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The Relationship between Inositol Trisphosphate Receptor Density and Calcium Release in Brain Microsomes

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SUMMARY

Calcium release in response to D-myo-inositol-1,4,5-trisphosphate (IP_3) was compared in two microsomal preparations derived from cerebellum and forebrain, regions of the brain that differ greatly in their density of [^{3}H]IP_3 binding sites. The proportion of accumulated calcium released by IP_3 was the same in both microsomal preparations when a saturating dose of IP_3 was used. However, the concentration of IP_3 or a nonhydrolyzable analog required to elicit half-maximal release was lower in cerebellum than in forebrain microsomes. Because cerebellum microsomes contain approximately 15 times the binding density of forebrain microsomes, the data suggest that the Ca^{2+} release response is proportional to the occupancy of IP_3 binding sites. This was also demonstrated by the observation that heparin, an inhibitor of IP_3 binding, blocked IP_3-mediated Ca^{2+} release from both cerebellum and forebrain microsomes. The potency of heparin was dependent on IP_3 concentration and was independent of receptor density. These data support the view that the receptor present in brain membranes represents the ligand-binding domain of a Ca^{2+} release mechanism.

The hormonal mobilization of intracellular Ca^{2+} stores is mediated by IP_3, a molecule generated as a result of receptor-mediated inositol lipid breakdown (1). A specialized organelle [calciosome (2)] is believed to be the Ca^{2+} store discharged by IP_3. Several studies have shown the presence of a specific receptor for IP_3 in microsomal membrane preparations (3–7). In all these cases, the reported affinity of the receptor (K_d) was in the range of 2–10 nM and the maximum binding capacity (B_{max}) was 50–200 fmol/mg of protein. In order to curtail metabolism of the radiolabeled IP_3 by phosphatases or kinases, the binding studies were performed at 4° in the absence of Mg^{2+} or ATP. In contrast to the extremely high affinity of the receptor measured under these conditions, IP_3 induces half-maximal Ca^{2+} release over the range 0.1–0.5 μM in most microsomal preparations incubated in the presence of MgATP (8). Consequently, the relationship of the high affinity receptors detected in microsomal preparations to the IP_3-triggered Ca^{2+} release mechanism present in these membranes remains to be clarified. It should be noted that in one study, performed on permeabilized hepatocytes and neutrophils, a discrepancy between IP_3 receptor occupation and Ca^{2+} release was not observed (9).

Recently, Worley et al. (10, 11) reported that rat cerebellum homogenates contain a very high density of IP_3 binding sites (B_{max} = 25 pmol/mg of protein) with a dissociation constant of 80 nM. These workers also found that heparin inhibited IP_3 binding to these sites and, using a heparin-agarose column, it proved possible to purify the receptor protein (12). The functional consequences of such a high IP_3 receptor density on the Ca^{2+} release properties of cerebellum microsomes are unknown and it remains to be established whether the cerebellum receptor protein is coupled to a Ca^{2+} channel or serves some other, as yet unidentified, role. In this paper we have compared the IP_3-mediated Ca^{2+} release properties of microsomes prepared from cerebellum with those prepared from forebrain, a region of the brain having a substantially lower density of binding sites than the cerebellum. The major difference in Ca^{2+} release between these two preparations was a greater sensitivity to IP_3 and more rapid initial rates of release in cerebellum microsomes. In addition, heparin was found to inhibit Ca^{2+} release from both preparations. The potency of heparin as an inhibitor was dependent on IP_3 concentration and independent of receptor density. The data support the view that the receptor present in brain microsomes represents the ligand-binding domain of a Ca^{2+} release mechanism.

Materials and Methods

Microsomes were prepared as described (13) from cerebellum and forebrain (predominantly cerebral cortex) dissected from rats. The isolation buffer contained 0.32 M sucrose, 10 mM Tris HEPES (pH

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ABBREVIATIONS: IP_3, D-myoinositol-1,4,5 trisphosphate; $GPIP_2$, gycerophophosphoinositol-4,5 bisphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N,N*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazoneethanesulfonic acid.

