

# Inositol 1,4,5-Trisphosphate-Gated Calcium Transport through Plasma Membranes in Nerve Terminals

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We developed new biochemical approaches to demonstrate the presence of inositol 1,4,5-trisphosphate (InsP<sub>3</sub>)-gated calcium channels in presynaptic plasma membranes (SPM) and their involvement in the presynaptic receptor-mediated Ca<sup>2+</sup> influx into nerve terminals. In perfusion experiments using SPM vesicles preloaded with <sup>45</sup>Ca<sup>2+</sup>, InsP<sub>3</sub> elicited the release of <sup>45</sup>Ca<sup>2+</sup> into perfusates in a saturable manner. The InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release from resealed SPM vesicles was more

potent than that from resealed vesicles using any other subcellular fractions. Here we also report the involvement of InsP<sub>3</sub>-gated mechanisms in the presynaptic receptor-mediated Ca<sup>2+</sup> influx into synaptosomes (nerve terminals) by use of such resealed vesicles reconstituted with purified G<sub>i1</sub>.

**Key words:** InsP<sub>3</sub> receptor; presynaptic receptor; G<sub>i1</sub>; reconstitution; resealed vesicles; Ca<sup>2+</sup>

A wide variety of stimulation of receptors by hormones and neurotransmitters results in increased phosphoinositide turnover and mobilization of Ca<sup>2+</sup> from intracellular stores (Berridge, 1993; Berridge and Irvine, 1984). Such post-receptor mechanisms involve the stimulation (or inhibition) (see Misawa et al., 1995) of phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate giving rise to diacylglycerol and inositol 1,4,5-trisphosphate (InsP<sub>3</sub>). It is well known that InsP<sub>3</sub> mobilizes Ca<sup>2+</sup> from microsomal organelles, such as rough (Henne et al., 1987) and smooth endoplasmic reticulum (Payne and Fein, 1987) and calciosome (Volpe et al., 1988) in various secretory cells. Thus, it is likely that InsP<sub>3</sub>-induced calcium mobilization from intracellular organelles is involved in hormone secretion with receptor stimulation.

On the other hand, it is also considered that neurotransmitter release occurs predominantly in nerve terminals in a calcium-dependent manner. Although PLC is reported to be present in nerve terminals (Gerfen et al., 1988) and is assumed to play an important role in presynaptic receptor-mediated regulation of neurotransmitter release, details on the InsP<sub>3</sub>-mediated calcium mobilization in nerve terminals remain to be determined.

We have reported that kyotorphin (tyrosine-arginine), a neuropeptide that is characterized as a releaser of met-enkephalin from brain slices (Takagi et al., 1979), increased intracellular concentrations of Ca<sup>2+</sup>, measured by Quin-II fluorometry, and stimulated the uptake of <sup>45</sup>Ca<sup>2+</sup> extracellularly added into brain synaptosomes (Ueda et al., 1986). However, because this <sup>45</sup>Ca<sup>2+</sup> uptake was not

affected by calcium channel blockers and kyotorphin had no effect on the membrane potential in synaptosomes (Ueda et al., 1986), it is unlikely that the voltage-dependent calcium channel is involved in this presynaptic mechanism. Most recently we have reported that kyotorphin stimulated PLC in synaptosomal membranes via G<sub>i1</sub> by reconstitution experiments (Ueda et al., 1989). These findings suggest that kyotorphin elicits calcium entry into synaptosomes via an action of InsP<sub>3</sub> at the plasma membranes rather than by means of calcium mobilization from intrasynaptosomal organelles. Taking into account the reports that InsP<sub>3</sub>-specific binding sites are also found in plasma membranes of hepatocytes (Guillemette et al., 1988), lymphocytes (Khan et al., 1992), and neurons (Worley et al., 1987) and that InsP<sub>3</sub> receptors are found immunohistochemically in plasma membranes of olfactory cilia (Cunningham et al., 1993) and in nerve terminals of deep cerebellar nuclei (Sharp et al., 1992), we speculated that InsP<sub>3</sub>-gated calcium channels other than voltage-operated ones are involved in the receptor-operated calcium transport through plasma membranes in nerve terminals. Indeed, there are reports that InsP<sub>3</sub>-gated calcium channels function in plasma membranes of human lymphocytes, mast cells, and liver (Kuno and Gardner, 1987; Guillemette et al., 1988; Penner et al., 1988). Here we attempted to obtain biochemical evidence for the presynaptic InsP<sub>3</sub>-gated calcium channels in nerve terminals and clarify the molecular basis of mechanisms in kyotorphin receptor-mediated calcium incorporation into synaptosomes through experiments using resealed presynaptic plasma membrane (SPM) vesicles.

## MATERIALS AND METHODS

**Materials.** InsP<sub>3</sub>, inositol 1,3,4,5-tetrakisphosphate (InsP<sub>4</sub>), inositol 1,4-bisphosphate (InsP<sub>2</sub>), inositol 4-monophosphate (InsP), and inositol (Ins) were purchased from Sigma (St. Louis, MO), and <sup>45</sup>CaCl<sub>2</sub> was purchased from DuPont NEN (Boston, MA). Kyotorphin was a gift from Dr. M. Kubota (Daiichi Pharmaceuticals, Tokyo, Japan) or purchased from Sigma. Other reagents were of analytical grade and were purchased from Sigma or Wako Pure Chemicals (Osaka, Japan).

**Preparation of subcellular fractions.** Male Sprague-Dawley rats weighing 200–250 gm were decapitated and the whole brains were homogenized in 10 vol of 0.32 M sucrose. The homogenates were centrifuged at

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1000 × g for 10 min, and the supernatant was further centrifuged at 12,000 × g for 20 min. Resulting pellets were used for preparation of myelin, synaptosomes, and mitochondria, and the supernatant was used for preparation of microsomes by further centrifugation at 100,000 × g for 60 min, according to Gray and Whittaker (1962). Further subsynaptosomal fractions were prepared by discontinuous density gradient centrifugation of lysed synaptosomes, composed of 0.4, 0.6, 0.8, 1.0, and 1.2 M sucrose (Whittaker et al., 1964). Synaptic vesicles were obtained from the interface between 0.4 and 0.6 M sucrose, SPM from that between 0.6 and 0.8 M and between 0.8 and 1.0 M sucrose, and presynaptic mitochondria from the pellet. [Na<sup>+</sup>/K<sup>+</sup>]-ATPase and NADPH cytochrome c reductase activities in each subfraction were measured according to Verity (1972) and Kasper (1971), respectively.

**Incorporation of <sup>45</sup>Ca<sup>2+</sup> into and <sup>45</sup>Ca<sup>2+</sup> release from subcellular preparations.** For preparation of resealed vesicles, each subcellular preparation per rat brain was hypo-osmotically lysed with 10 ml of 5 mM Tris-HCl buffer, pH 7.5, containing 1 mM MgCl<sub>2</sub>, 0.574 mM CaCl<sub>2</sub>, and 1 mM ethylene glycol bis (β-aminoethylether)N,N,N',N'-tetraacetic acid/EGTA (TMC buffer) by a Potter-Elvehjem homogenizer and centrifuged at 10,000 × g for 5 min. The free [Ca<sup>2+</sup>] in the TMC buffer was calculated to be 0.1 μM (Fabiato and Fabiato, 1979). The obtained pellets were resuspended in TMC buffer. Aliquots (10 mg of protein) were incubated in 10 ml of TMC buffer with <sup>45</sup>Ca<sup>2+</sup> (0.5 μCi) at 37°C. At various periods of incubation (0.5–35 min), an aliquot (100 μl) was removed and passed through a GF/C filter (Whatman, Maidstone, UK), followed by three washes with 3 ml of TMC buffer. For preparation of "previously resealed vesicles," the incubation with <sup>45</sup>Ca<sup>2+</sup> was preceded by prior incubation at 37°C for 30 min in its absence. In some experiments, to examine the ATP- and calmodulin-dependent <sup>45</sup>Ca<sup>2+</sup> incorporation, the free [Ca<sup>2+</sup>] was adjusted to 10 μM, a concentration required for activation of Ca<sup>2+</sup>-activated ATPase (calcium pump) by calmodulin using 0.109 mM CaCl<sub>2</sub> and 0.1 mM EGTA. Furthermore, in such experiments using unlysed microsomes, the preparation was preloaded with <sup>45</sup>Ca<sup>2+</sup> in the iso-osmotic buffer containing (in mM) KCl 145, NaCl 5, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 0.574, EGTA 1.0, HEPES 10, pH 7.4, in the presence or absence of 1 mM ATP under the condition of 37°C for 30 min. In the experiments for <sup>45</sup>Ca<sup>2+</sup> incorporation into unlysed synaptosomes or saponin-treated permeabilized synaptosomes, another iso-osmotic buffer containing (in mM) NaCl 145, KCl 5, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 0.574, EGTA 1.0, HEPES 10, pH 7.4, in the presence or absence of 1 mM ATP was used. In the latter experiments, saponin (30 μg/ml) was added to the synaptosomes just before <sup>45</sup>Ca<sup>2+</sup> incorporation. The accumulation of <sup>45</sup>Ca<sup>2+</sup> was determined by measuring radioactivity on the filter.

