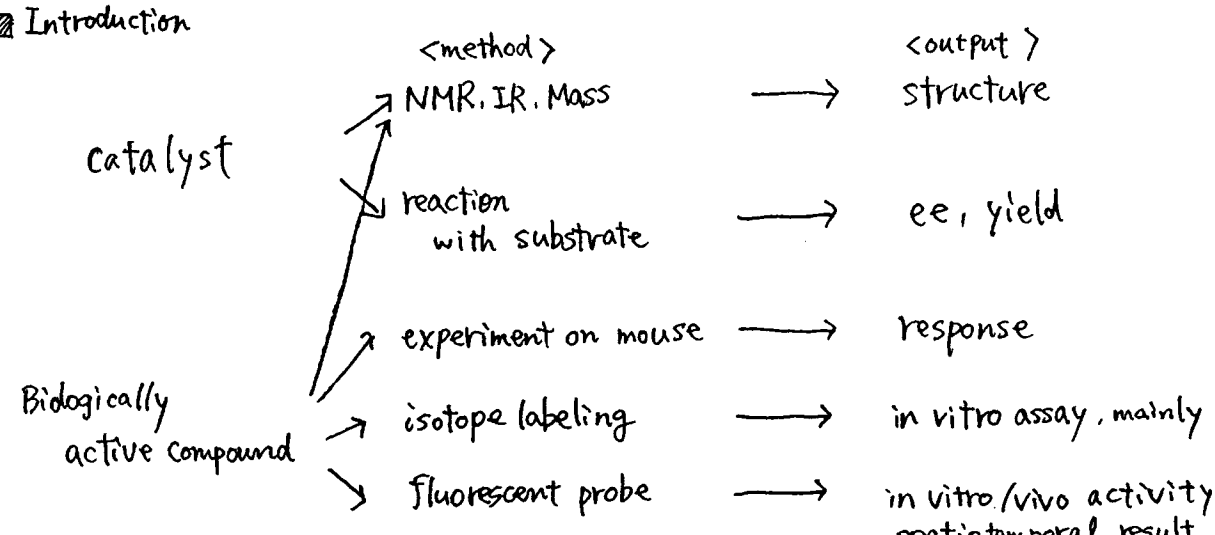
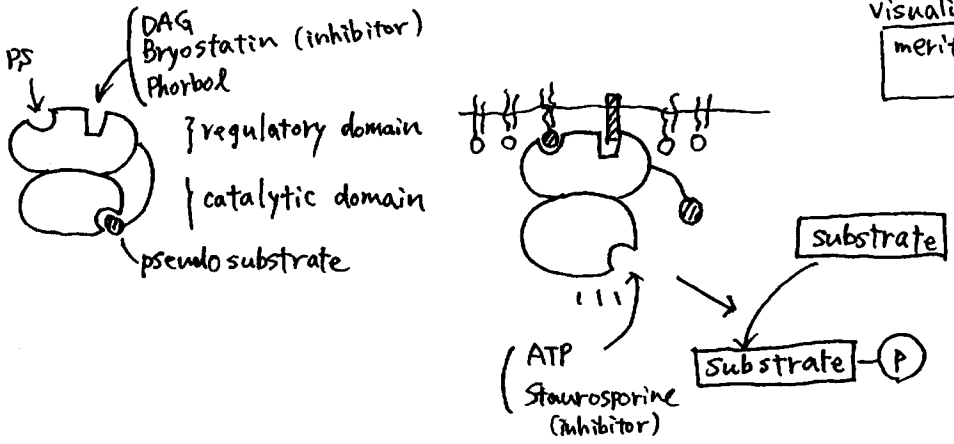
VISUALIZATION OF PKC BEHAVIOR BY USING FLUORESCENT PROBE

2004. 1. 28
MOTOKI Rie (B4)

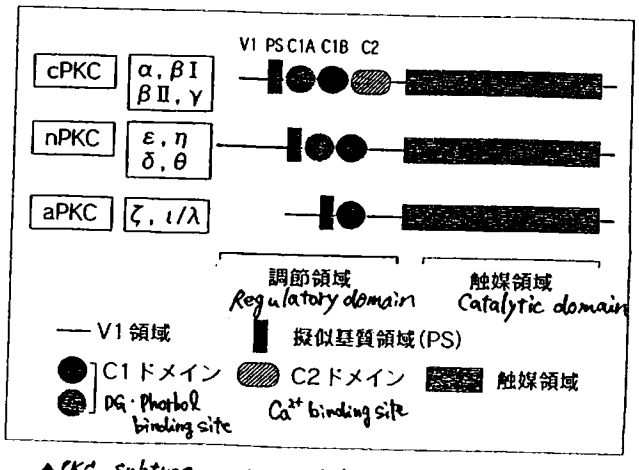
Introduction



- About PKC



- Today's topic
  - fluorescent phorbol ester
  - fluorescent inhibitor
  ] directly bind to PKC
  - fluorescent substrate of PKC (phosphorylation-induced reporter)
  - biological approach (using GFP)



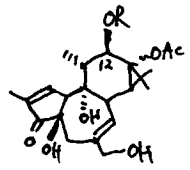
▲PKC subtype 蛋白質・核酸・酵素 (2003) vol48, p241

Fluorescent Tetradecanoylphorbol Acetate: A Novel Probe of Phorbol Ester Binding Domains

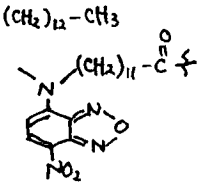
Margit Balázs, János Szöllösi, William C. Lee, Richard P. Haugland, Anthony P. Guzikowski, Mack J. Fulwyler, Sandor Damjanovich, Burt G. Feuerstein, and Harrihar A. Pershadsingh

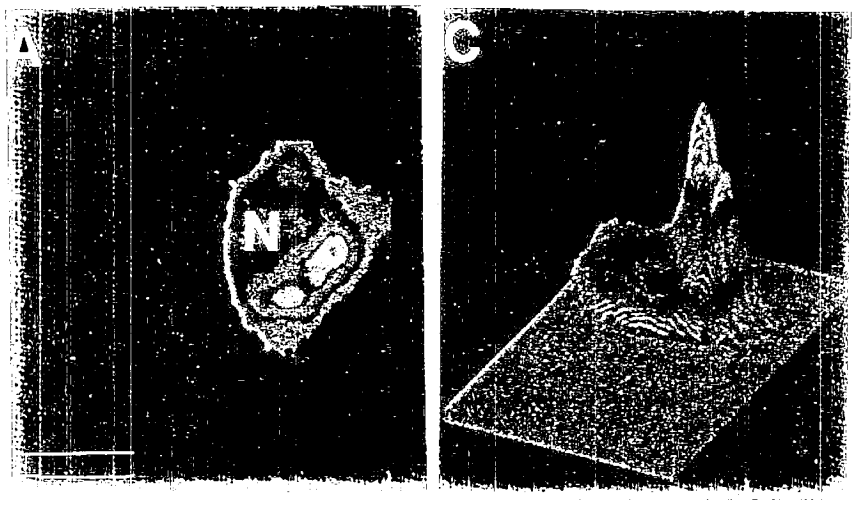
Departments of Laboratory Medicine (M.J.F., B.G.F., H.A.P.), Restorative Dentistry (W.C.L.), and the Brain Tumor Research Center (B.G.F.), University of California, San Francisco, San Francisco, California 94143; Department of Biophysics, University Medical School, Debrecen, H-4012, Hungary (M.B., J.S., S.D.); Molecular Probes Inc., Eugene, Oregon 97402 (R.P.H., A.P.G.)

Journal of Cellular Biochemistry 46:266–276 (1991)



TPA: $R = -C(=O)-(CH_2)_{12}-CH_3$

N-C12-Ac: $R =$ 



◀ Subcellular distribution of N-C12-Ac fluorescence (Pseudo-color digitized computer image)

N ··· nuclear

right → presented topographically as a three-dimensional plot (fluorescence intensity Σ axis)

* PKC presents in cytosol in high concentration

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 268, No. 21, Issue of July 25, pp. 15812-15822, 1993 Printed in U.S.A.