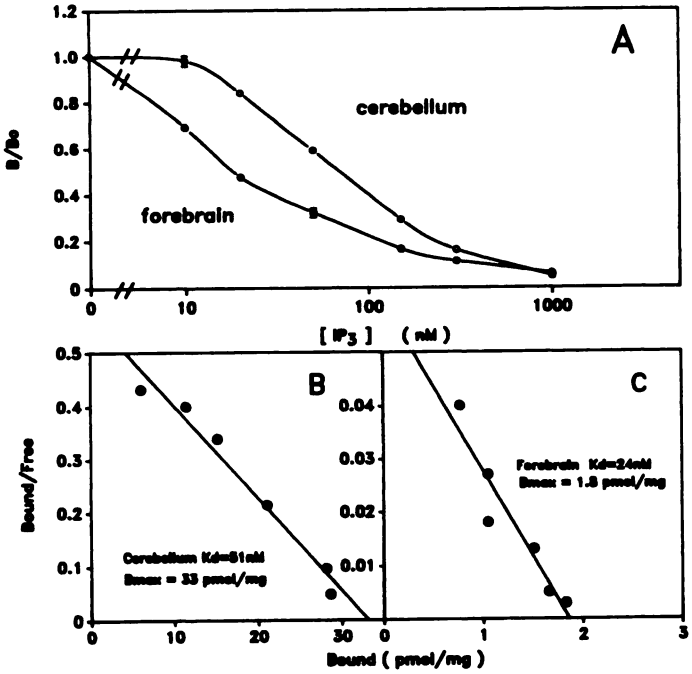

Fig. 1. Parameters of IP_3 binding to microsomes from cerebellum and forebrain. Microsomes from forebrain and cerebellum regions were incubated with [^{3}H]IP_3 (10 nM) and binding was measured in the presence of increasing concentrations of unlabeled IP_3. The amount of IP_3 bound (*B*) at any given concentration of IP_3 was expressed as a ratio of the amount bound in the absence of unlabeled ligand (B_o). Each *data point* is the mean of triplicate determinations. B and C show the Scatchard transformation of the data in A.

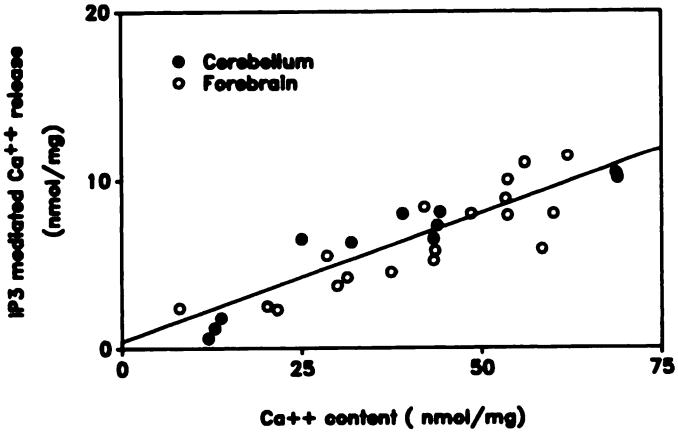

Fig. 2. Relationship between Ca^{2+} content and Ca^{2+} release. Forebrain and cerebellum microsomes were allowed to accumulate $^{45}Ca^{2+}$ in the presence of MgATP as described in Materials and Methods. A range of intravesicular contents were obtained by varying the extravesicular Ca^{2+} concentration in the range 0.2–10 μM using EGTA/Ca^{2+} buffers. After a 20-min incubation, triplicate 75 μl samples were removed and placed on nitrocellulose filters. An additional three samples were removed 20–30 sec after addition of 10 μM IP_3. Ca^{2+} release was measured as the difference in $^{45}Ca^{2+}$ content before and after IP_3 addition. A23187 (2 μM) was added and further samples removed at 25 min for the estimation of total $^{45}Ca^{2+}$ content of the vesicles. The data shown are from two cerebellum and three forebrain microsomal preparations.