The experiments of <sup>45</sup>Ca<sup>2+</sup> release were performed essentially as described (Ueda et al., 1987). Briefly, aliquots (300–500 μg protein) of lysed preparations were incubated with <sup>45</sup>CaCl<sub>2</sub> (0.5 μCi) at 37°C for 30 min and centrifuged at 5000 × g for 10 min. The pellets were resuspended in a small volume of TMC buffer, loaded on GF/C filters (diameter 6 mm), fixed in the chamber, and superfused in TMC buffer at a flow rate of 1 ml/min. The <sup>45</sup>Ca<sup>2+</sup> release from resealed vesicles was determined as "fractional release (%)" by measurement of the ratio of the <sup>45</sup>Ca<sup>2+</sup> release (cpm) to the total <sup>45</sup>Ca<sup>2+</sup> (cpm) in the preparation at the real time, as reported previously (Ueda et al., 1987). The total <sup>45</sup>Ca<sup>2+</sup> was calculated by summation of <sup>45</sup>Ca<sup>2+</sup> released into perfusates and remained in the preparation after the perfusion experiment. Other details in collection of perfusates, addition of drugs, and estimation of evoked release were also as described (Ueda et al., 1987).

**Reconstitution of pertussis toxin-treated membranes with purified G<sub>ii</sub>.** The pretreatment of SPM with preactivated pertussis toxin (PTX) and reconstitution of PTX-treated membranes with purified G<sub>ii</sub> or G<sub>o</sub> was performed as reported previously (Ueda et al., 1989). Briefly, freshly prepared SPM (2 mg protein) was pretreated with preactivated 50 μg/ml of PTX in a volume of 100 μl, followed by addition with purified G<sub>ii</sub> or G<sub>o</sub> (20 pmol/assay).

**<sup>45</sup>Ca<sup>2+</sup> influx into intact synaptosomes in membranes prepared from various regions of the rat brain.** Procedures of <sup>45</sup>Ca<sup>2+</sup> influx into synaptosomes from various brain regions have been reported previously (Ueda et al., 1986). Briefly, synaptosomes from various brain regions of the rat were prepared as described by Whittaker (1964). After the brain synaptosomes had been preincubated in HEPES-buffered medium (HBM) at 37°C for 10 min, 100 μM kyotorphin and <sup>45</sup>CaCl<sub>2</sub> (0.1 μCi) were added, the incubation extended for another 5 min, then terminated by adding 5 ml of cold HBM, incubating 5 mM EGTA instead of CaCl<sub>2</sub>. The preparation was then passed through a GF/C glass fiber filter (Whatman). This filter was washed three times with Ca<sup>2+</sup>-free HBM-EGTA (5 mM), and

the radioactivity was counted. Kyotorphin-evoked <sup>45</sup>Ca<sup>2+</sup> influx was represented as percentage of control without kyotorphin.

## RESULTS

### Accumulation of <sup>45</sup>Ca<sup>2+</sup> into resealed vesicles derived from SPM

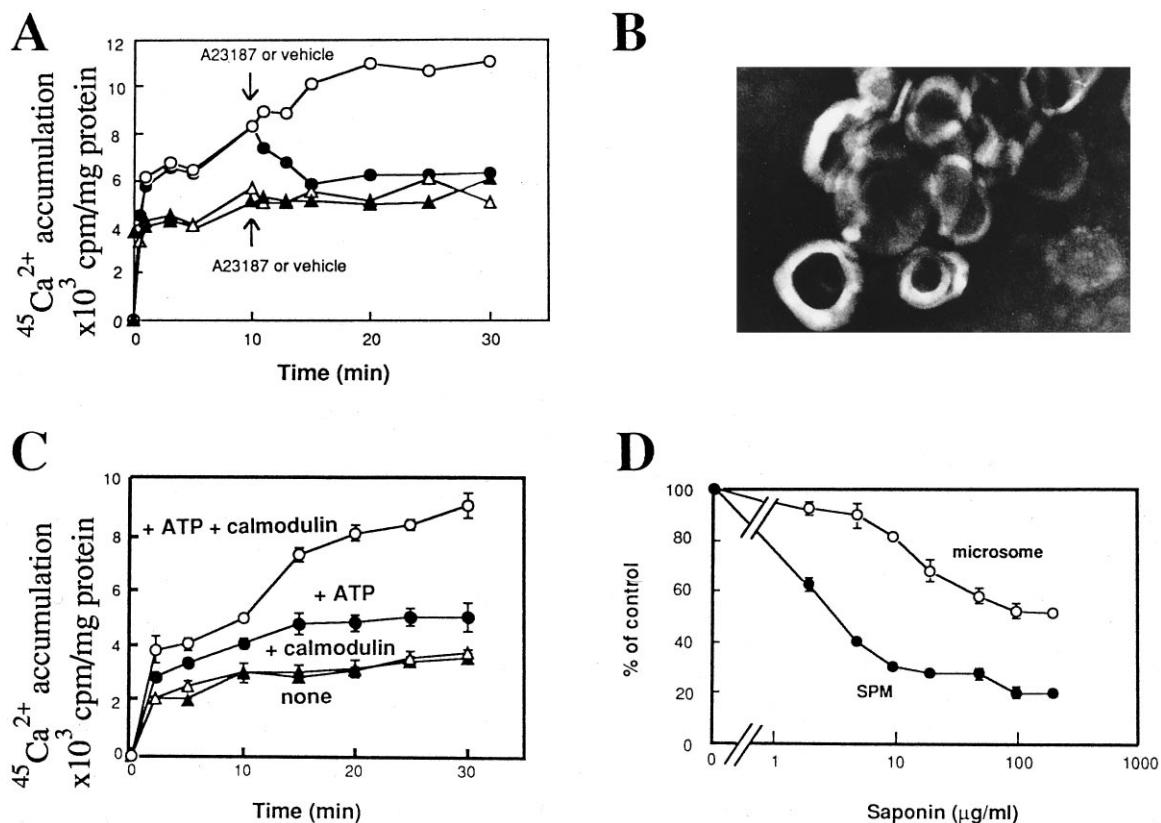
The first step in experiments of <sup>45</sup>Ca<sup>2+</sup> accumulation into resealed vesicles was to incubate the freshly prepared (lysed) SPM with <sup>45</sup>Ca<sup>2+</sup> in TMC buffer at 37°C. Aliquots were periodically removed and passed through GF/C filters to measure <sup>45</sup>Ca<sup>2+</sup> accumulation. As shown in Figure 1A, <sup>45</sup>Ca<sup>2+</sup> accumulation increased as the incubation time increased. There was a rapid increase in <sup>45</sup>Ca<sup>2+</sup> accumulation within 1 min, then a slow but linear increase within 20 min. The <sup>45</sup>Ca<sup>2+</sup> accumulation reached a plateau at 20–30 min. When 5 μM A-23187, a calcium ionophore, was added to the incubation medium at 10 min after the beginning of incubation, the level of <sup>45</sup>Ca<sup>2+</sup> accumulation decreased with further incubation (Fig. 1A). The <sup>45</sup>Ca<sup>2+</sup> level at 15–30 min after the start of the incubation was 6000 cpm/mg of protein in the presence of 5 μM A-23187, and it was 55–57% of vehicle control without A-23187 at 20–30 min. Because the <sup>45</sup>Ca<sup>2+</sup> level was the same with a higher concentration (10 μM) of A-23187 (data not shown), it is likely that such a decrease by 43–45% is attributed to the incorporation of <sup>45</sup>Ca<sup>2+</sup> inside during formation of resealed SPM vesicles.

In another set of experiments, the SPM was preincubated in the absence of <sup>45</sup>Ca<sup>2+</sup> at 37°C for 30 min, followed by further incubation with <sup>45</sup>Ca<sup>2+</sup> under the same condition, as mentioned above. In such preparations, the <sup>45</sup>Ca<sup>2+</sup> accumulation was markedly reduced, compared with the previous set of experiments. The <sup>45</sup>Ca<sup>2+</sup> accumulation reached a plateau at the level of 5000–5900 cpm/mg of protein at 10–30 min after the start of incubation with <sup>45</sup>Ca<sup>2+</sup>. Such a plateau level was as much as that observed in the previous set of experiments using A-23187. In addition, when 5 μM A-23187 was added to incubation medium at 10 min, there was no more decrease in the level of <sup>45</sup>Ca<sup>2+</sup> accumulation. Thus, it is suggested that <sup>45</sup>Ca<sup>2+</sup> was not actively incorporated into *previously resealed vesicles*, but just bound to SPM vesicles or aggregates. The formation of resealed vesicles (mostly unilamellar type) during the incubation of lysed SPM was confirmed in electron microscope studies with a negative staining method (Fig. 1B).

### Characterization of ATP-dependent <sup>45</sup>Ca<sup>2+</sup> incorporation into previously resealed SPM vesicles

When the *previously resealed vesicles* were incubated with <sup>45</sup>Ca<sup>2+</sup> in the presence of 1 mM ATP, there was an active incorporation of <sup>45</sup>Ca<sup>2+</sup> (Fig. 1C). Further addition of calmodulin at 5 μg/ml showed a marked potentiation of ATP-induced <sup>45</sup>Ca<sup>2+</sup> incorporation, whereas calmodulin alone had no significant effect (Fig. 1C).