New Fluorescent Probes for Protein Kinase C

SYNTHESIS, CHARACTERIZATION, AND APPLICATION*

(Received for publication, January 22, 1993, and in revised form, March 23, 1993)

Chii-Shiarng Chen and Martin Poenie‡

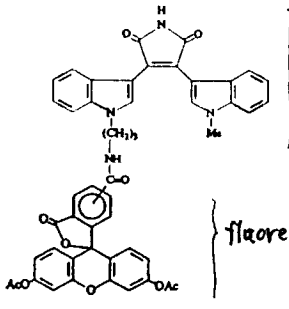
From the Department of Zoology, University of Texas, Austin, Texas 78712

in fixed cell

✕ disadvantage of fluorescent phorbol ···

→ high degree of nonspecific membrane staining

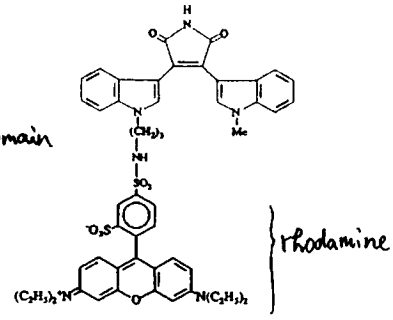
(because of hydrophobic side chain)



bisindolylmaleimide (PKC inhibitor) bind to catalytic domain

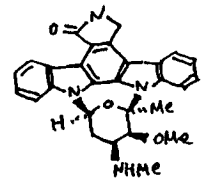
fluorescein

fim-1, 2



rhodamine

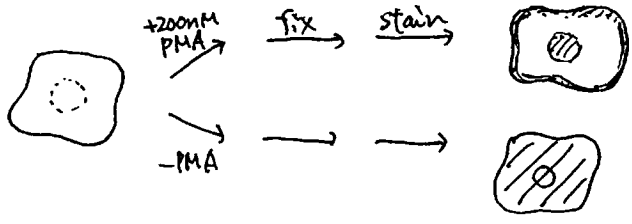
rim-1



Staurosporine

IC_{50} for PKCα 2.5nM

selectivity of PKC isoform is not so good ···



perinuclear staining↑
cytoplasm↓

J. Am. Chem. Soc.
2002, 124, 6258-6258

J|A|C|S COMMUNICATIONS

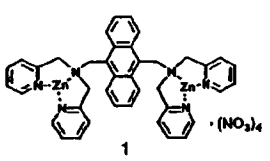
Published on Web 05/09/2002

First Artificial Receptors and Chemosensors toward Phosphorylated Peptide in Aqueous Solution

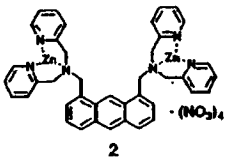
Akio Ojida,† Yasuko Mito-oka,† Masa-aki Inoue,† and Itaru Hamachi*,†,‡,§

PRESTO (Organization and Function, JST), Institute for Fundamental Research of Organic Chemistry (IFOC), Department of Chemistry and Biochemistry, Graduate School of Engineering, Kyushu University, Fukuoka, 812-8581, Japan

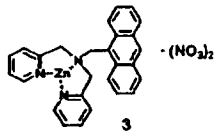
Received January 30, 2002



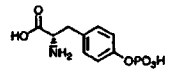
1



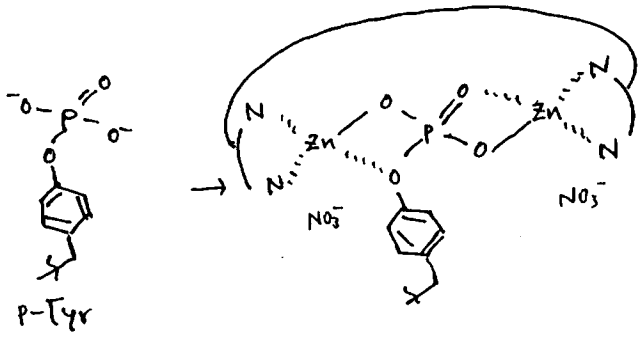
2



3



o-phosho-L-Tyrosine
(p-Tyr)



p-Tyr

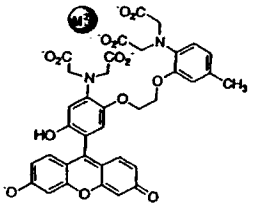
J|A|C|S COMMUNICATIONS

Published on Web 03/22/2002

Design and Synthesis of a Fluorescent Reporter of Protein Kinase Activity

Chien-An Chen,* Ren-Hwa Yeh,* and David S. Lawrence*

Department of Biochemistry, The Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461



← Ca^{2+} indicator

Tsien. et al

J. Biol Chem. 1989. 264. 8171-8178

J|A|C|S COMMUNICATIONS

Published on Web 11/04/2003

Versatile Fluorescence Probes of Protein Kinase Activity

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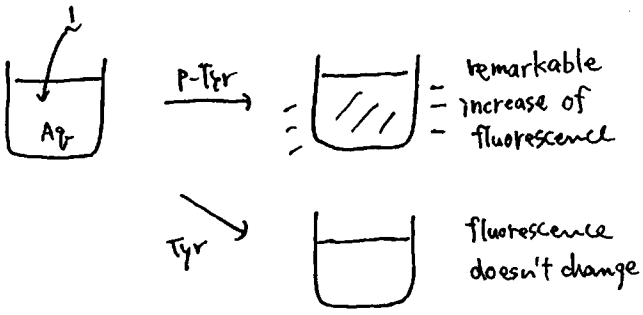
Received August 21, 2003; E-mail: imper@mit.edu

▶ New approach toward PKC probe
~ recognition of phosphorylation

cf. pioneering work : Czarnik. et al JACS. 1989. 111. 8735-

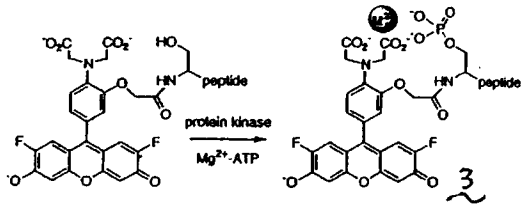


· 1 and 2 gave similar result.

· fluorescence change doesn't occur by addition of 3 into p-Tyr

· complexation-induced conformational rigidification of the receptor
↓
quantum yield ↑ !?

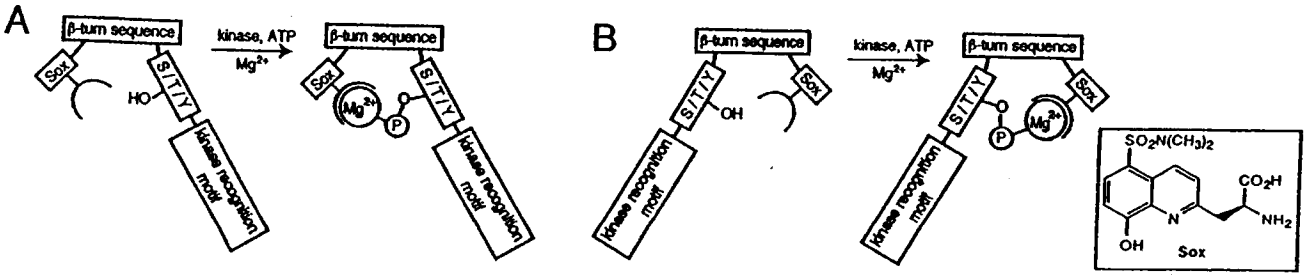
J. Am. Chem. Soc. 2002, 124, 3840-3841



peptide = fl-Phe-Arg-Arg-Arg-Arg-Ac
(fl = o)
PKC recognition sequence

· 140% increase in fluorescence intensity upon phosphorylation!