7.6), and 10 μM EGTA. Binding of [^{3}H]IP_3 to the microsomes (0.5 mg of protein/ml) was measured in a medium containing 120 mM KCl, 20 mM Tris HEPES (pH 8.3), 0.5 mM EGTA, and 10 nM [^{3}H]IP_3 (20 Ci/mmol). Bound and free label were separated after a 5-min incubation on ice using glass fiber filters (Gelman GF/C). The filters were rapidly

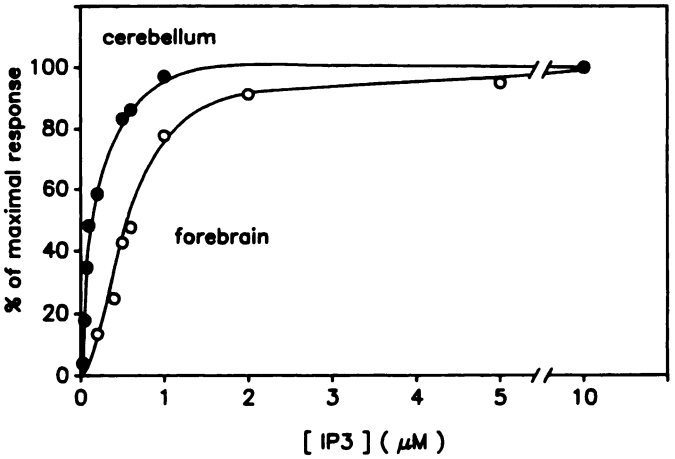

Fig. 3. Dose response of Ca^{2+} release to added IP_3. Microsomes were incubated at 1 mg of protein/ml in the chamber of a Ca^{2+}-sensitive minielectrode. The amounts of Ca^{2+} released in response to increasing concentrations of IP_3 were calibrated by addition of known amounts of Ca^{2+}. Maximal Ca^{2+} release (measured with 10 μM IP_3) was 6.3 ± 0.8 and 3.4 ± 0.6 nmol of Ca^{2+}/mg of protein from cerebellum and forebrain microsomes, respectively.

washed twice with 4 ml of a buffer containing 50 mM Tris HEPES (pH 8.3), 1 mg/ml bovine serum albumin, and 1 mM EDTA. Nonspecific binding was assessed in the presence of 10 μM unlabeled IP_3.

$^{45}Ca^{2+}$ fluxes were studied in a medium containing 120 mM KCl, 20 mM Tris HEPES (pH 7.2), 0.3 mM $MgCl_2$, 10 mM phosphocreatine, 10 units/ml creatine kinase, 3.75 μM antimycin A, 3.75 μM ruthenium red, 1 mM MgATP, and 0.5 mM EGTA. $CaCl_2$ was added to this medium to generate a range of free calcium concentrations measured directly with a Ca^{2+}-sensitive minielectrode calibrated as described previously (14). The medium was supplemented with $^{45}Ca^{2+}$ (1 μCi/ml; Amersham, Arlington Heights, IL). Incubations were performed at 30° for 20 min with 0.5 mg of microsomal protein/ml. Intravesicular Ca^{2+} content before and after IP_3 additions were determined with a filtration assay (0.4 μm; Millipore, Milford, MA) and a wash buffer containing 120 mM KCl and 20 mM Tris HEPES (pH 7.2). Ca^{2+} fluxes were also measured with a Ca^{2+}-sensitive electrode in a final volume of 0.2 ml, using the medium described above with the omission of EGTA and added Ca^{2+}.

High specific activity [^{3}H]IP_3 was synthesized and supplied by New England Nuclear (Boston, MA). Unlabeled IP_3 and glycerophosphoinositol-4,5-bisphosphate were obtained from Calbiochem (La Jolla, CA).

Results

At a concentration of 2.5 nM, the amount of [^{3}H]IP_3 bound to cerebral cortex (forebrain) membranes was reported to be 25% of that bound to cerebellum membranes (11). The difference in receptor density was further quantified by Scatchard analysis (Fig. 1) using microsomal vesicles prepared from cerebellum or forebrain regions. The maximum number of binding sites (B_{max}) was 15 times greater in cerebellum than in forebrain microsomes. A difference in binding affinity was also noted; forebrain microsomes bound IP_3 somewhat more tightly than cerebellum microsomes [K_d = 19 ± 4 (three experiments) and K_d = 64 ± 8 nM (six experiments), respectively].