To characterize the SPM vesicles, the effect of saponin on ATP-dependent incorporation was studied. Saponin is known to form micelles with cholesterol mainly found in plasma membranes, and to form small pores in such membranes (Inamitsu and Ohtsuki, 1984). In such experiments, *previously resealed SPM vesicles* were incubated at 37°C for 30 min with free [Ca<sup>2+</sup>] at 100 nM containing <sup>45</sup>Ca<sup>2+</sup> in the presence or absence of 1 mM ATP. The ATP-dependent <sup>45</sup>Ca<sup>2+</sup> incorporation (mean ± SEM) defined to be the difference between <sup>45</sup>Ca<sup>2+</sup> incorporations in the presence and absence of ATP was 699 ± 20 cpm/fraction (0.25 mg of protein) from three separate experiments. When various concentrations of saponin were added to resealed vesicles at 37°C for



**Figure 1.** Accumulation of  $^{45}\text{Ca}^{2+}$  into resealed vesicles derived from SPM. *A*, Freshly prepared SPM (○, ●) or previously resealed SPM vesicles (△, ▲) was incubated with  $^{45}\text{Ca}^{2+}$ , and aliquot (100  $\mu\text{l}$ ) at each incubation time was used for determination of  $^{45}\text{Ca}^{2+}$  incorporation, as described in Experimental Procedures. Results in the figure are representative profiles of the time course of  $^{45}\text{Ca}^{2+}$  accumulation. Vehicle (○, △) or 5  $\mu\text{M}$  A-23187 (●, ▲) was added to the assay tube at 10 min. *B*, An electron microscopical (negative-staining) image of the resealed SPM vesicles. *C*, Time course of ATP- and calmodulin-dependent  $^{45}\text{Ca}^{2+}$  accumulation into previously resealed SPM vesicles, which was prepared in Experimental Procedures. Vehicle (▲), 5  $\mu\text{g}/\text{ml}$  of calmodulin (△), 1 mM ATP (●), or 5  $\mu\text{g}/\text{ml}$  calmodulin plus 1 mM ATP (○) was added simultaneously with  $^{45}\text{Ca}^{2+}$  to the tube containing previously resealed SPM vesicles. Experiments were performed under the condition of free  $[\text{Ca}^{2+}]$  at 10  $\mu\text{M}$  using 0.109 mM  $\text{CaCl}_2$  and 0.1 mM EGTA. Each point of data represents the mean  $\pm$  SEM from three separate experiments. *D*, Blockade of  $^{45}\text{Ca}^{2+}$  accumulation into resealed SPM vesicles and unlysed microsomes by pretreatment with various concentrations of saponin. In both experiments using resealed SPM vesicles and unlysed microsomes, incubation was carried out at 37°C for 30 min under the condition of free  $[\text{Ca}^{2+}]$  at 100 nM in the presence or absence of 1 mM ATP. In resealed SPM vesicles, the ATP-dependent  $^{45}\text{Ca}^{2+}$  incorporation in control (without saponin) resealed SPM vesicles (0.25 mg of protein/fraction) was  $699 \pm 20$  cpm/fraction. Experiments using intact microsomes were performed as described in Experimental Procedures. The ATP-dependent  $^{45}\text{Ca}^{2+}$  incorporation in control microsomes (0.25 mg of protein/fraction) was  $209 \pm 9.3$  cpm/fraction. Data represent the mean  $\pm$  SEM from three separate experiments.

5 min before incorporation of  $^{45}\text{Ca}^{2+}$  in the presence of ATP, the ATP-dependent incorporation of  $^{45}\text{Ca}^{2+}$  was inhibited by saponin in a concentration-dependent manner (Fig. 1*D*). The  $\text{IC}_{50}$  of saponin was 3.5  $\mu\text{g}/\text{ml}$ . On the other hand, the ATP-dependent incorporation of  $^{45}\text{Ca}^{2+}$  into unlysed microsomes was  $2090 \pm 93$  cpm/fraction (0.25 mg of protein) from three separate experiments. As shown in Figure 1*D*, however, the ATP-dependent  $^{45}\text{Ca}^{2+}$  incorporation into microsomes was less sensitive to saponin treatment than that into SPM vesicles. The  $\text{IC}_{50}$  of saponin in microsomal preparations was  $>100$   $\mu\text{g}/\text{ml}$ .

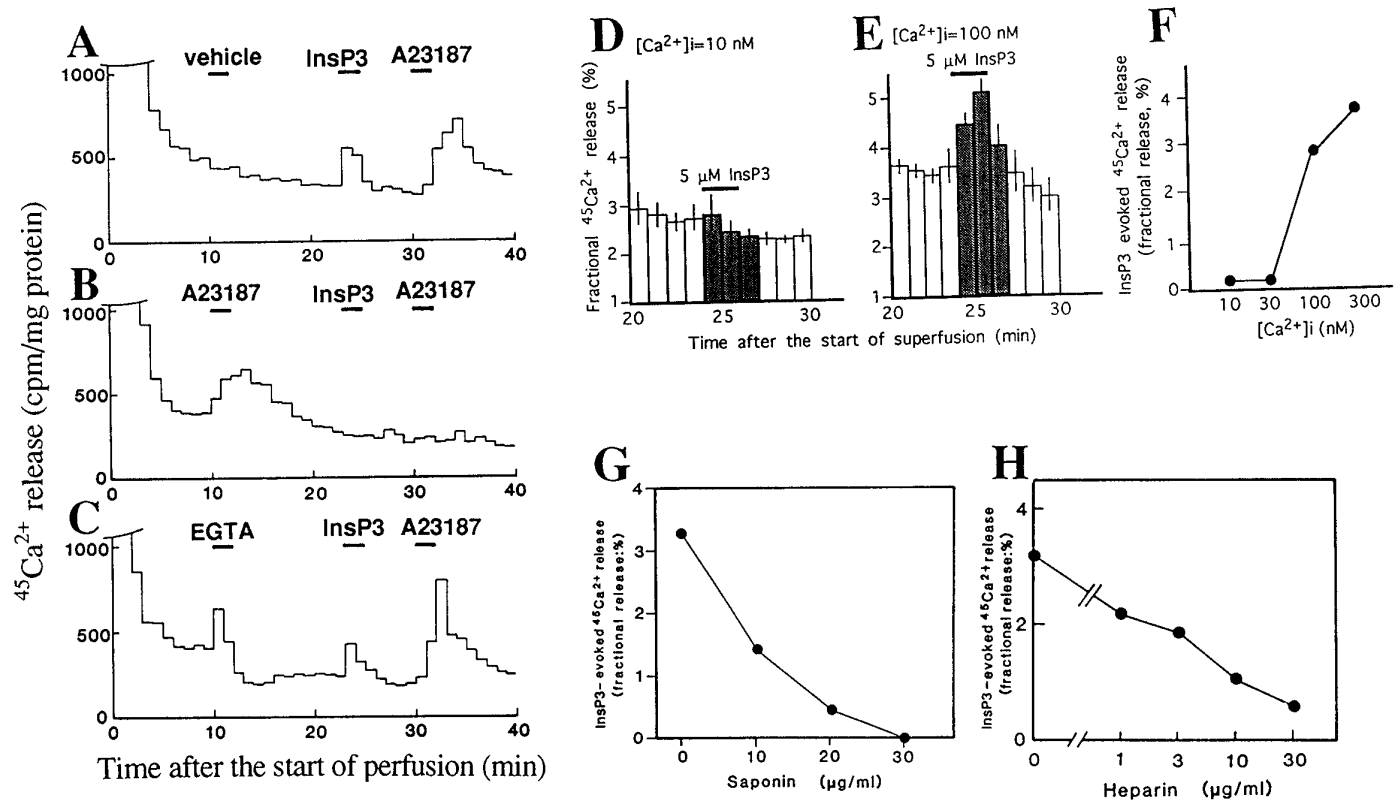
#### InsP<sub>3</sub>-evoked $^{45}\text{Ca}^{2+}$ release from resealed SPM vesicles and effects of A-23187 pretreatment on it

We examined the InsP<sub>3</sub>-mediated  $^{45}\text{Ca}^{2+}$  release from resealed SPM vesicles, prepared as follows: the freshly prepared SPM was incubated with  $^{45}\text{Ca}^{2+}$  in TMC buffer at 37°C for 30 min, placed on GF/C filters, and perfused in the TMC buffer. As shown in Figure 2*A*, the basal release of  $^{45}\text{Ca}^{2+}$  rapidly decreased and reached a plateau 20 min after the onset of perfusion. The  $^{45}\text{Ca}^{2+}$  release was increased by the addition to the medium of InsP<sub>3</sub> at 5  $\mu\text{M}$  at the 25th and 26th minute, and resting levels were restored

by its omission. A-23187, a calcium ionophore added at the 31st and 32nd minute, showed a similar but greater increase in  $^{45}\text{Ca}^{2+}$  release, even after treatment with InsP<sub>3</sub>. However, when A-23187 was pretreated at the 11th and 12th minute, there was no longer any increase in  $^{45}\text{Ca}^{2+}$  release by following InsP<sub>3</sub> challenge (Fig. 2*B*). The addition of EGTA, a calcium chelating agent, caused a similar  $^{45}\text{Ca}^{2+}$  release, and there was no effect on the  $^{45}\text{Ca}^{2+}$  release by following InsP<sub>3</sub> and A-23187 challenges (Fig. 2*C*). These findings suggest that EGTA releases  $^{45}\text{Ca}^{2+}$  into perfusates by taking off  $^{45}\text{Ca}^{2+}$ , which is adsorbed to vesicles, whereas both challenges with InsP<sub>3</sub> and A-23187 release  $^{45}\text{Ca}^{2+}$  from the inside of vesicles.

