

## Rapid annexin-V labeling in synaptosomes

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### Abstract

Distal neuronal terminals may be the site of apoptotic events and early synapse loss in neurodegenerative disease. To examine apoptosis in synaptic regions, we established a cell-free assay using a rat brain crude synaptosomal preparation (P-2 fraction) as a model system. The apoptosis marker annexin-V was used to measure phosphatidylserine (PS) exposure, and to ensure that only intact terminals were assayed, synaptosomes were dual labeled with a viability marker (calcein AM). Fluorescence was quantified by flow cytometry analysis. Annexin-V labeling increased rapidly in synaptosomes, following a 1 min incubation with staurosporine. However, increased caspase-3-like activity was not measured until 30 min with a fluorometric assay. The addition of a peptide inhibitor of caspase-3-like activity (Ac-DEVD-CHO) during homogenization was not able to block the initial increase in annexin labeling, but resulted in a partial blockade of annexin labeling after 30 min. These data demonstrate that PS externalization and caspase activation occur rapidly in this widely used neurochemical preparation.

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### 1. Introduction

The idea that neurons die via apoptosis in neurodegenerative diseases is an attractive premise (Sastry and Rao, 2000), in part because apoptotic cell death avoids the inflammation that results from the leakage of cell contents with necrotic cell death. The signal transduction mechanisms involved in the initiation of apoptosis are not clearly understood and vary between experimental systems; however, activation of the caspases and externalization of phosphatidylserine (PS) residues in the cell membrane appear to be early events in programmed cell death in most systems (Stennicke and Salvesen, 1998; van Engeland et al., 1998). Phospholipid asymmetry is maintained by aminophospholipid translocase in viable cells; PS exposure that results when asymmetry is lost is thought to trigger phagocytosis (Tang et al., 1996; Fadeel et al., 1999). The phospholipid binding protein annexin-V binds with high affinity to ex-

ternalized PS residues and has been widely used to detect early apoptosis in a number of model systems. PS externalization is usually considered to be a downstream event of caspase activation (van Engeland et al., 1998). However, it is interesting to note that in platelet activation, which shares some features in common with apoptosis, the loss of membrane asymmetry is caspase-independent (Wolf et al., 1999; Brown et al., 2000).

Demonstration of the central role of the caspases in the cellular apoptosis pathways has relied in part on a number of in vitro systems that include annucleate cytoplasts and cell free systems in which lysates from apoptotic cells are applied to intact cells or isolated nuclei (Chow et al., 1995; Mattson et al., 1998a,b; Ellerby et al., 1997). We have developed methods for analysis of in vitro nerve terminals using a synaptosome preparation in order to study mechanisms related to synapse loss, which is a key early event in Alzheimer's disease (Masliah et al., 1994; Mucke et al., 2000). By labeling with annexin-V and a viability marker (calcein AM) prior to flow cytometry (fluorescence-activated cell sorting; FACS), annexin-V labeling can be measured in intact synaptosomes. This assay is analogous to standard methods for detecting apoptosis in intact cells (Vermees et al., 1995; Schmid et al., 1994). In this study, we demonstrate

*Abbreviations:* P-2, crude synaptosomal fraction; FACS, fluorescence activated cell sorting; KRP, Krebs–Ringer phosphate buffer; PI, propidium iodide; PS, phosphatidylserine

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that both PS externalization and caspase activation occur within 30 min in synaptosomes.

## 2. Experimental procedures

### 2.1. Materials

Annexin-V-phycoerythrin (PE) was obtained from Pharmingen (San Diego, CA) and calcein acetoxymethyl ester (calcein AM) was obtained from Molecular Probes, San Diego, CA). Ac-YVAD-CHO and Ac-DEVD-CHO were obtained from Anaspec (San Jose, CA) and Kamiya Biomedical, respectively. Other reagents and protease inhibitors were obtained from Sigma.

### 2.2. Caspase activity

Caspase activity was measured using the fluometric Caspase Assay System, (Promega, Madison, WI). Aliquots of the crude synaptosomal preparation (P-2 fraction) were resuspended in cell lysis buffer (25 mM HEPES (pH 7.5), 5 mM MgCl<sub>2</sub>, 5 mM DTT, 5 mM EDTA, 2 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml pepstatin) and subjected to four freeze–thaw cycles. Cell lysates (400 µg protein per sample) were then incubated with and without 50 µM inhibitor (Ac-YVAD-CHO for caspase-1 or Ac-DEVD-CHO for caspase-3) for 30 min at 30 °C prior to addition of 50 µM substrate (Ac-YVAD-AMC for caspase-1 and Ac-DEVD-AMC for caspase-3). After 1 h at RT, fluorescence was measured in a Cytofluor II fluorescence reader at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Proteins were measured using Pierce BCA reagents.