Initially, a comparison of IP_3-mediated Ca^{2+} release from the microsomes was made at saturating doses of IP_3. However, it was found that microsomes from cerebellum accumulate more Ca^{2+} and consequently release more Ca^{2+} than microsomes from forebrain (data not shown). To normalize for these differences in Ca^{2+} uptake capacity between the two preparations, IP_3-

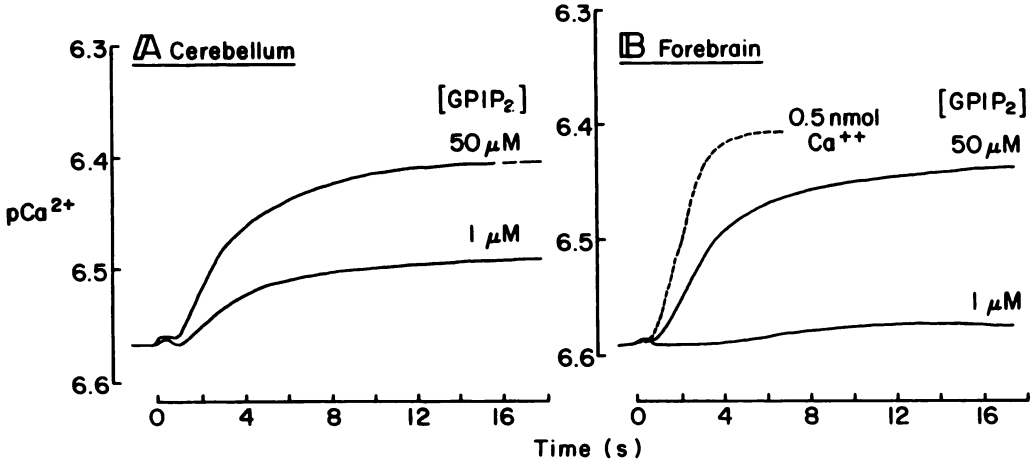

Fig. 4. Ca^{2+} release in response to $GPIP_2$. Microsomes were incubated in the chamber of a Ca^{2+} electrode (final volume, 0.2 ml) as described in Materials and Methods. The accumulation of endogenous Ca^{2+} was followed until a steady state was obtained. At this point, the indicated final concentrations of $GPIP_2$ were added from a 100× stock solution and Ca^{2+} released was monitored on a chart recorder. The release was calibrated by an addition of a known amount of Ca^{2+}, which also provides information on the mixing and response time of the electrode (see Ca^{2+} addition in B). Representative traces of Ca^{2+} release from cerebellum (A) and forebrain (B) are presented.

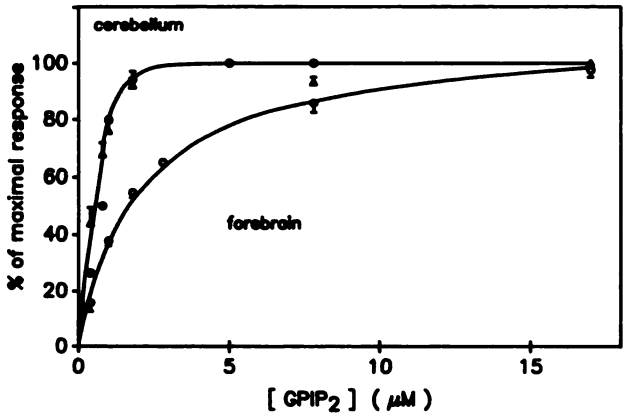

Fig. 5. Magnitude of Ca^{2+} release as a function of $GPIP_2$ concentration. Ca^{2+} release was measured with a Ca^{2+} electrode as described in Fig. 4. The data shown are the mean of two preparations of cerebellum (●) and three preparations of forebrain (○) microsomes. In another three preparations of cerebellum microsomes (▲), the endogenous Ca^{2+} in the medium was reduced by the addition of 4 μM EGTA. Maximal Ca^{2+} release obtained in the three conditions were (nmol/mg of protein): ●, 8.0; ○, 4.0 ± 0.7; ▲, 3.0 ± 0.6.