#### Characterization of InsP<sub>3</sub>-mediated $^{45}\text{Ca}^{2+}$ release from resealed SPM vesicles

To further characterize the InsP<sub>3</sub>-evoked  $^{45}\text{Ca}^{2+}$  release, the concentration of  $^{45}\text{Ca}^{2+}$  to be preloaded into newly resealed vesicles was varied. To normalize the variations among separate experiments, we evaluated the InsP<sub>3</sub> (or related compounds)-evoked  $^{45}\text{Ca}^{2+}$  release as a fractional release, a ratio (%) of the amount (cpm) of  $^{45}\text{Ca}^{2+}$  in each fraction to the total amounts



**Figure 2.** Characterization of InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release from resealed SPM vesicles. *A*, InsP<sub>3</sub>- or A-23187-evoked <sup>45</sup>Ca<sup>2+</sup> release. Data in the figure are representative results. Resealed SPM vesicles preloaded with <sup>45</sup>Ca<sup>2+</sup> were perfused at a flow of 1 ml/min in TMC buffer. Each 1 min perfusate was collected for measurement of radioactivity. Results represent <sup>45</sup>Ca<sup>2+</sup> (cpm) released/mg protein of SPM. Vehicle, InsP<sub>3</sub> (5 μM), or A-23187 (5 μM) was added to the perfusion medium at the indicated time. *B*, Blockade of InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release by pretreatment with A-23187. *C*, Lack of effect on InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release by pretreatment with EGTA. *D*, No significant InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release in the case with 10 nM [<sup>45</sup>Ca<sup>2+</sup>]<sub>i</sub> (*n* = 3). Results represent the fractional release (%) as described in Results. *E*, InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release (fractional release/%) in the case with 100 nM [<sup>45</sup>Ca<sup>2+</sup>]<sub>i</sub> (*n* = 3). *F*, [<sup>45</sup>Ca<sup>2+</sup>]<sub>i</sub> dependency of InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release (*n* = 3). InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release was described in Results. *G*, Concentration-dependent inhibition of InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release by saponin. *H*, Concentration-dependent inhibition of InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release by heparin.

(cpm) at real time (Ueda et al., 1987). The basal <sup>45</sup>Ca<sup>2+</sup> release (%) was represented as the sum of six fractional releases from the 22nd to the 24th minute and from the 28th to the 30th minute/2, and the InsP<sub>3</sub>-evoked increase (%) in the <sup>45</sup>Ca<sup>2+</sup> release was then represented as the sum of three fractional releases from the 25th to the 27th minute—the basal <sup>45</sup>Ca<sup>2+</sup> release. As shown in Figure 2*D*, there was no significant InsP<sub>3</sub> (5 μM)-evoked <sup>45</sup>Ca<sup>2+</sup> release in the case with [<sup>45</sup>Ca<sup>2+</sup>]<sub>i</sub> = 10 nM. When the [<sup>45</sup>Ca<sup>2+</sup>]<sub>i</sub> was increased to 100 nM, an identical concentration to free [Ca<sup>2+</sup>]<sub>o</sub> in perfusion medium (TMC), there was a marked <sup>45</sup>Ca<sup>2+</sup> release (Fig. 2*E*). As expected, the InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release was further increased at [<sup>45</sup>Ca<sup>2+</sup>]<sub>i</sub> = 300 nM (Fig. 2*F*).

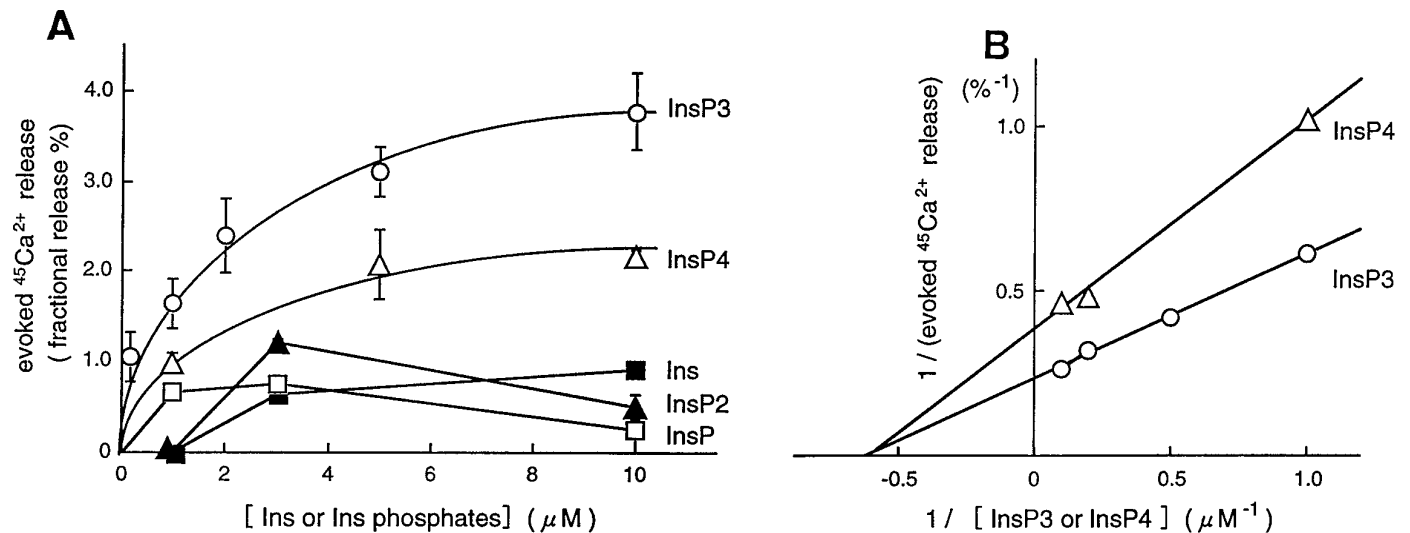
When resealed SPM vesicles were pretreated (5 min at 37°C) with saponin, the InsP<sub>3</sub> (5 μM)-evoked <sup>45</sup>Ca<sup>2+</sup> release was decreased (Fig. 2*G*). The IC<sub>50</sub> of saponin for InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release was 9 μg/ml, a value equivalent to data obtained with <sup>45</sup>Ca<sup>2+</sup> incorporation, as mentioned above. On the other hand, when 1–30 μg/ml of heparin, known to be a putative InsP<sub>3</sub> antagonist (Worley et al., 1987; Ehrlich and Watras, 1988; Kobayashi et al., 1988), was added to the perfusion medium from the 10th minute to the end of perfusion, the InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release was markedly inhibited (Fig. 2*H*). The IC<sub>50</sub> of heparin was 4.8 μg/ml, a value in good accord with its IC<sub>50</sub> in InsP<sub>3</sub> binding in cerebellar membranes (Worley et al., 1987).

### Kinetics of <sup>45</sup>Ca<sup>2+</sup> release evoked by InsP<sub>3</sub> and related compounds from resealed SPM vesicles

The InsP<sub>3</sub>-evoked increase in <sup>45</sup>Ca<sup>2+</sup> release from resealed SPM vesicles was concentration-dependent in ranges of 0.5–10 μM InsP<sub>3</sub> and InsP<sub>4</sub>, and these effects appeared to be saturable (Fig. 3*A*). The double-reciprocal plot showed that apparent *K<sub>m</sub>* and maximal response were 1.5 μM and 4.16% for InsP<sub>3</sub>, whereas they were 1.5 μM and 2.54% for InsP<sub>4</sub> (Fig. 3*B*). Ins, InsP, and InsP<sub>2</sub> evoked less marked releases compared with InsP<sub>3</sub> and InsP<sub>4</sub>. In addition, the concentration–response curves with InsP and InsP<sub>2</sub> were bell-shaped, and thereby kinetic analyses could not be performed. On the other hand, Ins evoked a weak but concentration-dependent <sup>45</sup>Ca<sup>2+</sup> release. It remains unclear whether this effect is attributed to the action on InsP<sub>3</sub>, InsP<sub>4</sub>, or other receptors. Details of these weak actions must be further characterized in subsequent studies.

### <sup>45</sup>Ca<sup>2+</sup> release evoked by InsP<sub>3</sub> from various resealed vesicles composed of different subcellular fractions

To examine the subcellular specificity of InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release, the effects of InsP<sub>3</sub> on the <sup>45</sup>Ca<sup>2+</sup> release were studied in various subcellular preparations (Table 1). As expected, the highest Na<sup>+</sup>/K<sup>+</sup> ATPase activity (a marker enzyme for plasma membranes) was observed in the fractions of microsomes and myelins. A modest level of activity was detected in the synaptosomal



**Figure 3.** Kinetics of <sup>45</sup>Ca<sup>2+</sup> release evoked by InsP<sub>3</sub> and related compounds from resealed vesicles of lysed synaptosomes preloaded with <sup>45</sup>Ca<sup>2+</sup>. *A*, Concentration-dependent curve of evoked <sup>45</sup>Ca<sup>2+</sup> release (% fractional release) by various concentrations of InsP<sub>3</sub> (○), InsP<sub>4</sub> (△), InsP<sub>2</sub> (▲), InsP (□), and inositol (■). Each experiment was performed in the same preparation so that the kinetics of test compounds can be compared. The data represent the mean ± SEM from three separate experiments. *B*, Double-reciprocal plots of InsP<sub>3</sub>- or InsP<sub>4</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release.

fraction. When the synaptosomal fraction was further separated into synaptic vesicles, SPM, and presynaptic mitochondria, highest activity was found in the SPM.