### 2.3. P-2 preparation

The P-2 fraction was prepared as described previously (Weiler et al., 1981); briefly, adult male Sprague–Dawley rats were decapitated and brains (minus cerebellum) were rapidly removed and placed in 10 vol. of ice cold 0.32 M Tris buffer (pH 7.4) with protease inhibitors (2 mM EGTA, 2 mM EDTA, 0.2 mM PMSF, 4 µg/ml leupeptin, 4 µg/ml pepstatin, 5 µg/ml aprotinin, 20 µg/ml trypsin I). The tissue was homogenized in a teflon/glass homogenizer (clearance 0.1–0.15 mm) by eight gentle up and down strokes at 800 rpm. The homogenate was spun at 1000 × *g* for 10 min to remove nuclei and cell debris. The resulting supernatant was centrifuged at 10,000 *g* for 20 min to obtain the crude synaptosomal pellet or P-2. The final pellet was washed with 2 ml of Krebs–Ringer phosphate buffer (KRP; 118 mM NaCl, 5 mM KCl, 4 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 16 mM sodium phosphate buffer (pH 7.4), and 10 mM glucose) and centrifuged (4 min, 2000 × *g*, 4 °C) before resuspension in KRP for incubations.

For experiments with caspase inhibitors, inhibitors were added to the sucrose during initial homogenization and to

the KRP buffer during incubations. Purified synaptosomes were isolated on a Percoll gradient according to a modification of previously described rapid method (Nagy and Delgado-Escueta, 1984). Briefly, the P-2 was layered onto a step gradient containing 4 ml each of 16, 10 and 6% Percoll (diluted in 0.32 M sucrose, pH 7.4), centrifuged (5 min, 32,000 × *g*); and the purified synaptosomes were collected from the 10/16 (%) interface.

### 2.4. Dye labeling

Aliquots of P-2 were suspended in KRP buffer with and without staurosporine and incubated for the indicated time at 32 °C; untreated P-2 samples were maintained in KRP on ice until labeled. For dye labeling, dye diluted in KRP buffer was added to 5–10 µl aliquots of P-2 (total volume 0.1 ml), incubated for 10 min at 4 °C, then diluted in 0.5 ml PBS for immediate flow cytometry analysis. For calcein AM, final dye concentration was 0.02 nM, and for annexin-V-PE final concentration was 150 ng/ml.

### 2.5. Flow cytometry

Flow cytometry was performed with a FACS 440 (Becton-Dickinson, Mountain View, CA) using a 488 nm argon ion laser. P-2 particle fluorescence was analyzed with logarithmic amplifiers set for four log decades. Sample flow rate was approximately 3000 events per second; 20,000–50,000 events were collected for analysis. A threshold was set on forward light scatter (channel 42) to exclude debris. Analysis was performed using FCS Express software (DeNovo software, Ont., Canada). A student's *t*-test for independent observations was used for all comparisons.

## 3. Results

### 3.1. Flow cytometry synaptosis assay

Crude synaptosomes are a heterogenous preparation containing free mitochondria, myelin, and membrane fragments in addition to functional and intact nerve terminals, and it is critical to exclude contaminants from data analysis when using this preparation. Our previous characterization (Gyllys et al., 2000) has shown that drawing an analysis gate on the largest particles (greater than 1 µm based on size standards) excludes most contaminants and results in focus on a population that is more than 90% neuronal (SNAP-25-positive). In addition, because PS exposure occurs in necrosis, it is essential for apoptosis assays to discriminate between the cell or particles with an intact membrane, and to measure annexin-V staining only in the intact cells. Our method uses calcein AM as a viability marker. Thus, apoptotic synaptosomal particle are dual-labeled, positive for both calcein AM and annexin-V.