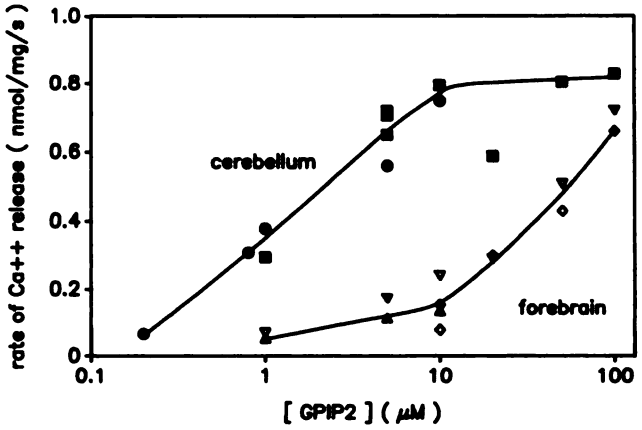

Fig. 6. Initial rate of Ca^{2+} release as a function of $GPIP_2$ concentration. The kinetics of Ca^{2+} release were determined from traces obtained in Fig. 4. The *individual symbols* refer to separate microsomal preparations.

mediated Ca^{2+} release was measured over a range of intravesicular Ca^{2+} contents (Fig. 2). The relationship between Ca^{2+} load and IP_3-mediated Ca^{2+} release was linear over the range examined and was not appreciably different between cerebellum and forebrain microsomes, when release was assayed at maximal IP_3 concentrations (10 μM). The IP_3 concentration dependence of Ca^{2+} release in the two preparations is shown in Fig. 3. A difference in sensitivity was noted, with half-maximal release being obtained at IP_3 concentrations of 0.18 and 0.65 μM from cerebellum and forebrain microsomes, respectively.

It is possible that differences in the rate of IP_3 metabolism of the two preparations could obscure attempts to measure the sensitivity of the release mechanism to added IP_3.[1] For this reason the experiments of Fig. 3 were repeated using $GPIP_2$, a poorly metabolized analog of IP_3. This compound has previously been shown to be effective at mobilizing Ca^{2+} over a higher concentration range than IP_3 (15, 16). Fig. 4 shows records of $GPIP_2$-mediated Ca^{2+} release from cerebellum and forebrain microsomes. To permit a comparison between the microsomal preparations, a low concentration of EGTA (4 μM) was used to limit uptake into the cerebellum microsomes such that Ca^{2+} release induced by maximal concentrations of $GPIP_2$ was approximately the same in both microsomal preparations (Fig. 4A versus 4B). Under these conditions, a suboptimal dose of $GPIP_2$ produced a faster and larger Ca^{2+} release from cerebellum microsomes.

The amount of Ca^{2+} released has been plotted as a function of $GPIP_2$ concentration in Fig. 5. The data for cerebellum microsomes were obtained at two different intravesicular Ca^{2+} loads. However, the dose response for $GPIP_2$ was similar under both conditions, with half-maximal release being obtained at 0.5 μM. By comparison, 1.8 μM $GPIP_2$ was required to produce half-maximal Ca^{2+} release from forebrain microsomes. Larger differences in the sensitivity of the release systems of the two preparations are apparent if the concentration dependence of the initial rates of Ca^{2+} release are compared (Fig. 6). In this case, half-maximal rates of Ca^{2+} release from cerebellum microsomes were obtained with approximately 1.3 μM $GPIP_2$. In contrast, forebrain microsomes required 35 μM $GPIP_2$ to achieve the same half-maximal rate as seen in cerebellum microsomes. Comparison of Figs. 5 and 6 also indicates that maximal amounts of Ca^{2+} can be released at relatively low $GPIP_2$ concentrations but that additional increases in concen-

[1] The measured rate of IP_3 metabolism was different for the two microsomal preparations. At 1 μM [^{32}P]IP_3, the rates of IP_3 metabolism by cerebellum and forebrain were 1.5 and 0.4 nmol/min/mg of protein, respectively.