On the other hand, NADPH cytochrome c reductase is known to be a marker enzyme for endoplasmic reticulum. This activity was highly found in the microsomal fraction and there was less marked activity in the synaptosomal fraction and its subfractions (Table 1). All subcellular fractions prepared here were hypo-osmotically lysed in TMC and immediately preloaded with <sup>45</sup>Ca<sup>2+</sup>, as mentioned above in the case with SPM. As shown in Table 1, the basal fractional <sup>45</sup>Ca<sup>2+</sup> release after InsP<sub>3</sub> challenges was similar among all these preparations. However, the InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release was bigger in the resealed SPM vesicles than in the other resealed vesicles. In this experiment, we measured only total amounts of <sup>45</sup>Ca<sup>2+</sup> uptake in each subcellular preparation for evaluating basal percentage release or InsP<sub>3</sub>-evoked percentage release, but such total amounts do not represent intravesicular <sup>45</sup>Ca<sup>2+</sup> concentrations. Because the incorporation of <sup>45</sup>Ca<sup>2+</sup> into such resealed vesicles is expected to have

occurred in a passive manner, however, the fractional percentage release obtained in the present study should be closely related to this intravesicular concentration. Indeed, the basal percentage release was quite similar among these preparations (Table 1). Therefore, it is likely that the difference of InsP<sub>3</sub>-evoked release is not attributed to the variation of <sup>45</sup>Ca<sup>2+</sup> uptake among these subfractional preparations, but to specific mechanisms for InsP<sub>3</sub> localized in synaptosomes or SPM.

Here we studied the InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release from intrasynaptosomal organelles. As shown in Figure 4*A*, neither ATP-dependent nor A-23187-sensitive <sup>45</sup>Ca<sup>2+</sup> accumulation was observed in unlysed synaptosomes. In such unlysed synaptosomes that had been incubated with <sup>45</sup>Ca<sup>2+</sup>, 5 μM InsP<sub>3</sub> had no effect on <sup>45</sup>Ca<sup>2+</sup> release (Fig. 4*B*). On the other hand, in saponin-permeabilized synaptosomes, there was a significant ATP-dependent and A-23187-sensitive <sup>45</sup>Ca<sup>2+</sup> accumulation (Fig. 4*C*), whereas 5 μM InsP<sub>3</sub> had no significant effect on <sup>45</sup>Ca<sup>2+</sup> release from the permeabilized synaptosomes loaded with <sup>45</sup>Ca<sup>2+</sup> in the presence of ATP (Fig. 4*D*). These findings suggest that some intrasynaptosomal micro-organelles are

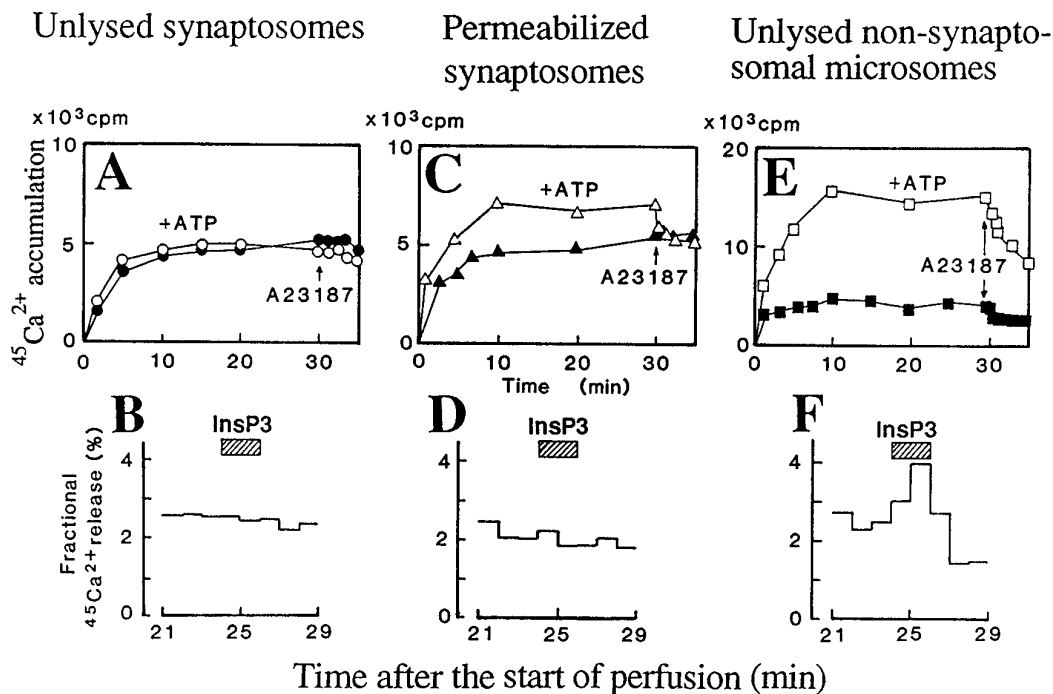
**Table 1. Na<sup>+</sup>-K<sup>+</sup> ATPase, NADPH cytochrome c reductase, and InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release in subcellular fractions of the rat brain**

Subcellular fractions	Na <sup>+</sup> -K <sup>+</sup> ATPase	NADPH cytochrome c reductase <sup>b</sup>	Basal release <sup>c</sup> (%)	InsP <sub>3</sub> -evoked <sup>d</sup> <sup>45</sup> Ca <sup>2+</sup> release (%)
Microsomes (P3)	2.27	1.33	2.30 ± 0.14	1.80 ± 0.18
Myelins (P2A)	2.22	0.83	1.86 ± 0.16	1.21 ± 0.15
Synaptosomes (P2B)	1.65	0.54	1.92 ± 0.11	3.61 ± 0.34
Mitochondria (P2C)	1.55	0.93	2.16 ± 0.25	1.54 ± 0.33
Synaptic vesicles (P2B1)	<0.01	0.35	2.51 ± 0.36	1.58 ± 0.33
Synaptic plasma membranes (P2B2/SPM)	1.67	0.33	2.02 ± 0.11	5.87 ± 1.24
Presynaptic mitochondria (P2B3)	0.97	0.33	1.80 ± 0.16	2.56 ± 0.44

<sup>a,b</sup>Ratios of activities of Na<sup>+</sup>-K<sup>+</sup> ATPase (*a*) and NADPH cytochrome c reductase (*b*) in each subcellular fraction to that of starting brain homogenates. The Na<sup>+</sup>-K<sup>+</sup> ATPase activity and NADPH-cytochrome c reductase in starting homogenates were 0.112 mmol/mg of protein/min and 5.98 nmol/mg of protein/min, respectively.

<sup>c,d</sup>Results represent basal (*c*) and InsP<sub>3</sub>-evoked (*d*) <sup>45</sup>Ca<sup>2+</sup> release (%), represented as described in the text under Results. Data obtained with 5 μM InsP<sub>3</sub> (*n* = 3–6 separate experiments) in various subcellular preparations (300–500 μg/assay) lysed and preloaded with <sup>45</sup>Ca<sup>2+</sup>.

In these experiments, preparations were divided into two groups [(P3, P2A, P2B, and P2C) and (P2B1, P2B2, and P2B3)], and experiments using each group were performed at the same time. Total <sup>45</sup>Ca<sup>2+</sup> amounts taken up into resealed vesicle preparations were 1.5–3 × 10<sup>4</sup> cpm/assay for the first group and 2–4 × 10<sup>4</sup> cpm/assay for the second group. Marked variations were not observed among subfractions in each group.



**Figure 4.** Lack of InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release from permeabilized synaptosomes. *A*, Lack of A-23187-sensitive <sup>45</sup>Ca<sup>2+</sup> incorporation into unlysed synaptosomes. *B*, Lack of InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release from unlysed synaptosomes. *C*, ATP-dependent and A-23187-sensitive <sup>45</sup>Ca<sup>2+</sup> incorporation into saponin-permeabilized synaptosomes. *D*, Lack of InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release from saponin-permeabilized synaptosomes. *E*, Potent ATP-dependent and A-23187-sensitive <sup>45</sup>Ca<sup>2+</sup> incorporation into microsomes. *F*, InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release from microsomes. Other details are given in the legends of Figures 1 and 2.

storage sites for <sup>45</sup>Ca<sup>2+</sup>, but they are unlikely targets for InsP<sub>3</sub>-evoked calcium mobilization. As mentioned before, a marked ATP-dependent and A-23187-sensitive <sup>45</sup>Ca<sup>2+</sup> accumulation was observed in unlysed microsomes that had been prepared from nonsynaptosomal microsomes, as shown in Figure 4*E*. As expected, InsP<sub>3</sub> evoked a significant <sup>45</sup>Ca<sup>2+</sup> release from such unlysed microsomes (Fig. 4*F*).

#### Kyotorphin-evoked <sup>45</sup>Ca<sup>2+</sup> release from resealed SPM vesicles and its guanine nucleotide dependency

Here we studied the receptor-mediated <sup>45</sup>Ca<sup>2+</sup> release from resealed SPM vesicles of the whole brain, as described above in the case with InsP<sub>3</sub>. Previously we have reported that kyotorphin evoked <sup>45</sup>Ca<sup>2+</sup> release in such resealed vesicles using SPM, and it was antagonized by leucine-arginine (Ueda et al., 1987), a kyotorphin receptor antagonist (Ueda et al., 1989). In the present experiments, we added GppNHp (an unhydrolyzable analog of GTP) together with kyotorphin in this system to study the involvement of G-proteins in such a receptor-mediated <sup>45</sup>Ca<sup>2+</sup> release in resealed SPM vesicles. Kyotorphin and GppNHp had potentiating effects to each other in evoking <sup>45</sup>Ca<sup>2+</sup> release from such preparations in a concentration-dependent manner, as shown in Figure 5, *A* and *B*. The *K<sub>m</sub>* value and maximal response by GppNHp alone were 3.0 μM and 5.0%, respectively. The addition of 100 μM kyotorphin decreased the *K<sub>m</sub>* value to 0.4 μM and slightly increased the maximal response to 8.3%. On the other hand, the *K<sub>m</sub>* value and maximal response by kyotorphin alone was 2.5 μM and 1.6%, respectively. The addition of 10 μM GppNHp resulted in no change in *K<sub>m</sub>* value (2.5 μM), but it did increase the maximal response to 7.8%. The <sup>45</sup>Ca<sup>2+</sup> release by 100 μM kyotorphin plus 10 μM GppNHp was completely blocked in the presence of 100 μM leucine-arginine (data not shown), as reported previously in experiments without GppNHp (Ueda et al., 1987). Thus, it is

suggested that the kyotorphin receptor-mediated <sup>45</sup>Ca<sup>2+</sup> release is possibly mediated through G-proteins.