J. Am. Chem. Soc. 125, 14248-14249
2003.



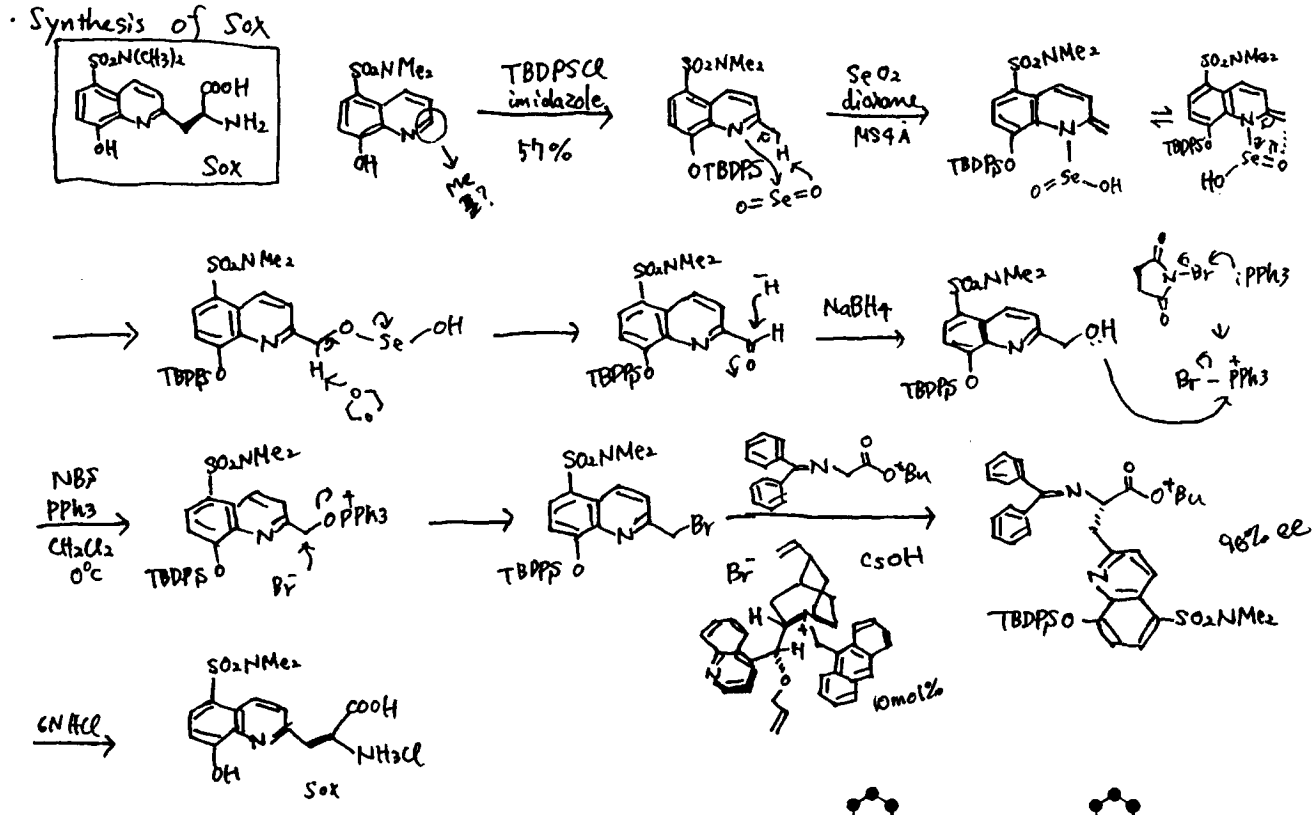
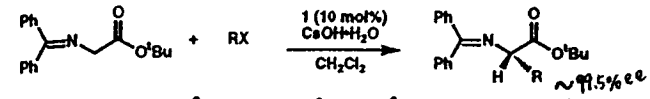
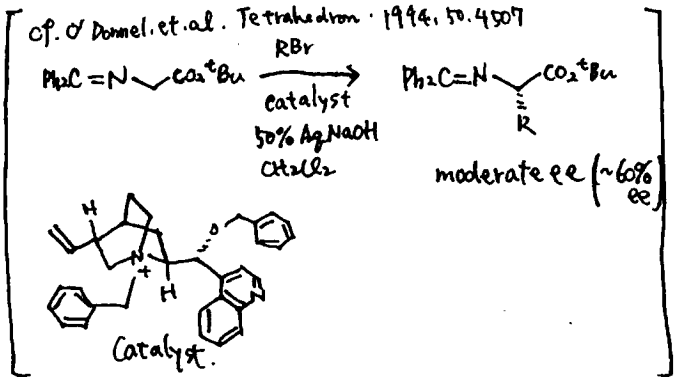


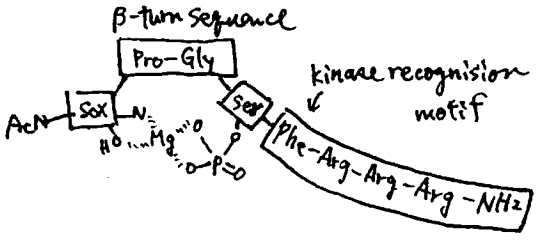
Table 1. Enantioselective Catalytic Phase Transfer Alkylation



E. J. Corey, et al JACS. 1997, 119, 12414–12415

cf. O'Donnel, et al. Tetrahedron · 1994, 50, 4507





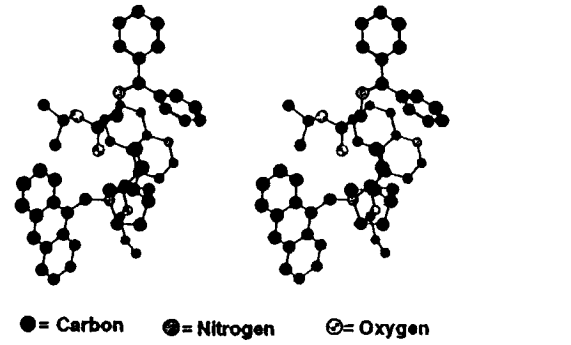


Figure 3. Stereopair representation of the preferred three-dimensional arrangement of the ion pair from 1 and the enolate 3. Alkylation of the enolate occurs by attack of the electrophile at *si* (front) face of the enolate for steric reasons, leading to the enantiomeric products shown in Table 1.

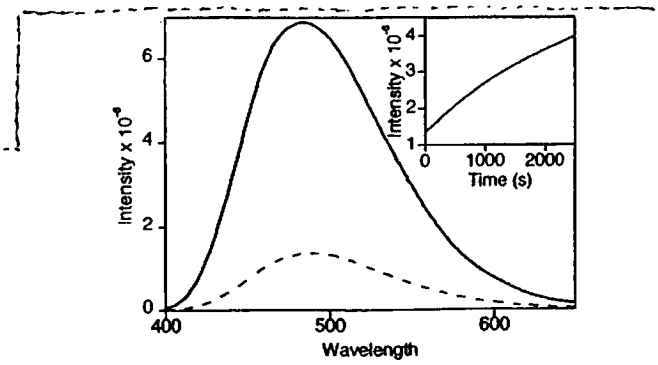


Figure 2. Spectra of peptide substrate (– –) and phosphopeptide product (—) (10 μM each) in PKC assay buffer containing 10 mM $MgCl_2$. Inset: Fluorescence intensity over the reaction time-course of Ac-Sox-Pro-Gly-Ser-Phe-Arg-Arg-Arg-NH_2 (10 μM) with PKC_α.

Table 1. Kinetic and Fluorescence Properties of Protein Kinase Substrates Containing Kinase Sensing Motif

Target Kinase	Phosphorylated Residue	Substrate Sequence[a]	K_M (μM)[b]	V_{max} (μmol/min/mg)[b]	Fluorescence Increase[c]
PKC_α	Ser	Ac-Sox-Pro-Gly-Ser*-Phe-Arg-Arg-Arg-NH_2	8.6 ± 2.9[d]	5.9 ± 1.9[d]	470%[e]
PKA	Ser	Ac-Leu-Arg-Arg-Ala-Ser*-Leu-Pro-Sox-NH_2	1.8 ± 0.5[f]	3.7 ± 1.6[f]	300%[e]
PKC_α	Thr	Ac-Sox-Pro-Gly-Thr*-Phe-Arg-Arg-Arg-NH_2			280%[g]
Abl	Tyr	Ac-Sox-Pro-Gly-Ile-Tyr*-Ala-Ala-Pro-Phe-Ala-Lys-Lys-Lys-NH_2			400%[g]

4/12

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 277, No. 13, Issue of March 29, pp. 11527–11532, 2002
Printed in U.S.A.