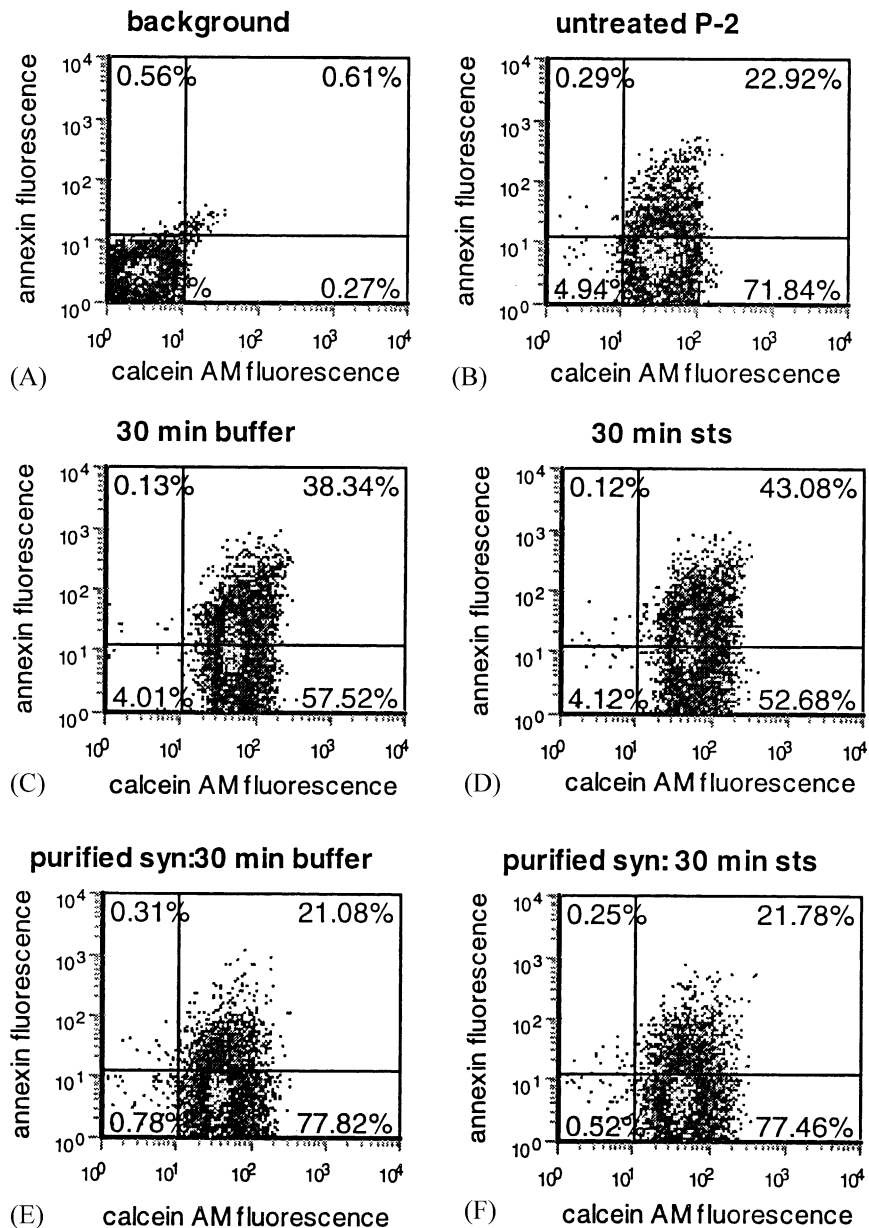


Fig. 1. Quadrant analysis from flow cytometry apoptosis assay for synaptosomes. Representative density plot showing calcein AM fluorescence vs. annexin-V fluorescence for large synaptosomal particles (data was collected for 20,000 particles from each sample, 10,000 events are plotted). Percentage of total particles is shown for each quadrant; particles positive for both markers are in upper right quadrant. (A) distribution of unstained P-2 preparation; (B) untreated P-2 dual-labeled for annexin and calcein AM; (C) dual-labeled P-2 preparation following 30 min incubation in KRP at 32 °C; (D) dual-labeled P-2 preparation following 30 min incubation with 100 nM staurosporine at 32 °C. No increase in annexin-V labeling was observed when gradient-purified synaptosomes were incubated with KRP buffer (E) or staurosporine (F).

Only the largest particles are included in the analysis. Fig. 1 shows a representative annexin-V/calcein plot for the crude synaptosomal preparation. The unstained P-2 fraction is shown in Fig. 1A and B shows the bivariate distribution of double-labeled particles in the untreated P-2. The upper right quadrant contains particles that the ‘apoptotic,’ positive for both annexin-V and calcein AM. Figs. 1C and D show an increase, greater than twofold, in the percentage of ‘apoptotic’ particles resulting from incubation of the crude

synaptosomes in KRP buffer and the pro-apoptotic protein kinase C inhibitor staurosporine (0.1 μM; 32 °C incubation). Annexin-V labeling does not increase above the level seen in untreated tissue when synaptosomes are first purified on a Percoll density gradient (Fig. 1E and F), even though in both preparations, greater than 90% of the large particles are calcein AM-positive (upper and lower right quadrants combined). Gradient purification requires at least one extra hour and additional manipulation; the absence of an annexin-V