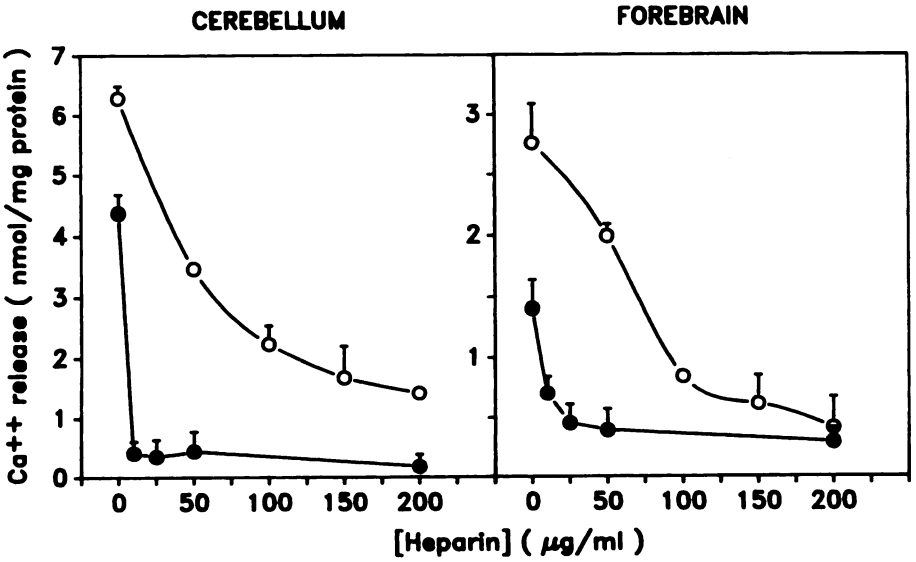

Fig. 7. Heparin effects on IP_3-mediated Ca^{2+} release. IP_3-mediated Ca^{2+} release from cerebellum (A) and forebrain (B) were measured by the $^{45}Ca^{2+}$ method, as described in Fig. 2, using either 10 μM (○) or 0.2 μM (●) IP_3. Porcine heparin (Sigma, M_r = 6000) at the indicated concentrations were present for the 20-min incubation period although the results were not altered if the heparin was added 1 min before IP_3. The data are the mean ± SE of triplicate measurements of Ca^{2+} release and is representative of two experiments.

tration can further enhance the rate of release. A similar discrepancy between the dose responses for rate and amount of Ca^{2+} release has been observed previously in permeabilized hepatocytes (17). Although other explanations are possible, the result is to be expected if Ca^{2+} can be released from individual vesicles that contain more than one release channel.

An alternative approach to assess the functional consequences of differences in IP_3 receptor density is to utilize an inhibitor of IP_3 binding. Heparin has been demonstrated to be a specific inhibitor of the binding site (11) and has also been found to block IP_3-mediated Ca^{2+} release from permeabilized pancreatic B cells (18) permeabilized hepatocytes (19) and isolated liver microsomes (20). These studies reported IC_{50} values in the range of 4 to 15 μg/ml heparin when release was promoted by maximal concentrations of IP_3. Fig. 7 shows that heparin also inhibited Ca^{2+} release from brain microsomes. In contrast to other systems (18–20), much higher concentrations of heparin were required to demonstrate an inhibitory effect using maximal concentrations of IP_3. However, concentrations of heparin as low as 10 μg/ml completely blocked release promoted by a suboptimal dose of IP_3. The inhibitory potency of heparin at low and high IP_3 concentrations were not markedly different for cerebellum and forebrain microsomes (Fig. 7, A and B).

Discussion

In this paper we have compared the properties of IP_3-mediated Ca^{2+} release of microsomes prepared from two regions of the brain that differ greatly in their density of IP_3 binding sites. In any microsomal preparation, many factors, in addition to the parameters governing IP_3 binding, are expected to determine the sensitivity, magnitude, and kinetics of the IP_3 response. These factors include the rate of IP_3 hydrolysis, the activity of the Ca^{2+} pump, the intravesicular Ca^{2+} content, and the fraction of the microsomal vesicle population containing mechanisms for both accumulating and releasing Ca^{2+}. When these factors are taken into consideration, the high density of IP_3 binding sites in cerebellum is associated with a greater sensitivity of the release response to added IP_3 or the poorly hydrolyzable analog $GPIP_2$. In contrast, responses to maximal concentrations of these compounds were approximately the same in cerebellum or forebrain microsomes when the data were normalized to take into account differences in Ca^{2+} accumulation.

