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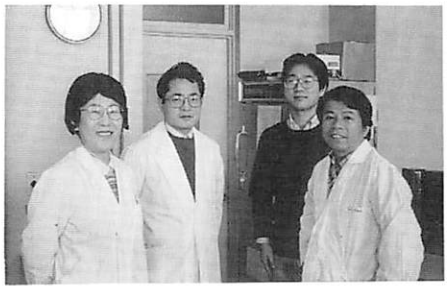
Pharmacology



DEPARTMENT OF PHARMACOLOGY

GENERAL SUMMARY

Our main interest is in developmental pharmacology of the nervous system. We are focusing on synaptogenesis and gene expression. The stimulation of a receptor on a developing neuron induces a series of new proteins such as ion channels and new receptors, by which the neuron subsequently acquires new functions and can receive new information.

We have three main projects for this line of experimentation; I. neurotrophic factors, II. Cloning of retina specific cDNAs and III. Signal transduction from receptors to ion channels. To approach this project we have adopted various methods used in current cell biology.

I. Neurotrophic factors

Differentiation of nerve cells is sensitively influenced by their environment such as neurotrophic factors and cell-cell interactions. Many neurotrophic factors and their receptors are involved in synaptogenesis. We have purified a neurite outgrowth factor (NOF) and produced polyclonal and monoclonal antibodies to clarify the biologically active domains of NOF and the developmental changes of NOF in the nervous tissues. A receptor for NOF was also isolated from smooth muscle membranes and characterized. A survival factor which prolongs the survival of cerebral cortical neurons was found in the cerebrum and studied.

II. Cloning of retina specific cDNAs

Many specific proteins are expressed in the nervous system and the characterization of these proteins is essential for an understanding of the function of the nervous system. We have selected the retina to find the specific proteins and to characterize their functions in the retina. So far we have characterized opsin, transducin gamma, pCR18 and R27. The latter two were found to code unknown proteins, and an effort was undertaken to evaluate their functions by in situ hybridization and immunohistochemistry by antibodies against synthetic peptides.

III. Signal transduction from receptor to ion channels

Neuroblastoma hybrid NG108-15 cells have unique bradykinin receptors which couple to No or Ni GTP binding proteins. Bradykinin increases the conversion rate from phosphatidylinositol 4, 5-bisphosphate to inositol 1, 4, 5-triphosphate and diacylglycerol. These two intermediates act as second messengers in these cells for further signal transduction to two distinct K^+ channels. Our scheme is that inositol phosphate indirectly opens Ca^{++}-dependent channels by increasing intracellular Ca^{++} concentrations, and diacylglycerol inactivates M-channels by protein phosphorylation due to the activation of protein kinase C.

(1) Interaction of monoclonal antibodies with a neurite outgrowth factor from chicken gizzard extract.

Y. Hayashi, H. Taniura and N. Miki

A neurite outgrowth factor (NOF), was purified to homogeneity from chicken gizzard extract and its monoclonal antibodies were produced. From 603 hybridoma clones, 55 positive hybridoma cell lines were detected. Four of them were selected for further study . These antibodies immunoprecipitated ^{125}I-labelled gizzard NOF molecules, whereas they did not inhibit the biological activity of NOF (or NOF-like molecules) derived from the tissues tested. The subcellular distribution of NOF in chick embryonal tissues was investigated using monoclonal antibody 4-2C. Both ciliary ganglionic (CG) neurons and gizzard muscle tissues were stained. The NOF content of embryonic CG, measured by an immunoblot assay, increased about 20-fold during embryogenesis from 6 to 15 days, whereas the sensitivity of neurons to NOF reciprocally decreased. At least two different sizes of NOF (700 and 800 kDa) were found in CG of various ages, with the 800/700 kDa ratio gradually increasing with age. Neurite outgrowth factor (NOF) from chicken gizzard was treated with elastase, and a protease-resistant fragment of 140kDa (F-140) with heparin-binding activity was isolated from the digested mixture. Although F-140 showed no neurite outgrowth activity, it bound dissociated cells from the dorsal root ganglia of chick embryos. The antiserum to F-140 inhibited the cell binding activity of native NOF in a dose-dependent manner, but not the neurite outgrowth activity.