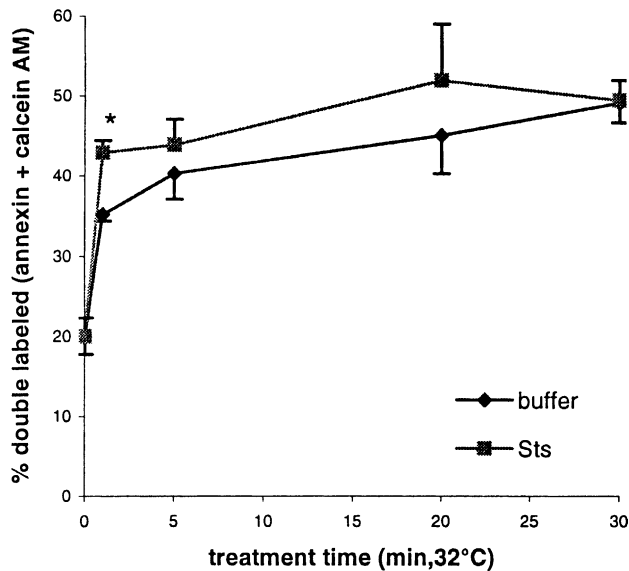


Fig. 2. Time course for production of 'apoptotic' (double-labeled) particles. Crude synaptosomes were treated for the time shown with either KRP buffer or staurosporine (Sts), then labeled with annexin-V and calcein AM for flow cytometry analysis. For each sample, data was collected from 20,000 particles in the 1–4  $\mu\text{m}$  size range; percentage double-labeled is number in upper right quadrant as shown in Fig. 1. Data are from four independent experiments. The symbol (\*) indicates  $P \leq 0.05$  compared to untreated P-2.

increase in purified synaptosomes likely results from loss of viability that occurs during the additional isolation steps.

### 3.2. The number of annexin-V-positive synaptosomes increases rapidly

Fig. 2 shows the time course for the production of annexin-V-positive synaptosomes. Data are from flow cytometric analysis, as described above, of the largest particles

in the P-2 fraction. At the beginning of the experiment, 20% ( $\pm 2.2$ ) of the intact particles are positive for annexin-V; this percentage increases twofold after 1 min by staurosporine treatment (0.1  $\mu\text{M}$ ), to 43% ( $\pm 1.49$ ). When synaptosomes are incubated in KRP buffer, annexin labeling increases to 35% ( $\pm 0.81$ ); the increase by staurosporine compared to buffer is significant following a 1 min incubation ( $P < 0.05$  compared to buffer treatment), but not at subsequent time points. The failure of staurosporine treatment to significantly increase 'apoptosis' levels above buffer controls after 1 min was surprising and indicates that activation of the pathway leading to phosphatidylserine exposure happens every early even in untreated synaptosomes. Increasing the staurosporine concentration to 300 nM did not result in any significant change in the annexin-V/calcein AM results (data not shown).

### 3.3. Caspase-3-like activity is increased in synaptosomes

The precise ordering of very early events in apoptosis is unclear and may vary between experimental systems (Green and Kroemer, 1998); however, PS externalization is thought to be a downstream event of early caspase activation (van Engeland et al., 1998), therefore caspase activity was measured in our synaptosome system with a fluorometric assay. Fig. 3 shows that neither caspase-1- (Fig. 3A) nor caspase-3-like activity (Fig. 3B) was increased after 5 min, but caspase-3-like activity was significantly increased after 30 min. Consistent with the annexin-V flow-cytometry analyses (Fig. 2), treatment with staurosporine (0.1  $\mu\text{M}$ ) does not result in a significant increase in caspase activity compared with buffer treated controls. At the concentration of substrate used (50  $\mu\text{M}$ ), caspase activity measures include caspase-4 and caspase-7 activity (Stennicke and Salvesen, 1998); however, only caspase-3 has been detected in rat brain (Shimohama et al., 2001).