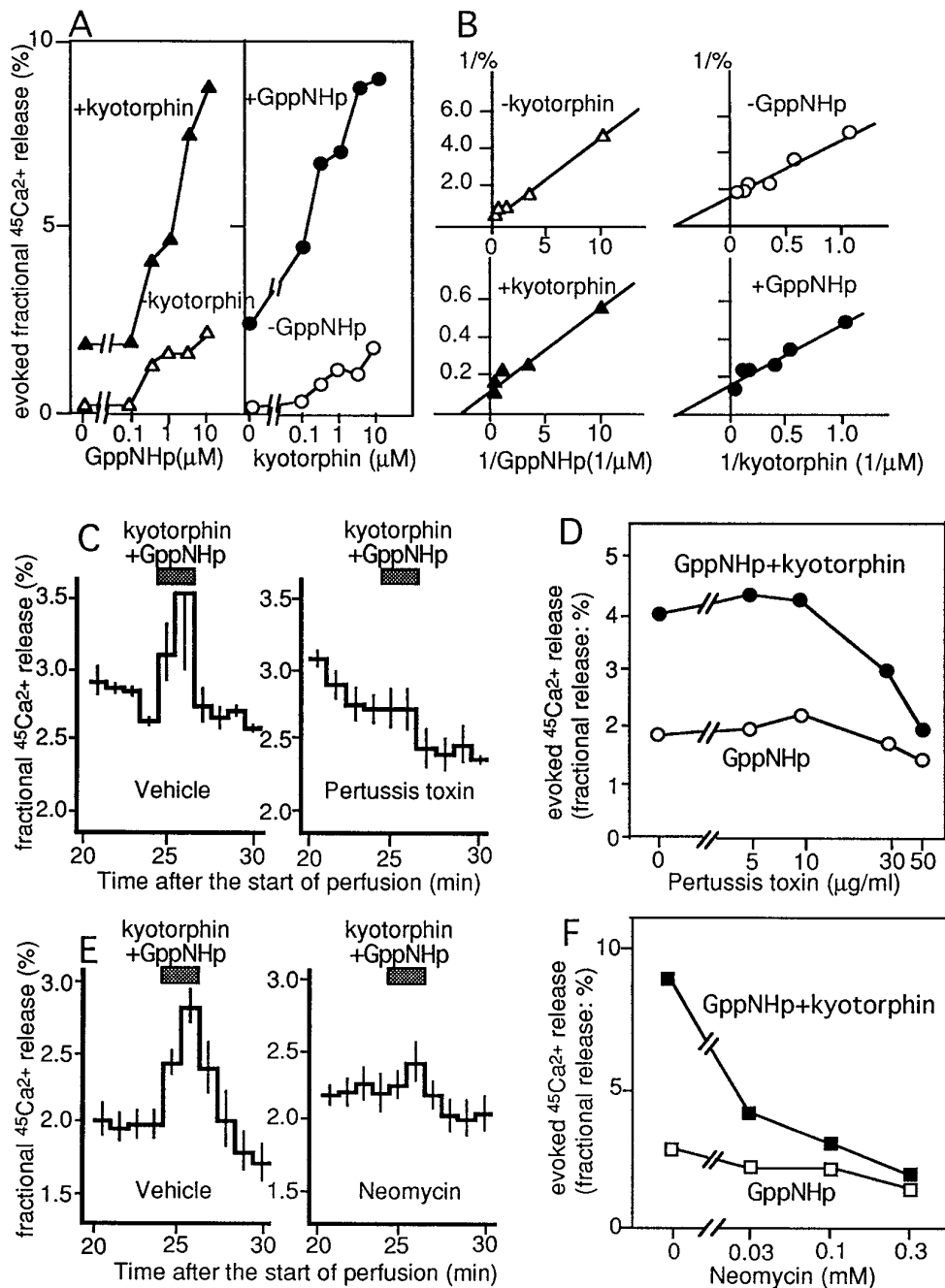
#### Blockade of kyotorphin-evoked <sup>45</sup>Ca<sup>2+</sup> release by PTX treatment and by addition with neomycin

To study the involvement of G-proteins in the kyotorphin-evoked <sup>45</sup>Ca<sup>2+</sup> release, SPM was pretreated with preactivated PTX. In such treatments, we used highly dense SPM (20 mg protein/ml) so as not to form resealed vesicles before <sup>45</sup>Ca<sup>2+</sup> incorporation. As shown in Figure 5, *C* and *D*, a marked reduction of <sup>45</sup>Ca<sup>2+</sup> release by 100 μM kyotorphin plus 10 μM GppNHp was observed at 30–50 μg/ml PTX, concentrations in good accordance with our previous experiments including PTX-catalyzed ADP ribosylation (Ueda et al., 1989).

On the other hand, the <sup>45</sup>Ca<sup>2+</sup> release evoked by 100 μM kyotorphin plus 10 μM GppNHp or by 10 μM GppNHp was concentration-dependently inhibited by 300 μM neomycin, which was added to the perfusion medium from the beginning of experiments (Fig. 5*E*). The IC<sub>50</sub> of neomycin was 30 μM (Fig. 5*F*), a comparable concentration in inhibiting PLC activity (Cockcroft and Gomperts, 1985).

#### Recovery of kyotorphin-evoked release of <sup>45</sup>Ca<sup>2+</sup> from resealed SPM vesicles that had been treated with PTX by reconstitution with purified G<sub>11</sub> but not with G<sub>o</sub>

The PTX (50 μg/ml)-treated SPM was reconstituted with G<sub>11</sub> or G<sub>o</sub>, which had been purified (>95% purity) from porcine brains (Katada et al., 1987) by incubation at 4°C for 60 min in the presence of 0.01% of CHAPS (a detergent), as described previously (Ueda et al., 1989). As shown in Figure 6, *A* and *B*, the <sup>45</sup>Ca<sup>2+</sup> release evoked by 10 μM kyotorphin and 100 μM GppNHp, but not by 100 μM GppNHp alone, was significantly

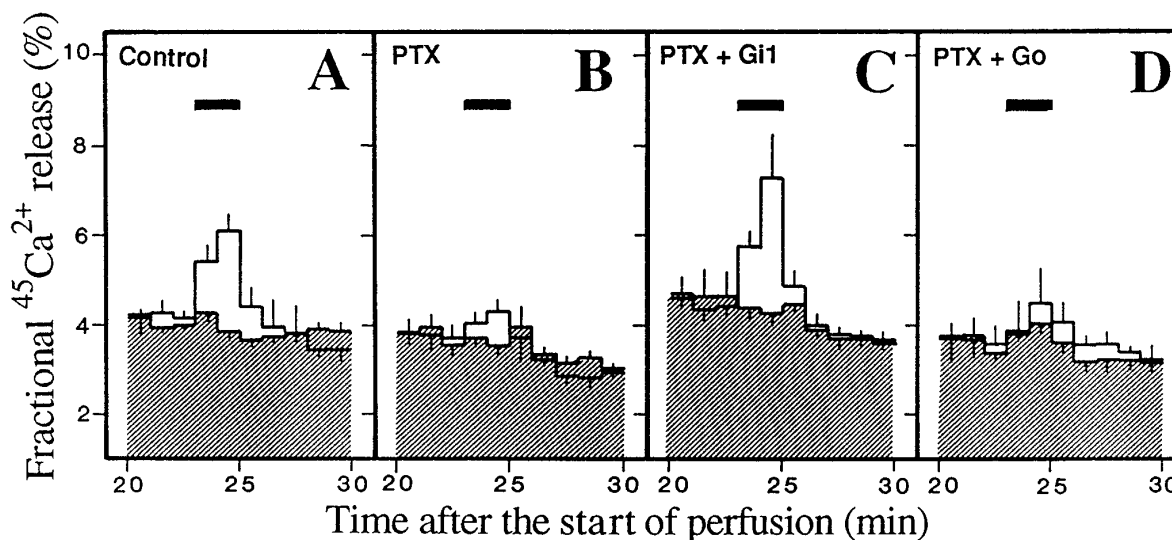


**Figure 5.** Kinetics of kyororphin- and GppNHp-evoked <sup>45</sup>Ca<sup>2+</sup> release from resealed SPM vesicles and involvements of PTX substrate G-proteins and PLC. *A*, Kyororphin- and/or GppNHp-evoked <sup>45</sup>Ca<sup>2+</sup> release were represented as with InsP<sub>3</sub>-evoked increase (%) in the <sup>45</sup>Ca<sup>2+</sup> release (see Results). *B*, Double-reciprocal plots of evoked <sup>45</sup>Ca<sup>2+</sup> release by various combinations of kyororphin and GppNHp. *C*, Effects of PTX (50 μg/ml) pretreatments of SPM on 100 μM kyororphin (plus 10 μM GppNHp)-evoked <sup>45</sup>Ca<sup>2+</sup> release. *D*, Blockade of kyororphin (plus 10 μM GppNHp)-evoked <sup>45</sup>Ca<sup>2+</sup> release by pretreatments of SPM with various concentrations of PTX. *E*, Effects of neomycin (0.3 mM) on 100 μM kyororphin (plus 10 μM GppNHp)-evoked <sup>45</sup>Ca<sup>2+</sup> release. *F*, Concentration-dependent inhibition of 100 μM kyororphin (plus 10 μM GppNHp)-evoked <sup>45</sup>Ca<sup>2+</sup> release by neomycin.