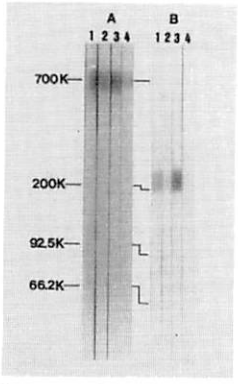


Fig.1 Western blotting analysis of NOF. Crude extract of adult gizzard (20μg protein/lane) was electrophoresed under non-reducing (A) and reducing (B) conditions followed by electrotransfer to a nitrocellulose sheet. Proteins on the membranes were immunostained with M-1-2G (lane 1), 5-10A (lane 2), 4-2C (lane 3) and 1-4D (lane 4).

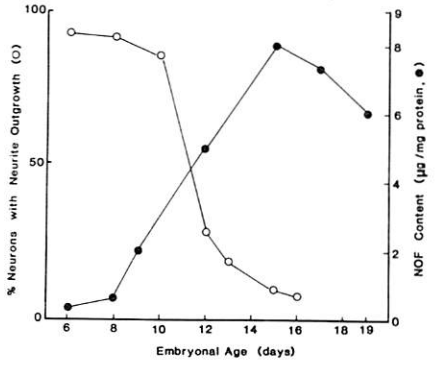


Fig. 2 Changes in the NOF contents (●) in CG and the sensitivities (○) of the neurons to NOF during embryogenesis. CG neurons from 6 to 16-day-old embryos were incubated in the culture wells previously coated with 5 μg of NOF. After 16h of incubation the neuritic responses of the neurons were examined. The NOF contents of CG from various aged embryos were estimated by a dot blot.

(2) An 82kDa membrane protein that inhibits the activity of neurite outgrowth factor (NOF).

H. Taniura, Y. Hayashi and N. Miki

A neurite outgrowth factor (NOF), an extracellular matrix glycoprotein of 700 kDa, promoted neurite outgrowth of cultured peripheral neurons. A component solubilized from chicken gizzard muscle membranes inhibited neuritic activity of NOF. The molecular size was determined to be about 82kDa by ligand blotting. The inhibitory ability was abolished by treatment with trypsin or heat. The active component was partially purified by using Sepharose CL-6B and Heparin Sepharose columns. We detected an 82kDa protein which bound to NOF by ligand blotting, using the fraction eluted from a Heparin Sepharose column. A major staining band by ligand blotting coincided with a major CBB staining band. To determine whether this 82kDa protein has inhibitory ability or not, we extracted the corresponding protein band from a SDS-gel and analyzed it. The extracted protein showed a single band of 82kDa and exhibited the inhibitory activity. It is suggested that this molecule is a membrane protein (or NOF receptor) which interacts with a domain for the neuritic activity of NOF and may modulate the NOF activity in vivo.