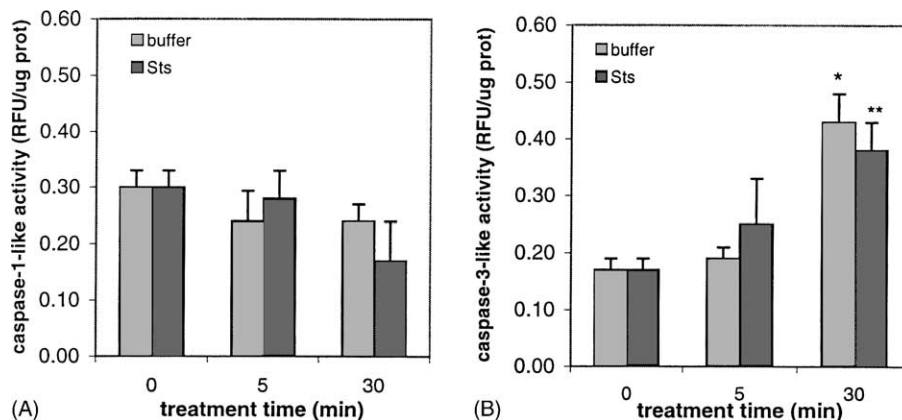


Fig. 3. Direct measurement of caspase activity in crude synaptosomes prepared from rat brain measured by fluorometric assay. Caspase activity is expressed as relative fluorescence units (RFU). (A) Time course of caspase-1-like activity with Ac-YVAD-AMC as substrate. Crude synaptosomes were treated for the time shown with either KRP buffer or staurosporine (Sts). (B) Caspase-3-like activity measured by fluorometric assay with Ac-DEVD-AMC as substrate. Data are from four independent experiments. The symbols (\*) and (\*\*) indicate  $P \leq 0.05$  and 0.01, respectively, compared to caspase activity measured at time 0.

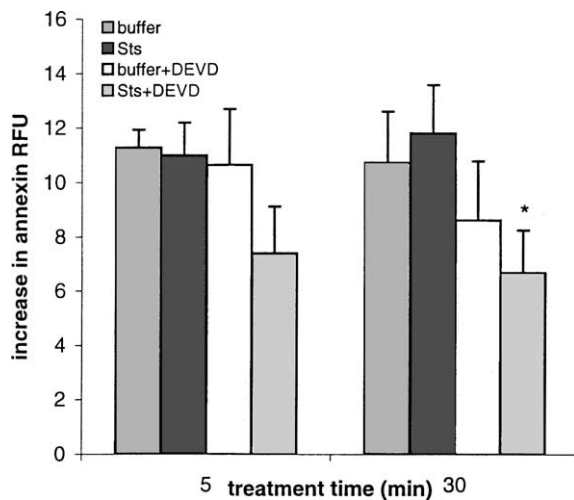


Fig. 4. Blockade of increased annexin fluorescence by inhibition of caspase-3-like activity. Crude synaptosomes were treated with either KRP buffer or in the presence and absence Ac-DEVD-CHO, then labeled with annexin-V and calcein AM for flow cytometry analysis. Annexin fluorescence is expressed as relative fluorescence units (RFU). Data are from four independent experiments. The symbol (\*) indicates  $P \leq 0.05$  compared to annexin-V increase by staurosporine alone.

#### 3.4. Increased annexin-V labeling is blocked by caspase inhibition

The relationship between PS exposure and caspase activation was further examined by addition of a caspase inhibitor during synaptosome preparation. Fig. 4 shows the effects of Ac-DEVD-CHO, a peptide developed as a caspase-3 inhibitor, on the increase in annexin-V staining in the presence of staurosporine or buffer incubation at 32 °C. Caspase inhibition resulted in a partial blockade of the increase in annexin-V staining after 30 min incubation with staurosporine (Fig. 4); the relative fluorescence (RFU) was decreased from 11.8 ( $\pm 1.8$ ) to 6.7 ( $\pm 1.6$ ) by the DEVD peptide. This difference is statistically significant compared to treatment with staurosporine alone ( $P < 0.03$ ). Decreased annexin-V staining by Ac-DEVD-CHO was observed after 5 min, but this trend did not reach significance, and inhibition of caspase-1 activity with the Ac-YVAD-CHO peptide did not affect annexin-V labeling at early time points. Taken together with the results of the caspase assay, the time-course of caspase activation and their inhibitory effects are consistent with caspase activation that follows PS externalization in the synaptosome preparation.

## 4. Discussion

Initial apoptotic events in neurodegeneration may occur in axonal, dendritic, or synaptic compartments far removed from the cell body, and may contribute to synapse loss before measurable cell death has occurred. The experiments described use flow cytometry analysis with annexin-V to

demonstrate rapid kinetics for the appearance of annexin-V labeling and caspase activation in synaptosomal membranes in this widely used in vitro neurochemical preparation.

A physiologic role for PS externalization in terminal regions is suggested by the visualization of annexin-V fluorescence accompanied by blebbing and caspase activation in the neurites of hippocampal neuron cultures exposed to amyloid- $\beta$  protein ( $A\beta$ )<sub>25–35</sub>. (Ivins et al., 1998; Cole et al., 1999), and the speed with which loss of membrane asymmetry and caspase activation occur reveals a potential for rapid terminal responses to toxic insults. However, it is important to note that the relationship of the present observations to in vivo neurodegeneration is not yet clear. Historically, cell-free systems have generated useful knowledge about the machinery of apoptosis. For example, in programmed cell death studies using annucleate cytoplasts, mitochondrial-mediated activation of apoptosis and bcl-2 protection from apoptosis have been shown to occur in the absence of a nucleus (Jacobson et al., 1994; Ellerby et al., 1997).

