedings* **37**, 1784.

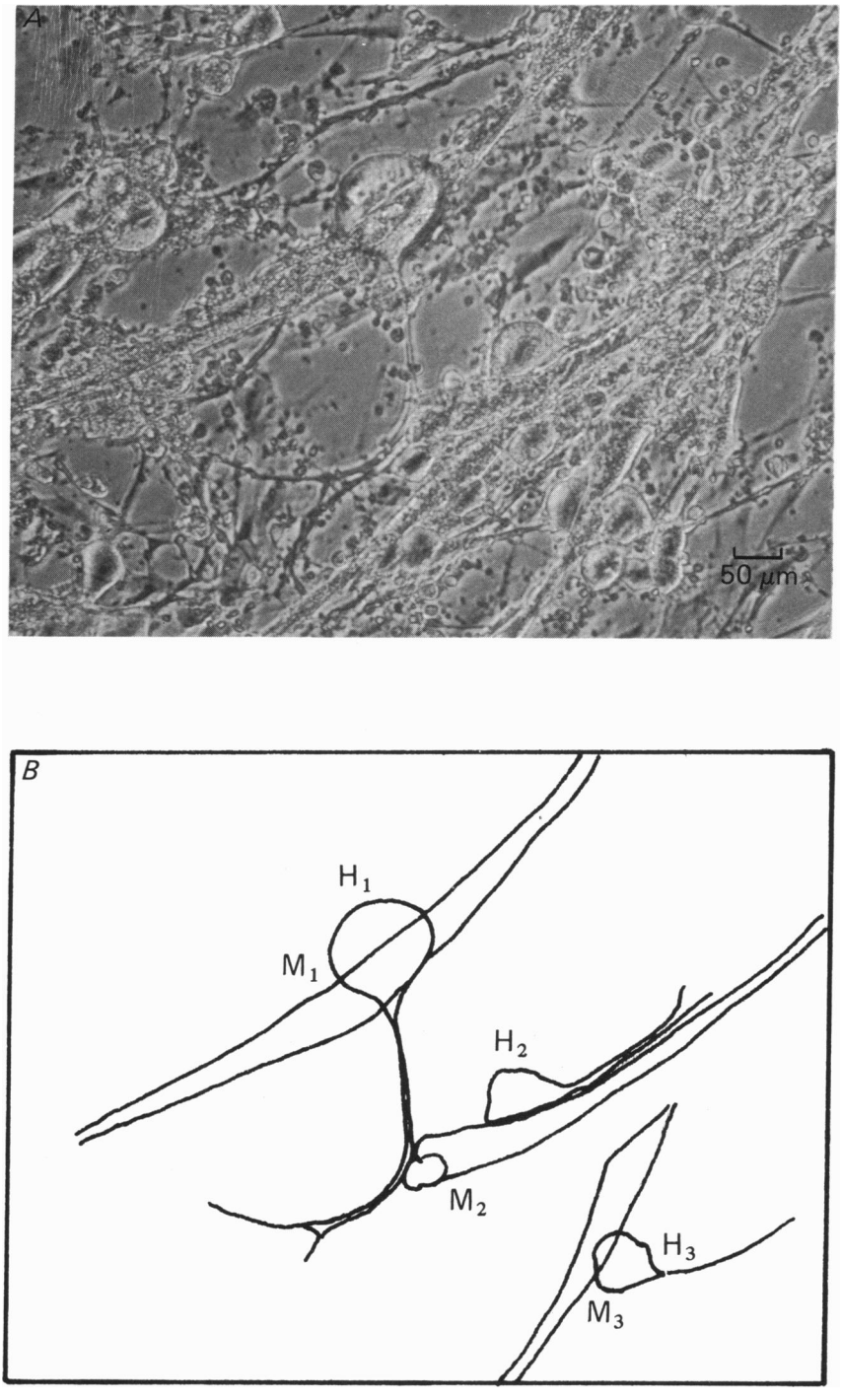
YANO, K., HIGASHIDA, H., HATTORI, H. & NOZAWA, Y. (1985*a*). Bradykinin-induced transient accumulation of inositol trisphosphate in neuron-line cell line NG108-15 cells. *FEBS Letters* **181**, 403–406.

YANO, K., HIGASHIDA, H., INOUE, R. & NOZAWA, Y. (1984). Bradykinin-induced rapid breakdown of phosphatidylinositol 4,5-bisphosphate in neuroblastoma × glioma hybrid NG108-15 cells. A possible link to agonist-induced neuronal functions. *Journal of Biological Chemistry* **259**, 10201–10207.

YANO, K., HIGASHIDA, H. & NOZAWA, Y. (1985*b*). Evidence for a Ca^{2+}-independent hydrolysis of phosphatidylinositol 4,5-bisphosphate in neuron-like cell line NG108-15 cells. *FEBS Letters* **183**, 235–239.

EXPLANATION OF PLATE

A, phase-contrast micrograph of NG108-15 hybrid cells co-cultured with rat myotubes for 21 days in the presence of 1 mM-dibutyryl cyclic AMP. Bar, 50 μm. *B*, traces of the upper photograph. H, NG108-15 cell. M, muscle cell. Cell contact is seen between each pair. The recording of Figs 4 and 5 were obtained from the H_1–M_1 cell pair.



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(*Facing p.* 222)