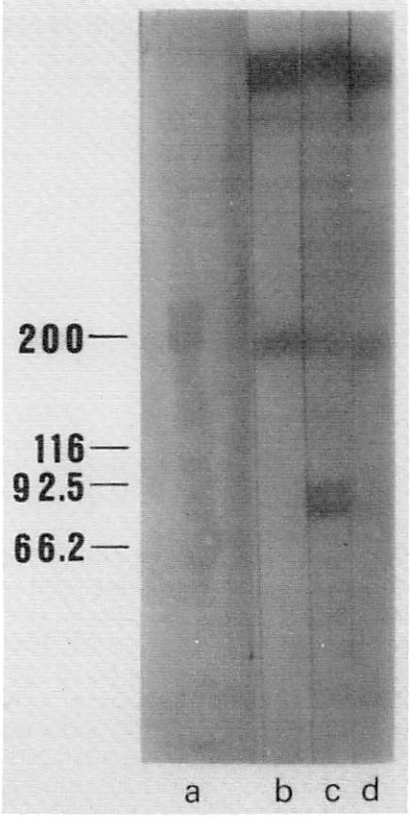
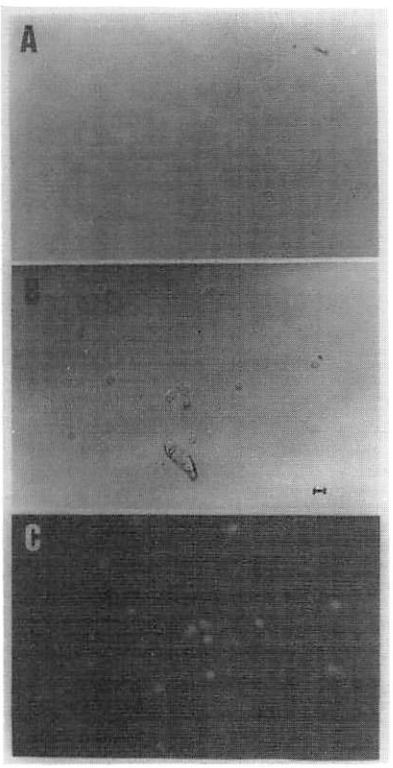


Fig. 1 NOF binding protein detected by ligand blotting. Nonidet P-40 (NP-40) extract was electrophoresed on a 3-15% polyacrylamide gradient gel, and proteins were transferred electrophoretically to a nitrocellulose sheet. The sheet was incubated with ligand NOF for 2 h at 37°C and then washed with 0.05% Tween 20 in PBS (−). The NOF binding protein was detected by monoclonal antibody (1-4D).
a, marker proteins detected by using biotin blot kit (BIO RAD).
b, incubated in the absence of ligand NOF.
c, incubated in the presence of ligand NOF.
d, incubated in the presence of NOF with excess of NP-40 extract.

(3) A neurotrophic factor for cerebrocortical neurons from chicken cerebral extract.

K. Yamagata, K. Koide, C-H. Kuo and N. Miki

The survival of cerebrocortical neurons from 6 to 8-day chick embryos was investigated in serum-free hormone-supplemented medium in poly-ornithine-coated wells. The addition of cerebral extract promoted the survival of cortical neurons in a dose-dependent manner, but induced almost no neurite outgrowth. The tissue extracts from eye and gizzard muscle also supported the survival, but were less active than the cerebral extract. The trophic activity was higher in the adult cerebrum than in the embryonic cerebrum. The trophic factor was partially purified from adult chicken cerebrum, and the molecular weight of the factor was estimated to be about 60kDa. The activity for the survival factor was fairly resistant to heat, acid or trypsin treatment. When the partially purified sample was, however, treated with trypsin (1 mg/ml) for 20h and applied to a TSK G2000 SW column, the activity moved from 60-70kDa untreated active fractions to fractions with about 10kDa. These physicochemical properties of the survival factor suggest a new class of macromolecular trophic factors in the brain.



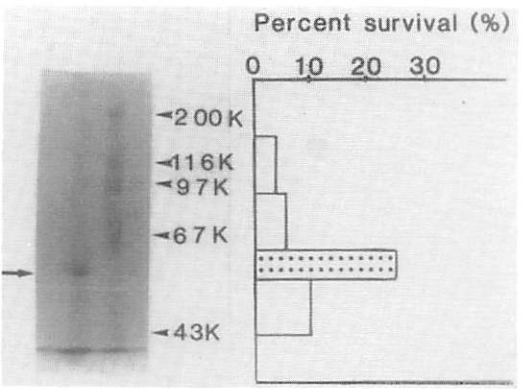


Fig. 1 Primary culture of cortical neurons from 7-day chicken embryo cells were cultured at low density (10^3 cells/well, 96 well Falcon plate) in serum-free defined medium for 20h in humidified 95% air/5% CO_2 at 37°C with no addition (A), with 1 mg/ml cerebral extract (B). Cortical neurons were stained with indirect immunofluorescence using anti 70K neurofilament antibody (C). Note that almost all surviving cells are neurons. Bar; 10μm

Fig. 2 SDS-PAGE profile of the most active fraction of the red agarose column. Sixty kDa polypeptide band was major and the corresponding area of the gel exhibited the neurotrophic activity for the cortical neurons.