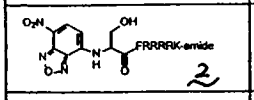
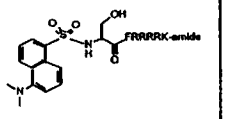
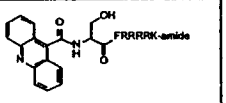
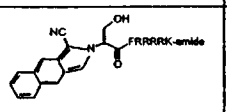
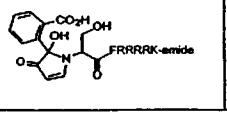
Real Time Visualization of Protein Kinase Activity in Living Cells*

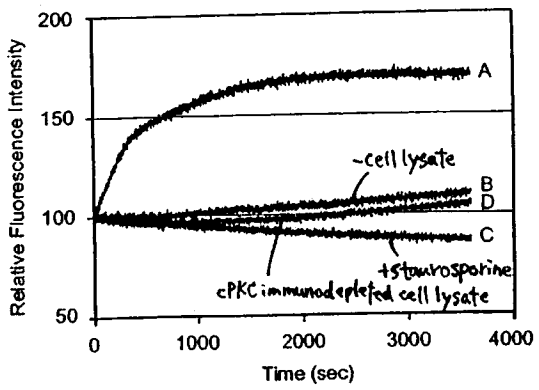
Received for publication, November 27, 2001, and in revised form, January 10, 2002
Published, JBC Papers in Press, January 14, 2002, DOI 10.1074/jbc.M111300200

J. Biol. Chem, 2002, 277, 11527-11532

Ren-Hwa Yeh, Xiongwei Yan, Michael Cammer, Anne R. Bresnick‡, and David S. Lawrence§

From the Department of Biochemistry, The Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461-1602

Fluorophore-peptide	Fluorophore Reagent	% Change	K_m (μM)	k_{cat} (min^{-1})
	NBD-Cl	150%	9.0 ± 1.0	380 ± 20
	Dansyl chloride	20%	28 ± 3	170 ± 10
	9-Acridinecarboxylic acid	20%	13 ± 2	20 ± 2
	Cyanide/Naphthalene -2,3-dicarbaldehyde	-	70 ± 1	220 ± 10
	Fluorescamine	-	280 ± 60	25 ± 4



PKC activity in mitotic HeLa cell lysates.

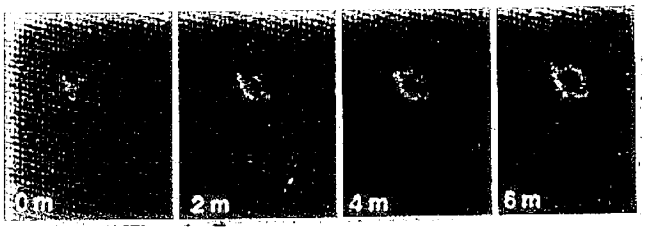


FIG. 4. TPA-induced time-dependent change in fluorescence intensity in HeLa cells with peptide 2.

J|A|C|S COMMUNICATIONS
JACS. 2003, 125. 13358-13359
Published on Web 10/11/2003

A Light-Activated Probe of Intracellular Protein Kinase Activity

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Received August 7, 2003; E-mail: dlawrenc@aecom.yu.edu

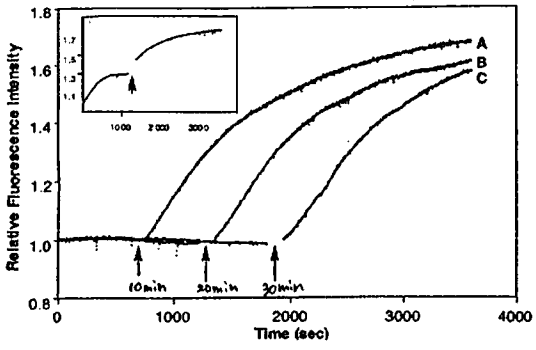
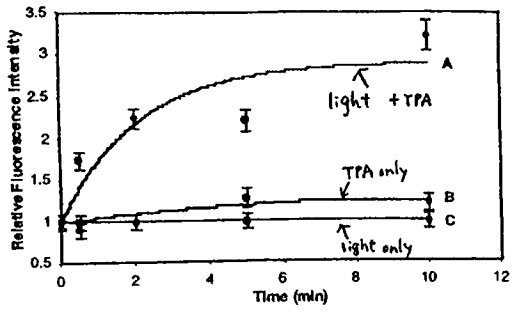
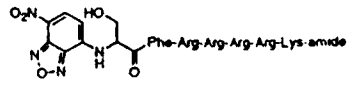
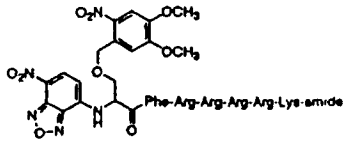


Figure 1. Time-dependent change in fluorescence before and after in situ illumination of caged peptide. The caged peptide 2 was incubated at 30 °C with PKCα and the change in fluorescence measured for 10 (A), 20 (B), or 30 (C) min. Samples were then irradiated at the indicated time points. (Insert) Partial photolysis of 2 followed by a second exposure to brief illumination.

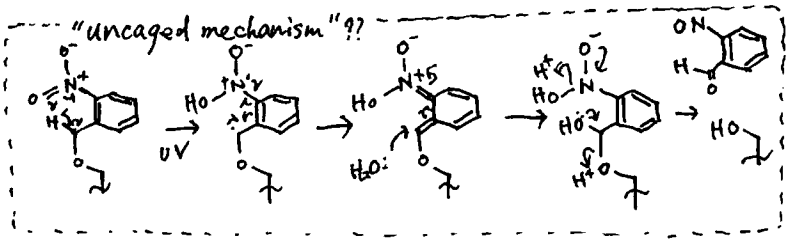




UV (300-400 nm)



inactive form
"caged"



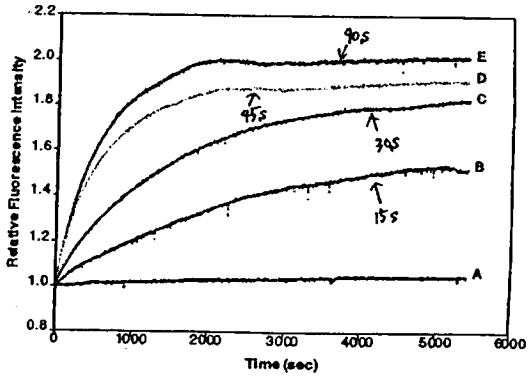


Figure 2. Fluorescence change as a function of irradiation time. Peptide 2 was illuminated for (A) 0, (B) 15, (C) 30, (D) 45, and (E) 90 s and PKCα-catalyzed activity subsequently sampled via fluorescence change.

Biological approach ～using GFP (Green Fluorescent Protein)～

*Advantage of GFP

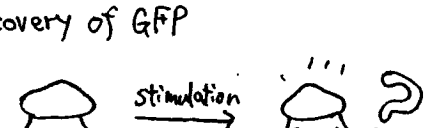
- Protein–GFP* — By expression of GFP-tagged protein, we can observe real-time movement of protein in living cell.
- → We can stain inside of organ
- Protein : GFP = 1 : 1 — To control ratio of Protein / GFP is possible

*disadvantage of GFP

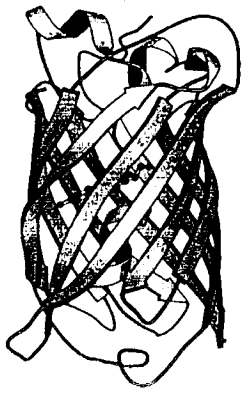
GFP — 238 amino-acid, 27KD

GFP is much more larger than chemically synthesized fluorophore (control experiment is necessary, because GFP may interfere ···)