Although the difference was significant at 1 min, the general lack of a staurosporine effect compared to buffer was unexpected, and suggests that homogenization results in activation of both necrotic and apoptotic protease cascades. Previously, apoptotic cell death was hypothesized to occur in neurodegenerative diseases, with necrotic death occurring in cerebral ischemia, traumatic brain injury and spinal-cord injury. However, entry into necrosis versus apoptosis pathways has been shown to depend on the intensity of the insult, with apoptosis predominating when an excitotoxic stimulus is mild (Bonfoco et al., 1995). More recently, based in part on substrate similarities, overlapping necrotic and apoptotic pathways have also been proposed to contribute to neurodegenerative cell death (Wang, 2000). The potential for combination therapy suggested by this hypothesis is supported by evidence that calpain and caspase inhibitor have additive neuroprotective effects (Nath et al., 1996; Pike et al., 1998). Further support for the hypothesis that necrotic pathways are activated in synaptosomes is found in recent results showing that synaptosomes are protected from in vitro lysis by both calpain and caspase inhibition (Gyls et al., 2002).

The finding that the early rise in annexin-V-positive particles could not be demonstrated reliably in purified synaptosomes may be related to increased integrity of the P-2 relative to a gradient-purified preparation, which requires extra time, manipulation and, in some protocols, exposure to hypertonic gradients. This explanation is supported by evidence that glutamic acid and high affinity choline uptake become labile and drop after 8–12 min of incubation (Wheeler, 1978; Jope and Jenden, 1977). On the other hand, synaptosomes have been described as stable for up to 6 h without deterioration in ATP level, ion gradients, or neurotransmitter release (Nicholls, 1993), and previous examinations of caspase activity using synaptosomes (Mattson et al., 1998a,b) and cell-free cytosolic extracts (Ellerby et al., 1997), showed increases with a time course of hours rather than min. The functional integrity of synaptosomal

preparations is not usually assessed, and despite discrepancies within the literature regarding viability of synaptosomal preparations, use of the P-2 offers the advantages of shorter preparation and time and greater tissue yield in addition to a shorter post-mortem interval.

PS exposure does not occur with apoptosis in all cell types; however, when PS exposure occurs, it is downstream of caspase activation (Naito et al., 1997; Fadeel et al., 1999; van Engeland et al., 1998). The lack of a significant increase in caspase-3-like activation before 30 min with the fluorometric assay in the present results is consistent with the explanation that PS externalization precedes caspase activation. However, our experiments with Ac-DEVD-CHO inhibitors suggest a trend for blockade of annexin labeling after 5 min, and a trend for increased caspase-3 activity is also visible after 5 min (Fig. 3). Taken together, these findings may indicate that caspase activation precedes PS exposure but is not detected by our assay. Additional mechanisms that might induce rapid PS externalization in synaptosomes include disruption of the aminophospholipid translocase that is responsible for membrane asymmetry (Tang et al., 1996), or activation of the phospholipid scramblase that externalizes PS residues in platelets (Zhou et al., 1997). The annexin-V labeling that we observe may also indicate lipid peroxidation, based on recent work showing that significant lipid peroxidation occurs in synaptosomes (Chakraborty et al., 2001), taken together with the observation that annexin-V binds to malondialdehyde (MDA) adducts in addition to PS residues (Balasubramanian et al., 2001). Because mitochondrial transmembrane potential is disrupted prior to PS exposure in anucleate cells (Castedo et al., 1996), mitochondrial dysfunction might be linked to rapid PS exposure in synaptosomes containing mitochondria. Each of these possibilities suggests that annexin-V labeling should be interpreted with caution as an apoptosis marker in some model systems.

Caspase-3 is considered to be a distal protease in a complex cascade, and the primary executioner caspase, and caspase-1 was initially identified as the protease that activates the pro-inflammatory cytokine IL1- $\beta$  (Stennicke and Salvesen, 1998). The therapeutic potential of caspase inhibitors has been of interest and is supported by observations in which these agents are neuroprotective. For example, studies in a rat model of hypoxic-ischemic brain injury show protection from delayed cell death by a non-specific caspase inhibitor, boc-aspartyl(OMe)-fluoromethylketone (Cheng et al., 1998), and related studies from our group have recently demonstrated that broad-spectrum caspase inhibition protects synaptosomes from *in vitro* lysis (Gyllys et al., 2002). The precise role played by individual proteases varies with the inducing agent and the experimental system and cell type; for example, caspase-1 inhibitors prevent programmed cell death in motoneurons (Milligan et al., 1995), but did not affect annexin-V labeling in synaptosomes in the present study.