blocked by PTX pretreatments. However, there was no marked reduction in the GppNHp-evoked release by PTX pretreatments. This finding might be explained by the data that PTX treatments block the functional coupling to receptors, but do not affect the intrinsic G-protein activity (Ueda et al., 1990). When PTX-pretreated SPM was reconstituted with purified G<sub>11</sub>, diluted in TMC, incubated with <sup>45</sup>Ca<sup>2+</sup>, and used for perfusion experiments, there was a complete recovery of kyororphin-evoked <sup>45</sup>Ca<sup>2+</sup> release (Fig. 6C). However, there was no significant recovery by reconstitution with purified G<sub>o</sub> (Fig. 6D). All these findings are consistent with our previous report that kyororphin receptor is coupled to G<sub>11</sub> in rat brain membranes (Ueda et al., 1989).

**Relationship between kyororphin-evoked <sup>45</sup>Ca<sup>2+</sup> release from resealed SPM vesicles and kyororphin-evoked <sup>45</sup>Ca<sup>2+</sup> influx into unlysed synaptosomes in various regions of the brain**

The kyororphin-evoked <sup>45</sup>Ca<sup>2+</sup> release from resealed SPM vesicles was high in the hippocampus and pons plus medulla, but low in the cerebellum. On the other hand, kyororphin-evoked influx of <sup>45</sup>Ca<sup>2+</sup> into unlysed synaptosomes from various brain regions was also high in the hippocampus and pons plus medulla, but low in the cerebellum. Accordingly, there was a significant positive correlation between regional distributions of <sup>45</sup>Ca<sup>2+</sup> release and <sup>45</sup>Ca<sup>2+</sup> uptake (*r* = 0.92; Fig. 7).



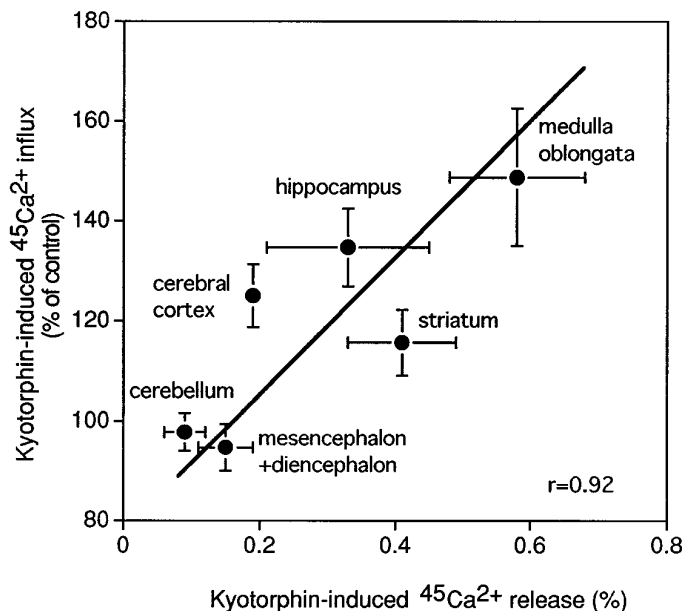
**Figure 6.** Recovery of kyotorphin-evoked  $^{45}\text{Ca}^{2+}$  release from PTX-pretreated and resealed SPM vesicles by reconstitution with  $G_{i1}$ . SPM was treated without (*A*) or with  $50\ \mu\text{g/ml}$  PTX (*B,C,D*). PTX-treated synaptosomes were reconstituted without (*B*) or with  $20\ \text{pmol/assay}$   $G_{i1}$  (*C*) or  $G_o$  (*D*). Test drugs were added to the medium at the time indicated by the bar. Open or shaded column represents the data in separate experiments with  $10\ \mu\text{M}$  GppNHp alone or with  $10\ \mu\text{M}$  GppNHp plus  $100\ \mu\text{M}$  kyotorphin, respectively. Results represent the mean  $\pm$  SEM from three separate experiments. Other details are given in the legend of Figure 2.

## DISCUSSION

In addition to the accepted view that InsP<sub>3</sub> mobilizes Ca<sup>2+</sup> from microsomal organelles, such as rough (Henne et al., 1987) and smooth endoplasmic reticulum (Payne and Fein, 1987) and calciosome (Volpe et al., 1988), in various secretory cells (including neurons), there is growing evidence that InsP<sub>3</sub> may have direct effects on calcium channels within the plasma membrane (for review, see Berridge, 1993; Fasolato et al., 1994; Clapham, 1995). A family of

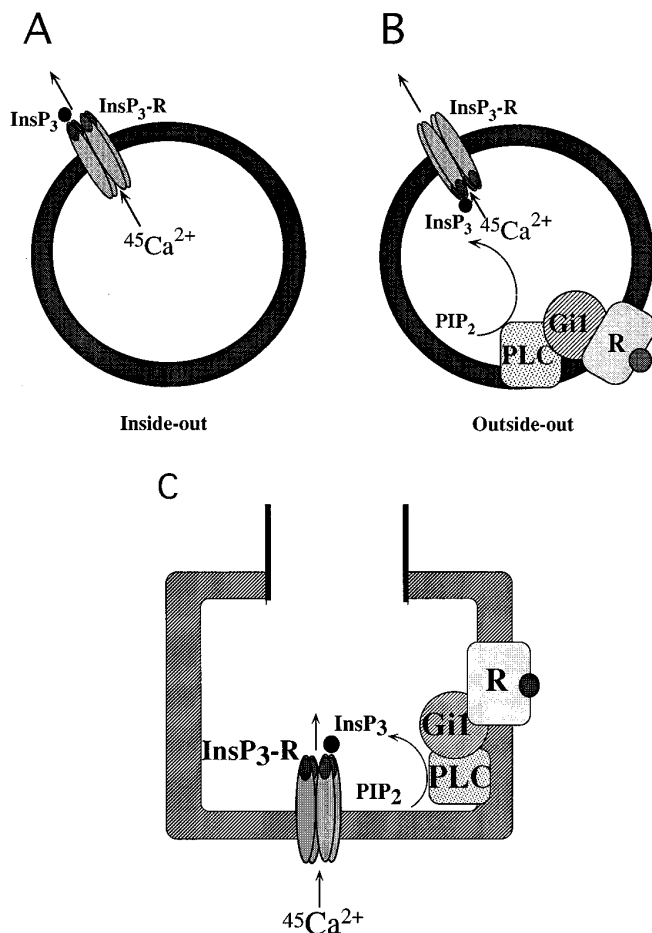
InsP<sub>3</sub> receptors has been identified with molecular diversity arising from both alternative splicing and separate genes (Furuichi et al., 1989; Sudhof et al., 1991; Ross et al., 1992). The immunoelectron microscopic analysis also revealed that InsP<sub>3</sub> receptors are also found in the plasma membrane as well as in endoplasmic reticulum (ER) (Cunningham et al., 1993; Sharp et al., 1992). However, it remains unclear whether these InsP<sub>3</sub> receptors found in different subcellular compartments are identical to one another. Most recently, findings suggest that different species of InsP<sub>3</sub> receptors are involved in such different actions through plasma membranes or ER membranes. For example, the InsP<sub>3</sub>-induced entry of calcium in lymphocytes may be mediated by a new InsP<sub>3</sub> receptor, which contains sialic acid and is localized in the plasma membrane (Khan et al., 1992). On the other hand, the binding protein at the plasma membrane of olfactory cells was equally sensitive to InsP<sub>3</sub> and InsP<sub>4</sub>. By contrast, the InsP<sub>4</sub>-sensitive calcium channel in the plasma membrane of endothelial cells was insensitive to InsP<sub>3</sub>. Thus, it may be true that multiple forms of InsP<sub>3</sub> receptor exist in various cells and that some species of such receptors are involved in calcium transport through the plasma membrane.

Here we demonstrated the InsP<sub>3</sub>-evoked Ca<sup>2+</sup> transport system in the plasma membrane of nerve terminals in the brain using unique experiments with resealed vesicles. Such preparations likely have both inside-out and outside-out types of vesicles, as shown in Figure 8, *A* and *B*. In the present experiments, the ATP-dependent incorporation of  $^{45}\text{Ca}^{2+}$  was potentiated by calmodulin (Fig. 1*C*). Because there is a report that Ca<sup>2+</sup>-dependent ATPase (Ca<sup>2+</sup> pump) in plasma membranes is activated by calmodulin (Verma et al., 1989), the present finding may provide important evidence, suggesting that the resealed SPM preparations have inside-out type of vesicles. However, we have no other evidence for the existence of inside-out vesicles independent of the biochemical assays. To our knowledge, the best evidence might be obtained from the immunoelectron microscopical study using specific antibodies against membrane-associated proteins (or their peptide motives), which are intra- and extracellularly located. This should be the subject of future experiments.