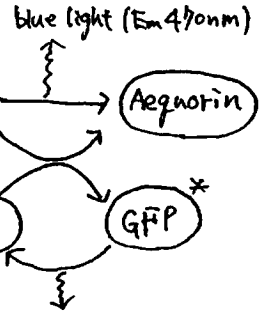
- Discovery of GFP



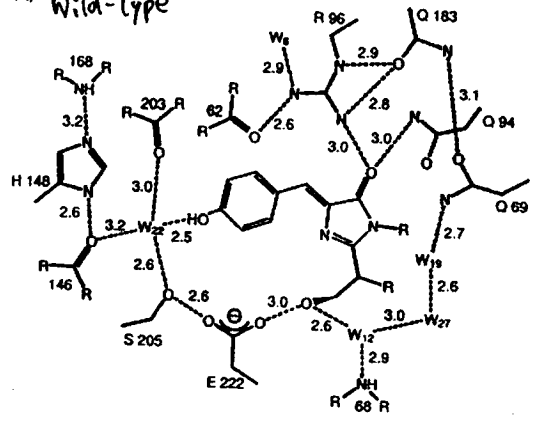
Aequorea victoria (オワンクラゲ)



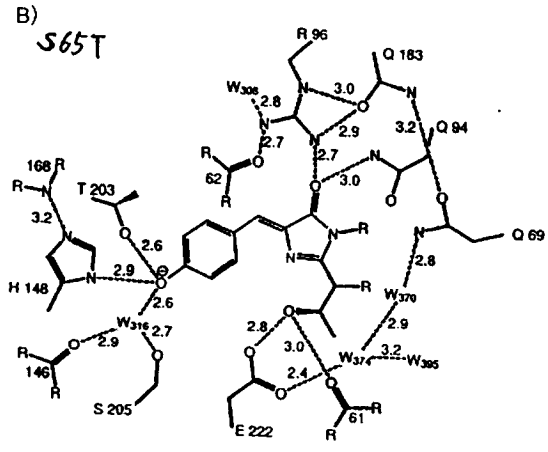
"β-can"
Science, 1996, 273, 1392

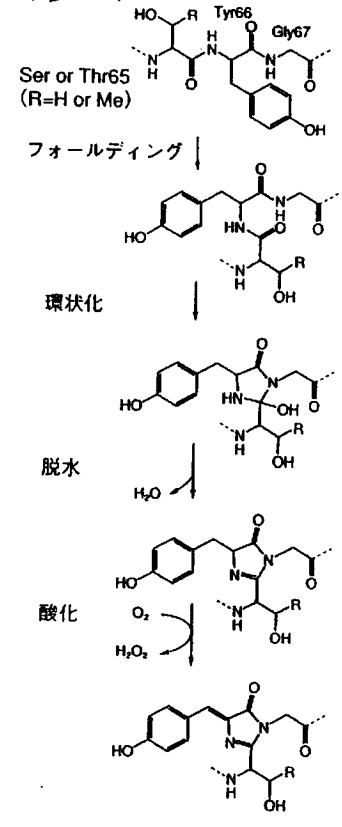


A) Wild-Type



B) S65T





参考「GFPとバイオイメージング」 羊土社, 宮脇

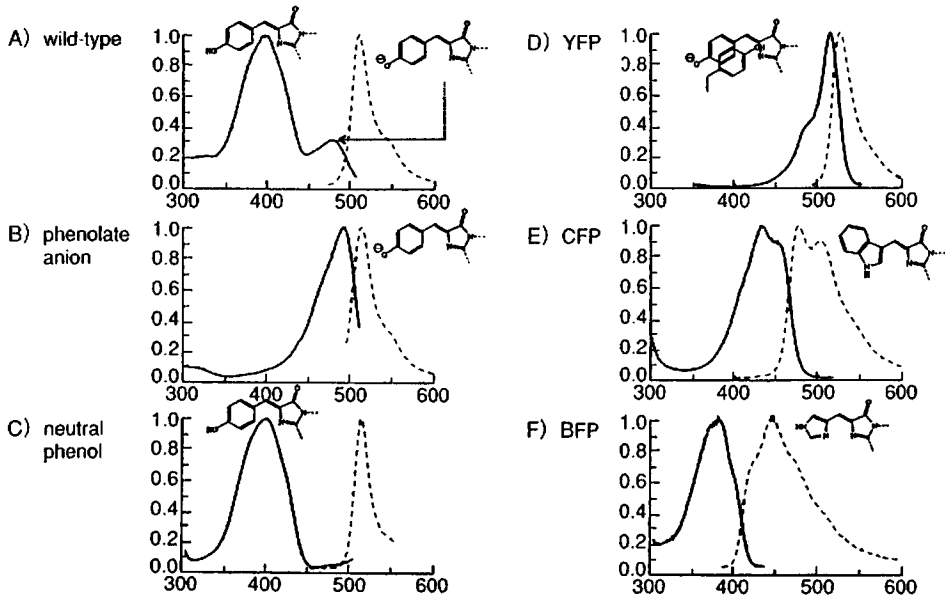


図6　改変GFPの励起（実線），蛍光（点線）スペクトルと，発色団（基底状態）の構造
縦軸：スペクトル強度，横軸：波長（nm），各グループに属する改変GFPの名称を図中に示した．

← artificial mutation based on X-ray structure

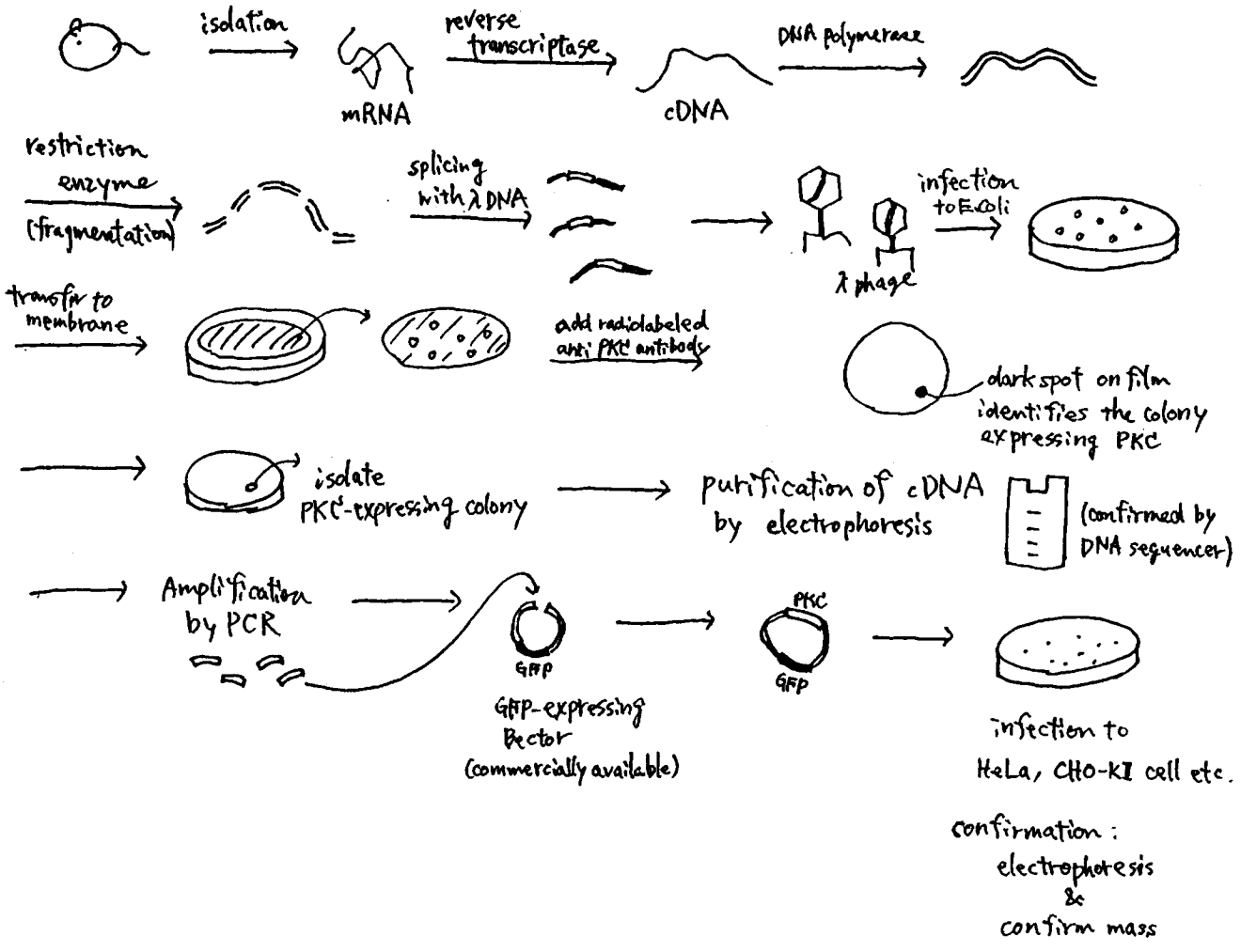
▨ RFP (DsRed)

discovered from Zoanthus (ストロンチウム)

non-fluorescent creature!!