Qualitatively, these results are expected if the Ca^{2+} release response is proportional to the number of occupied IP_3 receptors. In these circumstances, the greater the receptor density, the lower the concentration of IP_3 required to attain any given concentration of occupied receptor. A similar leftward shift in dose-response relationships has been observed in several systems in which the density of cell surface receptors has been experimentally increased (21–23). In brain microsomes, a 15-fold greater receptor density was associated with shifts of approximately 3-fold and 25-fold in the half-maximal concentrations of $GPIP_2$ required to alter the magnitude or initial rate of Ca^{2+} release respectively. Whether the observed differences in response are quantitatively appropriate to the differences in receptor density is difficult to estimate because of the complicating factors determining dose-response relationships in the vesicle system (discussed above) and also because binding assays were conducted under conditions designed to optimize binding (alkaline pH, no nucleotide or Mg^{2+}) and are therefore not directly comparable to the conditions used to measure Ca^{2+} fluxes. Nevertheless, the presence of a difference between the IP_3 responses of forebrain and cerebellum strongly supports the view that the measured receptor binding is closely associated with a Ca^{2+} release system. This is further supported by the recent observation that the *in vitro* phosphorylation of the receptor in cerebellum microsomes by cAMP-dependent protein kinase decreases the sensitivity of the Ca^{2+} release system for IP_3.[2]

An additional link between IP_3 binding sites and the Ca^{2+} release system comes from the data showing that heparin blocks IP_3-mediated Ca^{2+} fluxes from brain microsomes. Heparin has previously been shown to inhibit IP_3 binding to cerebellum membranes (11) and to inhibit the Ca^{2+} release response triggered by maximal concentrations of IP_3 in several nonneuronal tissues (18–20). However, the mechanism of action of this

[2] S. Supattapone, S. K. Danoff, A. Thiebert, S. K. Joseph, J. Steiner, and S. Snyder. cAMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. *Proc. Natl. Acad. Sci. USA* 85:8747–8750 (1988).

compound remains unknown. One possibility is that the highly negatively charged heparin interacts with a positively charged site(s) on the receptor protein that normally serves to bind IP_3. Such a hypothesis would predict that heparin and IP_3 should compete for the same binding site and, therefore, the inhibitory potency of heparin should depend on the IP_3 concentration. Evidence to support the latter prediction is presented in Fig. 7 and in the study of Ghosh *et al.* (24). It should also be noted that the action of heparin can be mimicked by other more highly charged heparinoids such as pentosan polysulphate or dextran sulphate (data not shown).

Tissues possessing high densities of binding sites have proved invaluable in studying the mechanism of ion channels. Examples are the electric organ of *Torpedo* electroplax for the acetylcholine receptor (25) and the T tubular membranes of skeletal muscle for voltage-dependent, dihydropyridine-sensitive Ca^{2+} channels (26). In the latter case, it has been suggested that only a small fraction of the high density of dihydropyridine binding sites detected in T tubular membranes are actually functionally coupled to a Ca^{2+} channel (27). Such possibilities cannot be excluded with reference to the cerebellar IP_3 receptor. The physiological role served by this receptor is unknown. Recent studies, using immunohistochemical techniques, have localized the receptor protein to the molecular layer of the cerebellum and primarily to the extensive dendritic arbor of the Purkinje neuron.[3] A similar localization has also been observed for protein kinase C and G_o, a GTP-binding protein (28). At present, it is not clear which membranes contain the receptor protein. Possible candidates include the calciosomes (2), specialized vesicle structures such as the membrane sacs seen in dendritic spines (29), or surface membranes. It is also not clear whether the cerebellum receptor protein is the same molecular entity as found elsewhere. Experiments directed to answering some of these questions and to achieving the functional reconstitution of the receptor protein are in progress.

Acknowledgments

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