These data indicate that PS is rapidly externalized in synaptosomal membranes, and that loss of membrane asym-

metry may precede caspase activation in this preparation. The present results demonstrate that the sensitivity of flow cytometric analyses can reveal rapid changes previously undetected in synaptosomes. Because synaptosomes contain mitochondria, which play a significant role in the initiation of apoptosis, future studies in this model system may illuminate the sequence of events in apoptosis and reveal differences between nerve terminals and intact neurons in the pathways leading to degeneration.

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## References

- Balasubramanian, K., Bevers, E.M., Willems, G.M., Schroit, A.J., 2001. Binding of annexin V to membrane products of lipid peroxidation. *Biochemistry* 40, 8672–8676.
- Bonfoco, F., Krainc, D., Ankarcrona, M., Nicotera, P., Lipton, S.A., 1995. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. U.S.A.* 92, 7162–7166.
- Brown, S.B., Clarke, M.C.H., Magowan, L., Sanderson, H., Savill, J., 2000. Constitutive death of platelets leading to scavenger receptor-mediated phagocytosis. *J. Biol. Chem.* 275 (8), 5987–5996.
- Castedo, M., Hirsch, T., Susin, S.A., Zamzami, N., Marchetti, P., Macho, A., Kroemer, G., 1996. Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. *J. Immunol.* 157, 512–521.
- Chakraborty, H., Ray, S.N., Chakrabarti, S., 2001. Lipid peroxidation associated protein damage in rat brain crude synaptosomal fraction mediated by iron and ascorbate. *Neurochem. Int.* 39, 311–317.
- Cheng, Y., Deshmukh, M., D Costa, A., Demaro, J.A., Gidday, J.M., Shah, A., Sun, Y., Jacquin, M.F., Johnson Jr., F.M., Holtzman, D.M., 1998. Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. *J. Clin. Invest.* 101, 1992–1999.
- Chow, S.C., Weis, M., Kass, G.E.N., Holmstrom, T.H., Eriksson, J.E., Orrenius, S., 1995. Involvement of multiple proteases during Fas-mediated apoptosis in T lymphocytes. *FEBS. Lett.* 364, 134–138.
- Cole, G.M., Yang, F., Chen, P.P., Frautschy, S.A., Hsiao, K., 1999. Caspase activation in dystrophic neurites in Alzheimer's disease and aged APPsw mice. In: Iqbal, K., Swaab, D.F., Winblad, B., Wisniewski, H.M. (Eds.), *Alzheimer's Disease and Related Disorders*. Wiley, Sussex, England, pp. 363–370.
- Ellerby, H.M., Martin, S.J., Ellerby, L.M., Naiem, S.S., Rabizadeh, S., Salvesen, G.S., Casiano, C.A., Cashman, N.R., Green, D.R., Bredesen, D.E., 1997. Establishment of a cell-free system of neuronal apoptosis: comparison of premitochondrial, mitochondrial, and postmitochondrial phases. *J. Neurosci.* 17, 6165–6178.
- Fadeel, B., Gleiss, B., Hogstrand, K., Chandra, J., Wiedmer, T., Sims, P.J., Hente, J.I., Orrenius, S., Samali, A., 1999. Phosphatidylserine exposure during apoptosis is a cell-type-specific event and does not correlate with plasma membrane phospholipid scramblase expression. *Biochem. Biophys. Res. Commun.* 266, 504–511.
- Green, D., Kroemer, G., 1998. The central executioners of apoptosis: caspases or mitochondria? *Trends Cell Biol.* 8, 267–271.