Ex 558nm , Em 583nm

▨ How to construct fluorescent PKC



THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 52, Issue of December 24, pp. 37233–37239, 1999
Printed in U.S.A.

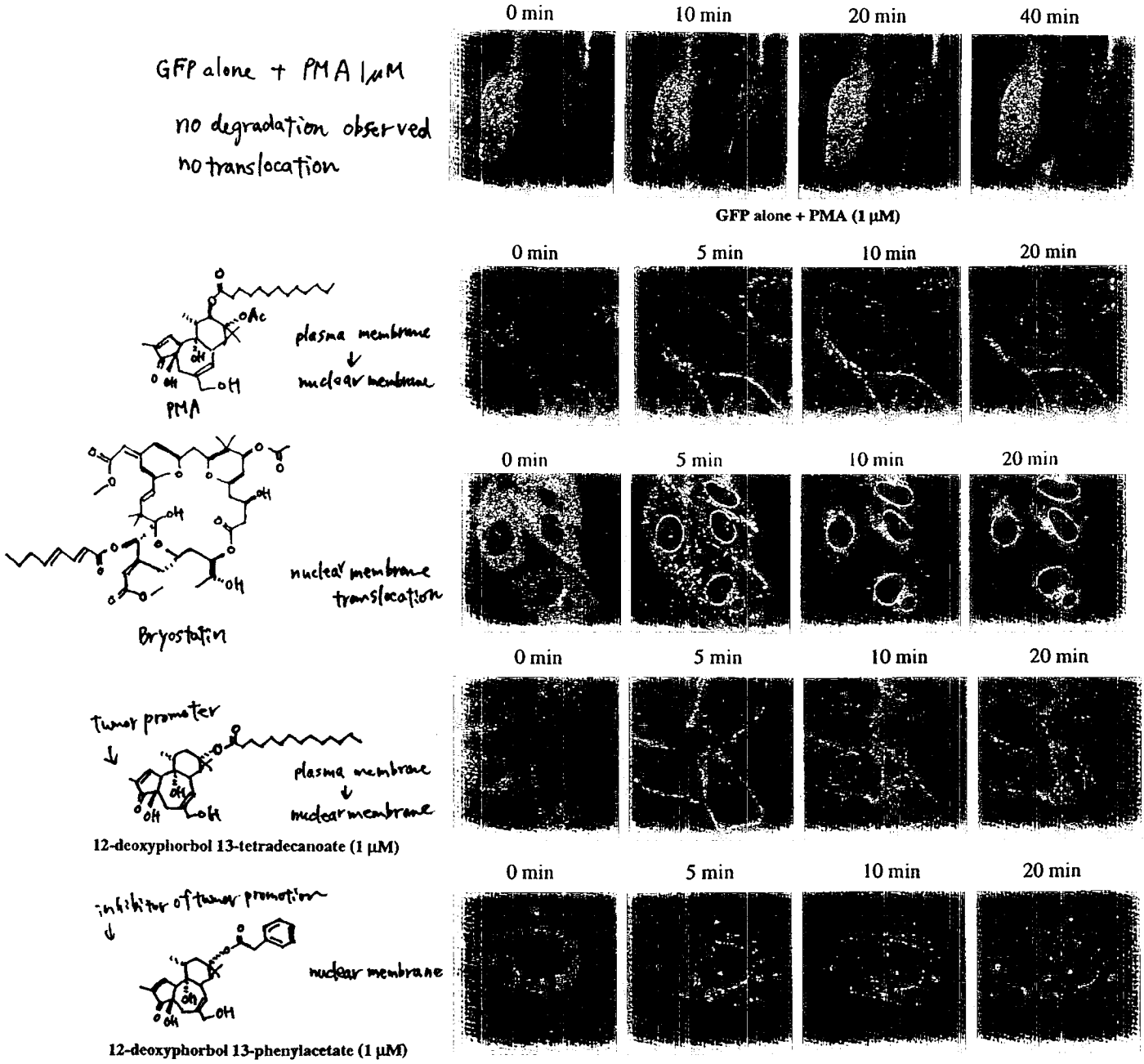
Differential Localization of Protein Kinase C δ by Phorbol Esters and Related Compounds Using a Fusion Protein with Green Fluorescent Protein*

(Received for publication, June 10, 1999, and in revised form, September 3, 1999)

J. Biol. Chem, 1999, 274, 37233-37239

Qiming J. Wang‡, Dipak Bhattacharyya‡, Susan Garfield§, Kassoum Nacro¶, Victor E. Marquez¶, and Peter M. Blumberg‡‖

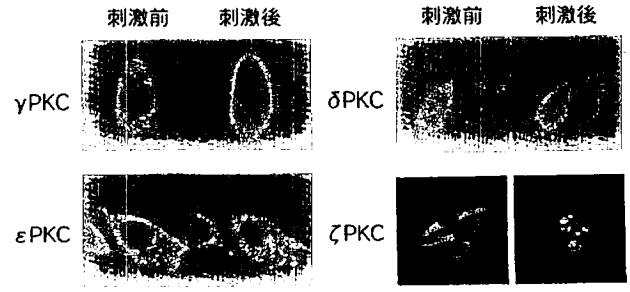
From the ‡Molecular Mechanisms of Tumor Promotion Section, Laboratory of Cellular Carcinogenesis and Tumor Promotion, §Laboratory of Experimental Carcinogenesis, and ¶Laboratory of Medicinal Chemistry, NCI, National Institutes of Health, Bethesda, Maryland 20892



Short Review "プロテインキナーゼCのダイナミックな機構"
「蛋白質 核酸 酵素」2003, 48, P1241- 白井 他

図2 アラキドン酸によるサブタイプ特異的なPKCトランスロケーション

γPKC-, δPKC-, εPKC-, ζPKC-GFPをCHO-K1細胞に発現し, アラキドン酸刺激を加えた. 3種のサブタイプ(γ, ε, ζ)はそれぞれ違う細胞内部位(細胞質膜, ゴルジ体, 核内)にトランスロケーションした. δPKC-GFPはアラキドン酸に応答しなかった.



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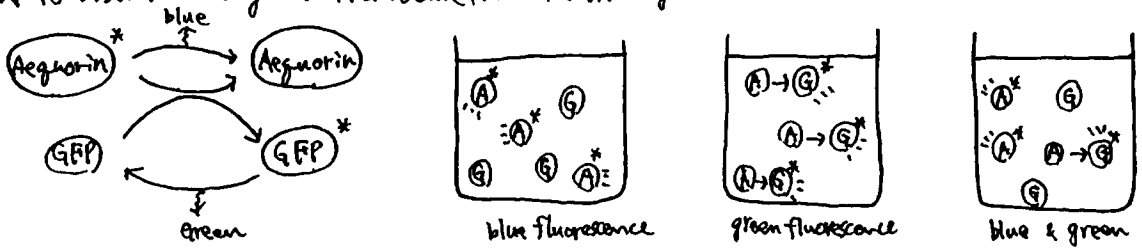
☆ To visualize signal transduction in living cells ...



- to generate energy transfer <FRET = Fluorescence Resonance Energy Transfer>

two spectrum must overlap

Donor ↔ Acceptor ~30Å distance between donor & acceptor have to be short

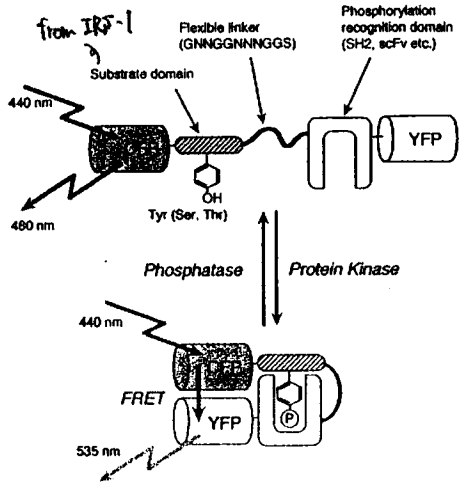
- ratio of donor/acceptor fluorescence reflects condition of molecular?