(4) Multiple opsin mRNA species in bovine retina.

C-H. Kuo, K. Yamagata, R. K. Moyzis,[1] M. W. Bitensky[1] and N. Miki

We have tried to isolate retina-specific cDNAs by differential hybridization to retina and brain, and selected three candidates (pCR-307 pCR-394 and pCR-470). One of them (pCR-394) was identified as an opsin cDNA. Opsin, a retina-specific 38kDa glycoprotein, is a major component of disc membranes in the rod outer segments and plays a key role in the photo-transduction process via the mechanism of light-induced conformational changes. Bovine opsin has 348 amino acid resudues which correpond to the 1044bp lenght of DNA. Opsin mRNA was estimated to be about 1.6% of total retinal mRNA by in vitro translation assay. In the Northern hybridization experiment, the opsin cDNA hybridized to two species of bovine mRNA, one approximately 18S (1800 nucleotides) and the other 22S (2600 nucleotides). Using pCR-394 as a probe two opsin clones, R-5 (about 1200 bp) and LR-8 (about 2500 bp), were isolated from a cDNA library which was prepared by the method of Okayama-Berg. The R-5 sequence was identical to the LR-8 sequence except for the location of the poly(A) tail which was 14 nucleotides downstream from the polyadenylation signal at 5'-side. The sequence TATTAATAAA at position 1376 of the bovine opsin cDNA seems to be a TATA box accompanied by a polyadenylation signal. This may raise the possibility that the opsin gene encodes yet another peptide. We found a polypeptide with start from a TAG triplet at 1803 and encode 87 amino acids (Fig. 1). The nucleotide sequences of R-5 and LR-8, as well as Northern and Southern hybridization experiments suggest that at least two species of opsin mRNA are expressed from a single gene. When the effects of illumination were examined by Northern hybridization, the ratio of the two opsin mRNA species changed between light- and dark-adapted eyes (Fig. 2).

1) Life Science Division, Los Alamos National Laboratory, Los Alamos, NM87545, U.S.A.

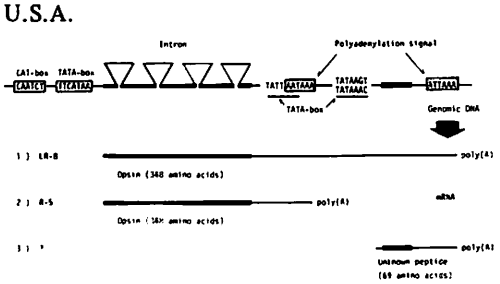


Fig. 1 Two opsin mRNAs are transcribed from a single genome DNA

Fig. 2 Effect of ocular illumination on opsin mRNA species.

Fresh bovine eyeballs were dark adapted and then divided into two groups. One group was kept in the dark and the other was exposed to light. Retinas were dissected under dark or light conditions and poly(A) RNA was isolated. Poly(A) mRNA prepared from dark- or light-adapted retinas were applied to lanes 1 and 2, respecively, and separated on an agarose gel. After blotting, the filter was hybridized with a coding region of opsin cDNA and washed.

(5) Isolation of a novel photoreceptor specific clone (MEKA cDNA).