- Gyllys, K.H., Fein, J.A., Cole, G.M., 2000. Quantitative characterization of crude synaptosomal fraction (P-2) components by flow cytometry. *J. Neurosci. Res.* 61, 186–192.
- Gyllys, K.H., Fein, J.A., Cole, G.M., 2002. Caspase inhibition protects nerve terminals from in vitro degradation. *Neurochem. Res.* 27, 465–472.
- Ivins, K.J., Bui, E.T.N., Cotman, C.W., 1998.  $\beta$ -Amyloid induces local neurite degeneration in cultured hippocampal neurons: evidence for neuritic apoptosis. *Neurobiol. Dis.* 5, 365–378.
- Jacobson, M.D., Burne, J.F., Raff, M.C., 1994. Programmed cell death and bcl-2 protection in the absence of a nucleus. *EMBO J.* 13, 1899–1910.
- Jope, R.S., Jenden, D.J., 1977. Synaptosomal transport and acetylation of choline. *Life Sci.* 20, 1389–1392.
- Masliah, E., Mallory, M., Hansen, L., DeTeresa, R., Alford, M., Terry, R., 1994. Synaptic and neuritic alterations during the progression of Alzheimer's disease. *Neurosci. Lett.* 174, 67–72.
- Mattson, M.P., Keller, J.N., Begley, J.G., 1998a. Evidence for synaptic apoptosis. *Exp. Neurol.* 151, 35–48.
- Mattson, M.P., Partin, J., Begley, J.G., 1998b. Amyloid  $\beta$ -peptide induces apoptosis-related events in synapses and dendrites. *Brain Res.* 807, 167–176.
- Milligan, C.E., Prevette, D., Yaginuma, H., Homma, S., Cardwell, C., Fritz, L.C., Tomaselli, K.J., Oppenheim, R.W., Schwartz, L.M., 1995. Peptide inhibitors of the ICE protease family arrest programmed cell death of motoneurons in vivo and in vitro. *Neuron* 15, 385–393.
- Mucke, L., Masliah, E., Yu, G.Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., McConlogue, L., 2000. High-level neuronal expression of A $\beta$  1–42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* 20, 4050–4058.
- Nagy, A., Delgado-Escueta, A.V., 1984. Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient material (Percoll). *J. Neurochem.* 43, 1114–1123.
- Naito, M., Nagashima, K., Mashima, T., Tsuruo, T., 1997. Phosphatidylserine externalization is a downstream event of interleukin 1 $\beta$ -converting enzyme family protease activation during apoptosis. *Blood* 89, 2060–2066.
- Nath, R., Raser, K., McGinnis, K., Nadimpalli, R., Stafford, D., Wang, K.K.W., 1996. Effects of ICE-like protease and calpain inhibitors on neuronal apoptosis. *NeuroReport* 8, 249–255.
- Nicholls, D.G., 1993. The glutamatergic nerve terminal. *Eur. J. Biochem.* 212, 613–631.
- Pike, B.R., Zhao, X., Newcomb, J.K., Wang, K.K.W., Posmantur, R.M., Hayes, R.L., 1998. Temporal relationships between de novo protein synthesis, calpain and caspase 3-like protease activation, and DNA fragmentation during apoptosis in septo-hippocampal cultures. *J. Neurosci. Res.* 52, 505–520.
- Sastry, P.S., Rao, K.S., 2000. Apoptosis and the nervous system. *J. Neurochem.* 74, 1–20.
- Schmid, I., Uittenbogaart, C.H., Giorgi, J.V., 1994. Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. *Cytometry* 15, 12–20.
- Shimohama, S., Tanino, H., Fujimoto, S., 2001. Differential subcellular localization of caspase family proteins in the adult rat brain. *Neurosci. Lett.* 315, 125–128.
- Stennicke, H.R., Salvesen, G.S., 1998. Properties of the caspases. *Biochim. Biophys. Acta* 1387, 17–31.
- Tang, X., Halleck, M.S., Schiegel, R.A., Williamson, P., 1996. A subfamily of P-type ATPases with aminophospholipid transporting activity. *Science* 272, 1495–1497.
- van Engeland, M., Nieland, L.J.W., Ramaekers, F.C.S., Schutte, B., Reutelingsperger, C.P.M., 1998. Annexin V-affinity assay: a review of an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 31, 1–9.
- Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., 1995. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled annexin V. *J. Immunol. Methods* 184, 39–51.
- Wang, K.K.W., 2000. Calpain and caspase: can you tell the difference? *Trends Neurosci.* 23, 20–26.
- Weiler, M.H., Gundersen, C.B., Jenden, D.J., 1981. Choline uptake and acetylcholine synthesis in synaptosomes: investigations using two different labeled variants of choline. *J. Neurochem.* 31, 789–796.
- Wheeler, D.D., 1978. Some problems inherent in transport studies in synaptosomes. *J. Neurochem.* 30, 109–120.
- Wolf, B.B., Goldstein, J.C., Stennicke, H.R., Beere, H., Amarante-Mendes, G.P., Salvesen, G.S., Green, D.R., 1999. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. *Blood* 94, 1683–1692.
- Zhou, Q., Zhao, J., Stout, J.G., Luhm, R.A., Wiedmer, T., Sims, P.J., 1997. Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids. *J. Biol. Chem.* 272, 18240–18244.