⇨ FRET must be applied to indicators to see biological activities!!

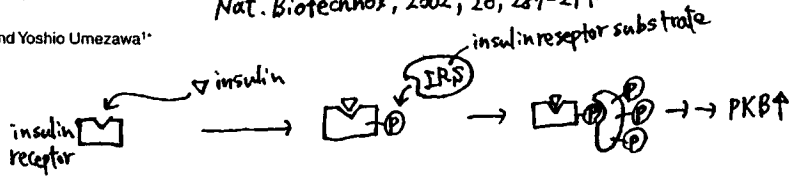
Fluorescent indicators for imaging protein phosphorylation in single living cells

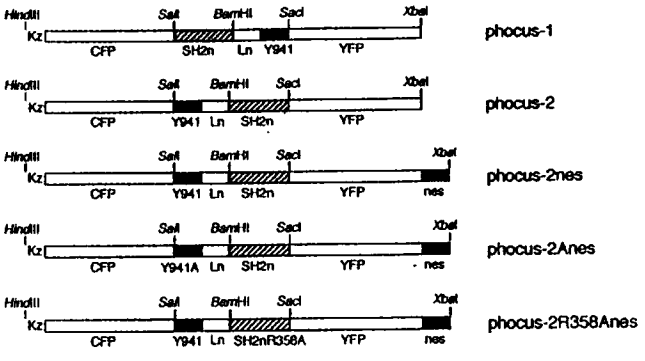
Moritoshi Sato[1], Takeaki Ozawa[1], Kouichi Inukai[2], Tomoichiro Asano[2], and Yoshio Umezawa[1*]

Nat. Biotechnol, 2002, 20, 287-294

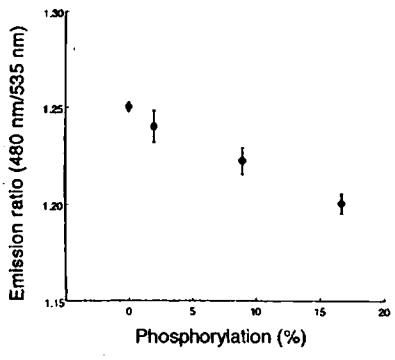


Phocus = fluorescent indicator of protein phosphorylation that can be custom-made

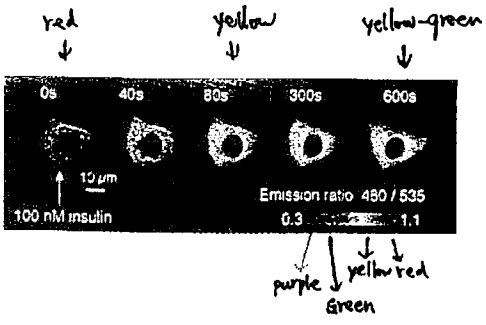


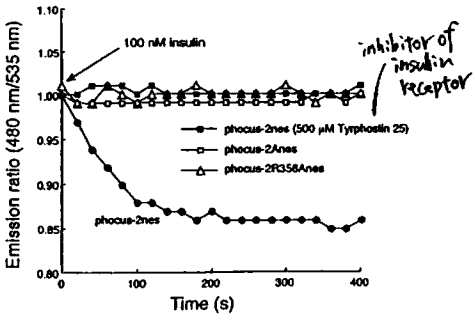


nes = nuclear-export signal sequence, LPPLERLTL (to avoid confusing nuclear transport of the indicator)

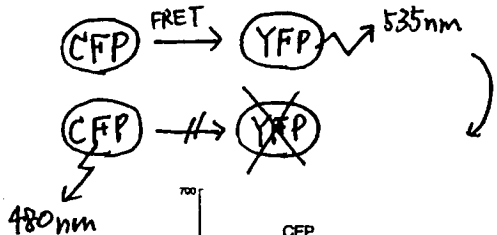


↑ determined by using anti p-Tyr

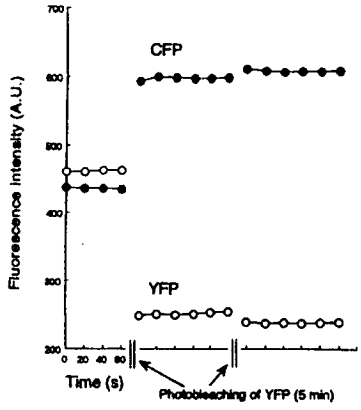


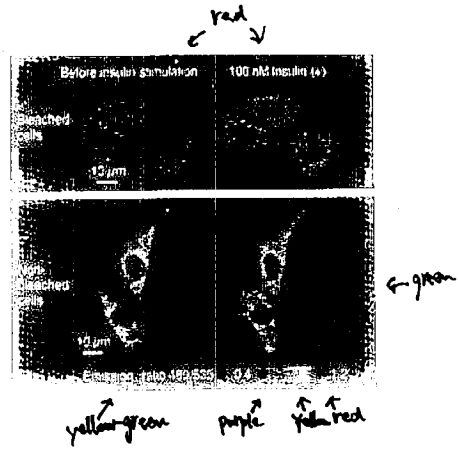


· to confirm the increase of the CFP/YFP emission ratio is caused by FRET ···



If acceptor (YFP) can be selectively photoinactivated CFP/YFP emission ratio must increase !!



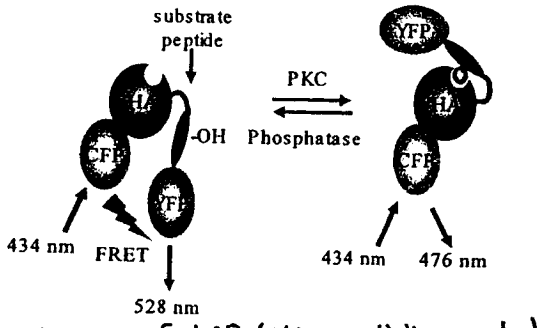


A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C

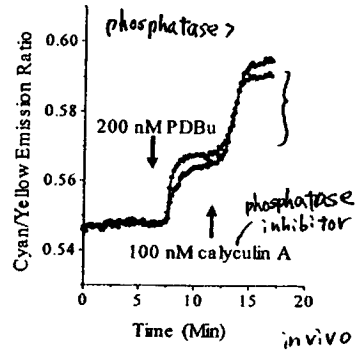
Jonathan D. Violin,[1,2] Jin Zhang,[1] Roger Y. Tsien,[1,3] and Alexandra C. Newton[1]

[1]Department of Pharmacology, [2]Biomedical Sciences Graduate Program, and [3]Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA 92093

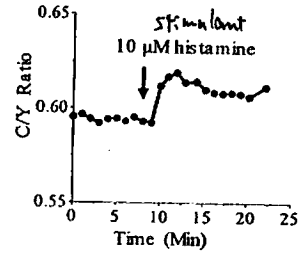
J. Cell. Biol. 2003, 161 899-909

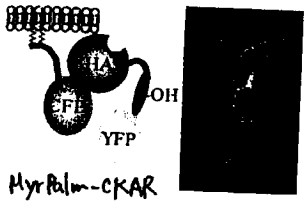


structure of CKAR (C kinase activity reporter)