C-H. Kuo, M. Akiyama and N. Miki

We have reported the isolation of clones (pCR-307, 394 and 470) which are candidates for retina-specific cDNAs. We demonstrated that the pCR-307 and pCR-394 encode transducin gamma subunit (Tγ) and opsin, respectively. This evidence suggests that differential colony hydridization is a useful method for isolating retina-specific cDNA clones. One of the remaining cDNA clones, pCR-470, was used as a probe to select a full length cDNA from another bovine retinal cDNA library constructed by the Okayama-Berg method. An R-27 clone (Fig. 1) was isolated in the third series of the colony hybridization experiment by the pCR-470. About 0.3% of the total colony exhibited hybridization signals by a HaeIII probe, suggesting an abundance of mRNA content in the retina. The HaeIII fragment, which includes coding region of the R-27, hybridized to 14.5S (1.3 k nucleotides) retina mRNA, but not to mRNAs from the brain, liver and kidney. R-27 cDNA shows almost a full length of about 1.3 kbp consisting of a 5' non-coding region (98 bp), a coding region (450 bp) and a 3' non-coding region (688 bp excluding the 75-85 bp poly(A) tail). We named a polypeptide encoded by the R-27 cDNA a MEKA protein, because its amino acid sequence starts from Met(M)-Glu (E)-Lys(K)-Ala(A). We found that there were no significant homologous proteins or nucleotide sequences with the MEKA protein or cDNA by computor homology research. The molecular weight and pI of the MEKA protein are calculated to be 17700 and 9.6, respectively. Regional localization of MEKA mRNA in the retina was investigated by in situ hybridization. Rat retinal sections were hybridized with ^{35}S-labelled anti-sense MEKA RNA. It was revealed that MEKA mRNA was transcribed only in the photoreceptor cells and accumulated in the inner segments just as opsin mRNA was (Fig. 2)

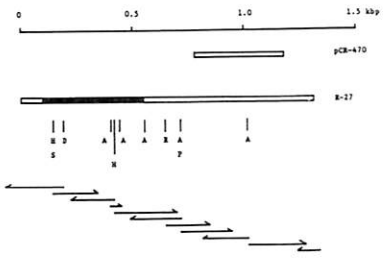


Fig. 1 Restriction enzyme maps of pCR-470 and R-27 (MEKA) cDNA.

Nucleotide sequence was determined by the dideoxynucleotide chain-termination method. The solid and open boxes indicate the coding and non-coding regions, respectively.

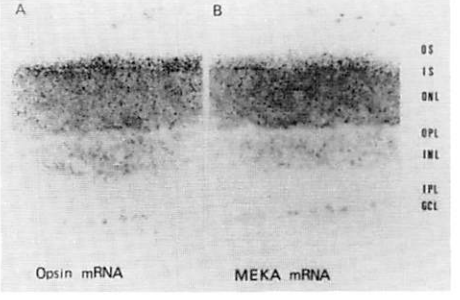


Fig. 2 In situ hydridization histochemistry with opsin and MEKA cRNA probes.

Rat retinal section were hybridized with anti-sense opsin (A) and MEKA (B) RNAs. Both of the probes showed grains most intensely at the inner segments (IS) of photoreceptor cells.

(6) Bradykinin-induced breakdown of phosphatidylinositol phosphate and accumulation of inositol trisphosphate in neuroblastoma x glioma hybrid NG108-15 cells.

H. Higashida, K. Yano[1] and Y. Nozawa[1]

External application of bradykinin to neuroblastoma X glioma hybrid NG108-15 cells produced a sustained depolarization preceded by a transient hyperpolarization. Bradykinin also increased the frequency of miniature endplate potentials recorded from cultured striated muscle cells. Effects of bradykinin on phospholipid metabolism in the hybrid cells were examined. Bradykinin induced specific incorporation of ^{32}Pi into phosphatidic acid and phosphatidylinositol without affecting [^{3}H] glycerol incorporation into these phospholipids within 10 min after its addition. The addition of bradykinin to hybrid cells prelabeled with ^{32}P caused a transient decrease (maximal effect seen at 10-30 s) in the radioactivity from phosphatidylinositol 4, 5-bis-phosphate (PI-4, 5-P_2).