**Figure 7.** Correlation between regional distributions of kyotorphin-induced  $^{45}\text{Ca}^{2+}$  release from resealed SPM vesicles and kyotorphin-induced  $^{45}\text{Ca}^{2+}$  influx into unlysed synaptosomes in the rat brain. Kyotorphin-induced  $^{45}\text{Ca}^{2+}$  release from resealed SPM vesicles was measured as in the legend of Figure 5*A*. Kyotorphin-induced  $^{45}\text{Ca}^{2+}$  influx into unlysed synaptosomes was measured in Experimental Procedures. In both experiments of  $^{45}\text{Ca}^{2+}$  influx and release,  $0.25\ \text{mg}$  of protein was used for each assay. Each point of data represents the mean  $\pm$  SEM from three to six separate experiments.





**Figure 8.** Proposed model of inside-out and outside-out types of resealed vesicles and working hypothesis of presynaptic InsP<sub>3</sub> receptor channel in plasma membranes of nerve terminals. *A*, Inside-out type of resealed SPM vesicles. *B*, Outside-out type of resealed SPM vesicles. *C*, In this model, there is a major calcium channel, a voltage-operated calcium channel (VOC), and a relatively minor calcium channel, InsP<sub>3</sub> receptor, in nerve terminals involved in the Ca<sup>2+</sup> influx. When agonist (kytorphin) binds to the presynaptic receptor, G<sub>i1</sub> and PLC are activated. Produced InsP<sub>3</sub> activates the InsP<sub>3</sub> receptor located in plasma membranes of nerve terminals, followed by gating of the calcium channel. Organelles in nerve terminals may not play important roles in the InsP<sub>3</sub>-evoked Ca<sup>2+</sup> mobilization.

As shown in Figure 1*D*, the ATP-dependent incorporation of <sup>45</sup>Ca<sup>2+</sup> in resealed SPM vesicles was much more efficiently inhibited by saponin than such an ATP-dependent incorporation into unlysed microsomes. Because saponin is well known to form micelles with cholesterol highly located in plasma membranes but not in ER (Inamitsu and Ohtsuki, 1984), it is evident that such an ATP-dependent <sup>45</sup>Ca<sup>2+</sup> incorporation into SPM preparations is mostly attributed to that into inside-out vesicles made of plasma membranes.

One of the major findings in this report is that InsP<sub>3</sub> plays a role in Ca<sup>2+</sup> transport through such plasma membranes. Because such effects in preparations of resealed SPM vesicles were relatively specific for InsP<sub>3</sub>, and the InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release was saturable in kinetic analysis, it is evident that the InsP<sub>3</sub> receptor is involved in such mechanisms. The <sup>45</sup>Ca<sup>2+</sup> release by InsP<sub>4</sub> was partial in potency, whereas it shows a saturability in kinetic analysis. From the finding that the *K<sub>m</sub>* value for InsP<sub>4</sub> is similar to that of InsP<sub>3</sub>, and the maximal response by InsP<sub>4</sub> is lower than that by InsP<sub>3</sub>, it is very likely that different InsP<sub>3</sub> and InsP<sub>4</sub> sites exist. This view is consistent with

the report using olfactory cells (Kalinowski et al., 1992), although further characterizations of InsP<sub>4</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> transport remain to be done. In Figure 3*A*, we showed weak effects by Ins, InsP, and InsP<sub>2</sub> compared with those by InsP<sub>3</sub> and InsP<sub>4</sub>. Because the concentration–response curves of InsP and InsP<sub>2</sub> were bell-shaped, kinetic analyses of these actions could not be performed. On the other hand, Ins evoked a weak but concentration-dependent <sup>45</sup>Ca<sup>2+</sup> release. The maximal response was 25% of InsP<sub>3</sub> action. But it remains unclear whether this effect is attributed to the action on InsP<sub>3</sub>, InsP<sub>4</sub>, or other receptors. Further studies must be done to fully characterize these weak responses.

Throughout various subcellular fractions, the InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release was most potent in resealed preparations using SPM. Although fractions of microsomes and myelins are expected to contain plasma membranes of neurons and glia, the InsP<sub>3</sub> actions in such preparations were much lower than that in the synaptosomal fraction (Table 1), which is expected to contain presynaptic nerve terminals and nerve ending particles (Whittaker et al., 1964). It is evident that SPM, but not other organelles in nerve terminals (including ER), is responsible for such InsP<sub>3</sub> actions because the InsP<sub>3</sub>-action was most potent in SPM preparations among synaptosomal subfractions (Table 1), and there was no significant InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release in saponin-permeabilized synaptosomes where <sup>45</sup>Ca<sup>2+</sup> had been previously taken up into intrasynaptosomal organelles through calcium pump (Fig. 4*C,D*). The finding that the InsP<sub>3</sub>-evoked <sup>45</sup>Ca<sup>2+</sup> release in SPM preparations was abolished by saponin treatment (Fig. 2*G*) also supports the view that presynaptic plasma membranes are responsible for InsP<sub>3</sub> actions. Most recently, several mechanisms via InsP<sub>3</sub> actions are reported to be involved in the calcium transport through plasma membranes (Fasolato et al., 1994). They are divided into two mechanisms via second messenger-operated channels (SMOC) and calcium release-activated channels (CRAC). The former mechanism is related to calcium channels directly gated by InsP<sub>3</sub> and to those gated by InsP<sub>3</sub> plus Ca<sup>2+</sup>, which is mobilized from ER by InsP<sub>3</sub>. The latter mechanism, on the other hand, includes the involvement of Ca<sup>2+</sup> influx factor (CIF). However, it is unlikely that both SMOC coupled to Ca<sup>2+</sup> mobilization from ER and CIF-regulated CRAC are involved in the present experiments, because resealed SPM vesicles are made of subfractionated membranes, where ER and CIF are expected to be absent. Thus, it is strongly suggested that InsP<sub>3</sub> mediates Ca<sup>2+</sup> transport via SPM in nerve terminals.

Another major finding is that such InsP<sub>3</sub>-mediated Ca<sup>2+</sup> transport mechanisms through presynaptic plasma membranes are linked to the presynaptic receptor, which is coupled to PLC via an activation of G<sub>i1</sub>. The present strategy using resealed vesicles has advantages in that the membrane is able to be treated with PTX and reconstituted with purified G-proteins before <sup>45</sup>Ca<sup>2+</sup> uptake and that outside-out-type vesicles possibly exist as well as inside-out ones. Previously, we have reported that kytorphin (tyrosine-arginine) releases methionine-enkephalin from brain slices (Takagi et al., 1979) by possible mechanisms through an increase in [Ca<sup>2+</sup>]<sub>i</sub> in brain slices or through a <sup>45</sup>Ca<sup>2+</sup> influx into synaptosomes (Ueda et al., 1986). After this report, we have demonstrated that kytorphin releases <sup>45</sup>Ca<sup>2+</sup> from such resealed vesicles of lysed synaptosomes as presented here (Ueda et al., 1987). Recently, it was revealed that kytorphin receptor is coupled to PLC through an activation of G<sub>i1</sub> in reconstitution experiments (Ueda et al., 1989). From such findings, we decided to clarify the possible involvement of InsP<sub>3</sub> in kytorphin receptor-mediated <sup>45</sup>Ca<sup>2+</sup> transport through SPM by reconstitution experiments.

The kyotorphin-evoked  $^{45}\text{Ca}^{2+}$  release was abolished in the presence of neomycin, an inhibitor of PLC (Fig. 5E,F). The evidence for the G-protein involvement in kyotorphin actions was demonstrated here, as follows. (1) Kyotorphin potentiated the  $^{45}\text{Ca}^{2+}$  release evoked by GppNHP, an unhydrolyzable analog of GTP (Fig. 5A,B). The change was observed in the decrease of  $K_m$  value for GppNHP, which is consistent with the functional coupling between many receptors and G-proteins (Gilman, 1987). (2) The kyotorphin-evoked  $^{45}\text{Ca}^{2+}$  release was abolished by PTX treatment of SPM membranes (Fig. 5C,D). (3) Such kyotorphin actions were recovered by reconstitution of PTX-treated SPM with purified  $G_{i1}$ , but not with  $G_o$  (Fig. 6), in good accord with our previous reconstitution experiments measuring GTPase and PLC activities (Ueda et al., 1989). Here we also measured  $^{45}\text{Ca}^{2+}$  influx into unlysed synaptosomes and  $^{45}\text{Ca}^{2+}$  release from resealed SPM vesicles. As shown in Figure 7, the distribution of kyotorphin-evoked  $^{45}\text{Ca}^{2+}$  release from resealed SPM vesicles was closely related to those of kyotorphin-mediated  $^{45}\text{Ca}^{2+}$  influx. Thus, it is evident that kyotorphin receptors mediate an activation of PLC through  $G_{i1}$  in such reconstitution experiments, followed by an opening of  $\text{InsP}_3$ -gated calcium channels located in the plasma membrane of nerve terminals.

There are many reports suggesting that  $\text{InsP}_3$ -sensitive calcium stores are present in ER and related to the hormone release in endocrine cells. In the CNS, the nerve terminal is a functional component related to neurotransmitter release. The concentration of  $\text{Ca}^{2+}$  in nerve terminals is closely related to the regulation of neurotransmitter release, and, hence, the receptor mechanism mediating calcium mobilization by  $\text{InsP}_3$  in nerve terminals might play an important role in the presynaptic regulation (Fig. 8C).

The present study provides evidence that the receptor operation of calcium ion channel activity is mediated by  $\text{InsP}_3$  through an activation of G-protein and PLC in neuronal systems, particularly in preparations closely related to presynaptic nerve terminals.

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