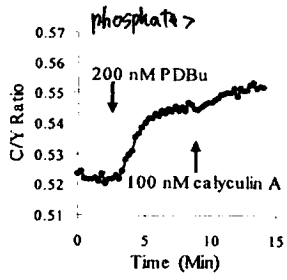
in vivo

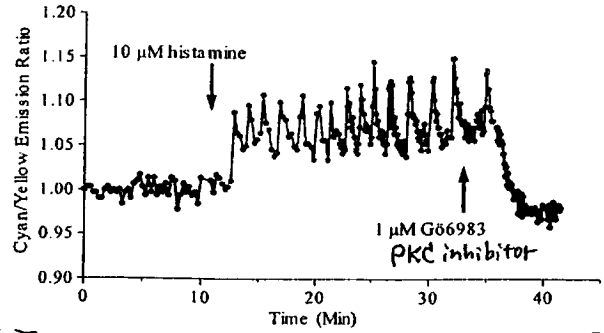


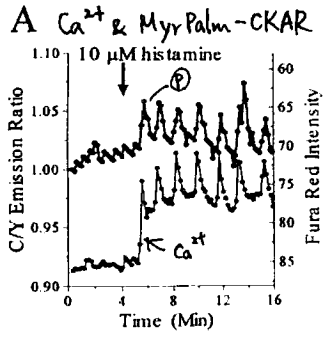


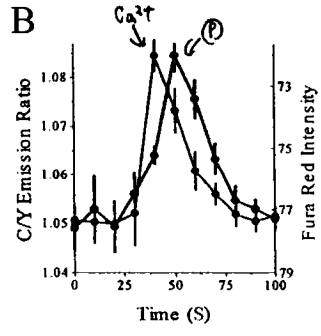
MyrPalm-CKAR

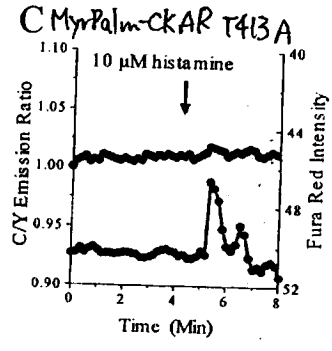
Targeting CKAR to plasma membrane.

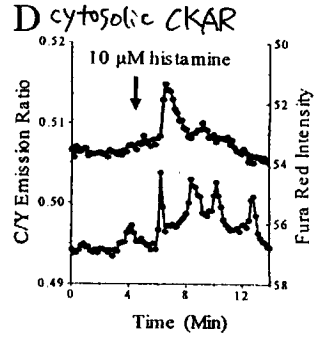












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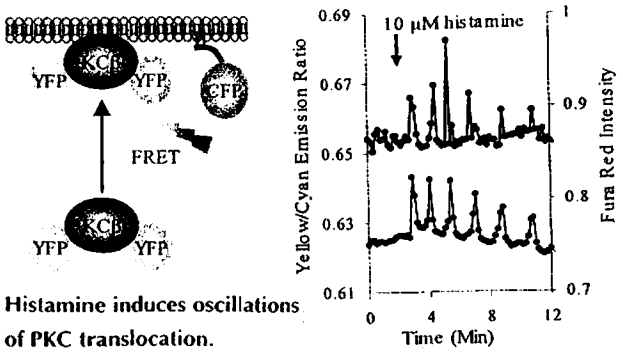


Histamine induces oscillations of PKC translocation.

- Ca^{2+} ↑ delay phosphorylation ↑ @membrane
- PKC membrane translocation ↔ Ca^{2+} phase lock
  Ca^{2+} controlled membrane association

* Does generation of DAG relate Ca^{2+} oscillation?

PIP_2 —PLC→ DAG + IP_3

PHδ : domain of PLCδ

PLC activity is independent of Ca^{2+} transients?

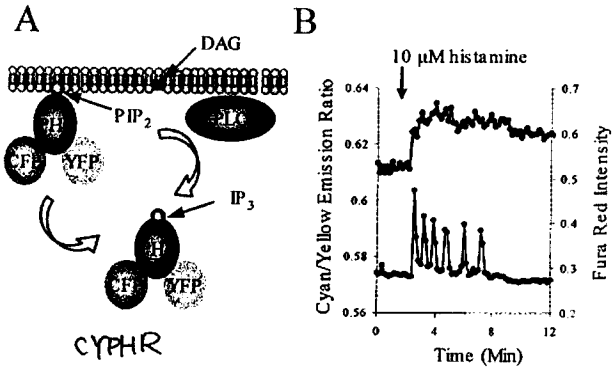
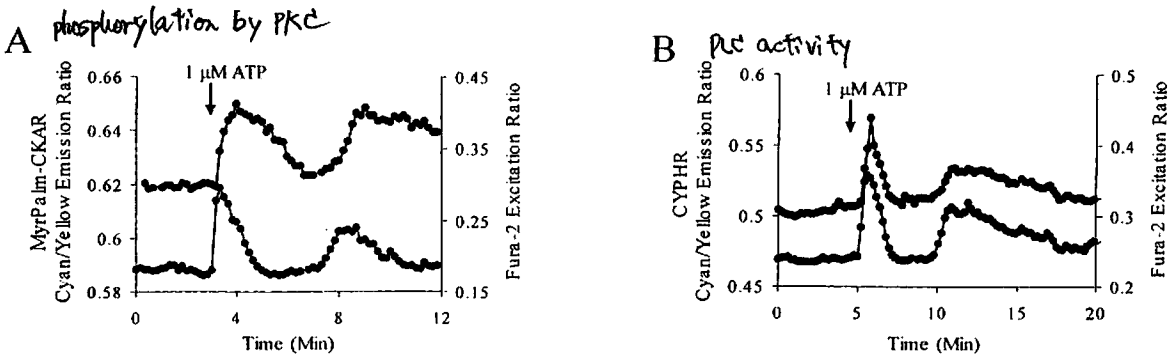


Figure 6. **Translocation of the PH domain of PLCδ reported by FRET reveals constant PLC activity during calcium oscillations.**

* How about the stimulation by ATP?



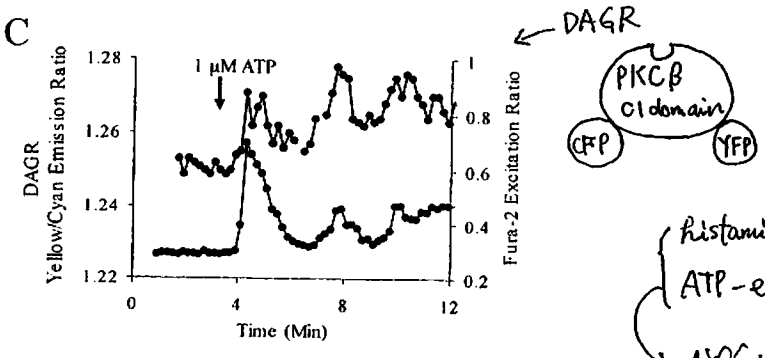


Figure 7. **PLC controls oscillations of PKC activity in MDCK cells.** 10 μM ATP induces calcium transients phase locked with responses of MyrPalm-CKAR (reports phosphorylation) (A), CYPHR (reports PLC activity) (B), and DAGR (reports DAG) (C). All data are representative of three to five experiments.

histamine-evoked PKC response in HeLa
ATP-evoked in MDCK
↳ different!
two distinct mechanism for temporal control of PKC activation exists!

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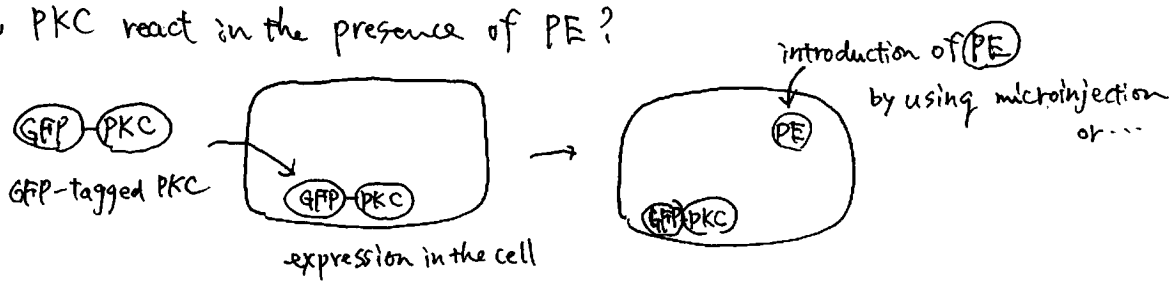
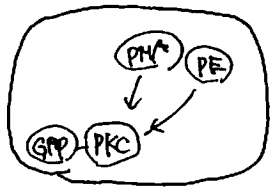
Application to my study

→ To reveal if our inhibitor really works in vivo ··· and ···

- How PKC react in the presence of PE?



- How PKC reacts in the presence of PE & PMA (kinds of inhibition assay)



- Is localization of PKC really important? (Is our strategy reasonable ?)