Using [^{3}H] inositol-labelled cells, bradykinin produced net increases in the level of [^{3}H] inositol phosphates, especially of [^{3}H] inositol trisphosphate which is formed transiently and most rapidly. The results indicate that bradykinin activates a phosphodiesterase to break down the generation of two recently recognized intracellular messengers, 1, 2-diacylglycerol and inositol trisphosphate.

1) Department of Biochemistry, Gifu University School of Medicine.

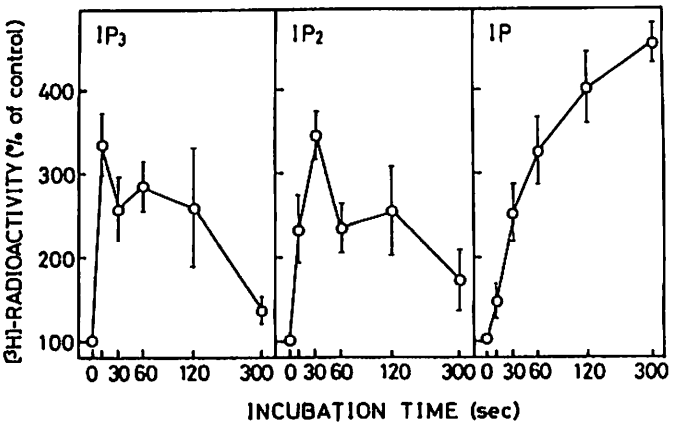


Fig. 1 Time course of the effect of bradykinin on the production of individual inositol phosphates in [^{3}H] inositol-labelled NG108-15 cells. [^{3}H] Inositol-labelled NG108-15 cells were incubated at 37°C for 5 min with or without 1×10^{-5} M bradykinin. Data are expressed as percentages of their respective controls. Control values at zero time for [^{3}H] IP, [^{3}H] IP_2 and [^{3}H] IP (dpm/10^6 cells) were 156±47, 369±74 and 660±48, respectively.

(7) 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 x glioma hybrid cells.

H. Higashida, W. Klee[1], and M. Nirenberg[2]

Injection of inositol 1, 4, 5-trisphosphate or Ca^{2+} into the cytoplasm of NG108-15 cells elicits cell hyperpolarization followed by depolarization. No such response was produced by equivalent intracellular injection of inositol 1, 3, 4-trisphosphate or inositol 1, 3, 4, 5-tetrakisphosphate. Thus, the hyperpolarizing phase of the cell response may be due to inositol 1, 4, 5-trisphosphate-dependent release of stored Ca^{2+} into the cytoplasm, which activates Ca^{2+} – dependent K^+ channels. Incubation of NG108-15 cells with pertussis toxin inhibits bradykinin-dependent cell hyperpolarization and depolarization. Bradykinin stimulates low km GTPase activity and inhibits adenylate cyclase in NG108-15 membrane preparations but not in membranes prepared from cells treated with pertussis toxin. Reconstitution of NG108-15 membranes from cells treated with pertussis toxin with nanomolar conentrations of a mixture of highly purified No and Ni restores bradykinin-dependent activation of GTPase and inhibition of adenylate cyclase. These results suggest that No and/or Ni mediate the transduction of signals from bradykinin receptors to phospholipase C and adenylate cyclase.

1) National Institute of Mental Health, U.S.A.
2) National Heart, Lung & Blood Institute, U.S.A.

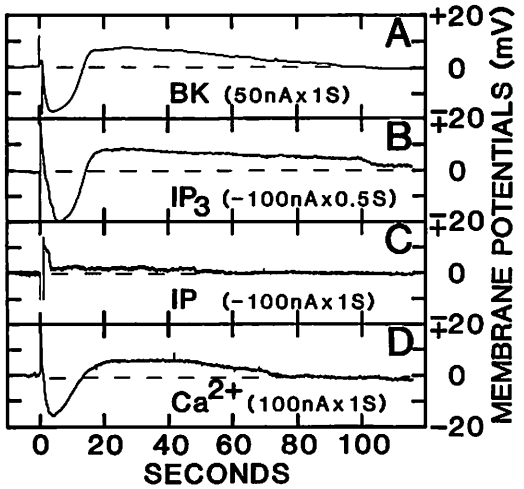


Fig. 1 Typical membrane potential changes of NG108-15 cells. (A) Cell hyperpolarization followed by depolarization elicited by extracellular application of bradykinin 50 nA for 1 sec. (B) Cell hyperpolarizing and depolarizing responses induced by intracellular injection of $InsP_3$. The recording electrode (3 M KCl) and a micropipette filled with 1 mM $InsP_3$ dissolved in water were inserted into an NG108-15 cell. At zero time -100 nA was passed through the $InsP_3$ pipette for 0.5 sec. (C) One mM inositol 2-phosphate dissolved in water was injected intracellularly (−100 nA for 1 sec). (D) Ca^{2+} was injected into the cytoplasm of an NG108-15 cell from a micropipette filled with a solution containing 0.5 M $CaCl_2$ by iontophoresis with 100 nA for 1 sec at zero time.

(8) Two polyphosphatidylinositide metabolites control two K^+ currents in neuroblastoma x glioma hybrid NG108-15 cells.

H. Higashida & D.A. Brown[1)]

Hydrolysis of the membrane phospholipid phosphatidylinositol-4, 5-bisphosphate (PtdIns (4, 5)P_2) produces two prospective intracellular messengers: inositol-1, 4, 5-trisphosphate ($InsP_3$), which releases Ca^{2+} from intracellular stores, and diacylglycerol (DG), which activates protein kinase C. We have shown here how the formation of these two substances triggered by one external messenger, bradykinin, leads to the appearance of two different sequential membrane conductance changes in the neuron-like NG108-15 neuroblastoma-glioma hybrid cell line. In these cells bradykinin rapidly hydrolyses PtdIns (4, 5)P_2 to $InsP_3$ and DG, raises intracellular Ca^{2+}, hyperpolarizes and then depolarizes the cell membrane. By voltage-clamp recording this shows that the hyperpolarization results from the activation of pharmacologically-identifiable species of Ca^{2+} dependent K^+ current. This is also activated by intracellular injections of Ca^{2+} or $InsP_3$ so it may be attributed to the formation and action of $InsP_3$. The subsequent depolarization results primarily from the inhibition of a different voltage-dependent K^+ current, the M-current that is also inhibited by DG activators. Hence a dual, time-dependent role for these two intracellular messengers in the control of nuronal signalling by a peptide is demonstrated.

1) Department of Pharmacology, University College London, U.K.

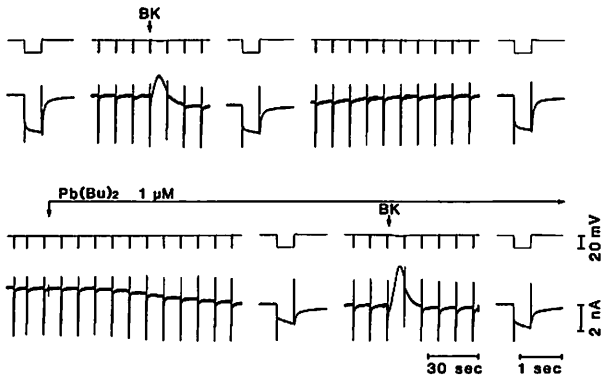


Fig. 1 Phorbol-12, 13-dibutyrate ($Pb(Bu)_2$, 1μM, Sigma) produced an inward current and occluded the inward current response to bradykinin (BK, 2μl of 10μM). Upper bradykinin alone; lower $Pb(Bu)_2$ and bradykinin. Continuous recording of membrane voltage (upper records) and membrane current (lower records) in a single cell. The cell was clamped at −41 mV and inward current responses to −20mV, 0.5 s duration hyperpolarizing commands recorded every 10s. The recorder was intermittently accelerated 25x to display these current transients.

Celebration for the 20th anniversary of Kanazawa University Cancer Research Institute (June 1, 1987).